Biochemical Characterization and Vaccine Potential of a Heme-Binding Glutathione Transferase from the Adult Hookworm *Ancylostoma caninum*

Bin Zhan,¹* Sen Liu,¹ Samirah Perally,³ Jian Xue,⁶ Ricardo Fujiwara,¹ Peter Brophy,³ Shuhua Xiao,⁴ Yueyuan Liu,¹ Jianjun Feng,¹ Angela Williamson,¹ Yan Wang,¹ Lilian L. Bueno,¹ Susana Mendez,¹ Gaddam Goud,¹ Jeffrey M. Bethony,¹ John M. Hawdon,³ Alex Loukas,¹,⁵ Karen Jones,² and Peter J. Hotez¹,*

Department of Microbiology and Tropical Medicine¹ and Biostatistics Unit,² The George Washington University and Sabin Vaccine Institute, Washington, DC 20037; Institute of Biological Science, University of Wales, Aberystwyth SY23 3DA, United Kingdom³; Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention, Shanghai 200025, China⁴; and Queensland Institute of Medical Research, Brisbane, Queensland 4006, Australia⁵

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Hookworm infection is a major cause of disease burden for animals and humans. An estimated 740 million cases of human hookworm infection occur worldwide (12). Most of the pathology attributed to hookworm infection results from intestinal blood loss caused by the adult stages of the parasite (21, 32). The adult hookworm is specially adapted to ingest red blood cells and feed on the intracellular contents and has evolved to produce a battery of molecules for this purpose (22, 42). For instance, the parasite uses its buccal capsule to attach to the intestinal mucosa and submucosa, where it mechanically ruptures capillaries and arterioles. From unique cephalic glands, the adult hookworm releases anticoagulants and anti-platelet-aggregating agents into the attachment site (10, 34). The parasite subsequently ruptures red blood cells through the action of a unique hemolysin (13) and then degrades the released hemoglobin (14, 35). The parasite-induced blood loss leading to iron deficiency anemia (35).

*Corresponding author. Mailing address: Department of Microbiology and Tropical Medicine, The George Washington University and Sabin Vaccine Institute, Ross Hall 736, 2300 Eye St NW, Washington, DC 20037. Phone: (202) 994-1766. Fax: (202) 994-2913. E-mail for Peter Hotez: mtmphpj@gwumc.edu. E-mail for Bin Zhan: mtmbxz@gwumc.edu.

The trichostrongyle *Hemonchus contortus* is a major cause of anemia and weight loss in small ruminants. Like hookworms, *H. contortus* produces numerous mechanistically distinct proteases that are thought to digest hemoglobin (27). Recently, adult *H. contortus* was shown to produce a novel glutathione S-transferase (*Hc-GST-1*), which has a high-affinity binding site for hematin (38). The hematin-binding GST is postulated to have a focused role in the detoxification or transport of heme (derived from the blood meal) and related compounds and is believed to represent a special adaptation to blood feeding (38).

Here, we report on the cloning, expression, immunolocalization, and heme-binding properties of a GST (*Ac-GST-1*) produced by the adult canine hookworm, *Ancylostoma caninum*. In addition, we report the results of a canine vaccine trial with recombinant *Ac-GST-1* (r*Ac-GST-1*) followed by *A. caninum* larval challenge and a hamster vaccine trial followed by heterologous *Necator americanus* larval challenge. Our data suggest that *Ac-GST-1* is a promising vaccine candidate and support the hypothesis that targeting the blood-feeding pathway of the adult hookworm is a promising vaccine strategy (28, 42).

**MATERIALS AND METHODS**

**Cloning of Ac-gst-1.** Larval (first-stage [L1] plus L2 and L3) and adult parasites of the canine hookworm, *A. caninum*, as well as their corresponding cDNAs and cDNA libraries, were obtained and prepared as described previously (17, 24). Partial cDNA sequence information on *Ac-gst-1* was obtained by searching the
Parasite Genomes Database through Wu-Blast2 (http://www.ebi.ac.uk/blast2/parasites.html) using the DNA sequence of Sj28, a gene encoding a GST vaccine antigen from Schistosoma japonicum (18). In order to clone the 5' and 3' cDNA regions of Ac-gst-1, gene-specific forward primers (Ac-GSTF1, AGA TGT TCG CTC TCG AC; Ac-GSTF2, TCC TCG AGA GAA TGA TGC TCT ACA ACA AGC TGA C) and reverse primers (Ac-GSTR1, TGT CTA GAA AGT GAG TCT CCG GTG TGC AC; Ac-GSTR2, GTA GTC GAT CTT ATG AGT TG) were designed based on partial expressed sequence tag sequence information for Ac-gst-1 obtained from the Parasite Genomes Database. The 5' and 3' regions of Ac-gst-1 cDNA were isolated from the first-strand cDNA of *A. caninum* third-stage infective larvae (L3) by a modified RNA ligase-mediated rapid amplification technique (GeneRacer; Invitrogen) using Ac-gst-1 gene-specific primers as described previously (45). The full-length cDNA sequence of Ac-gst-1 was obtained by aligning the 5' and 3' regions of Ac-gst-1 sequence.

