

IgE antibody during infection with the ovine abomasal nematode, *Teladorsagia circumcincta*: primary and secondary responses in serum and gastric lymph of sheep

J.F.HUNTLEY¹, H.D.F.H.SCHALLIG², F.N.J.KOOYMAN³, A.MACKELLAR¹, F.JACKSON¹ & W.D.SMITH¹

¹Moredun Research Institute, International Research Centre, Pentlands Science Park, Bush loan, Penicuik, Midlothian, EH26 0PZ, Scotland, UK

²Fort Dodge Animal Health, Biological Research and Development, PO Box 36, 1380 AA Weesp, The Netherlands

³Utrecht University, Faculty of Veterinary Medicine, Department of Parasitology, PO Box 80.165, 3508 TD Utrecht, The Netherlands

SUMMARY

A monoclonal antibody to ovine IgE was employed in an ELISA to investigate the IgE antibody responses in serum and gastric lymph to a primary infection of *Teladorsagia circumcincta*, and following challenge in previously infected sheep. During a primary response, IgE antibody to antigens derived from the infective third stage (L3) and adult (L5) worms were negligible, with low levels of IgE antibody detected in serum and lymph. In contrast, there was a pronounced IgE antibody response in 2/4 sheep to L3 antigens during 2–8 days after challenge of previously infected animals but low levels of IgE antibody to L5 antigens. This response was confirmed in a second but similar experiment, where relatively high levels of IgE antibody was detected to antigens from L3. Antibody levels were higher in lymph than in serum from the same animals, and Western blots of L3 antigen following SDS-PAGE under reducing conditions revealed several bands of MW26–96KD which reacted with the IgE antibody from gastric lymph. Immunohistochemical staining indicated that these IgE antibodies may be reacting with allergens associated with the surface cuticle of the worms.

Keywords gastrointestinal nematode, *Teladorsagia circumcincta*, IgE antibody, sheep, lymph

INTRODUCTION

Helminth infections in both man and rodents are almost invariably associated with a pronounced increase in total IgE levels, a proportion of which is specific IgE antibody (Jarrett & Miller 1982, Pritchard 1993). However, the role and significance of this antibody in terms of protection remains unclear, although there is evidence to support the hypothesis for a protective function of this antibody for at least some parasites (Pritchard *et al.* 1995, Bell 1996). In ruminants, while the elevation of circulating total IgE concentrations has been demonstrated (Thatcher *et al.* 1989, Baker & Gershwin 1993, Shaw *et al.* 1997, Huntley *et al.* 1998), there have only been a few reports on specific IgE antibody responses in cattle and sheep which occur during gastrointestinal nematode infections (Baker & Gershwin 1992, Shaw *et al.* 1997, 1998). In a previous study we demonstrated that the levels of total IgE were elevated in serum and gastric lymph, following a primary or secondary immune response to *Teladorsagia circumcincta*. In the present investigation we have re-analysed these samples for IgE antibody by enzyme-linked immunosorbent assay (ELISA) to adult and larval antigens of *T. circumcincta*, as part of ongoing studies to determine the worm allergens important in these responses.

MATERIALS AND METHODS

Experimental design

Samples from two experiments were employed in the present study; the first experiment has been described previously (Stevenson *et al.* 1994). Briefly, naive lambs received either a single oral dose with 50 000 infective third stage larvae (L3) and were killed 21 days post infection (seven animals), or were repeatedly infected (3000 L3 five

Correspondence: J.F.Huntley, Division of Parasitology, Moredun Research Institute, 408 Gilmerton Road, Edinburgh EH 17 7JH

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times weekly for seven weeks), treated with Fenbendazole (5 mg/kg.), challenged with 50 000 L3 and killed ten days later (eight animals). Unfortunately, cannulation of the gastric lymph duct was successful in only five of the primary response animals and collection of lymph for the full 21 day duration was obtained from only 2/5 animals and lymph from the remaining animals ceased flowing between days 6–13. Similarly, cannulation was achieved for only 4/8 secondary response lambs, and samples from only 3/4 animals of lymph were obtained for the duration (day 9 or 10) post challenge. Serum and gastric lymph samples were collected and stored at -20°C until use. Experiment 2 therefore provided additional gastric lymph samples from three sheep, which had been previously infected, cannulated and challenged with 50 000 L3 *T. circumcincta* as described for expt 1. The collected lymph was stored at -20°C .

Parasites

Infective larvae (L3) were from a strain of *T. circumcincta* which has been maintained at Moredun for several years. Adult worms (L5) were recovered from the abomasum of donor ewes between 13 and 18 days after infection with 50 000 infective L3, and washed five times in phosphate buffered saline, pH 7.2 containing streptomycin (5 mg/ml) and penicillin (500 I.U./ml).

