The effect of truncated infections with *Ostertagia ostertagi* on the development of acquired resistance in calves

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Abstract

The relative contribution of the third (L_3), fourth (L_4) and adult stages of *Ostertagia ostertagi* to the development of immunity was assessed in calves which were either continuously infected during 21 weeks or subjected to infections truncated by anthelmintic treatment at the L_3 or L_4 stage. A fourth group remained uninfected (control group). Faecal samples and blood samples were collected weekly for faecal egg counts and determination of pepsinogen and antibody levels. Only the continuously infected animals showed positive egg counts, which fell towards the end of the primary infection period. Pepsinogen and antibody levels remained high in the continuously infected group until the end of the primary infection period. At that time, they were significantly higher compared to the control calves, with intermediate values in the truncated infection groups. After the 21 weeks primary infection period all animals were dewormed. To evaluate the protection provided by the different immunisation protocols, all animals were challenged 1 week later with 156 000 *Ostertagia* L_3, spread over 12 consecutive days. The marked reduction in egg counts following challenge infection indicated a certain degree of immunity in the continuously infected calves, which was confirmed at necropsy by the reduced worm burdens, the high percentage of inhibited early L_4 larvae, the reduced size of the adult worms and the higher numbers of mucosal mast cells in this group. Numbers of globule leucocytes and eosinophils were not significantly different from the control group. Infections truncated by anthelmintic treatment elicited poor development of immunity as shown by the egg output after the challenge infection

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and the percentages of arrested larvae and the lengths of adult worms which were intermediate to those of the continuously infected calves and control animals.

1. Introduction

Ostertagia ostertagi is the most important gastrointestinal cattle nematode in temperate regions. Naturally acquired immunity to *Ostertagia* develops slowly and is not complete. Signs of developing immunity, such as reduced fecundity, stunted growth, retardation and arrest of development of the parasites, adult worm loss and, finally, resistance to establishment of larvae, are most evident only after the second grazing season or after prolonged or repeated experimental infections (Klesius, 1993). Control of gastrointestinal nematodes in ruminants is still largely based on the use of anthelmintic drugs. Since development of immunity to *Ostertagia* is difficult and requires an intensive long-term host–parasite interaction, care should be taken not to interfere with this process. Protection of first year calves against gastrointestinal nematodes should be balanced against allowing cattle to acquire immunity, considering the possible inverse relationship between the intensive use of modern anthelmintics and the build-up of immunity to gastrointestinal nematodes (Vercruysse et al., 1994). In recent years, more attention has been given to the development of alternative control methods (e.g. vaccination) which would overcome disadvantages like interaction between chemoprophylaxis and immunity, drug residues and the development of anthelmintic resistance. However, attempts to vaccinate calves against *O. ostertagi* have been unsuccessful so far (Bürger et al., 1968; Williams et al., 1974; Herlich and Douvres, 1979; Herlich and Tromba, 1982; Hilderson et al., 1995). To search more efficiently for future vaccines and to develop integrated control systems that allow acquisition of immunity, a better understanding of the development of immunity to *O. ostertagi* is required. The present experiment was carried out to examine the relative importance of the contribution of third larval (L_3), fourth larval (L_4) and adult stages to the development of acquired immunity to *Ostertagia*.

2. Materials and methods

2.1. Experimental design

Twenty-four helminth-free Holstein bull calves, 5–8 months old, were housed in individual pens and fed corn silage ad libitum and soya bean concentrate. Four groups of six calves were formed by blocked randomisation based on age. Group 1 was the uninfected control group, while groups 2, 3 and 4 were infected with *O. ostertagi* L_3 larvae for 21 weeks. In group 2 (L_3 truncated group) a weekly infection dose of 20 000 L_3 was followed by a treatment with oxfendazole (9 mg kg^{-1} body weight, BW) 4 days later, removing the *Ostertagia* population before the L_4 stage. The animals of group 3

(L_4 truncated group) were given 20 000 infective larvae every other week, followed by a treatment with oxfendazole 9 days after each infection, preventing the worm population from developing to the late L_4 stage. In group 4 (continuous infection group) weekly doses of 20 000 L_3 were spread over 3 days. These animals were not treated, allowing the larvae to mature to adult worms. After the 21-week primary infection period (days 0–144), all animals were dewormed with oxfendazole (9 mg kg^{-1} BW) on 3 consecutive days (days 147–149). To evaluate protection induced by the different immunisation protocols, all calves were challenged 1 week later with 156 000 *Ostertagia* L_3, spread over 12 consecutive days. For the first 3 days of the challenge period the calves received 4000 L_3 per day as a single dose. From day 4 to day 6 they were given 4000 L_3 twice daily. From day 7 to day 9 the animals were infected with 16 000 L_3 per day, divided into three doses, and during the last 3 days they were challenged with four doses of 6000 L_3 per day. On days 189 and 190 (35 and 36 days after the first challenge infection) all calves were necropsied.