Sequence analysis. DNA and predicted protein sequences were analyzed using ESEE version 3.1 (5). The sequences were aligned using CLUSTAL W (http://cluswalw.genome.jo) and prepared for display using BOXSHADE (http://bioweb.pasteur.fr/seqanal/interfaces/boxshade-simple.html).

RT-PCR amplification. Reverse transcription (RT)-PCR was used to determine the life history stages in which Ac-gst-1 mRNA is transcribed, as described previously (44). The specific primers Ac-GSTF2 and Ac-GSTR2, based on the Ac-gst-1 sequence between 35 and 54 bp and 309 and 328 bp, respectively, were used to amplify Ac-gst-1 cDNA. Genomic DNA of *A. caninum* was used as a control.

Expression and purification of the recombinant protein. The full-length cDNA of Ac-gst-1 was cloned in frame into the eukaryotic expression vector pPICZaA (Invitrogen) using XhoI and XbaI restriction sites. The correct open reading frame (ORF) was confirmed by sequencing using the linker flanking primers corresponding to the regions encoding the a-factor and 3'AOX1 genes. The recombinant plasmids were linearized by SacI digestion and transformed into *Pichia pastoris* strain X-33 by electroporation according to the manufacturer's instructions (Invitrogen). The transformants were selected on zeocin-resistant plates containing yeast extract-peptone-dextrose medium with sorbitol and identified by PCR amplification using the vector primers described above. A colony containing the appropriate insert was grown in a shaker flask, and expression of the recombinant fusion protein containing a polyhistidine tag (His tag) at the C terminus was induced with methanol, as described previously (14). The methanol concentration was maintained at 0.5% for 4 days at 28°C. The recombinant fusion protein was purified with a nickel affinity column as described previously (14).

Rabbit anti-rAc-GST-1 antiserum preparation, Western blotting, and immunocalization. Antiserum against rAc-GST-1 protein was prepared in a rabbit immunized with 100-μg aliquots of rAc-GST-1 at 3-week intervals as previously described (45). Antiserum was used in Western blotting to determine whether the corresponding native protein was present in larval or adult hookworms. Extracts of L3, combined L1 plus L2, eggs, adult worms, and adult were obtained by aligning the 5'/H11032 primers as described previously (45). The full-length cDNA sequence of *Ac-gst-1* was determined by measuring changes in intrinsic protein fluorescence as described elsewhere (38). In the binding assays, 1 μM rAc-GST-1 was used in 20 mM potassium phosphate buffer (pH 6.5) containing 100 mM sodium chloride at 25°C. Changes in fluorescence were recorded with a Shimadzu spectrophotometer (RF-5301 PC) with excitation and emission wavelengths for intrinsic protein fluorescence (tryptophan) of 280 and 320 nm, respectively. Increasing concentrations of hematin were added and incubated for 3 min prior to measurement.

Animal vaccine trials. *Pichia*-derived rAc-GST-1 was tested as a vaccine in a single canine trial (homologous *A. caninum* larval challenge) and in a single hamster trial (heterologous *N. americanus* larval challenge).

(i) Canine trials. The test and control animals were purpose-bred, parasitenaive male beagles with ages of 56 ± 7 days on arrival. Inclusion criteria for enrollment included body weight equal to or exceeding 2.5 kg, hematocrit equal to or exceeding 28.0, and white blood cell count not exceeding 20 × 10⁶/ml. All dogs in a trial were purchased from the same vendor (Harlan Farms) and maintained in the George Washington University (GWU) Animal Research Facility as previously described (20). An initial screening fecal examination was conducted shortly after the arrival of the dogs at the GWU Animal Research Facility to confirm the absence of canine hookworm infection. The experiments were conducted according to a protocol (protocol 48-12.1) approved by the George Washington University Institutional Animal Care and Use Committee. The dogs were housed in groups for approximately 1 month and at one to three animals per cage thereafter. Following trial challenge, all dogs were individually housed.

