CHAPTER 3

Immune expulsion of the trichostrongylid *Cooperia oncophora* is associated with increased eosinophilia and mucosal IgA

K. Kanobana, H.W. Ploeger, L. Vervelde

Department of Infectious Diseases and Immunology, Division of Parasitology and Tropical Veterinary Medicine, Faculty of Veterinary Medicine, Utrecht University

Reprinted from International Journal for Parasitology 32 (2002) 1389-1398
Copyright © (2002), with permission from Elsevier Sciences

What is the truth of all we know?
To hear the crap that comes out of the mouth…
The silent can say more….waiting and watching to see.
(G. Friday)

ABSTRACT

Previous experiments have shown that a primary infection with 100,000 infective larvae of the trichostrongylid *Cooperia oncophora* allows discrimination between different type of responder animals based on the speed by which the parasite is expelled from the host. In most of the animals (intermediate responders) the expulsion occurs 35-42 days after infection. This experiment was carried out to investigate which mechanisms contribute to the clearance of the parasite from the intestine. Sequential necropsy of the animals 14, 28 and 42 days after infection together with a segmental division of the small intestine, allowed us to characterise essential components associated with development of immunity and expulsion of the parasite from its niche. The results show that during the patent phase of the infection the parasite preferentially resides in the proximal gut. At day 42 after infection ongoing expulsion is characterized by a migration of the worms to the more distal part of the intestine. Expulsion of the adult worm population appears to be mast-cell independent and is associated with a significant increase in parasite-specific mucus IgA and IgG1 as well as with an influx of eosinophils in the intestinal lamina propria. Although we did not observe a specific lymphocyte recruitment into the intestinal mucosa, the accumulation of eosinophils seems to be mediated by CD4+ cells. We measured significant negative correlations between the number of eosinophils and the expulsion rate of the parasite expressed by sex ratio and ratio EPG. Parasite-specific mucosal IgA levels were negatively correlated to the fecundity of the worms, expressed as number of eggs per female worm. Our results describe the involvement of both eosinophils and mucosal IgA in the regulation of *C. oncophora* expulsion and suggest the development of a Th2 effector immune response.

INTRODUCTION

The trichostrongylid *Cooperia oncophora* is a lumen dwelling nematode of cattle. Experimental infections with 100,000 infective larvae in 3-month-old calves are highly reproducible and have the potential to allow discrimination between different responder animals based on parasitological variables and the speed by which the parasite is expelled from the host. [3, 84, 176, 231]. High responders, a small proportion of the host population, show almost no egg output and their worm burden at necropsy on day 42 after infection (p.i.) is low. Low responders show high egg output which is continued for weeks and results in high worm burdens at necropsy. In intermediate responders the egg output is initially similar to the low responders but between days 35-42 p.i, it declines rapidly. The worm burdens at necropsy vary from low to high numbers.

Previously, we demonstrated that intermediate and low responders also develop a distinct systemic immune response [127]. Regardless of their responder type, in all animals a Th2-biased immune response was developed. However, *C. oncophora*-specific serum IgG1 was significantly higher in intermediate responders than in low responders. Moreover, based on correlations between the systemic immune response and parasitological data, we proposed an effector role for the parasite-specific humoral response in the intermediate responders.

Here, we aimed to elucidate the immune effectors associated with the rejection of the parasite and the decrease in egg output observed in the intermediate responders. To this end, we performed a detailed study of the events occurring in the gut of infected animals in the course of the infection. Following a single primary infection with 100,000 infective larvae calves were necropsied sequentially at days 14, 28 and 42 p.i., corresponding with onset of egg excretion, peak egg output, and ongoing expulsion of adult worms, respectively, in most of the infected animals. Animals slaughtered at day 42 p.i. were subdivided in different responder types. The availability of those responder types provided the opportunity to compare the development of a successfull immune response in the intermediate responders with a non effective one in the low responders.

MATERIALS AND METHODS

Infective larvae

Third stage larvae (L3) from *C. oncophora* were obtained by coprocultures using standard procedures [30] and kept in water at 10°C until use. Larvae used for infection were less than 3 months old and originated from the Wageningen laboratory strain which has been maintained by regular passage through donor calves since 1967.

Animals and experimental design

Thirty-six female Holstein Friesian calves were raised under helminth free conditions on commercial farms and purchased at 3 months of age. Throughout the experiment the animals were

kept indoors at the animal facility of the Faculty of Veterinary Medicine in Utrecht. Animals were housed in one group and fed hay and water ad lib, as well as concentrates at a maximum of 500 g/day. Prior to infection animals were checked for general health condition and faeces was collected to test the worm-free status of the animals.
Twenty-four calves were infected orally with a single dose of 100,000 *C. oncophora* L3 and twelve calves were kept as controls. Faeces were collected from the rectum once a week during the first two weeks of the infection and thereafter three times a week. The number of eggs per gram faeces (EPG) was determined using a modified McMaster technique. A blood sample for serum was collected from the jugular vein one week before infection and weekly thereafter from day 7 to day 42 p.i.. At day 14, 28 and 42 p.i., 8 infected and 4 control animals were necropsied. The groups were named group A (necropsied at day 14 p.i.), group B (necropsied at day 28 p.i.) and group C (necropsied at day 42 p.i.).