Worm burdens

Worm counts were performed on abomasal washings and on digests of the abomasal mucosa as described elsewhere (Coop *et al.* 1977)

Larval (L3) and adult (L5) worm antigens

The soluble proteins from worms were extracted by grinding ≈ 1 g of L3 or L5 in a pestle and mortar with sharp sand and PBS, pH 7.2, to form a smooth homogenous paste at 4°C . This was harvested by diluting in ice cold PBS (final volume 5 ml), and centrifuged in an airfuge (Costar, USA) at maximum speed for ten min. The supernatant was collected and the protein concentration estimated by a commercial protein assay (Pierce BCA protein assay, Rockford, USA), before being aliquoted (100 μl) and stored at -20°C . L3 and L5 excretory/secretory (ES) antigens were prepared in a manner similar to that described in Young *et al.* (1995).

Surgical procedures and sample collection

The common gastric lymph duct was cannulated 4–7 days before infection as detailed elsewhere (Smith *et al.* 1981), and lymph was collected daily using the sampling and

reinfusion procedures also described previously (Smith *et al.* 1981). Blood was collected into 10 ml heparin-coated vacutainers by jugular venepuncture.

Preparation of samples

In a previous study, it was shown that the optimal demonstration of IgE antibody required preheating samples at 56°C for 30 min, prior to sequential precipitation with 30% and 50% saturated ammonium sulphate (SAS) (Kooyman *et al.* 1997). Briefly, samples (100 μl) of lymph or serum were heated at 56°C , and then precipitated with 30 and 50% (SAS). The final precipitate following the addition of 50% SAS was dissolved in 200 μl of PBS containing 0.05% Tween 20 (PBS/T20), before applying to the antigen coated wells in the ELISA plate.

Enzyme-linked immunoabsorbent assay for IgE antibody

Micro-ELISA plates (M129B, Dynatech Lab., Ltd) were incubated overnight (4°C) with (50 μl /well) *T. circumcincta* L3 or L5 antigen (10 $\mu\text{g}/\text{ml}$) in carbonate/bicarbonate buffer pH 9.6. Wells were washed three times in PBS/T20 before receiving 50 μl of the pretreated samples (see above). Plates were then sequentially incubated with 50 μl of a 1/50 dilution in PBS/T20 of the mouse antiovine IgE monoclonal, 1E7 (Kooyman *et al.* 1997), and with 50 μl (1/1000 dilution in PBS/T20) of goat antimouse -HRP conjugate (Dako Ltd, Denmark). Each incubation was for one h at room temperature and plates were washed six times in PBS/T20 between incubations. Colour reaction was developed with ortho-phenylenediamine (OPD) and H_2O_2 and stopped by the addition of 25 μl of 2.5 M H_2SO_4 (Voller *et al.* 1979). The colour reaction at OD 492 nm was measured in a Titertek Multiscan reader (Titertek Flow Laboratories). In order to compare results between assays, a positive control consisting of a pool of lymph containing IgE antibody was included on each plate, and the OD₄₉₂ of unknown samples were altered in proportion with changes of this standard. In practice, between test variability was minimal and the changes in the OD₄₉₂ was normally within 5% of the standard. Controls consisted of replacing serum or lymph with PBS/T20. In the ELISAs for both L3 and L5, very low background values were obtained (less than OD₄₉₂ of 0.03) when preinfected sera or gastric lymph was assayed, and little or no reaction product was demonstrated when the sera/lymph were replaced with diluent alone.

SDS-PAGE and Western blots

Discontinuous SDS-PAGE was with 0.75 mm thick 12% slab gels (Mini Protean II; Biorad, Richmond, CA, USA)

