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

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We report the cloning and expression of *Ac*-GST-1, a novel glutathione *S*-transferase from the adult hookworm *Ancylostoma caninum*, and its possible role in parasite blood feeding and as a vaccine target. The predicted *Ac*-GST-1 open reading frame contains 207 amino acids (mass, 24 kDa) and exhibited up to 65% amino acid identity with other nematode GSTs. mRNA encoding *Ac*-GST-1 was detected in adults, eggs, and larval stages, but the protein was detected only in adult hookworm somatic extracts and excretory/secretory products. Using antiserum to the recombinant protein, *Ac*-GST-1 was immunolocalized to the parasite hypodermis and muscle tissue and weakly to the intestine. Recombinant *Ac*-GST-1 was enzymatically active, as determined by conjugation of glutathione to a model substrate, and exhibited a novel high-affinity binding site for hematin. The possible role of *Ac*-GST-1 in parasite heme detoxification during hemoglobin digestion or heme uptake prompted interest in evaluating it as a potential vaccine antigen. Vaccination of dogs with *Ac*-GST-1 resulted in a 39.4% reduction in the mean worm burden and 32.3% reduction in egg counts compared to control dogs following larval challenge, although the reductions were not statistically significant. However, hamsters vaccinated with *Ac*-GST-1 exhibited statistically significant worm reduction (53.7%) following challenge with heterologous *Necator americanus* larvae. These studies suggest that *Ac*-GST-1 is a possible drug and vaccine target for hookworm infection.

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 through a carefully orchestrated cascade of hemoglobinases (43). This sequence of events is central to the pathogenesis of hookworm disease, which results almost entirely from hookworm-induced blood loss leading to iron deficiency anemia (35).

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).

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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 TTG CTG AC; *Ac*-GSTF2, TCC TCG AGA AAA GAA TGG TCC ACT ACA AGC TGA C) and reverse primers (*Ac*-GSTR1, TGT CTA GAA AGT GAG TCT CCG GTC TGG TC; *Ac*-GSTR2, GTA GTC CGT CAT CTG ATC 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* sequences.

Sequence analysis. DNA and predicted protein sequences were analyzed using ESEE version 3.1 (5). The sequences were aligned using CLUSTAL W (http://clustalw.genome.jp) 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 pPICZαA (Invitrogen) using XhoI and XbaI restriction sites. The correct open reading frame (ORF) was confirmed by sequencing using the vector flanking primers corresponding to the regions encoding the α-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-r*Ac*-GST-1 antiserum preparation, Western blotting, and immunolocalization. Antiserum against r*Ac*-GST-1 protein was prepared in a rabbit immunized with four 150-μg aliquots of r*Ac*-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 excretory/secretory (ES) products, as well as r*Ac*-GST-1, were boiled in sodium dodecyl sulfate (SDS) sample buffer. Five micrograms of each extract and 8 ng of r*Ac*-GST-1 were separated on a 4 to 20% gradient SDS precast polyacrylamide gel (Invitrogen) and subsequently electrotransferred onto a polyvinylidene difluoride membrane (Millipore) (37). Western blotting with a 1:20,000 dilution of rabbit anti-r*Ac*-GST-1 serum was performed as previously described (45).

For immunolocalization studies, adult *A. caninum* worms were prepared for staining as previously described (41). Briefly, sectioned worms were mounted on glass slides, and nonspecific binding sites were blocked with 5% fetal bovine serum in phosphate-buffered saline (PBS) containing 0.05% Tween 20 for 1 h. Antiserum to r*Ac*-GST-1 (1:500 in PBS) was applied to each section and incubated for 2 h at room temperature in a humidified chamber. Normal rabbit serum (at the same dilution) was used as a negative control. The sections were washed six times for 5 min each time in PBS and probed with anti-rabbit Cy3-conjugated immunoglobulin G (IgG) (heavy and light chains) (BD Biosciences) as described previously (41). The sections were viewed with an Olympus BX-60 fluorescence microscope using a 550-nm excitation filter block and emission at 565 nm.

