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Effect of CD4$^+$ T lymphocyte depletion on resistance of Gulf Coast Native lambs to *Haemonchus contortus* infection

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Abstract

It has been reported that CD4$^+$ T lymphocytes are important in acquired immunity to gastrointestinal nematode infection. Whether these lymphocytes are also involved in the immune response of naturally resistant Gulf Coast Native (GCN) sheep to *Haemonchus contortus* infection remains to be defined. The objective of this study was to determine the role of CD4$^+$ T lymphocytes in this resistance. Ten GCN lambs were randomly assigned to a control ($n = 5$) or a treatment ($n = 5$) group. The treatment consisted of a series of IV injections with mouse anti-ovine CD4$^+$ T lymphocyte monoclonal antibodies for a period of 3 weeks. After the second treatment, all lambs were experimentally infected with 10,000 *H. contortus* infective larvae by oral inoculation. All lambs were monitored for fecal egg counts, blood packed cell volumes, white blood cell differential counts and serum antibody responses on a weekly basis. Fluorescence-activated cell sorter (FACS) analysis was done biweekly to enumerate CD4$^+$ T lymphocytes in peripheral blood. Necropsies were performed at the end of the study and 10% of the contents of the gastrointestinal tract were preserved for nematode enumeration and identification. Also at necropsy, mesenteric lymph nodes were extracted and FACS analysis was run on lymphoid cells. Mean fecal egg counts on day 21 and 28 post-infection and nematode counts at necropsy of the treated group were significantly ($p < 0.05$) higher than that of the control group. Percent CD4$^+$ T lymphocytes in peripheral blood was significantly ($p < 0.05$) lower in the treatment group than in the control group from day 9 to the end of the study. No differences were found in blood packed cell volumes, white blood cell differential counts, antibody titer or lymph node CD4$^+$ lymphocytes between groups. Lambs depleted of their CD4$^+$ T lymphocytes were more susceptible to *H. contortus* infection than undepleted lambs. The results of this study suggest that CD4$^+$ T lymphocytes are associated with the natural resistance of GCN sheep to *H. contortus* infection.

Keywords: Sheep; *Haemonchus contortus*; CD4 Depletion; Immunity

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1. Introduction

It has been shown in epidemiological studies that Gulf Coast Native (GCN) sheep are more resistant to gastrointestinal nematode infection (mainly *Haemonchus contortus*) than Suffolk sheep (Bahirathan et al., 1996; Miller et al., 1998). This natural resistance is characterized by significantly lower fecal egg counts (FEC) and worm burdens, and higher blood packed cell volumes (PCV). The mechanisms underlying the natural resistance of GCN sheep are related to some components in the immune response (Peña et al., 2004). Dexamethasone treatment resulted in naturally resistant GCN (neonatal and weaner) lambs becoming more susceptible to *H. contortus* as evidenced by significantly higher FEC and worm burdens and lower PCV than control lambs. Several studies have demonstrated the importance of $CD4^+$ T lymphocytes in the immunity to gastrointestinal (GI) nematode infection (Katona et al., 1988; Urban et al., 1991; Gill et al., 1992, 1993; Koyama et al., 1995; Karanu et al., 1997). Monoclonal antibodies (mAb) to $CD4^+$ T lymphocytes have been shown to be invaluable in characterizing and defining the role of these lymphocytes in local immune responses to GI nematode infection. Using immunocytochemistry and mAb to $CD4^+$ and $CD8^+$ T lymphocytes, Kambara and McFarlane (1996) examined T lymphocyte populations from the intestinal lymph duct and blood of sheep infected with *Trichostrongylus colubriformis*. They indicated that parasite antigen-primed $CD4^+$ T lymphocytes were associated with protective immunity particularly in older animals.

