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PARTIAL PROTECTION OF LAMBS AGAINST *HAEMONCHUS CONTORTUS* BY VACCINATION WITH A FRACTIONATED PREPARATION OF THE PARASITE*

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ABSTRACT

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Florida Native lambs, <6 months of age, were successfully vaccinated against *Haemonchus contortus* with a high mol. wt fraction (>30 000 daltons) derived from a somatic extract of *H. contortus* larvae (SEL) and excretions and secretions (ES) of larvae isolated during in vitro development from the infective 3rd to 4th stage. A 59% reduction in adult worm numbers was obtained in vaccinates compared to naive lambs following challenge. The protection in vaccinated lambs was similar to that seen in lambs exposed to a primary infection of *H. contortus* larvae which had been cleared with anthelmintic prior to the challenge infection. The unfractionated SEL/ES preparation and a low mol. wt fraction gave no significant protection against challenge infection.

INTRODUCTION

Florida Native lambs, <6 months of age, are more refractory to a primary infection with *Haemonchus contortus* (Radhakrishnan et al., 1972) and develop a stronger acquired resistance to a secondary challenge infection than do conventional breeds such as Suffolk, Rambouillet, Dorset and Finn, (Courtney et al., 1985). Given the demonstrated ability of Florida Native lambs to consistently develop an acquired resistance following infection with *H. contortus*, this model system is suited to the testing of candidate vaccines in young lambs against infection with this parasite.

Attempts to vaccinate lambs against *H. contortus* with non-living material have given disappointing results (Mansfield et al., 1974; Neilson, 1975). Speculation on this failure has included, among other reasons, qualitative and quantitative aspects of the preparation of the parasite used, the

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presentation modes applied and the nature of concurrent adjuvantation.

While X-irradiated larvae of *H. contortus* failed as a potential vaccine in lambs <6 months of age (Urquhart et al., 1966), several massive infections with viable infective larvae, each truncated at the 4th stage with an efficient anthelmintic, stimulated a high measure of protection in young lambs (Christie and Brambell, 1966). The present experiments involved the isolation and concentration of antigens associated with development of the parasite in vitro through those stages shown to be immunogenic in vivo (Christie and Brambell, 1966). The protective properties of preparations of the parasite composed of somatic and excretory components derived from *H. contortus* while cultured in vitro from the infective third to fourth stage (Neilson, 1969) were studied in lambs <6 months of age. Adjuvants (Wells et al., 1982) and inoculation parameters (Murray et al., 1979) were based on previous successful vaccinations against other parasites.

MATERIALS AND METHODS

Sheep

Florida Native lambs were raised worm-free on concrete floored pens following recommended sheep management practices. The lambs were weaned at 60 days of age and individual samples of feces and blood were taken at weekly intervals after weaning.

Parasitological techniques

A strain of *H. contortus* isolated in 1979 at Wooster, Ohio (Courtney et al., 1985), was used in all experiments. Experimental lambs were infected by depositing the requisite number of infective larvae on a moist filter paper which was folded and inserted in a gelatin capsule immediately prior to oral delivery with a bolus gun. Fecal egg per gram (EPG) counts were made by a modified McMaster technique (Whitlock, 1948). At necropsy, aliquots totaling 10% of the abomasal contents were examined for adult worms. The abomasal mucosa was scraped from the muscularis and digested with 200 ml of 1% HCl at 37°C for 3 h. After digestion and the addition of 10% formalin, aliquots representing 10% of the total volume were examined for immature worms. These procedures for counting the number of adult and immature worms were similar to those described by Courtney et al. (1985).

Statistical analysis

Fecal egg count data were transformed by square roots. Worm count data were transformed by $\log_{10}(x + 1)$ and analyzed by analysis of variance. Differences were considered significant at $P < 0.05$.

Hematology

A microhematocrit method (Schalm et al., 1975) was used to measure packed cell volumes (PCVs) and the hemoglobin genotype of each lamb was determined by electrophoresis of hemoglobin extracted from blood (Gebott and Peck, 1978).

