Protective Immunity to a Blood-Feeding Nematode (*Haemonchus contortus*) Induced by Parasite Gut Antigens

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Blood-feeding nematodes cause some of the most important parasitic diseases of humans and domestic animals (1, 9, 22, 28). Control of these parasites is complicated by the cost of anthelmintics, a high capacity of some blood-feeding nematodes to develop drug resistance (7, 23, 29), and the lack of effective vaccines.

Immunity to blood-feeding nematodes can be induced in both young and adult hosts (3, 12, 15, 18–20). However, the only effective vaccine developed so far involves the use of irradiated third-stage larvae to protect against *Ancylostoma caninum* (15–17). The use of irradiated larvae to vaccinate against other blood-feeding nematodes has achieved variable success (10, 12, 25). Furthermore, the long-term viability of larvae represents a major obstacle to this approach. The identification of antigens capable of inducing a protective immune response is currently the most significant problem to overcome for immunologic control of these parasites.

The parasite gut provides a potential source of protective antigens from blood-feeding nematodes (18, 20, 21, 30). It is thought that the blood-feeding process could deliver an immune response directed against gut surface antigens of the parasite. Excellent progress in immunizing against tick infections by using this approach has been made (20, 21, 30). In addition, immunization experiments using putative parasite gut antigens of the blood-feeding nematode *Haemonchus contortus* induced significant protection against challenge infections (18). Here we demonstrate directly that parasite gut antigens induce a protective immune response to *H. contortus* challenge in goat kids. Our data indicate that gut membrane proteins were the dominant antigens recognized by antibody from immune serum and demonstrate that the epitopes of some gut antigens are conserved in other blood-feeding nematodes.

**MATERIALS AND METHODS**

**Parasites and gut antigen.** The strain of *H. contortus* used in this research was obtained from Ray Gamble (8) (U.S. Department of Agriculture, Beltsville, Md.). The strain of *Ostertagia ostertagi* was originally isolated from Louisiana (26) and was obtained from John Dame (University of Florida, Gainesville). Procedures for maintaining these parasites in the laboratory by passage through host animals were previously described (11). Because of their relatively large size, adult female worms were dissected to obtain gut samples. Gut samples consisted of intestine excluding the esophagus and were stored in phosphate-buffered saline (PBS) (pH 7.4) at −20°C. Adult small strongylo nematodes (species undetermined) were obtained from the large intestine of a horse submitted to the Washington State Animal Disease and Diagnostic Laboratory.

**Sample preparations for antigen analysis.** Gut samples from adult worms or whole, third-stage larvae were disrupted in a ground-glass homogenizer in the presence of proteinase inhibitors (1 mM phenylmethylsulfonylfluoride–1 mM L-tosyl-p-lysylchloromethylketone) and either Nonident P-40 or Triton X-114. Triton X-114 extraction of integral membrane proteins followed described protocols (4). Gut samples (1 mg of protein) were homogenized in 1% Triton X-114–150 mM NaCl–10 mM Tris-HCl, pH 7.4, on ice. The lysate was centrifuged at 100,000 × g for 30 min at 4°C to pellet detergent-insoluble material. After the supernatant was layered onto a sucrose cushion, cloud formation was initiated by heating at 37°C for 5 min. Phase separation was accomplished by centrifugation at 2,000 × g for 5 min. Aqueous, sucrose, and detergent phases were collected separately, and the aqueous phase was extracted two additional times with Triton X-114 as described above. Individual Triton X-114 fractions were adjusted to 3% Triton X-114 in a buffer identical to that used for lysis, and the phase separation procedure was repeated. The resulting Triton X-114 samples were stored at −20°C. The aqueous phase was concentrated with a Centriprep-10 (Amicon) filter prior
to storage. Protein concentrations were determined by the bicinchoninic acid assay (Pierce).

Western immunoblots. Gut lysates were boiled in sample buffer (2% [wt/vol] SDS [sodium dodecyl sulfate], 2.5% 2-mercaptoethanol [vol/vol], 25 mM Tris-HCl [pH 6.8], 15% glycerol [vol/vol], a few crystals of bromphenol blue), and antigens were separated on a 7.5 to 17% gradient SDS-polyacrylamide gel. After gel electrophoresis, proteins were transferred to a nitrocellulose filter (30 V for 16 h) in a buffer containing 25 mM Tris-HCl (pH 8.5), 190 mM glycine, and 20% methanol (vol/vol). Filters were blocked in a buffer containing 10 mM Tris-HCl (pH 7.5), 170 mM NaCl, 0.1 mM PMSF, and 1% horse hemoglobin (wt/vol); rinsed in the same buffer; and reacted with antisera from immunized and control goats at various dilutions. Antiserum were diluted in blocking buffer containing 0.1% SDS (wt/vol), 0.1% Triton X-100 (vol/vol), and 1 mM EDTA. Filters were rinsed in buffer four times for 3 min and reacted with 125I-labeled protein G. After excess protein G was rinsed away, filters were dried and autoradiographed. Molecular weights of proteins were determined from a minimum of three different experiments.