Mouse mAb antibodies have also been successfully used to deplete $CD4^+$ T lymphocytes in cattle and sheep. Naessens et al. (1998) effectively depleted cattle of $CD4^+$ and $CD8^+$ T lymphocytes using mAb to bovine T lymphocyte antigens. They observed that when depletion was effected by intravenous injections of murine antibody isotypes that activate complement (IgG_{2a}), the targeted cells disappeared from peripheral blood in less than 1 h. In contrast, when non-complement binding antibody (IgG_1) was used, the target cells remained in circulation for several days coated with mAb and were slowly removed until their near-total disappearance more than 1 week after the treatment. Gill et al. (1992) successfully depleted sheep of their $CD4^+$ T lymphocytes and showed that depleted lambs did not mount an antibody response to ovalbumin (OVA) and they did not show a skin reaction to T lymphocyte mitogens. When anti-$CD4^+$ T lymphocyte mAb was administrated to a genetically selected line of Merino lambs resistant to *H. contortus*, their expression of resistance was abrogated as indicated by significantly higher fecal egg counts (FEC) and nematode burdens found in the $CD4^+$ T lymphocyte depleted lambs compared with those of controls (Gill et al., 1993). In addition, host responses associated with resistance to *H. contortus* including mucosal mast cell hyperplasia, tissue eosinophilia, and antibody responses to *H. contortus* were also significantly suppressed in the lymphocyte depleted lambs. Based on the lack of antibody response, it was concluded that T lymphocyte help is required for the generation of anti-parasite antibody in *Haemonchus*-infected sheep. Karanu et al. (1997) indicated that $CD4^+$ T lymphocyte depletion partially abrogated immunity induced by gut antigen immunization against challenge infection with *H. contortus*. Those findings are consistent with Howard et al. (1989) where calves depleted of their $CD4^+$ T lymphocytes showed a reduced ability to mount an antibody response to human O red blood cells and OVA. Accordingly, Wofsy et al. (1985) demonstrated that mice treated with monoclonal antibody to L3T4 cells were unable to generate an IgG response to either bovine serum albumin or OVA.

The objective of this study was to determine the role of $CD4^+$T lymphocytes in the natural resistance of GCN lambs after an experimental *H. contortus* infection.

2. Materials and methods

2.1. Animals

Ten 5-month-old GCN lambs raised on pasture at the Central Station Sheep Farm, Louisiana Agricultural Experiment Station, Baton Rouge, LA, were randomly assigned to a treatment ($n = 5$) or control ($n = 5$) group. All animals were dewormed with albendazole (Valbazen®, Pfizer, 10 mg/kg) and levamisole (Levasole®, Schering-Plough, 8.8 mg/kg) 6 weeks prior to administering experimental infections. They were maintained in concrete-floored pens for the duration of the study where they were

fed a lamb growing ration and water was available at all times.

2.2. Monoclonal antibody treatment

Mouse anti-ovine $CD4^+$ monoclonal antibodies from the 44.97 hybridoma cell line (Center of Animal Biotechnology, The University of Melbourne, Melbourne, Australia) were produced in tissue culture (Louisiana State University Medical Center Core Laboratory, New Orleans, LA, USA). Lambs in the treatment group were injected IV with 2 mg of anti-$CD4^+$ mAb on days −3 and −1 before all lambs received an experimental infection of 10,000 *H. contortus* L_3 (day 0 of study) by oral inoculation. Injection treatments continued with 2 mg on day 1; 4 mg on days 3, 5, 7 and 10; 2 mg on days 14 and 17.

2.3. Fecal and blood samples

Individual fecal and blood samples were collected on days −14, −7, 0, 7, 14, 21 and 28. Fecal samples were collected directly from the rectum and processed to determine FEC using a modified McMaster technique with 2 g of feces (Whitlock, 1948). Results were reported as egg per gram (EPG). Peripheral blood was collected in 7 ml EDTA vaccutainer tubes via jugular venipuncture. WBC was determined by an automated hematology analyzer (Inc. Baker System 9110 + Plus). Differential leukocyte counts were made on microscope slide smears stained with modified Wright's stain.