Necropsy procedure and parasitology
To obtain a more detailed insight in the localisation of the worm burden in the small intestine in relation to the population dynamics, the small intestine was divided in six segments (table 1). All individual segments were sampled for histological examinations and mucus isolation. Additional sampling of S1, S3 and S6 was done for the isolation of lamina propria lymphocytes.

TABLE 1. Segmental division of the small intestine

Segment	Localisation	anatomical nomenclature
S1	first meter after the pylorus	duodenum
S2	next 5 meters	proximal jejunum
S3	next 5 meters	middle jejunum 1
S4	next 5 meters	middle jejunum 2
S5	next 5 meters	distal jejunum
S6	last part of the small intestine	ileum

The remaining of the intestinal segments was used for worm counts. Intestinal washings were done according to the methods described by Eysker and Kooyman [74]. All segments were processed separately. Two percent of the worm burden of each segment was counted and sex and developmental stages were determined. Worm fecundity was assessed by counting the number of eggs per female worm. Twenty worms per segment were enumerated. Worms were fixed in 70% alcohol and, at time of egg counting rinsed in tap water to remove the excess of alcohol. Individual female worms were placed in a 96-well flat-bottom plate (Greiner high binding, cat 655093; Greiner Labortechnik, Frickenhausen, Germany) and lysed to release the eggs using a 100 µl of a 0.4% sodium hypochlorite solution. Eggs were enumerated using an inverted microscope 5 to 10 minutes after lysis of the adult females (magnification 16 x).

Isolation of intestinal lamina propria lymphocytes
Lamina propria lymphocytes (LPL) were isolated using an enzymatic method according to Van den Broeck et al. [228] with minor modifications. Sections of approximately 5 cm were removed from the proximal part of the duodenum (S1), the proximal jejunum (S3) and the ileum (S6) and checked for the absence of Peyers patches before processing. Erythrocytes were removed from the final cell suspension by an erythrocyte shock lysing solution and viability of the cells was assessed by

Trypan blue exclusion. After each incubation step, a sample of the gut tissue was taken for histological examination as control for the isolation procedure.

Isolation of mesenterial lymphnode lymphocytes (MLNL)
One mesenterial lymphnode at the beginning of S3 was collected aseptically in ice cold sampling medium. MLNL were isolated as described by Schallig et al. [190]. One part of the cells was resuspended in RPMI supplemented with P/S, 10% FCS, 2 mM glutamine and 2 mM β-mercapthoethanol at a concentration of $2*10^6$ cells/ml until further use in lymphocyte proliferation tests (LPT). The remaining cells were resuspended at a concentration of $1*10^7$ cells/ml in RPMI supplemented with P/S. The latter cell suspension was used for flow cytometric analysis.

Phenotyping of lymphocyte subpopulations
Flow cytometric analysis of LPL and MLNL was performed using the same panel of monoclonal antibodies and staining method as described before [127]. Interference with other cells was excluded by gating for lymphocytes, based on forward and side scatter pattern of the cells. Results are expressed as percentage positive cells after subtraction of the isotype control percentages. Percentages of the lymphocyte subpopulations were based on a total of 10,000 events within the gate set for lymphocytes.

In vitro lymphocyte response to *C. oncophora* antigen extracts
C. oncophora L3 crude worm antigen (L3), Adult crude worm antigen (Ad) and Adult excretory/secretory products (ESP) were prepared as described previously [127]. *C. oncophora*-specific proliferation was measured after stimulation of MLNL with 10 µg/ml L3, Ad or ES antigen. The LPA was performed as described by Schallig et al. [197] with minor modifications. Cells were incubated for 5 days at 37°C and 5% CO2. Twenty-four hours before harvesting a final pulse with 0.5 µCi (methyl) ^{3}H-thymidine was added to each well.

Detection of eosinophils and mast cells
Tissues were fixed in 4% paraformaldehyde in PBS (pH 7.4) or Carnoy's fluid for 24 hours. Histochemical quantification of eosinophils has been described by Vervelde et al. [235]. Eosinophils were only present in the lamina propria and cells were counted on a minimum of 25 graticule fields (0.0676 mm^2) throughout two or three sections. For the histochemical quantification of mast cells, Carnoy's fixed tissues were stained overnight with toluidine blue at pH 0.1 [118] and counterstained with light green. The counts were made separately in the different layers of the mucosae and a minimum of 10 graticule fields (0.1764 mm^2) were counted in the lamina propria of 2 or 3 sections of the tissue. A mininum of 5 graticule fields were counted in the lamina submucosa as well as in the lamina muscularis interna and externa. The total number of mast cells was obtained after summation of the number of cells in the different layers. Results are expressed as number of cells/mm^2.