In situ antigen localization. Adult and third-stage larvae of *H. contortus*, adult *O. ostertagi*, and adult small horse strongyles were fresh-frozen in embedding medium (O.C.T.; Miles) for cryosectioning. Sections were cut 5 mm thick, applied to poly-L-lysine-coated microscope slides, fixed in methanol-acetone-water (1:6:3), and used in direct fluorescence-antibody-binding assays. Antibodies from immunized and control goat sera were isolated as described previously (24) by ammonium sulfate precipitation and then by DEAE anion-exchange chromatography. Isolated immunoglobulin G (IgG) was conjugated with fluorescein isothiocyanate (FITC) (6), and unreacted FITC conjugate was eliminated by dialysis. FITC-labeled antibody was adjusted to 0.2 mg/ml in PBS containing a 1:10 dilution of normal goat serum and reacted with cryosections for 30 min at room temperature. After three 15-min washes in PBS, cryosections were examined with a fluorescence microscope.

Immunization and challenge experiments. For immunization, 25 mg of whole gut was homogenized in 1 ml of PBS with a ground-glass tissue homogenizer. The homogenate was then mixed with an equal volume of Freund’s complete adjuvant and sonicated at 50 W until emulsified. The antigen preparation was divided among six goats for primary immunization by intramuscular injection. Ovalbumin (1 mg per goat) was used as the control antigen and was prepared in adjuvant as described above. Immunized and control goats received booster immunizations with their respective antigens, using Freund’s incomplete adjuvant. Saanen kid goats used in the first and third immunization trials were part of a breeding herd maintained at Washington State University in confinement. Experimental kid goats were all maintained in isolation facilities from birth. Kid goats were shown to be fecal egg count negative by sugar flotation (2) at the beginning of the experiment. Immunized and control kids received a primary immunization shortly after weaning at 2 months old. They were boosted seven times at 2-week intervals, and kids received their final boost when about 5 months old.

Three weeks after the final immunization, each goat was challenged orally with 10,000 third-stage larvae of *H. contortus*. After week 3, when infections became patent, fecal egg counts were determined twice weekly for 3 weeks. Packed cell volumes were also obtained for kids during this period. At the end of the observation period, abomasum were obtained and processed for worm counts. The entire contents of each abomasum were saved, and then abomasum were soaked individually in tap water overnight to release fourth-stage larvae from the mucosa. For each goat, all worms contained in 10% of the abomasal content and mucosal digest were counted and the total worm counts were calculated. Feecidity of adult female worms was assessed by counting the eggs contained in the uteri of 10 females from each goat, except for some immunized goats which had insufficient worms to obtain 10 females.

Mean parasite egg and worm counts were compared between immunized and control groups by using Student’s t test (27). Means were considered significantly different at *P* < 0.05. When variances of means tested were significantly different (*P* < 0.05 by using the f test), data were normalized by log_{10} transformation and retested. In all cases, results from r tests with transformed data were in agreement with those using nontransformed data.

Yearling Pygmmy goats were used in the second immunization trial. These goats were obtained as kids from local goat herds. All were fecal egg count negative, with the exception of one which had nine nematode eggs per gram of feces (the species was undetermined). The one goat with low egg counts was treated with fenbendazole, which eliminated the eggs in feces. This goat was assigned to the control group 6 weeks after treatment to allow time for drug elimination. Pygmmy goats were separated into immunized and control groups containing six goats each, and this immunization trial was conducted as described for the first immunization trial.

In a third trial, confinement-raised Saanen goats were initially immunized at 6 months of age and received a total of four immunizations for both the immunized and control groups. In this case, fecal egg counts, but not worm counts, were obtained for goats after challenge.