2.4. Necropsies

All animals were necropsied on day 28. The abomasum, small intestine and large intestine were collected for nematode enumeration and identification in accordance with established procedures (Miller et al., 1987). The recovery procedure was modified in that organ contents were brought to a volume of 5 l and a 500 ml aliquot was taken and preserved with formalin.

2.5. Flow cytometry

2.5.1. Peripheral blood lymphocytes

Peripheral whole blood was collected on days −1, 2, 6, 9, 13, 16, 20 and 27 via jugular venipuncture into 7 ml heparin vaccutainer tubes. $CD4^+$ lymphocytes were stained by an indirect procedure and enumerated on a flow cytometer (FACScan, Becton Dickison). The indirect staining procedure was done as follows: 50 μl of blood was incubated for 30 min at room temperature and in the dark with 50 μl of mouse anti-CD4 mAb diluted 1/50 in phosphate buffered saline (PBS). Cells were then washed with 2 ml of 1× PBS and centrifuged for 5 min at 1400 rpm. After centrifugation, the supernatant was decanted and 50 μl of goat anti-mouse IgG FITC conjugate (1:200 diluted in 1× PBS) was added to the tubes. The samples were then vortexed and incubated for 30 min at room temperature in the dark. After the second incubation, 1× NH_4Cl lysis buffer was added to the tubes to lyse the erythrocytes and the samples were centrifuged again at 1400 rpm for 5 min. The supernatant was decanted and the cells were washed with 1× PBS and centrifuged. The supernatant was decanted, the pellet was vortexed and the cells were fixed with 200 μl of 1% formaldehyde-PBS for flow cytometer analysis (Jackson and Warner, 1986).

2.5.2. Mesenteric lymph node lymphocytes

Mesenteric lymph nodes were removed at necropsy and transferred to plastic petri dishes, cut and mashed through a strainer. The strainer was rinsed into another petri dish with PBS to wash out the cells and the remaining liquid was transferred into a 50 ml centrifuge tube. The cells were centrifuged at 1500 rpm for 5 min and after decanting the supernatant, the pellet was resuspended in PBS and centrifuged again. After three more washes and centrifugations in PBS, the cells were stained using the same procedure described in Section 2.5.1, for flow cytometer analysis.

2.6. ELISA for H. contortus whole worm antigen (WWA)

ELISA tests were run on day −7, 0, 7, 14, 21 and 28 using a modification of the procedure described by Smith et al. (1999). Briefly, microtiter plates were coated with 50 μl of WWA obtained from mature *H. contortus* worms. The antigen was diluted in carbonate (pH 9.6) coating buffer to 10 μg/ml. The plates were incubated at room temperature

overnight and then washed with PBS containing 0.05% Tween-20 (washing buffer). Serum samples, diluted 1/500 in serum diluent, were added to the plates and then incubated for 2 h at room temperature. Serum from a mature ewe with a high infection level was used as a positive control and was also diluted 1/500 in serum diluent and incubated for 2 h at room temperature. All serum samples (test and positive control) were done in triplicate. After another wash, 50 μl of rabbit anti-sheep IgG alkaline phosphatase conjugate (Kirkegaard and Perry, MD, USA) diluted 1:1000 in blocking buffer was added and incubated for 2 h at room temperature. The plates were then washed and dried, and 75 μl of pNPP substrate (Kirkegaard and Perry, MD, USA) was added. The plates were incubated in the dark for 1 h and the color reaction was stopped with 75 μl of 5% EDTA. The plates were read at 405 nm with an automatic ELISA plate reader.