Detection of *Cooperia* specific mucus Ig
A 3 to 5 cm piece of each intestinal segment was sampled for the extraction of intestinal mucus. Until storage at –20°C the samples were kept on ice to avoid proteolytic breakdown. Mucus was isolated based on the method described by McClure et al. [154] with modifications. Tissues were thawed and mucus was scraped off with a glass slide. Best results were obtained when the tissue was not completely defrosted. The scrapings were collected in a falcontube on ice. Three ml ice cold PBS supplemented with protease inhibitors (1 tablet for 10 ml; Complete®, Roche) was added to each sample. The samples were shaken for 1 hour at 4°C and centrifuged for 30 minutes at 4°C

and 3,000 x g. Finally, the supernatant was spun down for 30 minutes, 4°C at 15,000 x g. Protein concentration was determined with the Bradford assay. ELISA's were performed with samples adjusted to a final concentration of 5 mg protein per ml supernatant. *Cooperia* Ad IgG1, IgG2 and IgA specific ELISA's were performed as described before [127].
Total IgE levels were measured in the mucus using a sandwich ELISA as described before [133]. Mucus samples at a protein concentration of 10 mg/ml were used. All samples were inactivated for 1 hour at 56°C to increase the affinity of the monoclonal antibody [136].

Statistical analysis
Statistical analysis was carried out using SPSS statistical package (version 10.0). Differences between groups were tested with the non-parametric Friedman or with the Wilcoxon test for dependent samples. Independent samples were analysed with Kruskas-Wallis. Immunologial data were correlated with parasitological variables expressing host responsiveness, which were sex ratio, ratio EPG, number of eggs per female worm and the worm burden. The sex ratio was defined as the % male worms in the total adult worm burden. This parameter is useful to monitor worm expulsion because male worms are expelled first [3, 48]. Based on the worm distribution at day 14, day 28 and day 42 p.i. we used the sex ratio in S2, which is the predilection site of the adult worm population. The ratio EPG was defined as: mean EPG between day 35 and day 42 p.i. (end of egg excretion pattern)/mean EPG between day 21 and day 28 p.i. (peak of egg excretion pattern). This parameter features the effect of the host immune response on both worm survival and fecundity. Previously we demonstrated that this parameter is significantly positively correlated with the number of eggs per female worm [123]. The eight infected animals slaughtered at day 42 p.i. were classified into different responder types based on ratio EPG and worm counts as described previously [127]. All correlations between immunological and parasitological parameters were evaluated with the Pearson's correlation coefficient.

RESULTS

Parasitology

The egg excretion pattern of all the infected animals was comparable with previous experiments and within normal values. Worm counts at days 14, 28 and 42 p.i. showed that *C. oncophora* preferentially homed in the proximal jejunum (S2) (fig. 1).

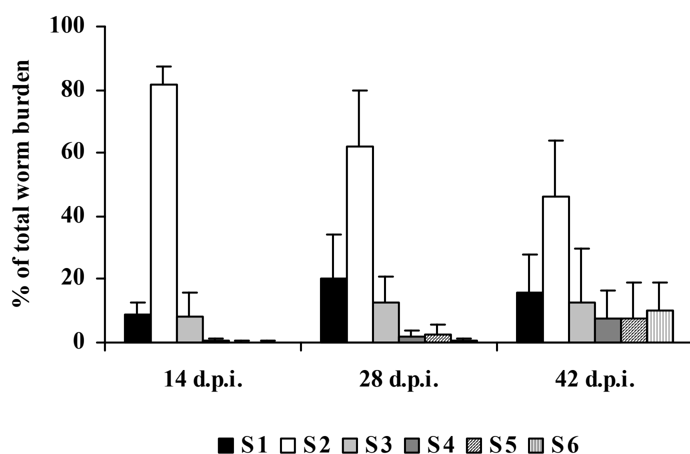

FIGURE 1 Worm distribution in the small intestine after a single-dose infection of 100,000 L3 *C. oncophora* at days 14, 28 and 42 p.i.. Data shown are mean values of 8 infected animals (+ SD).

At day 14 p.i., worms were only recovered from the proximal gut (S1, S2 and S3). The percentage recovery of the infection dose ranged between 56 and 117% at day 14 p.i., between 52 and 116% at day 28 p.i., and between 12 and 88% at day 42 p.i.. The sex ratio in the whole small intestine was consistently in the range of 46-51% with the exception of one animal which had almost no worm burden and a sex ratio of 9% at day 42 p.i.. Comparison of the sex ratio in the proximal and distal gut showed that at day 28 p.i. the % male worms was higher in the proximal gut than in the distal gut (49 versus 40%) while at day 42 p.i. the opposite was found (41 versus 45%).
Based on the faecal egg output and the necopsy results the animals slaughtered at day 42 p.i. were classified into 2 low and 5 intermediate responders [123]. None of the calves could be classified as a high responder and one calf showed a distinct EPG pattern which did not fit any of the classifications. Figure 2A shows that worm fecundity was highest at day 28 p.i., which coincided with the peak in egg output. Forty two days after infection, the worm fecundity decreased significantly compared with day 28 p.i. (P<0.01) but remained higher than day 14 p.i. (P<0.01). Analysis of the different responders showed a significantly lower fecundity in the intermediate responders compared with the low responders.