RESULTS

To assess the immune responses of immunized goats to gut antigens, postchallenge sera from Saanen kids (Table 1, experiment 1) were used in Western blots (Fig. 1). The profiles of proteins were similar, although not identical, for each of the goats in the immunized group. Dominant antigens recognized by most immune sera included 33-, 37-, 40-, 43-, 50- to 53-, 56-, and 98-kDa proteins. Although broad bands may contain more than one protein, resolving these will require further analysis. Antibody from kid 6 exhibited the strongest reaction and recognized two additional gut antigens of 103 and 173 kDa. While antibody from control goats showed little reactivity to *H. contortus* gut proteins, weak reactivity on longer exposures of Western blots was detected.

To determine whether antibodies in sera of immunized goats were induced against integral membrane proteins, whole gut samples were extracted with Triton X-114. Triton X-114-soluble and aqueous-soluble fractions were analyzed with sera from immunized goat 6 and control goat 13 (Table 1, experiment 1) on Western blots (Fig. 2). Proteins of 40, 43, 56, and 98 kDa were enriched in the Triton X-114 phase compared with the aqueous phase. Other proteins of 33, 36, and 50 kDa (the 33- and 36-kDa proteins are more apparent in longer exposures) appeared to be distributed equally in both phases. Partial partitioning of these gut proteins into Triton X-114 likely reflects specific characteristics such as heavy glycosylation or conformation (5, 14).

To localize parasite gut antigens recognized by immunized goats, antibody from immunized and control kids (6 and 13, respectively) were used in situ antibody-binding assays.
IgG isolated from serum was labeled with FITC and reacted with cryosections of adult *H. contortus* worms (Fig. 3). The gut of *H. contortus* is a syncytium composed of an external cellular layer and a luminal microvillous layer (Fig. 3D). Antibody-binding activity of the immunized goat was localized predominantly in the microvillous layer of the gut, and very little reactivity was detected in other parts of the gut. Labeled control antibody did not bind detectably to gut antigens.

Next, the ability of immunized goats to resist challenge infections was analyzed. In the first immunization trial, 2-month-old Saanen kid goats were tested. The final booster immunization was given when the kids were 5 months old, and they were challenged at about 6 months of age. The results of challenge infections are shown in Fig. 4 and Table 1. Mean fecal egg counts were significantly (*P* < 0.05) reduced in kids immunized with *H. contortus* gut antigens from the time of patency throughout the sampling period (Fig. 4). The percentages of decrease in the immunized group ranged from 86 to 95% when compared with the control group.

Mean worm numbers were reduced by 65% in the immunized group. The reduction in adult worms in immunized kids was not due to a delay in the development of fourth-stage larvae in the mucosa, since there was no increase in the number of these larvae for the immunized compared with the control group. Although surviving female worms in the immunized group had a mean of 29% fewer eggs occurring in their uteri than female worms from the control group, the difference was not statistically significant (*P* > 0.05) in this experiment. No significant decrease in packed cell volumes in the control or immunized groups in this or the subsequent experiments was observed.

The immunization and challenge experiment was repeated with yearling Pygmy goats, and the results are summarized in Table 1. In this experiment, mean fecal egg counts, worm counts, and uterine egg counts were significantly reduced by 95% (*P* < 0.05), 89% (*P* < 0.05), and 52% (*P* < 0.001), respectively, in the immunized compared with the control group. In this case, the significant reduction in uterine egg counts for the immunized group may indicate reduced fecundity of female worms in these goats. While the numbers of worms in the abomasa and eggs in the feces were lower for control Pygmy goats compared with control Saanen kids.

### Table 1. Reduction in fecal egg counts, worm counts, and worm fecundity in kids vaccinated with gut antigens from *H. contortus*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Mean (SD) count</td>
<td>Mean (SD) count</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>Immunized</td>
</tr>
<tr>
<td>EPG&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3,373 (2,714)</td>
<td>170 (157)</td>
</tr>
<tr>
<td>Adult worms</td>
<td>2,777 (1,074)</td>
<td>973 (918)</td>
</tr>
<tr>
<td>Fourth-stage larvae</td>
<td>5.0 (2.6)</td>
<td>1.7 (1.3)</td>
</tr>
<tr>
<td>Uterine eggs&lt;sup&gt;d&lt;/sup&gt;</td>
<td>257 (91)</td>
<td>183 (63)</td>
</tr>
</tbody>
</table>

<sup>a</sup> n = 6 goats.

<sup>b</sup> Probability values for means were compared by Student's *t* test, as described in Materials and Methods.

<sup>c</sup> EPG (eggs per gram) values show only for 3 weeks postpatency.

<sup>d</sup> Eggs from 10 worms for each kid were counted when available. For experiment 1, n = 60 and 55 for control and immunized groups, respectively; for experiment 2, n = 51 (control) and 39 (immunized).