The dogs were vaccinated with either 100 μg of *Pichia*-expressed rAc-GST-1 formulated with the adjuvant system 03 (AS03; a gift from GlaxoSmithKline Biologicals) or AS03 only as a control, as described previously (28), under a protocol approved by the GWU Institutional Animal Care and Use Committee. The vaccines were administered intramuscularly every 3 weeks on days 0, 21, 42, and 63. An additional group of five dogs was immunized subcutaneously with 1.000 irradiated *A. caninum* larvae in 500 μl of phosphate-buffered saline using the same immunization schedule. Two weeks after the final vaccination, the dogs were challenged with *A. caninum* larvae by applying 500 infective larval larvae in a final volume of 50 μl to the footpad (28). Quantitative hookworm egg counts were performed three times a week starting 14 days after infection. Four weeks after infection, the dogs were euthanized to recover hookworms at necropsy from the small intestine and colon as described previously (24). Following the analysis of variance, Dunnett's test was chosen to compare each experimental mean with the control mean while controlling the experimental error rate.

(ii) Hamster trials and *N. americanus* L3 challenge. The hamster vaccine trial was performed under a protocol approved by the Institute of Parasitic Diseases of the Chinese Center for Disease Control and Prevention (IPD-CCDCP). The IPD-CCDCP has maintained *N. americanus* in a colony of Mesocricetus auratus for more than 100 passages (25). Golden hamsters aged 5 weeks were obtained from the Shanghai Animal Center, Chinese Academy of Sciences. Recombinant Ac-GST-1 was used to immunize 20 hamsters by subcutaneous injection. The dose of rAc-GST-1 given to the hamsters was 25 μg, which was administered once every 2 weeks for a total of three injections. The hamster vaccine trial was conducted shortly after the arrival of the dogs at the GWU Animal Research Facility as previously described (20). Another group of 20 hamsters were immunized with Alhydrogel as a negative control. One week after the last immunization, the hamsters were challenged subcutaneously with 150 L3 larvae of *N. americanus* maintained in hamsters. Twenty-five days postchallenge, hamsters in each group were euthanized, and the worms were recovered from the intestines. The t test was used for statistical analysis.

Measurement of canine humoral and cellular immune response. The dogs were bled on days after each vaccination. Serum was separated and used to measure levels of antibody isotype (IgG1, IgG2, and IgE) response by an indirect enzyme-linked immunosorbent assay (ELISA) as described previously (24). The lymphoproliferation assay was performed using whole-blood culture as previously described (33). Whole-blood cultures were either unstimulated or stimulated with concanavalin A (ConA) (2.5 μg/ml) or rAc-GST-1 (2.5 μg/ml). Cellular proliferative responses were expressed as a stimulation index (mean proliferation of stimulated cultures/mean proliferation of unstimulated cultures). Canine interleukin 4 (IL-4), IL-10, and gamma interferon (IFN-γ) cytokine levels were measured in supernatants from whole-blood cell cultures. This was done by incubation of blood cells with hookworm adult extract antigen (25 μg/ml), rAc-GST-1 (25 μg/ml), or 1 μg/ml of phorbol 12-myristate 13-acetate (Sigma-Aldrich Co.) and then measuring canine cytokines using a capture ELISA (R&D Sys-
tems, Minneapolis, MN) according to the manufacturer’s instructions. The t test was used for statistical analysis.

**RESULTS**

Cloning of Ac-gst-1 cDNA. The 5′ and 3′ regions of Ac-gst-1 were isolated from *A. caninum* L3 first-strand cDNA based on the partial expressed sequence tag sequence (BM077922) obtained from the Parasite Genomes Database. A full-length 743-bp cDNA consensus sequence of Ac-gst-1 was obtained by linking the 5′ and 3′ regions generated by 5′ Gene-Racer. The full-length Ac-gst-1 cDNA comprises an ORF encoding 207 deduced amino acids. There is a nematode spliced leader sequence (SL1) at the 5′ end, followed by a translational start codon (ATG). The predicted Ac-GST-1 protein has a calculated molecular mass of 23.75 kDa and a theoretical pI of 5.87. One putative N-linked glycosylation site (Asn-X-Ser) was located between amino acids 144 and 146. InterPro database searching at EMBL-EBI revealed that Ac-GST-1 is a member of the GST superfamily containing typical GST N-terminal (IRP004045) and C-terminal (IRP004046) domain structures, including conserved tyrosine residues in the N-terminal domain (Tyr-4 and Tyr-8).