SEL and ES preparations of the parasite

Batches of 20×10^6 *H. contortus* infective larvae were cultured in vitro to the early L4 stage by the methods of Neilson (1969). Briefly, the larvae were washed five times in the culture salt solution containing 1000 units penicillin, 1 mg streptomycin and 10 μg fungizone ml^{-1}. After washing, the larvae, suspended in sterile water at a concentration of 10^4 ml^{-1} in a gassing jar, were held at 39°C for 1 h while gassed with CO_2. Following exsheathment, the larvae were suspended at a concentration of 10^3 larvae ml^{-1} in the culture salt solution containing 400 units penicillin, 400 μg streptomycin and 4 μg fungizone. The 72-h culture was performed in flasks, gassed with a mixture of 50% CO_2 and 50% air, held at 39°C and shaken on a gyratory shaker to maintain the larvae in suspension. The culture flasks were monitored daily for bacterial contamination and on the rare occasions when this occurred such flasks were discarded. After 72 h culture, >90% of the larvae had reached the 4th stage. The larvae and discarded 2nd and 3rd stage sheaths were separated from the medium by pressure filtration on an Amicon 8 μm filter.

The larvae and sheaths were suspended in 0.1 M Tris-HCl buffer, pH 8.0, containing protease inhibitors, L-1-tosylamide-2-phenyl-ethylchloromethyl ketone, 50 μg ml^{-1} and N-α-p-tosyl-L-lysine-chloromethyl ketone-HCl, 25 μg ml^{-1} and homogenized by several passages through a French Pressure Cell. Both protease inhibitors are also effective bacteriostats and eliminated bacterial contamination during the extraction and concentration of the SEL and ES preparations. After a 48-h extraction period the somatic extract of larvae (SEL) was centrifuged at $30\,000 \times g$ and the supernatant concentrated by pressure filtration in a stirred Amicon cell fitted with a UM-05 ultrafilter under 50 psi of nitrogen to yield a final protein concentration of 1.0 mg ml^{-1} for the SEL preparation.

The used culture medium, containing parasite excretions and secretions (ES), was concentrated by pressure filtration on a UM-05 Amicon ultrafilter as described above to yield a final protein concentration of 0.1 mg ml^{-1} for the ES preparation. All manipulations for the isolation and concentration of the SEL and ES preparations were conducted at 4°C.

Fractionation of the SEL and ES preparation

Equal volumes of the SEL and ES preparations were mixed. The mixture was separated into high and low molecular weight (MWt) fractions by

pressure ultrafiltration on a stirred Amicon cell fitted with a YM-30 filter under 50 psi of nitrogen. The retentate was washed in the filtration apparatus with 20 times its volume of Tris-HCl buffer, pH 8.0, containing protease inhibitors to ensure maximum removal of molecules with a MWt <30 000. This retentate was concentrated on the YM-30 filter to give 1.0 mg ml^{-1} of protein. The filtrate was concentrated on a UM-05 Amicon filter until the protein concentration reached 1.0 mg ml^{-1}. All manipulations for the fractionation and concentration of the SEL and ES preparations were conducted at 4°C. The Amicon YM-30 and UM-05 filters have general MWt retentivities of 30 000 and 500, respectively. The effectiveness of the separation into high MWt (>30 000) and low MWt (<30 000) fractions was analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (Laemmli, 1970) and a silver staining technique (Merril et al., 1981).

Adjuvants

Following the techniques of Wells et al. (1982), the appropriate parasite preparation was mixed with saponin and muramyl dipeptide before intraperitoneal injection, while BCG and *Bordetella pertussis* were injected intramuscularly.

Enzyme linked immunosorbent assay (ELISA)

The ELISA was based on the method of Voller et al. (1976). Equal volumes of unfractionated SEL and ES preparations were mixed, centrifuged at 100 000 × *g* for 60 min at 4°C, dialyzed against coating buffer for 24 h at 4°C and diluted with coating buffer to give a final concentration of 100 μg protein ml^{-1} before storage at −70°C.