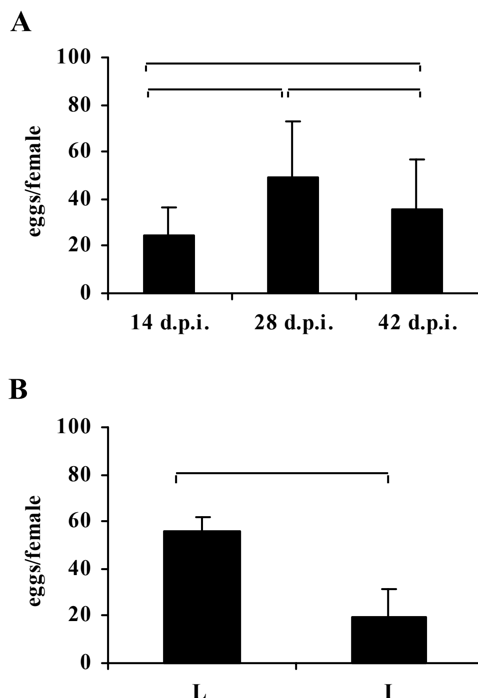

FIGURE 2 Mean number of eggs/female worm (+SD) for infected animals at day 14, 28 and 42 p.i. (A) and for low (L) and intermediate (I) responders (B). Data shown are mean number of 20 individual worms per calf. Lines indicate significant differences between groups (P<0.01).

Phenotypic characterisation of duodenal, jejunal and ileal LPL

In fig. 3 the mean percentage of the lymphocyte subsets in S1 is depicted for day 14 and day 28 p.i.. The results at day 42 p.i. were similar to those at day 28 p.i. and therefore omitted

from the figure. The %CD3+ cells was significantly higher in S3 than in S1 (P<0.01) and S6 (ileum) (P<0.05) (data not shown). This holds for both infected and control animals. The same pattern was observed for the %CD2+, CD4+ and CD8+ cells. On the contrary, the %TCR1+ cells was comparable in S1 and S3 (9.8+/-5.4 and 11.1+/-5.6 respectively) but significantly lower in S6 (7.2+/-4.5; P<0.05).

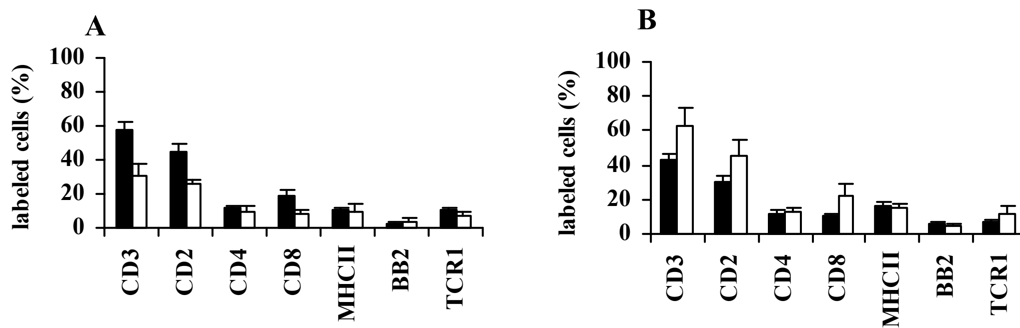

FIGURE 3 Phenotypic analysis of lymphocyte subsets of the lamina propria of the small intestinal segment S1 (duodenum) at day14 (A) and day 28 p.i. (B). Data are shown as mean % positive cells of 8 infected animals + SD (black bars) and of 4 control calves + SD (open bars).

Although no quantitative differences were measured, a negative correlation was present between the %TCR1+ cells and CD4+ cells in S1 (R=-0.62; P<0.1) and in S3 and S6 (R=-0.79 and R=-0.81 respectively; P<0.05) at day 42 p.i. in the infected animals. This was not found for the control animals (data not shown).

Phenotypic characterisation of MLNL

Subset analysis of lymphocytes from the draining lymphnode of S3 revealed no major differences in the T-cell populations. The TCR1+ population was expanded in the infected animals at day 14 p.i. (11+/-4 compared to 7+/-5 and 8+/-3 at day 28 and 42 p.i. respectively).

Comparison of infected and control animals at the different time points revealed significant changes in the BB2+ population and the MHCII+ (P<0.05) (fig. 4). Analysis of the infected animals showed a marked increase in %BB2+ cells at day 28 p.i. compared with day 14 (P<0.1) and day 42 p.i. (P<0.05). At day 28 p.i. the %BB2+ cells in the lymphnode was negatively correlated with the total worm burden (R=-0.79; P<0.05).

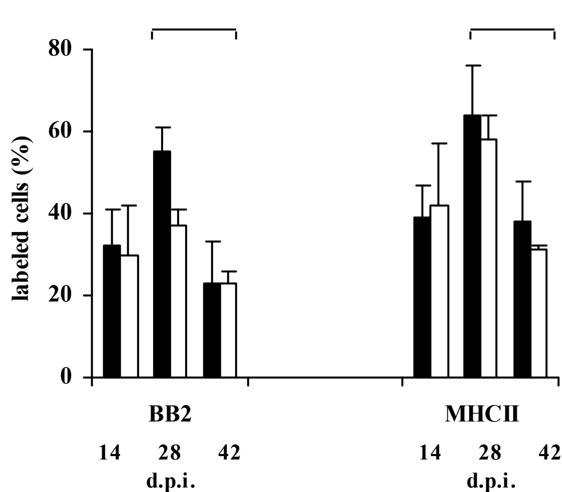

FIGURE 4 Phenotypic analysis of lymphocyte subsets of mesenterial lymph node lymphocytes (jejunum, S3) at day 14, 28, and 42 p.i.. Data are shown as mean % positive cells of 8 infected animals + SD (black bars) and of 4 control calves + SD (open bars). Lines indicate significant differences between groups (P<0.05).