An alignment of the putative Ac-GST-1 ORF with GST homologues from other nematodes, *S. japonicum* and *human* GST-α3 is shown in Fig. 1; it demonstrates that Ac-GST-1 exhibits 65% identity with HpGST2 (*Heligmosomoides polygyrus*), 58% identity with *Hc-GST-1* (*H. contortus*), Ce-GST-5 (Ce-GST-5; NP_496357), Hc-GST-1, and Sj28 (S. japonicum; P26624) and human GST-α3 (A49365). The sequences were aligned using CLUSTAL W and prepared for display using BOXSHADE. Identical amino acids are shaded in black, and similar amino acids in gray. Conserved tyrosine residues (Tyr-4 and Tyr-8) are marked with asterisks. The percentage of sequence identity to Ac-GST-1 is shown at the end of each sequence.

**RT-PCR analysis.** The Ac-gst-1-specific primers Ac-GSTF2 and Ac-GSTR2, based on the Ac-gst-1 sequence between 35 and 328 bp, were employed to amplify Ac-gst-1 cDNA reverse transcribed from mRNAs of different developmental life history stages of *A. caninum* (Fig. 2). A 294-bp cDNA product was amplified from the cDNAs obtained from *A. caninum* eggs, L1 plus L2, L3, and adult hookworms, indicating that Ac-gst-1 mRNA is transcribed throughout development. A band with an approximate size of 700 bp was observed in the reaction with genomic DNA, suggesting the amplification of an intron(s) (Fig. 2).

**Detection and immunolocalization of native Ac-GST-1 in *A. caninum*.** Recombinant Ac-GST-1 was expressed in *Pichia* strain X-33, purified by nickel affinity chromatography, and used to raise antiserum for detection of the native protein in the parasite. On a Western blot, a single strong band migrating with an apparent mass of 30 kDa was recognized by the antiserum in the extract of adult *A. caninum* (Fig. 3). A band of the same size was also observed in the ES products of adult hookworms. The molecular masses of native Ac-GST-1 observed in Western blots were approximately 6 kDa greater than the predicted molecular mass of the ORF. This may indicate post-translational modifications, such as glycosylation at Asn-144.
The molecular mass of \( \alpha \)C-GST-1 was approximately 3 kDa higher than that of the native protein, presumably because of a His tag expressed at the C terminus. The absence of additional major bands in the lane containing the recombinant protein suggested there was no evidence for major contamination of the purified \( \alpha \)C-GST-1 with \textit{Pichia} host cell contaminants. This was confirmed by an ELISA and Western blot test that employs an antibody to \textit{Pichia} pastoris proteins (Immunoenzymatic assay kit and Western blot kit; Cygnus Technologies, Southport, NC) (data not shown). In contrast to adult hookworms and their ES, no band on Western blots was recognized that anti-\( \alpha \)C-GST-1 antiserum bound strongly to the adult parasite hypodermis (Fig. 4A) and muscle (Fig. 4B) and weakly to the gastrointestinal tract (Fig. 4B).

**Enzymatic and ligand binding activities of \( \alpha \)C-GST-1.** The enzymatic activity of \( \alpha \)c-GST-1 with a His tag catalyzing the conjugation of GSH to CDNB substrate was estimated to be 844.93 + 23.43 nmol/min/mg (mean of duplicates ± standard deviation), indicating that \( \alpha \)C-GST-1 functions as a glutathione transferase in vitro (Table 1). No activity was observed with the same quantity of an irrelevant control, recombinant \textit{Ancyllostoma}-secreted protein 2 from \textit{Necator americanus} (Na-ASP-2).