Assessment of enzymatic activity. The enzyme activity of r*Ac*-GST-1 was determined according to the method of Habig and Jakoby (15) with 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. The enzyme assay was carried out in a final volume of 0.6 ml containing 45 μg r*Ac*-GST-1, 0.1 M phosphate buffer (pH 6.5), 1 mM CDNB, and 1 mM reduced glutathione (GSH). The reaction was initiated by the addition of the aromatic substrate. The change in absorbance due to the formation of the glutathione conjugate of CDNB was recorded at 340 nm once every minute at 25°C. The enzyme activity was expressed as nmol/min/mg protein.

Ligand binding assays. Ligand binding to r*Ac*-GST-1 was determined by measuring changes in intrinsic protein fluorescence as described elsewhere (38). In the binding assays, 1 μM r*Ac*-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 spectrofluorometer (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 r*Ac*-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, parasite-naïve 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 20×10^6/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 larval challenge, all dogs were individually housed.

The dogs were vaccinated with either 100 μg of *Pichia*-expressed r*Ac*-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* L3 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* L3 by applying 500 infective L3 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, and adult hookworms were recovered 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 a Chinese strain of *N. americanus* in golden hamsters (*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 r*Ac*-GST-1 given to the hamsters was 25 μg, which was administered once every 2 weeks for a total of three injections. The adjuvant selected for the hamster trial was Alhydrogel (the IPD-CCDCP has no contractual arrangements with GlaxoSmithKline for receiving AS03). Another group of 20 hamsters were immunized only 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 responses. The dogs were bled 9 days after each vaccination. Sera were 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 r*Ac*-GST-1 (25 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), r*Ac*-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-

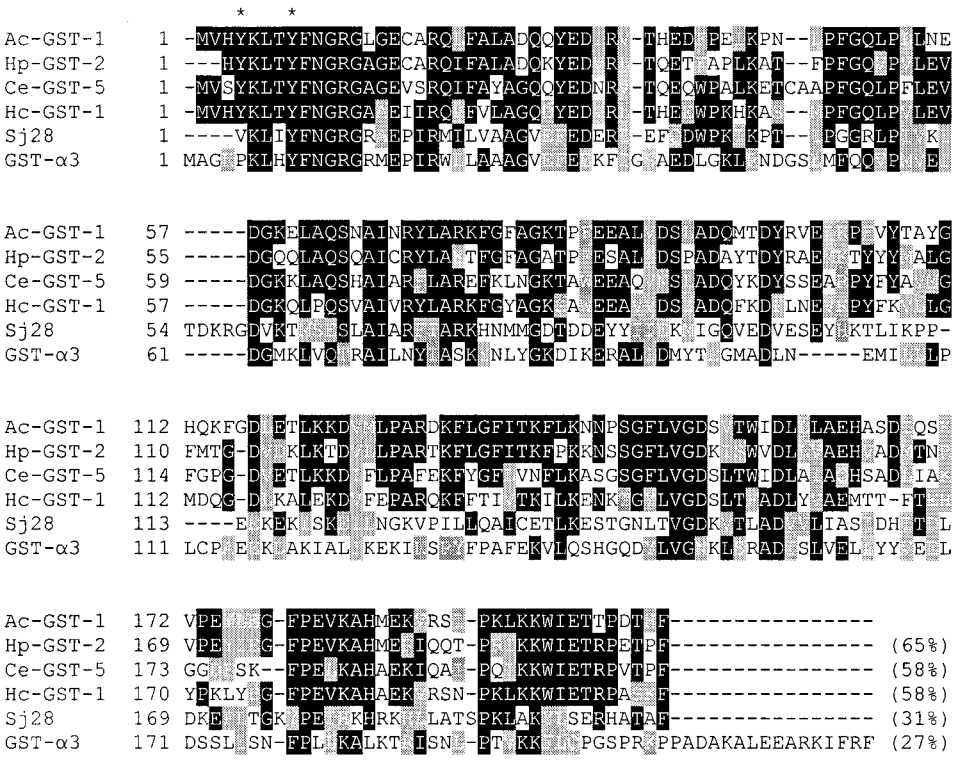


FIG. 1. Alignment of the deduced amino acid sequence of *Ac*-GST-1 with those of putative homologues from *Heligmosomoides polygyrus* (HpGST2; GenBank accession no. AAF36480), *C. elegans* (*Ce*-GST-5; NP_496357), *H. contortus* (*Hc*-GST-1; AAF81283), and *S. japonicum* (*Sj*28; P26624) and human GST-α3 (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.

tems, Minneapolis, MN) according to the manufacturer's instructions. The *t* test was used for statistical analysis.