In vitro lymphocyte proliferation to *C. oncophora* antigens

C. oncophora Ad and ESP-specific proliferation was observed in mesenterial lymphnode lymphocytes at day 14, day 28 and day 42 p.i., whereas proliferation in response to L3 was only detected at day 28 p.i.. Stimulation indices of the response to *C. oncophora* Ad are depicted in table 2. An increased SI was measured at day 28 p.i. with ESP and Ad in all infected animals, while 6 out of the 8 infected animals showed an increased response to *Cooperia* L3. At day 42 p.i. only 2 infected animals had an increased response to Ad antigen and 3 animals to ESP. Proliferation was always higher in response to ESP (range SI: 14-12,238) compared with Ad (range SI: 5-330) and L3 antigen (range SI: 4-2,053) (data not shown).

TABLE 2 Stimulation indices (cpm experimental/cpm medium) after stimulation of mesenterial lymphnode lymphocytes with *C. oncophora* adult antigen (10 µg/ml). At each time point data from 8 infected and 4 control animals are depicted (nd=not done).

Calves	Days post infection		
	14	28	42
Infected	2	76	0
	1	112	2
	1	86	1
	5	73	1
	1	330	1
	1	8	1
	2	77	11
	1	28	5
Control	3	2	1
	1	1	1
	1	18	nd
	1	9	2

Quantification of eosinophils

The number of eosinophils was counted in the proximal jejunum (S2) at the different time points after infection. At day 42 p.i. the infected animals had a significantly higher number of eosinophils compared with the control animals (P<0.01) (fig. 5A). Moreover, the number of eosinophils of infected animals necropsied at day 42 p.i. were significantly higher than in the infected animals necropsied at day 14 and day 28 p.i. (P<0.05).

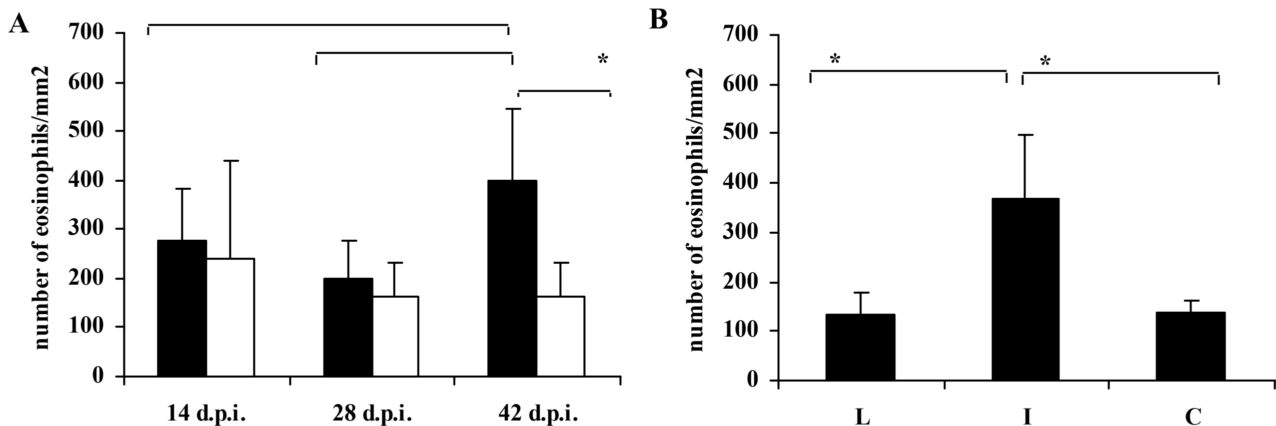

FIGURE 5 The mean number of eosinophils (+SD) in infected (n=8, black bars) and control (n=4, open bars) animals is depicted for day 14, 28 and 42 p.i.(A) and for low responders (L), intermediate responders (I) and control animals (C) (B). Lines indicate significant differences between groups (P<0.05, * P<0.01).

Analysis of the eosinophil numbers of the different responder types (day 42 p.i.) revealed that the increased number of eosinophils was caused by a higher number in the intermediate responders, while in the low responders the number of eosinophils was comparable with the control animals (fig. 5B).

At day 42 p.i. the increased number of eosinophils was negatively correlated with the sex ratio in S2 (R=-0.69, n=8, P<0.1) and the ratio EPG (R=-0.77, n=8, P<0.05) (fig. 6). Moreover, a positive correlation was observed with the % of CD4+ cells in the corresponding mesenterial lymphnode (R=0.76, P<0.05) (data not shown).

In order to investigate whether changes were induced in the distal gut during expulsion, we assessed the number of eosinophils in S6. Overall the number of eosinophils was higher compared with the proximal gut but there were no significant differences between infected and control animals. Nor were there differences between the low and intermediate responders (mean +/-SD: 1,008+/-340 and 969+/-336 respectively).