2.7. Lymphoproliferation assay

Lymphoproliferation assays were performed on peripheral blood mononuclear cells collected on day 7. Lymphocytes were isolated from whole blood using Ficoll-Plaque. After three washes with PBS, 1 ml of RPMI-1640 was added, and cell concentration was determined with a haemocytometer using Trypan Blue to obtain a final suspension of 2×10^6 cells/ml. Phytohemagglutinin (PHA), concanavalin A (ConA), and pokeweed (PW) mitogens were used to test T lymphocyte function. ConA and PW were used at 4, 2 and 1 μg/ml and PHA was used at 8, 4 and 2 μg/ml. Mitogens were diluted in RPMI-1640 and added to respective wells in 100 μl volumes. RPMI-1640 was added to the control wells, and then 100 μl of the cell suspension was added to the plates. All cultures were done in triplicate. The plates were incubated at 39 °C in a humidified incubator with 5% CO_2 for 3 days. The plates were pulsed with 0.5 μCi [^{3}H] thymidine/well for 4 h and cells were harvested for liquid scintillation counting.

2.8. Statistical analysis

Statistical analysis was done with raw data for PCV, WBC/differential counts, lymphocyte counts, antibody titers and $CD4^+$ T lymphocyte counts and with log-transformed (to stabilize variance) data for FEC and total nematode counts. One of the animals in the treatment group did not respond to the treatment and showed normal (control level) $CD4^+$ T lymphocyte percent throughout the study; therefore, it was not included in the statistical analysis. The means of the groups were compared using Proc Mixed and Proc GLM for repeated measures in SAS. Variables in the models were treatment group and time. For FEC, analysis was done for two time periods. The first time period (T1) was from day −14 to day 7 when the relationship between treatment groups was linear. The second time period (T2) was for days 14–28 when the relationship between groups was quadratic. Tukey test was used to compare differences in mean nematode counts between groups. Means are presented as untransformed means ± S.E.M. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Fecal egg counts

The mean FEC of all lambs remained low through day 14 and increased in both groups on days 21 and 28 (Fig. 1). The treatment × time interaction for T1 and T2 was not significant ($p = 0.3002$ and $p = 0.2681$,

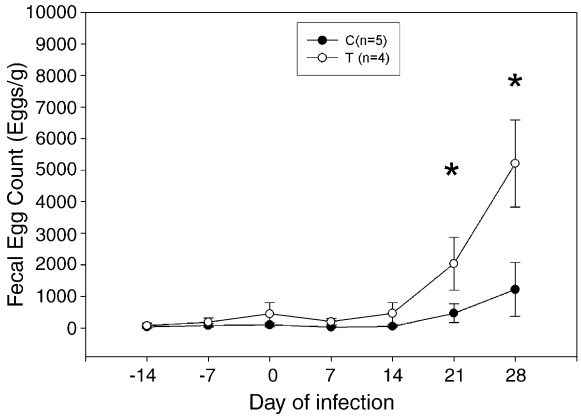

Fig. 1. Mean fecal egg counts comparing $CD4^+$ depleted (T) Gulf Coast Native lambs to undepleted (C) lambs. Day 0: experimental infection of 10,000 *Haemonchus contortus* L_3. (*) Means are significantly different ($p < 0.0001$).

respectively). However, on days 21 and 28, the treated group was significantly ($p = 0.0331$ and $p = 0.0185$, respectively) higher than the control group.

3.2. Nematode counts

Haemonchus contortus was the only species identified in the abomasum and more than 99% were adults. No nematodes were found in the small or large intestine of either group. The mean nematode count in the treatment group (1743 ± 356) was significantly ($p = 0.049$) higher than that of the control group (698 ± 308).

3.3. FACS

3.3.1. Peripheral blood $CD4^+$ lymphocytes

The control group $CD4^+$ T lymphocyte mean percent was 30.1% (range 28.0–34.2%) and the range of the deleted treatment group animal was 24.6–30.0%. Even though this was slightly lower than the control group, the difference cannot be considered as due to the effect of the treatment, as two of the control animals had similar $CD4^+$ T lymphocyte percent ranges (25.5–30.0% and 22.0–29.6%). The effect of $CD4^+$ T lymphocyte depletion was not seen until day 9 (Fig. 2). The mean percent of $CD4^+$ stained lymphocytes remained similar in both groups through day 6. From day 9 to the end of the study, $CD4^+$ stained lymphocytes in the treatment group were essentially absent from peripheral circulation. The treatment × time interaction was significant ($p < 0.0001$).