Nucleotide sequence accession number. The full-length nucleotide sequence of *Ac-gst-1* has been submitted to GenBank with accession number AY605283.

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′ and 3′ GeneRacer. 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*) and *Ce*-GST-5 (*Caenorhabditis elegans*), 31% with *Sj*28 (*S. japonicum*), and 27% with human GST-α3.

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.

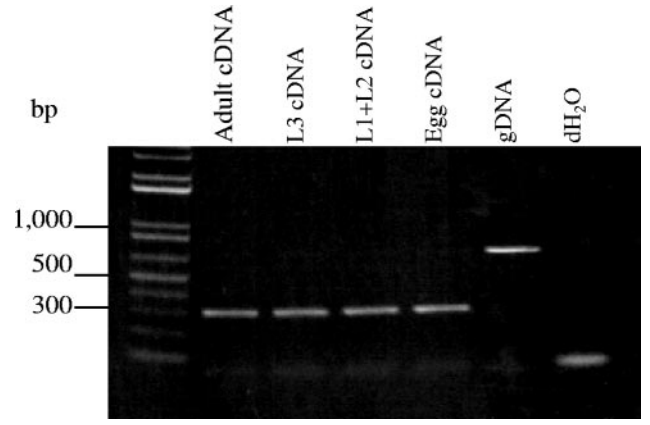


FIG. 2. Stage specificity of *Ac-gst-1* mRNA. RT-PCR was performed on total RNA isolated from the adult worms, L3, L1 plus L2, and eggs of *A. caninum*. Specific primers for *Ac-gst-1* (*Ac*-GSTF2 and *Ac*-GSTR2) were used for amplification. Adult hookworm genomic DNA (gDNA) was used as a control. Lane dH_2O (distilled H_2O), negative (no-template) control.

The molecular mass of r*Ac*-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 r*Ac*-GST-1 with *Pichia* host cell contaminants. This was confirmed by an ELISA and Western blot test that employs an antibody to *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 by the anti-r*Ac*-GST-1 rabbit serum in the extracts of L3, L1 plus L2, and eggs (Fig. 3). Immunolocalization studies revealed that anti-r*Ac*-GST-1 antiserum bound strongly to the adult parasite hypodermis (Fig. 4A) and muscle (Fig. 4B) and

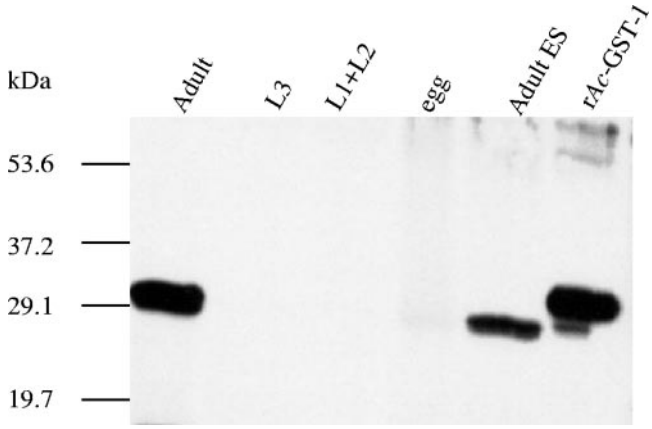


FIG. 3. Identification of the native *Ac*-GST-1 by Western blotting with antiserum raised to r*Ac*-GST-1. Five-microgram extracts from adults, L3, L1 plus L2, eggs, and ES products of the adult worm *A. caninum* were homogenized in SDS-polyacrylamide gel electrophoresis sample buffer, subjected to electrophoresis, and transferred to a polyvinylidene difluoride membrane. Eight nanograms of r*Ac*-GST-1 protein was used as a positive control. The Western blot was probed with rabbit antiserum against r*Ac*-GST-1.

weakly to the gastrointestinal tract (Fig. 4B). No significant staining was observed using normal rabbit serum (Fig. 4C).