Faeces for parasitological examination and blood for biochemical and serological analysis were collected weekly. Extra samplings were carried out after treatment and challenge and at necropsy.

2.2. Parasite strain

The strain of *O. ostertagi* was initially isolated from a commercial dairy farm in 1987 and was stored in liquid nitrogen until 1993. Thereafter it was maintained in the laboratory by passage through parasite-naive calves. Third stage larvae were collected from faecal cultures by the Baermann technique and stored at 10°C until further use. Larvae administered to the calves were less than 10 weeks old.

2.3. Parasitological techniques

The faecal egg output was estimated using a modified McMaster technique (Thienpont et al., 1979) with a sensitivity of 50 eggs per gram (EPG). If negative, a flotation technique was used (sensitivity 25 EPG). Geometric group means of EPG were calculated.

Necropsy, abomasal washings and abomasal digests (HCl–pepsin) were carried out according to standard techniques (Ministry of Agriculture, Fisheries and Food, 1986; Ritchie et al., 1966). In total, 2% of the worm burden was counted, and geometric group means of worm counts were calculated. Adult *O. ostertagi* worm lengths were measured ($n = 50$ per animal).

2.4. Serological parameters

Jugular vein blood samples were collected with vacutainer tubes and sera were stored at −20°C until analysis. Serum pepsinogen concentration was determined according to Berghen et al. (1987) and expressed in milli-units (mU) of tyrosine.

Ostertagia IgG antibodies against crude L_3, L_4 and adult antigen extracts (4 μg ml^{-1}) were determined in an enzyme-linked immunosorbent assay. Bovine sera were

administered at one fixed serum dilution (1:400) in phosphate buffered saline (pH 7.2, 0.5% Tween-20) in duplicate. Anti-bovine IgG coupled to horseradish peroxidase was used as a conjugate and o-phenylenediamine 0.1% in citrate buffer (0.038 M, pH 5.0) served as peroxidase substrate. Values were expressed as mean optical density.

2.5. Histology

Abomasal tissue for histological examination was taken at necropsy before washing of the abomasum. For mucosal mast cell counts tissue samples were fixed in Carnoy's fixation fluid at 4°C. Six hours later the fixation fluid was replaced by 70% alcohol. Paraffin sections were stained with toluidine blue (pH 0.5). Globule leucocytes and eosinophils were enumerated after fixation in 4% paraformaldehyde at 4°C (replaced by 70% alcohol after 6 h) and staining with carbol chromotrope (Lendrum, 1944). All cell counts were performed on 10 graticule fields with a total surface of 2 mm^2.

2.6. Statistical analysis

Data are presented as arithmetic mean ($\pm$ SEM) or geometric mean ($\pm$95% confidence limits) values. Firstly, we examined two a priori hypotheses: indicators of worm fitness, except percentage inhibition (i.e. total worm burden, size and egg output), should decline with increasing initial experience of infection (duration of immunising infection, i.e. group 1 > group 2 > group 3 > group 4); and indicators of hosts' cellular responses (mastocytosis, eosinophilia, globular leucocyte counts) and percentage inhibition of worms should increase with increasing experience of infection (i.e. group 1 < group 2 < group 3 < group 4). However, it was also possible that immunity might be elicited after a certain threshold of infection was exceeded and in this respect our a priori prediction was that the group experiencing the longest immunising infection might differ from challenge controls in so far as total worm burdens and adult worms would be smaller (group 1 > group 4) and indicators of hosts' cellular responses greater (group 1 < group 4). All a priori predictions were examined by the specific one-way ANOVA by ranks (Meddis, 1984; Barnard et al., 1993) using a University of Nottingham computer program, and z scores are given as relevant. When a priori predictions were not possible (pepsinogen and antibody levels), we applied a general one-way ANOVA by ranks, to determine whether there was a significant difference across the four experimental treatment protocols (referred to as experimental group effect) and the parameter H is given. Probability (P) values ≤ 0.05 were considered to indicate significant differences.