The high degree of amino acid alignment between \( \alpha \)C-GST-1 and \( Hc \)-GST-1, a heme-binding GST from \textit{H. contortus} (38), suggests that the former could also exhibit heme-binding properties. On a Western blot, rabbit anti-\( \alpha \)C-GST-1 serum also showed cross-reaction with recombinant \( Hc \)-GST-1 (data not shown). Recombinant six-His-tagged \( \alpha \)C-GST-1 was observed to bind to hematin and its precursor, protoporphyrin IX, by a double-reciprocal-plot quenching of intrinsic fluorescence assay (Fig. 5A and B). The dissociation constant \((K_d)\) values for hematin and for protoporphyrin IX are 2.3 ± 0.38 \( \mu \)M and 19.98 ± 4.48 \( \mu \)M, respectively. This indicates that \( \alpha \)C-GST-1 has almost 10-times-higher binding activity to hematin than to protoporphyrin IX, the structurally related precursor that does not contain iron. The 50% inhibitory concentration of hematin for the GSH-CDNB conjugation catalyzed by \( \alpha \)C-GST-1 was determined to be 3.23 ± 0.1 \( \mu \)M, indicating that hematin and CDNB share the same binding site on the GST.

**Canine antibody and cellular responses to \( \alpha \)C-GST-1 immunization.** Dogs vaccinated with \( \alpha \)c-GST-1 formulated with AS03 adjuvant produced high IgG1 and IgG2 antibody responses in an indirect ELISA (Fig. 6). The highest geometric mean antibody titers for IgG1 (1:13,500) and IgG2 (1:40,500) were observed after the fourth vaccination. Both IgG1 and IgG2 titers declined after the canines were challenged with L3. \( \alpha \)c-GST-1-specific IgE titers were low (<1:500) (data not shown). Control dogs (adjuvant AS03 alone) did not generate detectable anti-\( \alpha \)C-GST-1 immune responses (data not shown). Peripheral blood leukocytes (PBLs) from dogs vaccinated with \( \alpha \)c-GST-1 proliferated in the presence of \( \alpha \)C-GST-1, while PBLs from dogs immunized with adjuvant alone did not proliferate. The mean stimulation index for the vaccinated dogs was 2.36 times higher than that for the negative control dogs \((P < 0.05)\) (Fig. 7A). PBLs from both groups were stimulated equally with ConA, indicating that the PBLs were functional and responsive to nonspecific mitogens at all time points tested (Fig. 7A). PBLs from dogs vaccinated with \( \alpha \)c-GST-1 produced 9.1 ± 2.9 ng/ml of IFN-\( \gamma \) after antigen-specific stimulation compared to control dogs (AS03 only), which produced only 3.3 ± 1.4 ng/ml \((P < 0.05)\) (Fig. 7B). The peripheral leukocytes from the vaccinated group also produced significantly higher IFN-\( \gamma \) levels after being stimulated with adult worm extract than those of controls \((P < 0.04)\). In contrast, there were no significant differences in the production of IL-4 (Fig. 7B) or IL-10 (data not shown) induced by \( \alpha \)C-GST-1, even though IL-4 was significantly induced by hookworm adult extract in the vaccinated dogs \((P < 0.04)\).

**Postchallenge reductions in hookworm burdens and fecal egg counts.** Two weeks after the immunization series was completed, both \( \alpha \)C-GST-1/AS03-vaccinated dogs and AS03-injected dogs were challenged percutaneously with 500 \( A. \) cani-
num L3 larvae. The numbers of adult *A. caninum* parasites recovered from the small and large intestines of the vaccinated dogs were reduced by 39.4% compared with those from dogs immunized with adjuvant alone. Reductions in fecal egg counts (32.3%) were also observed in the feces of dogs vaccinated with rAc-GST-1 compared with those of adjuvant control dogs (Table 2). However, because the variation in the numbers of worms and eggs recovered from both the vaccinated and control groups was high, the reduction rates were not statistically significant. Dogs vaccinated with irradiated L3 larvae exhibited a mean 48.9% worm burden reduction and 56% egg reduction. The former was statistically significant \((P < 0.05)\) using Dunnett’s test compared with control dogs.

In a second trial using an *N. americanus* challenge model in golden hamsters, the rAc-GST-1/Alhydrogel-vaccinated hamsters exhibited a 53.7% adult worm reduction in the intestines compared to Alhydrogel-injected control hamsters \((P < 0.05)\) (Table 3). Two of the vaccinated hamsters died unexpectedly during the trial. It was noted that both of the hamsters exhibited substantially reduced body weights, which appeared to result from decreased feeding because of a broken tooth. The results indicate that rAc-GST-1 exhibits cross-protective immunity against *N. americanus* in hamsters.