The positive reference serum was pooled hyperimmune sera from four sheep multiply infected with a total of 200 000 *H. contortus* larvae. The negative reference serum was pooled from eight uninfected sheep maintained under worm-free conditions. Rabbit anti-sheep immunoglobulins (Ig) were obtained commercially and conjugated to alkaline phosphatase (Type VII-S, 900-1100 units mg^{-1} protein, Sigma Chemicals).

Optimal reagent dilutions for ELISA of vaccinated or infected sheep antibody were determined in a checkerboard fashion. The preparation of parasitic antigen was used at a concentration of 2 μg protein ml^{-1}. The positive, negative and test sheep sera were diluted 1 : 500. The phosphatase conjugated rabbit anti-sheep Ig was diluted 1 : 400.

The assay was performed in Linbro polystyrene microtiter plates. After an appropriate reaction period when the antigen-positive reference serum wells gave an absorbance value of about 1.0 spectrophotometrically at 405 nm, the absorbance values of all other wells on the same plate were measured.

Experimental plans

The following experiments were performed on the lamb crops from a flock of Florida Native ewes over two consecutive years.

Forty eight lambs, 10—12 weeks of age from the first year's crop were distributed among six groups, each of eight animals, balanced for sex and hemoglobin type. The experimental plan is outlined in Table I. Lambs in Groups 1, 2 and 3 were injected intraperitoneally with unfractionated SEL/ES, the low MWt fraction and the high MWt fraction of the SEL/ES preparation, respectively, at a dose rate of 1.0 mg protein of the appropriate preparation per lamb. The dose of 1.0 mg protein of the SEL/ES preparation per lamb was chosen as a previous unpublished dose response experiment, covering a dose range of 0.001 mg protein to 10 mg protein unfractionated SEL/ES preparation per lamb, indicated that maximum antibody titers, measured by ELISA, were attained with the 1.0 mg protein dose. The adjuvants, saponin and muramyl dipeptide, were mixed with SEL/ES preparations or buffer and injected intraperitoneally, each lamb receiving 1.0 mg and 0.1 mg of saponin and muramyl dipeptide, respectively. The adjuvants *B. pertussis* and BCG were injected intramuscularly, each lamb receiving 0.5 mg and 0.2 mg, respectively. Lambs of Group 4 were injected with the Tris-HCl buffer and adjuvants while lambs in Group 5 received only the Tris-HCl buffer. A second treatment was given to each lamb 28 days following the first injection.

Lambs in Group 6 were each infected with 5000 larvae and drenched orally with 10 mg kg^{-1} levamisole 28 days later to eliminate the infection. Simultaneously, all lambs in the other groups were dosed similarly with

TABLE I

Design of experiments on vaccination against *H. contortus* with lambs from the 1st and 2nd year crops

Group	No. of lambs	Vaccine composition	No. of doses	Dose interval[1] (days)	Days to challenge
1	8	Unfractionated SEL/ES + adjuvants	2	28	35
2	8	Low MWt fraction of SEL/ES + adjuvants	2	28	35
3 + 7	16	High MWt fraction of SEL/ES + adjuvants	2	28	35
4 + 8	13	Buffer + adjuvants	2	28	35
5	8	Buffer	2	28	35
6 + 9	15	Primary infection[2]	—	—	35

[1] First vaccine dose given on Day 1.

[2] Each lamb infected with 5000 larvae on Day 1 and treated with 10 mg kg^{-1} levamisole on Day 28.

levamisole to equalize treatments. All lambs in all groups were challenged with 10 000 infective larvae of *H. contortus* 35 days following the primary sensitization or infection. Samples of blood and feces were collected weekly from each lamb over a 6-week period prior to necropsy.

Twenty lambs from the second year's crop were distributed among three groups to give 8, 5 and 7 lambs in Groups 7, 8 and 9, respectively. Group 7 lambs received the high MWt preparation and were a replicate of Group 3 above. Likewise, lambs in Group 8 (buffer plus adjuvants) and Group 9 (primary infection) were replicates of Groups 4 and 6, respectively.