3. Results

3.1. Faecal egg counts

Faecal egg counts (Fig. 1) remained negative in groups 1, 2 and 3 during the primary infection period, with the exception of a few animals which occasionally had low egg
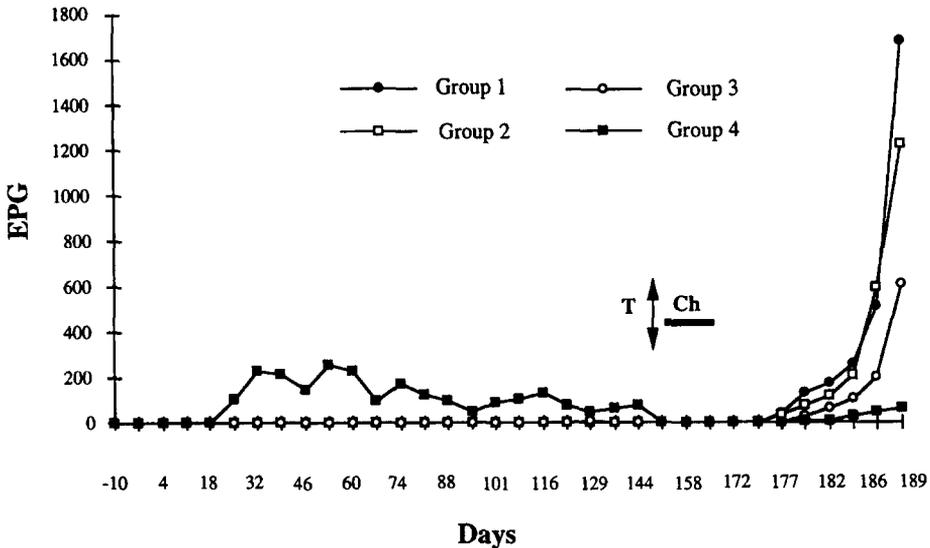
Fig. 1. Geometric mean faecal egg counts of groups 1–4. T, treatment. Ch, challenge infection.

counts, always < 50 EPG (group 1: one calf positive on days 116 and 144, one on day 136; group 3: one calf positive on day 123, four on day 129). The animals in group 4 began to shed strongyle eggs on day 25 (EPG = 102). The highest egg counts were observed during the first 2 months of the experiment with geometric means varying from between 143 EPG (day 46) to 256 EPG (day 52). Thereafter egg output fell (geometric mean egg counts between 48 and 172 EPG) until treatment, after which all animals ceased shedding eggs.

After challenge infection egg counts rose quickly in the control group and in both the truncated infection groups (groups 2 and 3) but remained low in the continuously infected group ($\leq$ 60 EPG). At necropsy on day 189, the geometric mean egg counts were 1670, 1223, 608 and 60 EPG for groups 1, 2, 3 and 4, respectively. Statistical analysis of these data, testing the a priori prediction that EPG in group 1 > group 2 > group 3 > group 4 gave $z = 3.79$, $P < 0.0001$.

3.2. Worm burdens

A geometric mean worm burden of 92 335 *Ostertagia* was recovered from group 1 at necropsy, representing a challenge infection take of 59% (Table 1). There was no apparent reduction in worm counts in groups 2 and 3 (mean worm burdens of 91 454 and 94 540, respectively) but group 4, the continuously infected group, harboured a mean of only 67 035 worms, an apparent reduction of 27.4% compared to group 1. However, the predicted relationship of declining worm burdens with increasing experience of infection was not borne out by statistical analysis ($z = 1.54$, $P =$ NS, not significant). The lower geometric mean count in group 4 suggested a threshold effect, but a direct comparison

Table 1
Geometric mean *Ostertagia ostertagi* worm burdens, percentage of inhibited L_4 larvae, arithmetic mean lengths of adult worms and arithmetic mean cell counts of groups 1–4

Group number	Total worm burden	% L_4	Length (mm)		Mast cells	Cell counts	
			Female	Male		Globule leucocytes	Eosinophils
1	92335	41	7.06	5.94	39	12	65
2	91454	49	6.87	5.80	25	2	66
3	94540	57	6.89	5.88	43	3	66
4	67035	73	6.45	5.63	73	7	34

of group 4 with group 1 confirmed that there was no significant difference (For group 4 < group 1, $z = 1.6$, $P = NS$).

3.3. Inhibition of L_4 larvae and size of adult worms

The percentage of L_4 larvae (Fig. 2 and Table 1), reflecting inhibition of developing worm burdens, showed a highly significant increase with increasing experience of infection (For a priori hypothesis group 1 < group 2 < group 3 < group 4, $z = 4.22$, $P < 0.0001$).