3.3.2. Mesenteric lymph node $CD4^+$ lymphocytes

Mean $CD4^+$T lymphocyte percent between treatment and control groups were similar (data not shown).

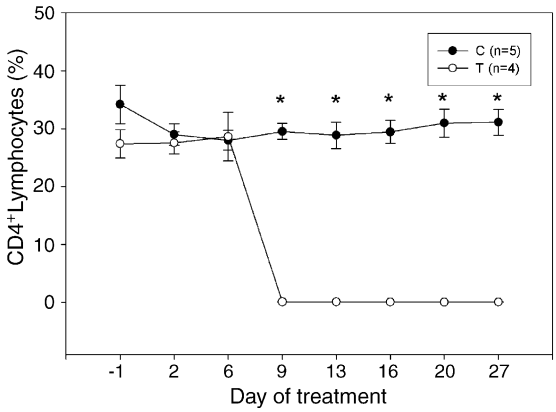

Fig. 2. Mean percent $CD4^+$ lymphocytes in $CD4^+$ depleted (T) Gulf Coast Native lambs and undepleted (C) lambs. (*) Means are significantly different ($p < 0.0001$).

3.4. Peripheral blood tests

Throughout the study, PCV, WBC differential and antibody titer to *H. contortus* WWA were similar (data not shown).

Because $CD4^+$ lymphocytes were still circulating on day 6, proliferation assays were done on day 7 to test functionality of the lymphocytes. Results showed that the treatment affected $CD4^+$ lymphocyte functionality as evidenced by significantly ($p < 0.05$) lower responses seen to mitogen stimulation in the treatment group compared to the control group (Table 1).

4. Discussion

The potential immunological basis for resistance of GCN sheep to *H. contortus* infection has been described previously (Peña et al., 2004). Several

Table 1
Mean stimulation index in response to phytohemagglutinin (PHA), concanavalin A (Con A), and pokeweed (PW) mitogen stimulation in $CD4^+$ depleted Gulf Coast Native lambs and undepleted lambs after six anti-CD4 mAb treatments over 12 days

Treatment	*N*	Mitogen (μg/ml)								
		PHA			ConA			PW		
		8	4	2	4	2	1	4	2	1
None	5	87.5 a	82.2 a	73.5 a	140.2 a	129.9 a	112.3 a	87.7 a	83.7 a	75.6 a
CD4 mAb	4	17.0 b	13.8 b	8.7 b	22.5 b	20.4 b	17.7 b	17.3 b	17.3 b	16.5 b

Means with unlike letters are significantly different (PHA8, $p = 0.0301$; PHA4, $p = 0.0131$; PHA2, $p = 0.0003$; ConA4, $p = 0.0307$; ConA2, $p = 0.0124$; ConA1, $p = 0.0063$; PW4, $p = 0.0134$; PW2, $p = 0.0169$; PW1, $p = 0.0189$).

studies have established the importance of $CD4^+$ T lymphocytes in immunity to GI nematodes (Koyama et al., 1995; Katona et al., 1988; Urban et al., 1991; Gill et al., 1993, 1992; Karanu et al., 1997). Similar to what was reported by Gill et al. (1993) for genetically derived resistant Merino lambs, naturally resistant GCN lambs in the present study that were depleted of $CD4^+$ T lymphocytes, became susceptible to infection with *H. contortus*. In these studies, lambs treated with anti-$CD4^+$ mAb showed a significantly higher FEC and nematode burden than the control lambs. However, in contrast with Gill et al. (1992, 1993), antibody titers in the present study did not differ between groups. In the Gill et al. (1993) study, the anti-$CD4^+$ mAb used was complement fixing (IgG_{2a}) and $CD4^+$ T lymphocytes were depleted within 48 h (before experimental infection). In the present study, a non complement-binding anti-$CD4^+$ mAb (IgG_1) was used and $CD4^+$ T lymphocyte depletion was not seen until 9 days after initial treatment. Therefore, $CD4^+$ T lymphocytes were still circulating when the lambs were challenged. It may be suggested that during this period circulating $CD4^+$ T lymphocytes were providing help to B lymphocytes to produce immunoglobulins to *H. contortus* WWA resulting in a normal antibody response in treated lambs. These results are consistent with those of Howard et al. (1992) in which calves depleted of their $CD4^+$ T lymphocytes produced an antibody response to bovine viral diarrhea similar to that of controls.