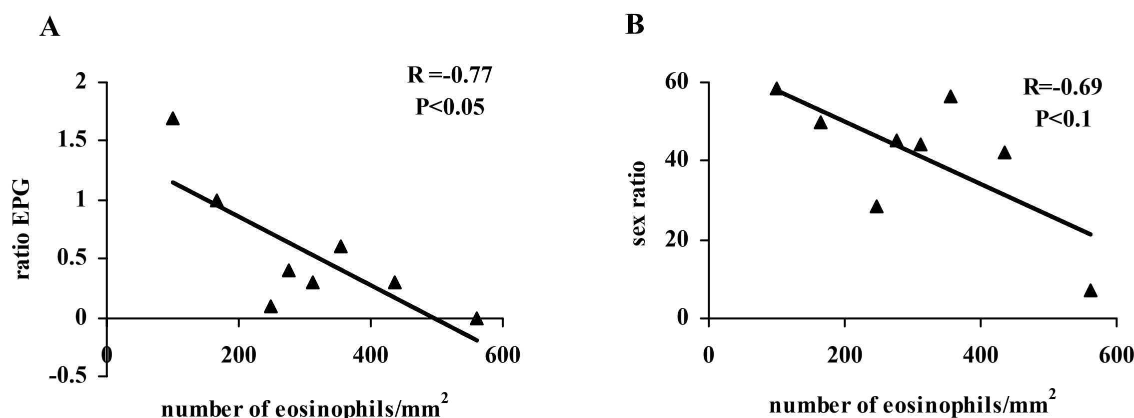

FIGURE 6 Correlations between the number of eosinophils/mm^2 in S2 and ratio EPG (A) and sex ratio in S2 (B) for the infected animals at day 42 p.i..

Quantification of mast cells

There were no significant differences between infected and control calves at any time point, and the total number of mast cells between the responder types did not differ. A significant positive correlation was measured between the number of mast cells in the LP and the total serum IgE response [123] at day 42 p.i. (R=0.71, n=10, P<0.05) (data not shown). The same trend was present with the total number of mast cells (R=0.43 and n=9) and the number of mast cells in the SM (R=0.45 and n=10), though not significant.

C. oncophora-specific Ig responses in the intestinal mucus

Exposure to a primary *C. oncophora* infection with 100,000 L3 larvae resulted in a gradual increase in *C. oncophora* Ad-specific mucus IgA and IgG1 in S2. The *C. oncophora*-specific IgG1 and IgA titres at day 42 p.i. were significantly higher than at day 28 and at day 14 p.i. (both P<0.05). Only a slight increase in mucus IgG2 was observed in some infected animals. In order to assess the Th2 phenotype of the local humoral response the IgG1/IgG2 ratio was calculated. At day 42 p.i. this ratio was higher than at day 14 and day 28 p.i. (9.3+/-6 versus 2+/-0.3 and 3+/-0.2). Low responders had a maximal ratio of 2, while in the intermediate responders the ratio was always higher than 5.

For both parasite-specific mucus IgA and IgG1 the intermediate responders had significantly elevated antibody levels compared with the low responders and the control animals (P<0.05) (fig. 7).

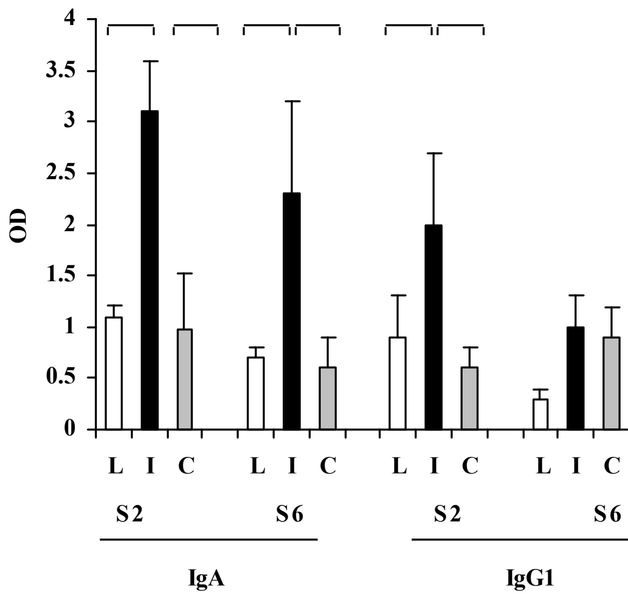

FIGURE 7 Mean OD (+SD) of *C. oncophora* Ad mucus IgA and IgG1 in S2 and S6. Low responders (L; open bars), intermediate responders (I; black bars) and control calves (shaded bars). Lines indicate significant differences between groups (P<0.05).

No correlations were observed between *C. oncophora*-specific mucus IgG1 or IgG2 and parameters suggesting worm expulsion. However, the mucus IgA in the infected calves was negatively correlated to the ratio EPG (R=-0.85, n=8, P<0.05) and the number of eggs per female worm in S2 (R=-0.85, n=8, P<0.05) (fig. 8). A similar negative trend was observed between *C. oncophora*-specific mucus IgA and the sex ratio (R=-0.56, n=8) as well as the worm burden in S2 (R=-0.56, n=8). In S2 the increase in IgA but not in IgG1 concurred with the rise in eosinophil numbers (R=0.65, n=12, P<0.05).