There was also a strong effect on the size attained by adult nematodes (Fig. 3 and Table 1). The length of both male and female worms declined significantly with increasing experience of infection (female worms, $z = 3.38$, $P < 0.0001$; male worms, $z = 2.21$, $P = 0.0136$).
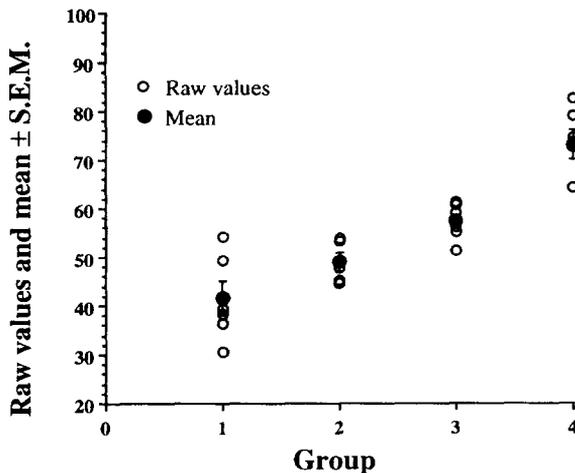

Fig. 2. Individual and group mean percentages of inhibited *O. ostertagi* L_4 larvae.

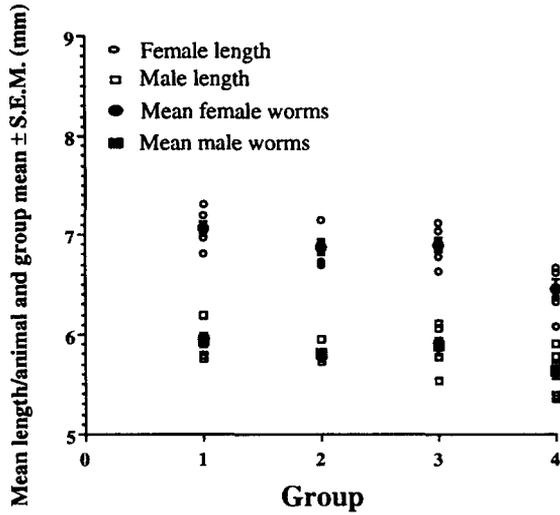Fig. 3. Female and male adult *O. ostertagi* worm lengths per animal, and group means.

3.4. Serological parameters

At the outset of the experiment the pepsinogen levels of all animals were low, with group means between 416 mU and 455 mU tyrosine (Fig. 4). In groups 1, 2 and 3 pepsinogen levels remained low during the whole primary infection period with the exception of a small increase between day 120 and 140 in group 3. In contrast, pepsinogen levels in the continuously infected calves (group 4) rose quickly from day 18
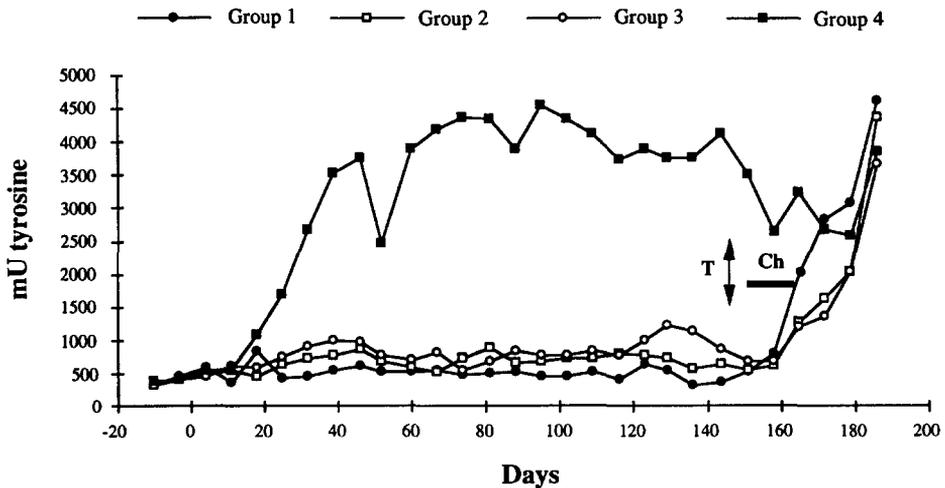

Fig. 4. Mean pepsinogen concentrations of groups 1–4. T, treatment. Ch, challenge infection.