**TABLE 1. Glutathione transferase activity of rAc-GST-1**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sp act (μmol/min/mg)</th>
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<tbody>
<tr>
<td>rAc-GST-1</td>
<td>844.93 ± 23.43</td>
</tr>
<tr>
<td>rNa-ASP-2</td>
<td></td>
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<tr>
<td>dH₂O</td>
<td>0</td>
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</tbody>
</table>

\(a\) The results represent the mean of the two determinations ± SD.

\(b\) dH₂O, distilled H₂O.

**DISCUSSION**

Here, we report on the cloning and function of a unique glutathione transferase from the hookworm, *A. caninum*; its possible role in blood feeding; and its potential as a vaccine antigen. This is the first report of the cloning of a full-length cDNA encoding hookworm GST.

Like other members of the GST superfamily, including nematode and trematode GSTs, Ac-GST-1 contained typical GST N-terminal (IRP004045) and C-terminal (IRP004046) domain structures. Ac-GST-1 also contained both Tyr-4 and Tyr-8 in the N-terminal region, which are highly conserved in mammalian and helminth GSTs (19). Tyr-8 is predicted to be essential for catalytic activity (8). The full-length Ac-GST-1 shares 31% identity at the amino acid level with Sj28, a protective schistosome GST that is undergoing clinical trials as a human vaccine for schistosomiasis (6).

Although Ac-gst-1 mRNA was detected in several developmental life history stages of *A. caninum* (including L1, L2, L3, adult, and egg), and the Ac-gst-1 cDNA was cloned from L3 cDNA, Ac-GST-1 protein was detectable by Western blotting only in adult hookworm somatic extracts and adult ES products. This suggests that Ac-gst-1 mRNA might be transcribed but not translated in the non-blood-feeding stages or expressed at such a low level that it cannot be detected with antibody. Helminth GSTs have been reported from every developmental stage (31, 39, 40); however, one helminth GST from the lung fluke, *Paragonimus westermani*, was expressed only by adult flukes (19). The adult-specific expression of Ac-GST-1 protein suggests that the protein may play an important role in the survival of the adult worm in the host during blood feeding.

Immunolocalization with specific antiserum confirmed that Ac-GST-1 mainly localized in the hypodermis and muscle tissue of the adult worm. Ac-GST-1 was also observed weakly in the intestine, consistent with the finding of Ac-GST-1 in the ES.
The detection of Ac-GST-1 in the hypodermis, intestine, and adult ES products suggests that it not only functions as an intracellular cytosolic housekeeping enzyme, but is also acting at the host-parasite interface and could function in blood feeding and other parasitic roles.

The glutathione transferases are detoxification enzymes that catalyze the conjugation of the tripeptide GSH to endogenous and xenobiotic electrophilic toxins. Based on this enzymatic activity, many parasitic helminth GSTs play an important role in detoxifying the secondary products of lipid peroxidation produced via immune-initiated free-radical attack on host or parasite membranes (3, 7, 26). In this sense, helminth GSTs are immunomodulatory. However, similar to the Hc-GST-1 produced by the adult blood-feeding helminth H. contortus, the Pichia-expressed Ac-GST-1 possessed little glutathione-dependent peroxidase activity or conjugating activity with cytotoxic carbonyls, products of lipid peroxidation (results not shown). Therefore, both Ac-GST-1 and Hc-GST-1 may not be much involved in immunomodulation compared to GSTs from cestode and digenean parasites (3, 4).

The GSTs from blood feeding nematodes (Ac-GST-1 and Hc-GST-1) were noted to contain a high-affinity binding site for hematin and heme-related compounds (38) (Fig. 5). In contrast, a GST homologue from the closely related free-living nematode C. elegans has a weak affinity for hematin (38). Together with the adult-stage-specific expression of Ac-GST-1, as well as its secretion in ES products, our findings suggest that, just like Hc-GST-1, adult A. caninum worms employ GST-1 in processes linked to blood-feeding behavior, including the detoxification of heme released during hemoglobin digestion.

Like many blood-feeding parasites, such as Plasmodium sp., Schistosoma sp., and Hemonchus sp., that depend on the globin portion of hemoglobin for nutrition (42), hookworms have evolved a carefully orchestrated cascade of hemoglobin digestion (43). However, this stepwise degradation of hemoglobin results in the release of heme, which is a potent enzyme inhibitor and generator of toxic reactive oxygen species (29). Adult A. caninum worms may produce Ac-GST-1 to assist in removing and/or scavenging heme or heme-related compounds generated during hemoglobin digestion. The GST from Plasmodium falciparum has also been shown to bind and detoxify free iron and therefore is not toxic. However, the binding affinity to protoporphyrin IX is much lower than the binding affinity to hematin (Fig. 5). This observation further suggests that Ac-GST-1 might selectively bind to heme rather than the nontoxic precursor.