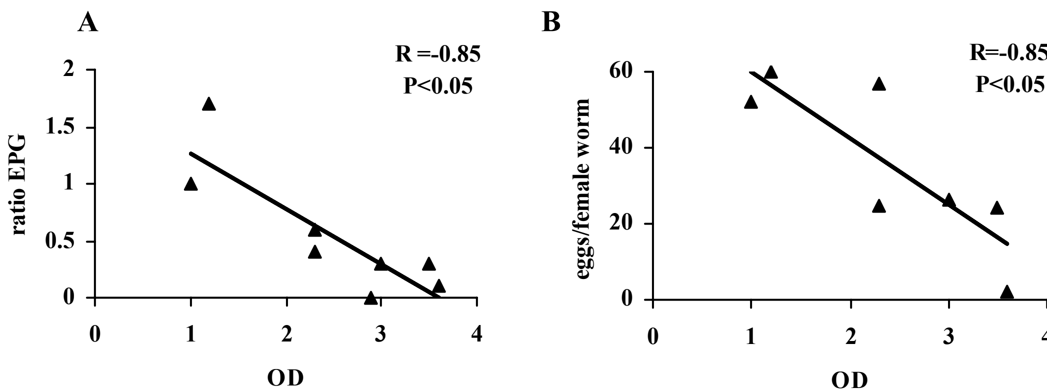

FIGURE 8 Correlations between *C. oncophora* Ad-specific mucus IgA in S2 and ratio EPG (A) and number of eggs/female worm (B) for the infected animals at day 42 p.i..

Both *C. oncophora* Ad-specific mucosal IgA and IgG1 levels during the infection were significantly positively correlated with the *C. oncophora* Ad serum IgA and IgG1 response, respectively (IgA: R=0.63, n=36, P<0.001 and IgG1: R=0.69, n=36, P<0.001).
The humoral response in S6 was assessed to evaluate whether the response in the proximal and the distal segment would be similar and whether the presence of a higher worm burden in the distal segment in intermediate responders would induce a rise in *C. oncophora* specific titres. Both *Cooperia*-specific IgA and IgG1 were increased in S6 in intermediate responders compared to low responders though at a lower level than in S2 (fig. 8). The *Cooperia*-specific mucus IgA levels in both segments were positively correlated. This did not hold for the *Cooperia*-specific mucus IgG1 levels. No difference was found in the mucus IgG2 in S6 between infected and control animals. A significant negative correlation was found between mucus IgA in S6 and the ratio EPG (R=-0.71; P<0.05). A trend was also observed for IgG1 (R=-0.68; P<0.1). No correlations were observed with the total and segmental worm burden. Analysis of variance revealed no relationship between the antibody titres and the eosinophil numbers in the distal gut.

Total IgE in mucus

We could detect total IgE in the mucus of S2 of the infected animals, however within-group variation was high. At day 14 p.i. total IgE levels ranged from 44 to 2464 mU/ml, at day 28 p.i. from 46 to 2169 mU/ml and at day 42 p.i. from 38 to 3847 mU. The observed variability could not be related to any parameter of infection and no differences were measured between the different responder types. Moreover, in some of the control animals high IgE levels were detected.

DISCUSSION

This experiment was carried out to study the dynamics of both parasitological and immunological events occurring in the gut of *C. oncophora* infected calves and which eventually result in clearance of the parasite from the gut. An infection of 100,000 L3 larvae was chosen as this protocol has been studied most extensively [3, 84, 176, 231] and induces protective immune responses. Moreover, based on the speed by which the parasite is expelled from the host, infected animals can be subdivided in high, intermediate and low responders [127]. The difference between susceptible and resistant calves is further emphasised by a different recognition of *Cooperia*-specific proteins after Western blot analysis [231]. We investigated the contribution of both the cellullar and humoral component of the immune response to the rejection of the parasite and analysed the results in view of the parasitological response to the infection.

Segmental division of the small intestine revealed that during the patent phase of the infection the parasite preferentially resides in the first 6 meters of the intestine. Generation of immunity in intermediate responder animals induced migration of the adult worm population to the distal part of the intestine. Armour et al. [9] described a similar phenomenom in animals trickle infected with *C. oncophora* during twelve weeks, however, their infection protocol did not allow discrimination between susceptible and resistant calves. At day 42 p.i. a decreased fecundity was found in the worms of the proximal gut which was caused by a lower fecundity in the intermediate responders. In the other animals the fecundity was comparable to day 28 p.i., the peak in egg output. Interestingly, the fecundity of the worms in the distal segment was not reduced and always higher than the fecundity of the worms in the proximal segment. Reduced fecundity has been implicated as a major regulatory force for gastrointestinal populations in sheep but this was a derivative of worm length [204, 209, 211]. In our study no differences in worm length were observed (data not shown). Our fecundity and localisation data suggest that part of the effector mechanisms/immune responses act on the egg laying machinery and part on the expulsion of the worms from their niche. On the other hand, vital worms which are still producing eggs, could escape the detrimental environment of the immune proximal gut.