onwards to reach a plateau between 3519 mU and 4546 mU tyrosine from day 39 until treatment. Individual values as high as 7104 mU tyrosine were observed. Statistical analysis of the values on day 144 (just before administration of anthelmintic) by a general test (one-way ANOVA) indicated that there was a highly significant experimental group effect ($H = 16.949$, $P = 0.001$). After treatment with anthelmintic the pepsinogen levels in group 4 fell to 2643 mU tyrosine, whereas the other groups remained
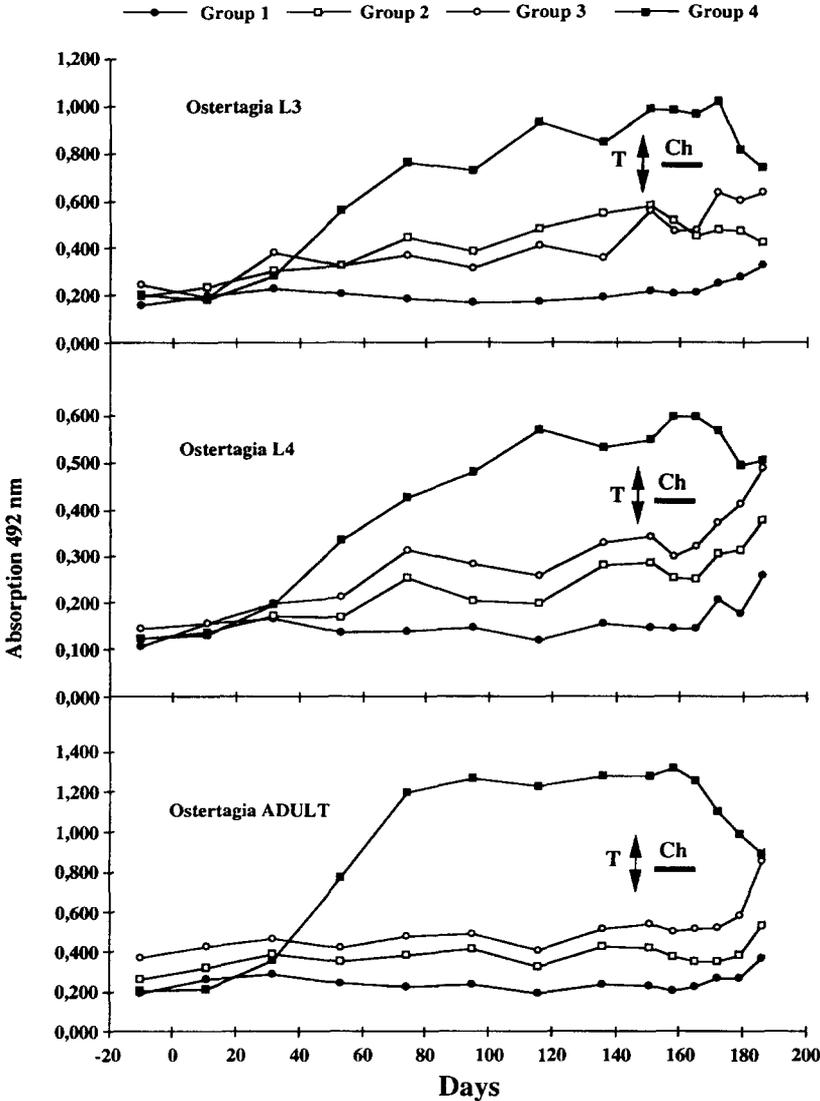