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**FIG. 1.** Antigens in *H. contortus* gut homogenate recognized on Western blots by antibody from protected kids. Gut antigens were used at 20 μg of protein per lane, and a 1:1,000 dilution of sera from immunized (lanes 3 to 7 and 9) and control (lanes 10 to 13, 16, and 18) kids was reacted with individual filters. Molecular weight standards are shown.

**FIG. 2.** Identification of integral membrane protein antigens in *H. contortus* gut homogenate. Ten micrograms of protein of total gut homogenate (lanes 1 and 4) and Triton X-114-soluble (lanes 2 and 5) and aqueous-soluble (lanes 3 and 6) fractions were Western blotted with serum (1:1,000 dilution) from kid 6 (immunized, lanes 1 to 3) or kid 13 (control, lanes 4 to 6). Molecular weight standards are shown at the left.
FIG. 3. Reactivity of antibody from an immune goat to microvilli of the *H. contortus* gut. Cryosections of adult *H. contortus* were reacted with FITC-labeled IgG from kid 6 immunized with *H. contortus* gut homogenate (A and C) or kid 13 immunized with ovalbumin (B). The gut lumen is more clearly seen in panel C. A section of adult *H. contortus* showing the gut, which is composed of a peripheral syncytial layer and a microvillous layer (m), is also shown (D). Bar, 200 μm.

(Table 1), the differences in these parameters for the immunized groups were similar in both experiments.

A third experiment involving Saanen kid goats assessed only fecal egg outputs in the immunized and control groups. In this case, the immunized group had a mean of 107 ± 97 eggs per gram and the control group had a mean of 831 ± 298 eggs per gram, representing a mean reduction of 87% (*P* < 0.05) for the immunized group.

Because of the significant protection observed, the expression of gut antigens in another life cycle stage (third-stage larvae) of *H. contortus* was determined. The FITC-labeled antibody from goat 6 bound to internal organs of third-stage larvae, while no binding of an FITC-labeled goat antibody from a control goat was detected (results not shown). Although the small size of these larvae prevented identification of the specific organs involved, the gut is the dominant internal organ occurring in this life cycle stage. In Western blots, antibody from immune serum recognized antigens of 33, 45, 50, and 98 kDa in homogenates of third-stage larvae which were also present in the adult *H. contortus* gut (Fig. 5). An antigen of 173 kDa was detected in third-stage larvae and not detected in the adult gut in this Western blot; however, a protein of this molecular mass was detected in the adult gut in other experiments (Fig. 1, lane 6). This protein could be expressed at higher levels in larvae compared with adults.

To determine whether gut antigens of *H. contortus* might be conserved in related hemaphagous nematodes, antisera against *H. contortus* gut homogenate were used on cryosections and Western blots of gut from *O. ostertagi*, which is the primary stomach worm of cattle. In cryosections, FITC-conjugated IgG reacted predominantly with the microvillous region of the *O. ostertagi* gut (Fig. 6), which is identical to the location for *H. contortus*. In Western blots, four *O. ostertagi* gut antigens of 50, 98, 105, and >200 kDa were clearly detected with immune serum to *H. contortus* gut antigen (Fig. 5). Two of these *O. ostertagi* gut antigens (50 and 98 kDa) had apparent molecular masses similar to gut antigens of *H. contortus*. Adult small strongyle nematodes (species not determined) from a horse were also tested in the in situ assay, and fluorescent antibody to *H. contortus* gut cross-reacted with the microvillous gut region of these nematodes (results not shown).
FIG. 6. Cryosections of adult O. ostertagi were reacted with FITC-labeled IgG from kid 6 immunized with H. contortus gut homogenate (A) or kid 13 immunized with ovalbumin (B). Bar, 200 μm.

DISCUSSION

The most important contribution of this research is direct evidence that gut antigen of a blood-feeding nematode can induce significant protection against challenge infections in young animals. Other antigen preparations have been shown to induce protective immunity against H. contortus. One of these was a preparation of high molecular mass (>30,000 kDa), soluble somatic antigen extract (30,000 × g) and excretory-secretory products from fourth-stage larvae (19). Another was derived by differential centrifugation from whole adult H. contortus, representing material which pelleted between 10,000 and 50,000 × g and contained putative gut antigens (18). Both of these antigen preparations induced significant protective immunity against lambs <6 months old. However, the tissue origin of protective antigens was not determined in either study. The reductions in mean worm counts (65%) and mean fecal egg counts (95%) shown here support the earlier proposal that gut antigens are prospective targets for vaccination against H. contortus and other blood-feeding nematodes.