The results presented in this study demonstrated that infection did not induce the recruitment of a specific lymphocyte subset in the intestinal mucosa. We could possibly have missed a transient early increase or decrease in one of the lymphocyte subsets as our analysis was restricted to a few time points after infection. Infection of 4-month-old calves with *Ostertagia ostertagi* did induce an influx of Ig- bearing cells, TCR1+ cells and IL-2R+ cells in both the abomasal mucosa and the draining lymphnodes [4]. Similar findings were done by Balic et al. [14] after infection of sheep with *Haemonchus contortus,* however, in this experiment the increase was not sustained until the adult infection. This can possibly be explained by the observation that both nematodes are more pathogenic and invasive. We do not exclude that an incoming *Cooperia* infection is associated with the same kinetics early after infection, but, based on our results, we predict that the patent phase of the infection as well as the expulsion of *C. oncophora* are not associated with a specific recruitment of lymphocytes in the intestinal mucosa.

Similarly, calves exposed to a primary *C. oncophora* infection showed no major changes in the T lymphocytes subsets of the mesenterial lymphnode. The increased number in TCR1+ cells in the infected animals at day 14 and day 28 p.i. supports the hypothesised role attributed to γδ T-cells in directing the immune profiles during a primary infection [79]. An exact role for this cell population in nematode infections is still not clarified. It has been proposed that expansion of this subset is associated with the rejection of *Trichostrongylus colubriformis* in sheep [154]. This is not likely in our experimental model as by day 42 p.i. the

expansion of this population is gone. Twenty-eight days after infection we observed a significant increase in both BB2+ and MHCII+ cells. Recent data have shown that B cells are recquired for a succesfull priming of a rapid Th2 response in *T. muris* infected mice [28]. In that model the B cell involvment in protective immunity could be through the role of accessory antigen presenting cells or via antibody production. In the current study the increase in B cell numbers coincided with the onset of antibody production both in the serum and the mucus of the infected animals.

Histological analysis of S2 revealed a significant influx of eosinophils in the lamina propria of the infected animals at day 42 p.i.. Eosinophil recruitment is known to be dependent on cytokines secreted by CD4+ Th2 cells. The positive correlation observed with the percentage CD4+ cells in the mesenterial lymphnode lymphocytes and the eosinophil numbers in the gut, fits within this hypothesis. The eosinophilia was more pronounced in the intermediate responders and significantly negatively correlated with the sex ratio and with the ratio EPG which suggests a role for eosinophils in the rejection of the adult worm population from the gut. The finding is surprising in that until now during primary nematode infections eosinophils have mainly been associated with expulsion of larval stages rather than adults. Increased number of eosinophils was seen during primary infection of sheep with *H. contortus* [45], *T. circumcincta* [213] and *N. battus* [245]. Only during a primary infection with *N. battus* the increased number of eosinophils concurred with the time of adult nematode expulsion [245]. In our study, at day 14 p.i. the number of eosinophils was slightly but not significantly higher in the infected animals compared to the control animals. This moderate eosinophil response might have been in response to the larvae. Analysis of S6 in the animals slaughtered at day 42 p.i. showed only a slight increase in the infected calves compared to the controls. This indicates that the immune reaction of the host was not disseminated throughout the whole small intestine and that the eosinophil response was specifically located in the proximal gut, at the site of infection.

Mast cell numbers showed a high individual variability which could not be related to the infection status of the animals. Presumably this cell population is not involved in the regulation of a primary *C. oncophora* infection. Although in this study the analysis was restricted to enumaration of the number of cells without investigation of their activation status, several studies in sheep [114, 116, 72] and rodents [104, 168] demonstrate that resistance to nematode infection and expulsion of parasites can be both mast-cell dependent or independent, depending on the host-parasite system studied.

Exposure to a single dose infection of 100,000 L3 *C. oncophora* larvae resulted in a gradual increase in *C. oncophora* Ad-specific IgA and IgG1 and an increased IgG1/IgG2 ratio in the infected animals. At day 42 p.i. the intermediate responders had significantly higher *C. oncophora* mucus IgG1 and IgA than the low responders. This was also found in serum [127],

but in all animals mucus IgA was higher than IgG1, while in serum we observed the opposite. The possible effector role of IgA at mucosal surfaces seemed confirmed by the significant negative correlation with the ratio EPG and the number of eggs/female. The rise in mucus IgA but not IgG1 concurred with the increased number of eosinophils in S2. A possible interaction between both has been postulated previously [60]. IgA/antigen complexes could bind through the Fc-alpha receptor on eosinophils and provoke the release of anti-inflammatory mediators and cytokines which have a detrimental effect on the worm population. Upregulation of Fc receptors and complement receptors on the eosinophil membrane occurs after activation. Moreover, it has been demonstrated that sIgA at mucosal surfaces has increased ability to activate eosinophils compared to serum IgA [166]. Based on our results, we hypothesise that both eosinophils and mucus IgA are involved in an effective immune response against adult *C. oncophora*. The efficacy of the immune response in reducing the level and the fecundity of the parasite burden is clear in the intermediate responders. The parasitological phenotype of these animals can be expanded with the immunological features which are characterised by high numbers of eosinophils and high parasite-specific IgA titres at the time of parasite rejection.

AKNOWLEDGEMENTS

This research was supported by the Technology foundation STW, applied science division of NWO and the technology programme of the Ministry of Economic Affairs. The authors would like to thank their colleagues from the laboratory of Parasitology for the technical assistance during the necropsies. We would also like to thank Dr. M. Eysker and Prof. A. W. C. A. Cornelissen for the critical reading of the manuscript.