RESULTS

The unfractionated SEL and ES preparations and the low MWt fraction did not result in a reduction in the number of adult worms recovered at necropsy following challenge infection. Animals receiving the vehicle buffer with or without adjuvantation, Groups 4 and 8 and Group 5, respectively, had similar worm burdens indicating no non-specific protection due to the adjuvants. For this reason the data on worm counts from these three groups of lambs were combined in Table II.

Sensitization with the high MWt fraction stimulated a level of resistance to challenge infection similar to that induced by a prior infection with the parasite. Further groups of lambs from the Year 2 lamb crop (Groups 7, 8 and 9 in the experimental design shown in Table I) confirmed the similar level of protection between previously infected lambs and those injected with the high MWt fraction of the SEL/ES preparation. These data from Year 1 and 2 lambs were combined for presentation in Table II.

Combined EPG data from Year 1 and Year 2 lambs of Groups 3 and 7, 4, 5 and 8 and 6 and 9 are shown in Fig. 1. Significantly lower counts

TABLE II

Adult worm counts of lambs vaccinated against *H. contortus*[1]

Group	No. of lambs	Arithmetic mean	Transformed mean ± SD $\log_{10}(X + 1)$[2]	Percentage protection
1	8	952	2.50 ± 0.71^a	14
2	8	846	2.53 ± 0.69^a	24
3 + 7	16	453	2.18 ± 0.67^b	59
4 + 5 + 8	21	1110	2.72 ± 0.65^a	—
6 + 9	15	435	2.11 ± 0.73^b	61

[1] All lambs necropsied at 6 weeks after the challenge infection of 10 000 *H. contortus* larvae per lamb.

[2] Values with different superscripts were significantly different ($P < 0.05$).

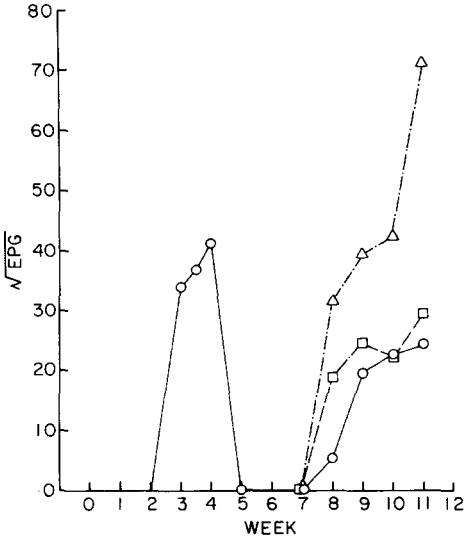

Fig. 1. Fecal egg counts of Florida Native lambs following a challenge infection of 10 000 *H. contortus* larvae per lamb at Week 5. □-----□ Groups 3 and 7, high MWt vaccine given in 2 doses on Days 1 and 28; ○——○ Groups 6 and 9, primary infection of 5000 *H. contortus* larvae on Day 1 and cleared with anthelmintic on Day 28; △—•—△ Groups 4, 5 and 8, challenge infection control.

TABLE III

ELISA antibody titers (mean ± SD) of lambs vaccinated against *H. contortus*

Group	Antibody titers		
	Days from start of experiments		
	1	35	44
1	0.21 ± 0.03	1.24 ± 0.12	1.30 ± 0.14
2	0.23 ± 0.02	1.13 ± 0.09	0.96 ± 0.07
3 + 7	0.30 ± 0.04	1.01 ± 0.11	1.15 ± 0.09
4 + 5 + 8	0.31 ± 0.06	0.32 ± 0.04	0.36 ± 0.07
6 + 9	0.28 ± 0.03	1.01 ± 0.14	0.84 ± 0.11

were obtained in previously infected lambs (Groups 6 and 9) and lambs sensitized with the high MWt fraction of SEL/ES preparation (Groups 3 and 7) compared to lambs of the groups injected either with buffer and adjuvants or buffer alone (Groups 4, 5 and 8).

Sensitization of lambs with the unfractionated SEL/ES preparation and its high and low MWt fractions gave ELISA antibody titers which showed no correlation with resistance to the challenge infection (Table III).