Enzymatic and ligand binding activities of r*Ac*-GST-1. The enzymatic activity of r*Ac*-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 *Ac*-GST-1 functions as a glutathione transferase in vitro (Table 1). No activity was observed with the same quantity of an irrelevant control, recombinant *Ancylostoma*-secreted protein 2 from *Necator americanus* (*Na*-ASP-2).

The high degree of amino acid alignment between *Ac*-GST-1 and *Hc*-GST-1, a heme-binding GST from *H. contortus* (38), suggests that the former could also exhibit heme-binding properties. On a Western blot, rabbit anti-r*Ac*-GST-1 serum also showed cross-reaction with recombinant *Hc*-GST-1 (data not shown). Recombinant six-His-tagged *Ac*-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 μM and 19.98 ± 4.48 μM, respectively. This indicates that r*Ac*-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 r*Ac*-GST-1 was determined to be 3.23 ± 0.1 μM, indicating that hematin and CDNB share the same binding site on the GST.

Canine antibody and cellular responses to r*Ac*-GST-1 immunization. Dogs vaccinated with r*Ac*-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. *Ac*-GST-1-specific IgE titers were low (<1:500) (data not shown). Control dogs (adjuvant AS03 alone) did not generate detectable anti-r*Ac*-GST-1 immune responses (data not shown). Peripheral blood leukocytes (PBLs) from dogs vaccinated with r*Ac*-GST-1 proliferated in the presence of r*Ac*-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 r*Ac*-GST-1 produced 9.1 ± 2.9 ng/ml of IFN-γ 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-γ 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 r*Ac*-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 *Ac*-GST-1/AS03-vaccinated dogs and AS03-injected dogs were challenged percutaneously with 500 *A. cani-*

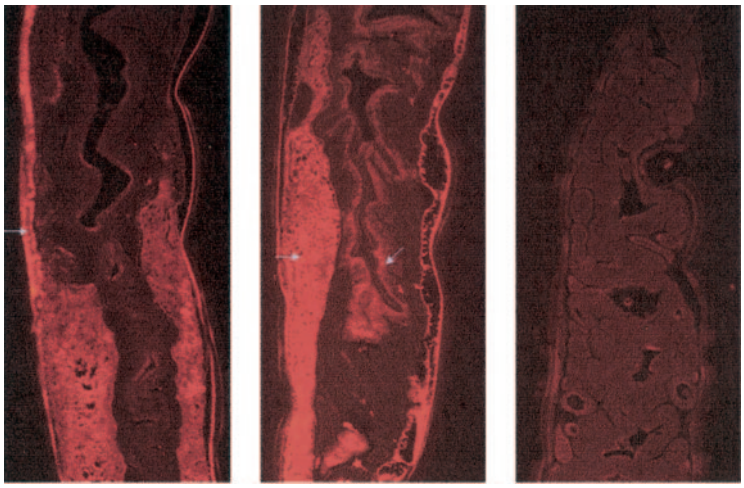


FIG. 4. Immunolocalization of *Ac*-GST-1 in sections of adult *A. caninum*. Fluorescence was detected with a Cy3-conjugated anti-rabbit IgG serum. Longitudinal sections of adult *A. caninum* worms probed with anti r*Ac*-GST-1 serum showed *Ac*-GST-1 localized intensively to the hypodermis (A) and muscular tissue (B) and weakly to the intestine (B) (arrows). Normal rabbit serum with the same dilution was used as a control (C).

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 r*Ac*-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 r*Ac*-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 r*Ac*-GST-1 exhibits cross-protective immunity against *N. americanus* in hamsters.

TABLE 1. Glutathione transferase activity of r*Ac*-GST-1

Protein	Sp act (μmol/min/mg)[a]
r*Ac*-GST-1	844.93 ± 23.43
r*Na*-ASP-2	0
dH_2O[b]	0

[a] The results represent the mean of the two determinations ± SD.
[b] dH_2O, distilled H_2O.