The schistosome GST is currently a leading vaccine candidate for human schistosomiasis (9, 36) and is undergoing a
Because of its putative importance in blood feeding, Ac-GST-1 is a potential drug and vaccine target. The hookworm vaccine trials reported here indicate that Pichia-expressed rAc-GST-1 is highly immunogenic and induces not only Th2-associated antibody (IgG1) and cytokine (IL-4) responses, but also a strong Th1-like response indicated by the production of IFN-γ, as well as IgG2 antibody response. Although a link between IFN-γ and IgG2 exists in humans and mice, the link has not been established for dogs. However, experimental evidence from published canine studies does indicate an association between isotype production and possible Th1/Th2 shifts (1, 11, 30). Recombinant Ac-GST-1 also induced a strong proliferative response in PBLs. Dogs vaccinated with rAc-GST-1 exhibited a marked reduction in adult hookworm burdens and egg counts compared with the control dogs, although they were not sufficient to show statistical significance because of variation in worm recovery and egg counts. This partly reflected the small sample size of dogs used in the trial, which was limited in scope because of the expense and ethics of employing larger numbers of this sensitive species. However, hamsters vaccinated with rAc-GST-1 showed 53.7% reduction in hookworm burden after being challenged with the human hookworm N. americanus. The statistical significance of the hamster trial partly reflects the comparatively large numbers of animals (20 in each group) used in the study. The results from the hamster trial indicate that Ac-GST-1 and its N. americanus orthologue presumably share sufficient identity to produce immunological cross-reactivity. However, we cannot rule out the possibility that the improved protection in hamsters also reflected differences in the adjuvant employed.

Our overall strategy to vaccinate against human hookworm disease is based on employing two different antigens, one that targets the larval stage to reduce the numbers of L3 larvae entering the gastrointestinal tract, the other targeting adult blood feeding (20, 23). The L3-secreted protein, ASP-2, is our lead larval vaccine antigen (14), while a cathepsin B hemoglobinase has shown promise as an adult vaccine antigen (28). These studies suggest that members of the GST family could also be used as adult hookworm vaccine candidate molecules.

Western blotting with rabbit anti-rAc-GST-1 could not recognize mouse GST in the liver cytosol (data not shown), indicating limited or no cross-reaction between hookworm and mammalian GSTs. The identification and cloning of an Ac-GST-1 homologue from N. americanus, the major human hookworm worldwide, is now in progress.

### Table 2. Reduction of adult A. caninum hookworms and fecal egg counts from dogs vaccinated with rAc-GST-1 and radiated L3

<table>
<thead>
<tr>
<th>Vaccine group</th>
<th>No. of dogs</th>
<th>Worms Mean ± SE</th>
<th>% Reduction</th>
<th>EPG* Mean ± SE</th>
<th>% Reduction</th>
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<tbody>
<tr>
<td>AS03 control</td>
<td>5</td>
<td>137 ± 29</td>
<td></td>
<td>3,500 ± 1149</td>
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</tr>
<tr>
<td>Ac-GST-1</td>
<td>5</td>
<td>83 ± 13</td>
<td>39.4</td>
<td>2,370 ± 626</td>
<td>32.3</td>
</tr>
<tr>
<td>Irradiated L3</td>
<td>5</td>
<td>70 ± 23</td>
<td>48.9</td>
<td>1,540 ± 741</td>
<td>56.0</td>
</tr>
</tbody>
</table>

*EPG, number of eggs per gram of feces.

### Table 3. Reduction of adult N. americanus hookworms recovered from the intestines of hamsters vaccinated with rAc-GST-1

<table>
<thead>
<tr>
<th>Vaccine group</th>
<th>No. of hamsters</th>
<th>Worms Mean ± SE</th>
<th>% Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adjuvant control</td>
<td>20</td>
<td>33.9 ± 3.4</td>
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<tr>
<td>Ac-GST-1</td>
<td>18</td>
<td>15.7 ± 2.3</td>
<td>53.7*</td>
</tr>
</tbody>
</table>

*P < 0.05.
REFERENCES


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