Fig. 5. Mean IgG antibodies of groups 1–4 against *O. ostertagi* L_3, L_4 and adult stage antigens, expressed as optical density at 492 nm. T, treatment. Ch, challenge infection.
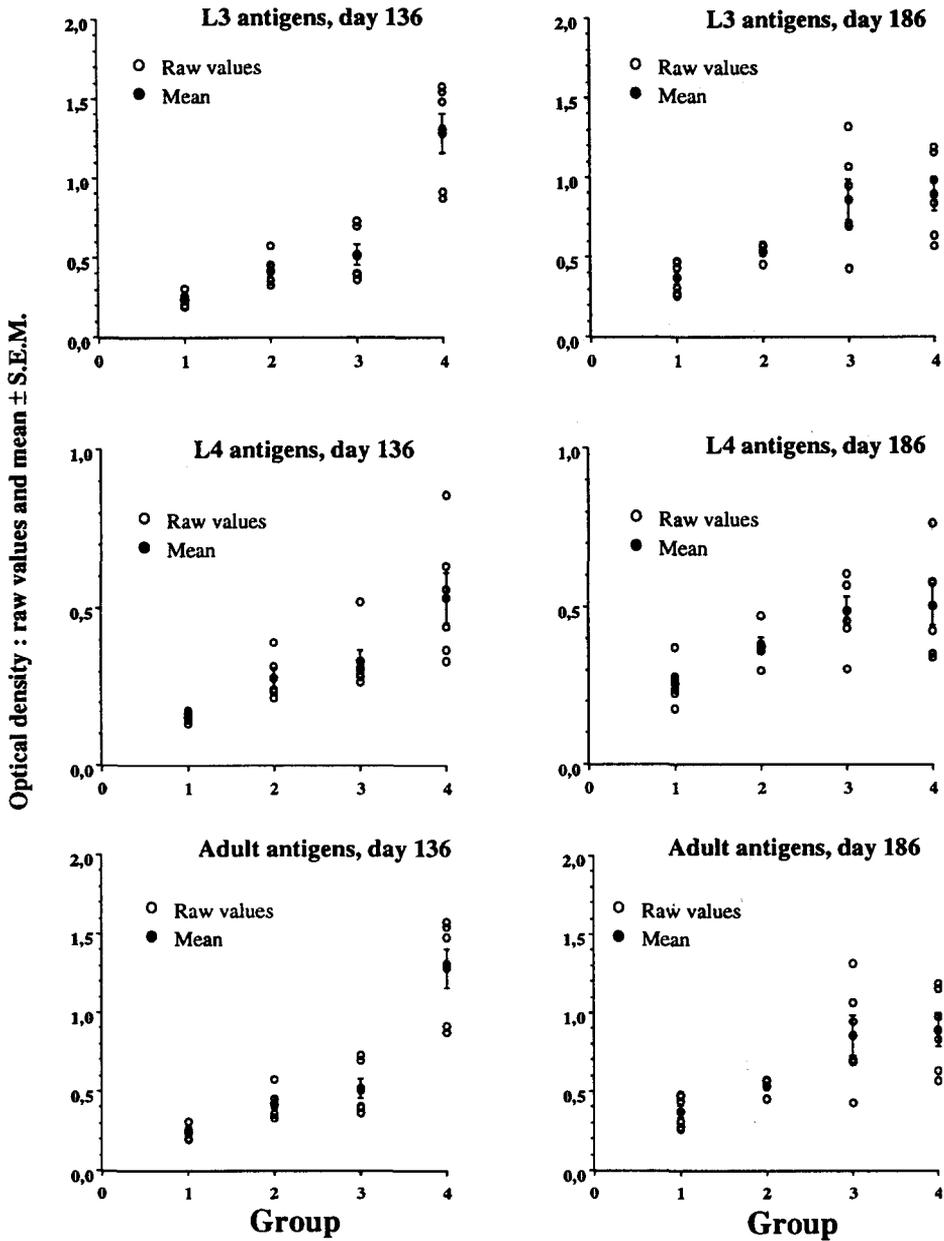
Fig. 6. Individual and group mean IgG antibodies of groups 1–4 against *O. ostertagi* L_3, L_4 and adult stage antigens on days 136 (end of primary infection period) and 186 (after challenge infection), expressed as optical density at 492 nm.

unaffected. Following challenge infection pepsinogen levels rose most rapidly in the challenge control group (group 1), to a maximum of 4617 mU tyrosine shortly before necropsy (day 186). The levels also increased in groups 2 and 3 but more slowly achieving maxima of 4368 and 3668 mU tyrosine, respectively (day 186). In group 4 pepsinogen levels rose initially to 3234 mU tyrosine, but this was followed by a reduction to 2585 mU tyrosine, and then a small increase to reach the pre-treatment level by day 186 (3834 mU tyrosine). The values recorded on day 186 were analysed by a general test (one-way ANOVA) and this revealed that there was no significant experimental group effect at this time ($H = 3.35$, $P = $ NS).