DISCUSSION

The unfractionated and low MWt fraction of the SEL/ES preparation of *H. contortus* used in the present vaccination trial gave no significant protection in Florida Native lambs but did stimulate the production of antibodies detected by ELISA. However, the high MWt (>30 000 daltons) components of the SEL/ES preparation did stimulate a partial protective response similar to that induced by a single prior infection with the parasite. There are indications that secretions associated with parasites (Rickard et al., 1977) or purified somatic components isolated from certain anatomical sites of parasites (Despommier et al., 1977) are more successful in stimulating host resistance when used as vaccines than are complex unfractionated preparations of parasites. Various reasons given for this include the presence of immunosuppressive components in the complex mixtures and antigenic competition among components which diminishes stimulation of immune responses harmful to the parasite.

Antibody levels detected by ELISA bore no relationship to the resistant status of the host. This may be a consequence of the complex nature of the parasite antigen preparation used in the tests. The presence of irrelevant and cross-reacting antigens in the parasite preparation could have masked the expected responses stimulated in the host to functional antigens. In a related study, serum antibody titers failed to correlate with resistance stimulated in sheep by a vaccine containing irradiated larvae of *H. contortus* (Smith and Christie, 1978). Gastrointestinal nematodes of ruminants are located within or very close to the intestinal mucosa. The developing parasitic larval stages of *H. contortus* reside in the mucosa and associated gastric glands of the sheep abomasum (Miller, 1984). Local immune responses are therefore of importance where acquired resistance to intestinal parasites is concerned and the greater susceptibility of young compared to adult animals to these pathogens results from an inability of the former to generate an effective anamnestic immune response in the gut (Smith et al., 1985).

Repeated larval infections over a period of time induce significant refractoriness to subsequent *H. contortus* infections in sheep (Christie et al., 1978). This results from an inability of incoming exsheathed *H. contortus* larvae to enter the gastric mucosa due to a host-mediated immune mechanism (Miller et al., 1983). Stimulation of this immune exclusion through vaccination would be of value in controlling this nematodiasis, particularly if it could be achieved in newly weaned lambs.

The present vaccination regimen utilizing the high MWt fraction of SEL/ES gave partial yet significant reduction in worm burdens of Florida Native lambs <6 months of age given a single challenge with the parasite. The regimen involved intraperitoneal injection of the preparation as this injection route gave best protection in studies with the rat *Nippostrongylus brasiliensis* model system (Murray et al., 1979). The IgA system of sheep

is most effectively stimulated by the injection of antigens into the peritoneal cavity which provided a long lasting stimulation of the serosal surface of the gut (Husband et al., 1979).

Recent studies have implicated local IgA antibody responses in the abomasum as contributing to host immunity against this parasite. Multiply infected sheep showed elevated specific IgG and IgA in the abomasal mucosa (Smith, 1977a). IgG was derived from the blood whereas the IgA was produced locally. Intramuscular inoculation of sheep with *H. contortus* larval antigen raised specific IgG but not IgA antibodies in the abomasal mucus (Smith, 1977b). There was a close temporal relationship between the rise in abomasal mucosa IgA anti-larval antibody levels in previously infected sheep given a challenge infection (Charley-Poulain et al., 1984). The significance of specific anti-larval IgA antibody, locally produced and locally active in the abomasum, was demonstrated by Smith et al. (1984) where they transferred immune lymphocytes from resistant to susceptible, histocompatible sheep, produced by embryo splitting. In this study Smith cannulated the common gastric lymph duct and could collect and monitor immunocompetent cells and antibodies of infected sheep. A most significant finding of these studies was the induction in a naive recipient of strong local IgA response and a marked reduction in worm count.

The choice of adjuvants was based on the findings of Wells et al. (1982). Where experimental host animals are scarce and expensive it is not feasible to test numerous combinations of antigen doses routes and frequency of administration and adjuvantation. Better protection than presently obtained may be possible in lambs using different vaccination parameters. While it is unlikely that total refractoriness to reinfection will ever be stimulated by vaccines composed of worms and their metabolic products derived from in vitro culture, it is reasonable to expect significant diminution in the number of worms becoming established.

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