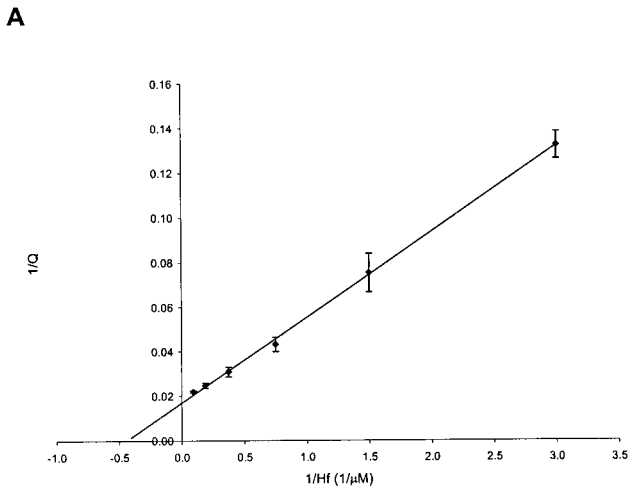
DISCUSSION

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 *Sj*28, 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



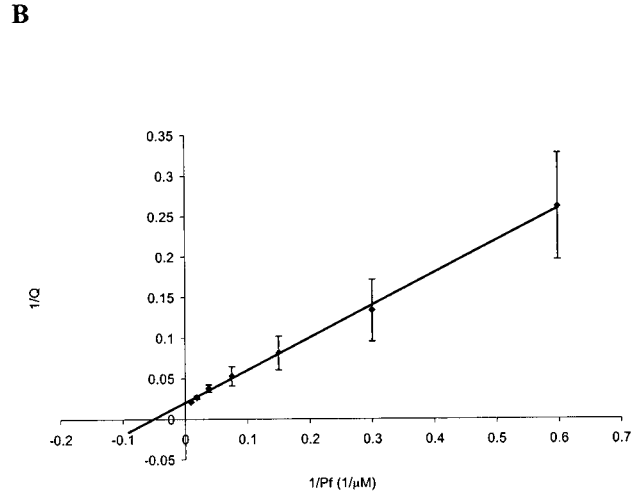


FIG. 5. Binding of r*Ac*-GST-1 to hematin (A) and its precursor, protoporphyrin IX (B), as demonstrated by double-reciprocal-plot quenching of intrinsic fluorescence in r*Ac*-GST-1 (Q) against the concentrations of free hematin (Hf) and free protoporphyrin IX (Pf). The intrinsic fluorescence K_d values for binding hematin and protoporphyrin IX are 2.3 ± 0.38 μM and 19.98 ± 4.48 μM, respectively. The data shown are averages of three determinations with standard deviations.

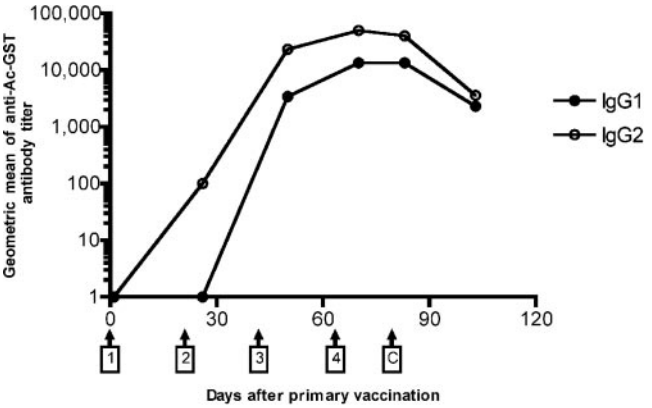


FIG. 6. Geometric mean titers of the IgG1 and IgG2 antibody responses of vaccinated dogs against r*Ac*-GST-1 formulated with AS03 adjuvant. Vaccination times (1, 2, 3, and 4) and challenge day (C) are marked with arrows.

products of the adult hookworm. 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 r*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 digegean 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 hematin or heme-related compounds generated during hemoglobin digestion. The GST from *Plasmodium falciparum* has also been shown to bind and detoxify heme compounds and serves as an efficient buffer for detoxifying it (2, 16). *Ac*-GST-1 also binds protoporphyrin IX, the structurally related precursor of hematin that does not contain free iron and therefore is not toxic. However, the binding affinity to protoporphyrin IX was almost 10 times lower than the binding affinity to hematin (Fig. 5). This observation further suggests that *Ac*-GST-1 might selectively bind and detoxify hematin rather than the nontoxic precursor.