Antibodies (IgG) against the three stages of *O. ostertagi* remained at a constant low level in group 1 animals throughout the initial period when the other groups were being immunised. The continuously infected animals in group 4 showed the most marked responses to all three antigens (Fig. 5 and Fig. 6). Antibody concentrations against adult antigen increased rapidly from day 32 onwards and reached a plateau on day 74. Those against L_3 and L_4 antigens increased more slowly, reaching a plateau by about day 116. The animals receiving the truncated infections (groups 2 and 3) showed intermediate responses. On day 136, shortly before administration of anthelmintic, there was a significant experimental group effect in respect of all three antigens (general test by one-way ANOVA: for L_3, $H = 19.047$, $P = 0.001$; for L_4, $H = 17.37$, $P = 0.001$; for adult, $H = 19.047$, $P = 0.001$). After treatment and challenge infection, the antibody concentrations to all three antigens fell in group 4 animals. Responses to L_4 and adult worm antigens rose in group 1, 2 and 3 animals, whereas the response to L_3 antigen rose in group 1 and group 3 animals but fell slightly in group 2. Statistical analysis by the general test (one-way ANOVA) of antibody concentrations in sera collected on day 186 indicated that there was still a significant experimental group effect (for L_3, $H = 14.067$, $P = 0.003$; for L_4, $H = 11.871$, $P = 0.008$; for adult, $H = 14.067$, $P = 0.003$).
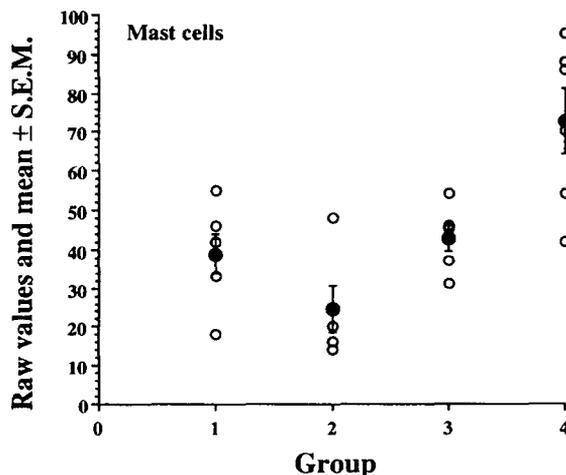

Fig. 7. Individual and group mean mast cell counts of groups 1–4.

3.5. Cellular responses

Mucosal mast cell, globule leucocyte and eosinophil responses were quantified and the group mean numbers per graticule field are presented in Table 1. The most intense mastocytosis was observed in group 4 animals, and least cells were evident in group 2. Although mastocytosis increased significantly with increasing experience of infection (Fig. 7; $z = 2.49$, $P = 0.0064$), as predicted, the relationship arose mainly as a consequence of the strong response of group 4 compared with the other groups. As can be seen from Fig. 7, there was little difference between groups 1, 2 and 3 in respect of mast cell counts.

No such relationships were evident for globule leucocytes ($z = -0.49$, $P =$ NS) or for esinophil counts ($z = -1.09$, $P =$ NS). The data suggested that both globule leucocyte and eosinophil counts were lower in animals experiencing truncated or full immunising infections compared with challenge control animals, but these were not statistically significant.

4. Discussion

The results demonstrated that trickle infections with 20 000 L_3 per week for 21 weeks partially immunized calves against a heavy larval challenge, as shown by the faecal egg counts and the necropsy findings. The reduced worm burdens (although not significant), the smaller size of the adult worms and the high percentage of inhibited early L_4 larvae, together with the reduced egg output are widely accepted criteria for evaluating acquisition of resistance (Klesius, 1988). The results of the histological examination of the abomasal mucosa are not so straightforward. The abomasal sections of the continuously infected calves showed higher numbers of mucosal mast cells than the sections of the other groups. Both mast cells and globule leucocytes are associated with immunity to gastrointestinal parasites of several species, including ruminants (Miller, 1984), and their accumulation has been described previously in *Ostertagia* infected cattle (Ritchie et al., 1966; Murray et al., 1970; Snider et al., 1981; Wiggin and Gibbs, 1987). This is in contrast with the globule leucocyte counts in the present experiment where the highest number was found in the control, previously uninfected, group, although there was considerable variation within animals of all groups. Since, in ruminants, globule leucocytes have been shown to be the degranulated end-stage of mucosal mast cell maturation (Huntley et al., 1984), the presence of these cells in the control group suggests that the extended challenge regime employed was sufficient to induce a local mast cell response. Such a response to the challenge may have ameliorated differences in the tissue mast cell status which existed immediately after the primary infection regimes. Mean counts of eosinophils tended to be lowest in the continuously infected group 4. This corresponds with other data suggesting that *O. ostertagi* itself is partly responsible for the eosinophil accumulation in the abomasum of infected cattle, by means of a chemotactic factor present in excretory/secretory products (reviewed by Klesius, 1993) but, as for globule leucocyte counts, differences between groups were not statistically significant and the present results highlight the difficulties

in assessing retrospectively the events of worm immunity and inflammatory cell responses by histological analysis.