In the present study there were no differences in the PCV between treated animals and controls. Even though the treated group had a significantly higher nematode burden than the control group, the difference in the number of nematodes and the relatively short infection period (4 weeks) might not have been enough time for anemia to develop and affect the PCV.

Because depletion was not achieved until 12 days after anti-$CD4^+$ mAb treatment started, lymphoproliferation assays were done to determine the effect of mAb on T lymphocyte functionality. The results of those tests showed that there was a decreased functionality in the treatment group compared to those of the control group. These results concur with those of Howard et al. (1989) where the responses of peripheral blood mononuclear cells (PBMC) preparations to ConA, PHA, and PW were significantly reduced in BoT4 ($CD4^+$) depleted calves. These results may suggest that the coating of the lymphocytes by the mAb might have affected their functionality by altering the surface membrane. Considering the role of $CD4^+$ T lymphocytes in resistance to *H. contortus* infection, the decreased functionality of $CD4^+$ T lymphocytes observed might explain the higher establishment of nematodes in the treated group compared to the controls. It has been established in several laboratory animal models that CD4 T lymphocytes are required for immunity to GI nematode infections. Koyama et al. (1995) demonstrated that in vivo depletion of $CD4^+$ T lymphocytes resulted in the suppression of the expulsion of *Trichinella muris* in BALB/c mice. In a similar study, Katona et al. (1998) deplete mice of $CD4^+$ T lymphocytes that resulted in prevention of spontaneous cure of *Nippostrongylus brasiliensis* infection and inhibition of $CD4^+$ T lymphocyte function including induction of IgE response and intestinal mucosal mast cell hyperplasia. They also demonstrated that both the persistence of increased serum IgE levels in mice infected with *N. brasiliensis* after the peak period of IgE secretion and the development of a secondary IgE response to re-infection were dependent on the continued presence of $CD4^+$ T lymphocytes. Urban et al. (1991) indicated that $CD4^+$ T lymphocytes were also required for the induction and maintenance of an IgE response following *Heligmosomoides polygyrus* infection in mice. In addition, adult nematode fecundity was increased in the depleted mice compared to the control non-depleted. The indication was that $CD4^+$ T lymphocytes play a critical role in the stability and fecundity of GI nematode populations.

Although infection level and circulating $CD4^+$ lymphocyte numbers and functionality were significantly decreased in the treated group, there was no difference in lymph node $CD4^+$ lymphocytes between groups. One explanation may be that the dose of monoclonal used in our study was not enough to eliminate T lymphocytes in the lymphoid organs. Naessens et al. (1998) indicated that the dose of 2 mg anti-$CD4^+$ mAb/kg body weight was necessary for efficient elimination of T lymphocytes in the lymphoid organs of cattle. The dose (2 mg total) used in this study did not deplete $CD4^+$ T lymphocytes in the lymph nodes as shown by the high percentage of

$CD4^+$ T lymphocytes present in the lymph nodes at necropsy of the treated lambs.

5. Conclusion

GCN lambs depleted of their $CD4^+$ T lymphocytes became more susceptible to *H. contortus* infections as demonstrated by significantly higher FEC and worm burdens than untreated lambs. Therefore, this suggests that $CD4^+$ T lymphocytes are an integral part of their natural resistance to *H. contortus* infection.

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