Significant reductions in fecal egg counts and worm numbers were also achieved in immunized yearling goats, and a significant reduction in fecal egg counts (the only parameter examined) was observed in a third experiment. While previous exposure to H. contortus cannot be ruled out for the yearling Pygmy goats, a comparison of immunized and control goats clearly indicates that gut antigens induced the protective immunity observed. Even though packed cell volumes were not significantly decreased in control goats in any of the experiments, the level of adult worm reductions in immunized goats suggests that gut antigens will be capable of inducing protection against clinical disease.

The variability in reductions of fecal egg counts and worms among the immunized goats most likely reflects differences in the immune responses of individual goats. Currently, this variability does not limit the potential of gut antigens in vaccine development for the following reasons. A significant decrease of fecal egg counts in young animals, even with relatively high individual variability, would translate into a significant decrease in subsequent pasture exposure to H. contortus for the herd. Also, the antigen source used in these trials is complex and not amenable for use on a large scale. As specific protective antigens are identified and produced, presentation protocols, which may reduce the variability observed, can be optimized.

An analysis of the antibody response of immune goats indicates that a vigorous immune response was generated against gut membrane antigens. Antibodies from an immune serum selectively reacted with the microvillous region of the gut, and these antibodies recognized several gut antigens, some of which were integral membrane proteins defined by solubility in Triton X-114. It is unlikely that these antigens originate from the esophagus, since there was no reactivity to this region of the worm in situ antibody-binding assays. While only one protected goat serum was tested in some analyses, the similar gut antigen profiles recognized by antibodies from all of the immunized goats from experiment 1 indicate that it is representative of the group. We have also conducted preliminary experiments in which FITC-labeled antibody from immune serum reacted to the surface of microvilli in freshly dissected pieces of H. contortus gut (results not shown). These results are encouraging, since gut membrane surface antigens would be accessible to an immune response, and protective immunity against Boophilus ticks has been induced with gut surface antigens of this blood-feeding parasite (20, 21, 30).

Although antibody was used to identify gut antigens recognized by a protective host immune response, it was not assumed that the immunity was antibody mediated. There is evidence for mucosal immunity and both cell-mediated and humoral immunity to gastrointestinal nematodes (13), with the significance of each depending upon parasite and host species. Protective immunity induced by gut antigens may be different from that in natural immunity, especially if the parasite damage is restricted to the gut and caused by immune mechanisms delivered during blood feeding. For instance, physical or biochemical constraints imposed by feeding and digestive processes of the parasite may eliminate some cellular and/or antibody-dependent immune mechanisms, including mucosal immunity. Alternatively, the location of antigens recognized by the immune response may not be limited to the gut. In our in situ studies, some antibody to gut antigen reacted weakly with the inner body wall of H. contortus. On the basis of our method of antigen preparation, this result suggests that cross-reactive gut epitopes occur in other tissues of the parasite, and preliminary results with monoclonal antibodies to gut surface epitopes support this possibility. In some cases, monoclonal antibodies to gut surface epitopes bind specifically to a variety of tissues, including some recognition of cuticular antigens (unpublished data). Consequently, immune mechanisms induced by gut antigens may be directed at regions other than the gut and could involve mechanisms similar to those induced in natural infections.

Results indicating that gut antigens expressed in adult H. contortus were present in third-stage larvae may be important. Experiments described here were not designed to test whether preadult parasite stages were affected by immunity to adult gut antigens. However, our results suggest this is possible, since both third- and fourth-stage larvae are tissue dwelling. The presentation of protective gut antigens expressed in these tissue stages could stimulate anamnestic immune responses in vaccinated animals when they are exposed to natural challenge. Therefore, selecting specific antigens expressed in the adult gut and in larvae may be an important strategy in vaccine development.

Species cross-reactivity of gut antigens is also of special interest. Blood-feeding nematodes in the order Strongyloida
belong to three superfamilies, including the Anclyostomatoidea, Strongyloidea, and Trichostrongyloidea. Nematodes from the last two superfamilies were shown in this study to share cross-reactive microvillus gut antigens. Currently, no vaccines are available for nematodes in any of these superfamilies, and control procedures have limitations similar to those for H. contortus. Since gut membrane antigen epitopes may be phylogenetically conserved, protective gut antigens from one blood-feeding nematode could have direct application either to vaccination against other such parasites or in identifying gut surface antigens from these parasites.

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