The schistosome GST is currently a leading vaccine candidate for human schistosomiasis (9, 36) and is undergoing a

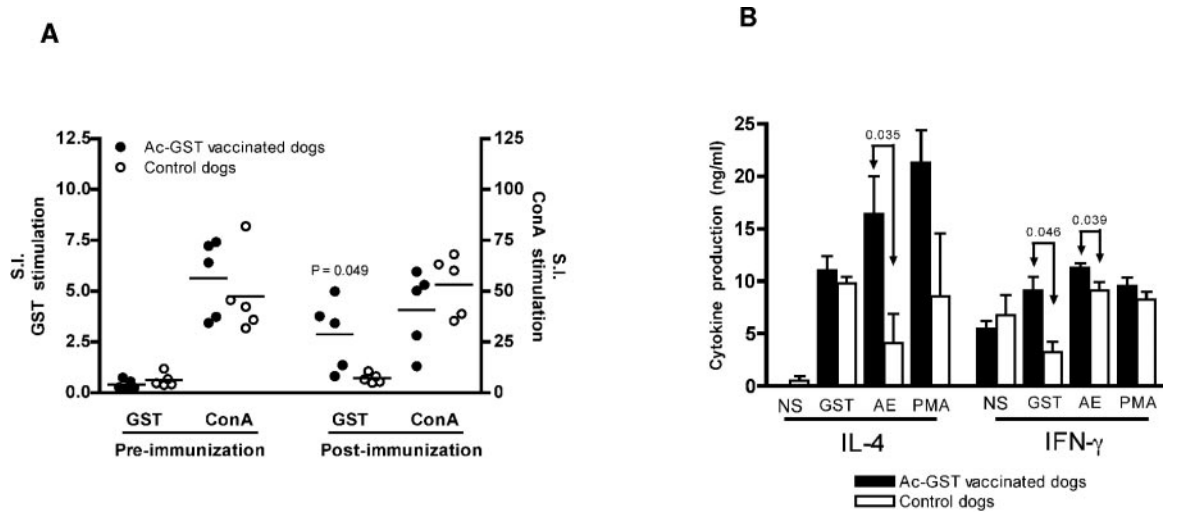


FIG. 7. Cellular immune responses of dogs to vaccination with r*Ac*-GST-1. (A) r*Ac*-GST-1-specific proliferation responses of peripheral leukocytes from vaccinated dogs. The results are shown as the stimulation index (S.I.) for each dog, and the geometric mean value for each group is indicated by a bar. The *P* value between results for dogs vaccinated with r*Ac*-GST-1 and adjuvant alone is given above the mean. ConA was used as a mitogen control at all time points tested. (B) Detection of IL-4 and IFN-γ in peripheral blood leukocytes of r*Ac*-GST-1-vaccinated dogs primed with NS (no stimulus), GST (r*Ac*-GST-1), AE (adult worm extracts), and PMA (phorbol 12-myristate 13-acetate). The numbers shown above the bars are the *P* values between results obtained for dogs vaccinated with r*Ac*-GST-1 and adjuvant alone. The error bars represent standard deviations.

phase II clinical trial (6). 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 r*Ac*-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 r*Ac*-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 r*Ac*-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-r*Ac*-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 r*Ac*-GST-1 and radiated L3

Vaccine group	No. of dogs	Worms		EPG[a]	
		Mean ± SE	% Reduction	Mean ± SE	% Reduction
AS03 control	5	137 ± 29		3,500 ± 1149	
Ac-GST-1	5	83 ± 13	39.4	2,370 ± 626	32.3
Irradiated L3	5	70 ± 23	48.9[b]	1,540 ± 741	56.0

[a] EPG, number of eggs per gram of feces.
[b] $P < 0.05$.

TABLE 3. Reduction of adult *N. americanus* hookworms recovered from the intestines of hamsters vaccinated with r*Ac*-GST-1

Vaccine group	No. of hamsters	Worms	
		Mean ± SE	% Reduction
Adjuvant control	20	33.9 ± 3.4	
Ac-GST-1	18	15.7 ± 2.3	53.7[a]

[a] $P < 0.05$.

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