The build-up of immunity in the continuously infected calves was predicted by the high pepsinogen and antibody levels at the end of the trickle infection period (days 144 and 151, respectively). Ploeger et al. (1994, 1995) demonstrated that *Ostertagia* antibody titres and pepsinogen levels at the end of the first grazing season or early in the housing period are valuable tools for estimating levels of exposure to infection in the first grazing season. Further, previous work showed that the build-up of immunity in the first grazing season can only be achieved if there is sufficient larval challenge (Ploeger et al., 1990). Therefore, the levels of antibodies and pepsinogen after the primary infection period could be used indirectly to predict whether or not animals had acquired immunity. In contrast, in an experiment with an identical infection schedule for 18 weeks (Hilderson et al., 1993), a decrease of pepsinogen levels from day 67 onwards, indicating that fewer worms reached the adult stage, was interpreted as a sign of developing immunity. This was not confirmed by a reduction in worm burdens after challenge, although a higher percentage of worms were inhibited L_4 larvae.

Preliminary signs of immunity developing were also observed in the calves of both truncated infection groups in the present study. Antibody levels at the end of the sensitization period were modestly increased compared to the control group, especially against L_3 or L_4 antigen. Indications that at least a start was made in animals of the truncated groups towards acquired immunity are also given by certain parasitological parameters after challenge infection. Egg output was lower in group 2 (mean 1223 EPG) and in group 3 (mean 608 EPG) than in the control group (mean 1670 EPG) and the percentage of arrested larvae and the lengths of adult worms in the truncated infection groups were intermediate between those of groups 1 and 4. The egg counts and percentage of inhibition suggest that the L_4 truncated group was more immune than the L_3 truncated group, but total worm counts did not confirm this. Several workers have suggested that larval stages are important in the process of development of immunity against gastrointestinal nematodes. Studies with *Haemonchus contortus* (Eysker, 1981) and *O. ostertagi* (Christensen et al., 1992; Monrad et al., 1992) indicated that hypobiotic larvae were involved in the development of immunity. In truncated infection experiments with *Heligmosomoides polygyrus* in mice (Wahid and Behnke, 1992), *Trichostrongylus colubriformis* in sheep (Emery et al., 1992) and *O. ostertagi* in cattle (Gasbarre, 1988) L_4 larvae also have been shown to confer immunity against challenge infections. Therefore the absence of more obvious signs of acquired immunity in group 2 and especially in group 3 animals is somewhat surprising. Possible protective antigens may have been masked or suppressed by others. Suppression of lymphocyte reactivity to phytohaemagglutinin and to concanavalin A in *O. ostertagi* infected calves has been reported (Klesius et al., 1984; Gasbarre, 1986; Cross et al., 1986; Snider et al., 1986; Wiggin and Gibbs, 1989, 1990). Another possibility is that the young adults emerging from the abomasal glands (causing epithelial disruption and inflammation) provide the continuous antigenic stimulus necessary for development of immunity, rather than the arrested larvae per se. This could explain why in the present experiment a reduction in *Ostertagia* worm burden (although not significant) was only observed in calves which maintained elevated pepsinogen levels until the end of the primary infection period,

while in the study of Hilderson et al. (1993) worm burdens were not reduced after a decrease of pepsinogen levels from day 67 onwards.

In the experiments of Christensen et al. (1992) and Monrad et al. (1992) inhibited larvae were present in the hosts for 4 months or more. In the present study, group 3 was subject to ten infections of 9 days, representing only ± 50 days of contact with the L_4 stage. Perhaps the duration of contact with the parasite is equally as important as the parasitic development stage (Emery et al., 1992). The continuous infection in group 4 represented 144 days of host–parasite contact, compared to only 90 days in group 3 and 84 days in group 2. This concept is only useful if the duration of infection is the major stimulus to immunity (Emery et al., 1992), as adult parasites produce greater daily quantities of antigen, and as the total number of infectious larvae received by the L_4 truncated animals (group 3) was only half that of the other groups. If duration of parasite–host contact is important for development of immunity under field conditions, intensive anthelmintic treatment may compromise the build-up of resistance by reducing the number of 'worm days'. However, this is in contrast to the observations of Gasbarre (1988) that as little as three priming infections, truncated at the L_4 stage, were sufficient to provide immunity against *O. ostertagi*. He suggested that the protective immune response was induced by the death of the larvae in the abomasal glands, which naturally implies the presence of protective antigens in this developmental stage.

Finally, the possibility must be considered that a challenge infection of 156 000 L_3, even given as an increasing trickle infection, is too high. Worm counts from tracer calves which grazed together with second grazing season cattle at turn-out, reflected natural challenge infection levels far below that of the artificial challenge given in this study (unpublished data). In conclusion, 5–8-month-old calves continuously infected with 20 000 L_3 per week were partially immune against a heavy challenge infection after 21 weeks. No indications were found that larval stages of *Ostertagia* provided a better source of protective antigens, since infections truncated at the L_3 or L_4 stage did not induce the same level of protection as continuous infections.

Acknowledgements

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