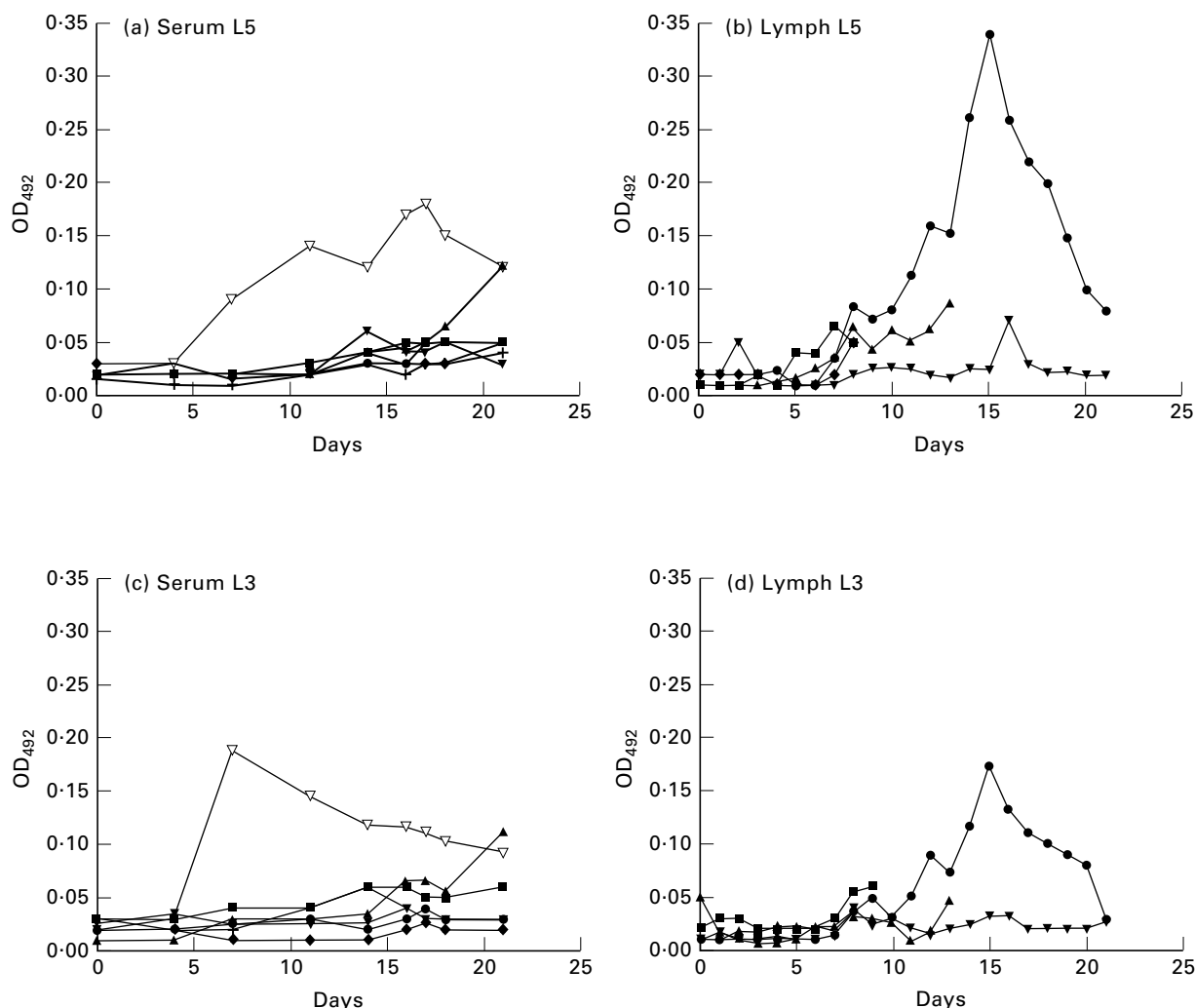


Figure 1(a)-(d). The IgE antibody response to a primary infection with 50 000 *Teladorsagia* larvae from expt. 1. Figure 1(a): Serum response to L5 antigen. Figure 1(b): Lymph response to L5 antigen. Figure 1(c): Serum response to L3 antigen. Figure 1(d): Lymph response to L3 antigen. Each symbol represents the values from individual lambs, and shows seven and five animals for sera and lymph, respectively.

and samples were electrophoresed at a constant 200 V for 45 min. Samples of L3 and L5 worm antigen were treated with reducing buffer and boiled for three min before they were loaded onto the gels at 5 µg/lane. Proteins from SDS-PAGE gels were transferred onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) using a semidry transfer apparatus at 0.9 mA per cm² of gel for one h at room temperature. After transfer, the nitrocellulose membranes were incubated in PBS containing 0.5% Tween 80 and 0.5 M NaCl (PBS/T80) for one h to block nonspecific protein adsorption. The blots were then incubated with gastric lymph taken prior to infection (pool of lymph collected from four animals before infection) or five days post challenge of previously infected sheep (pool of lymph from three sheep in expt. 2), pretreated as for the ELISAs, and diluted 1

in 20 in PBS/T80 for one h. The membranes were washed 6 × 5 min in PBS/T80 before incubation with monoclonal IE7 (diluted 1/50 in PBS/T80). Following a further six washes in PBS/T80, the membranes were incubated with goat anti-mouse-HRP (diluted 1 in 1000 in PBS/T80) (Dako). After a final six washes in PBS/T80, peroxidase activity was revealed with diaminobenzidine/urea/H₂O₂ substrate (Sigma Ltd, Poole, UK). Protein molecular weight markers were employed (Boehringer mannheim, Low-range markers), to estimate the molecular weight of reactive bands.

Immunolocalization of IgE antibody

T. circumcincta L3 were fixed in modified Bouins fixative for 16 h and in 70% alcohol for 24 h before being dehydrated

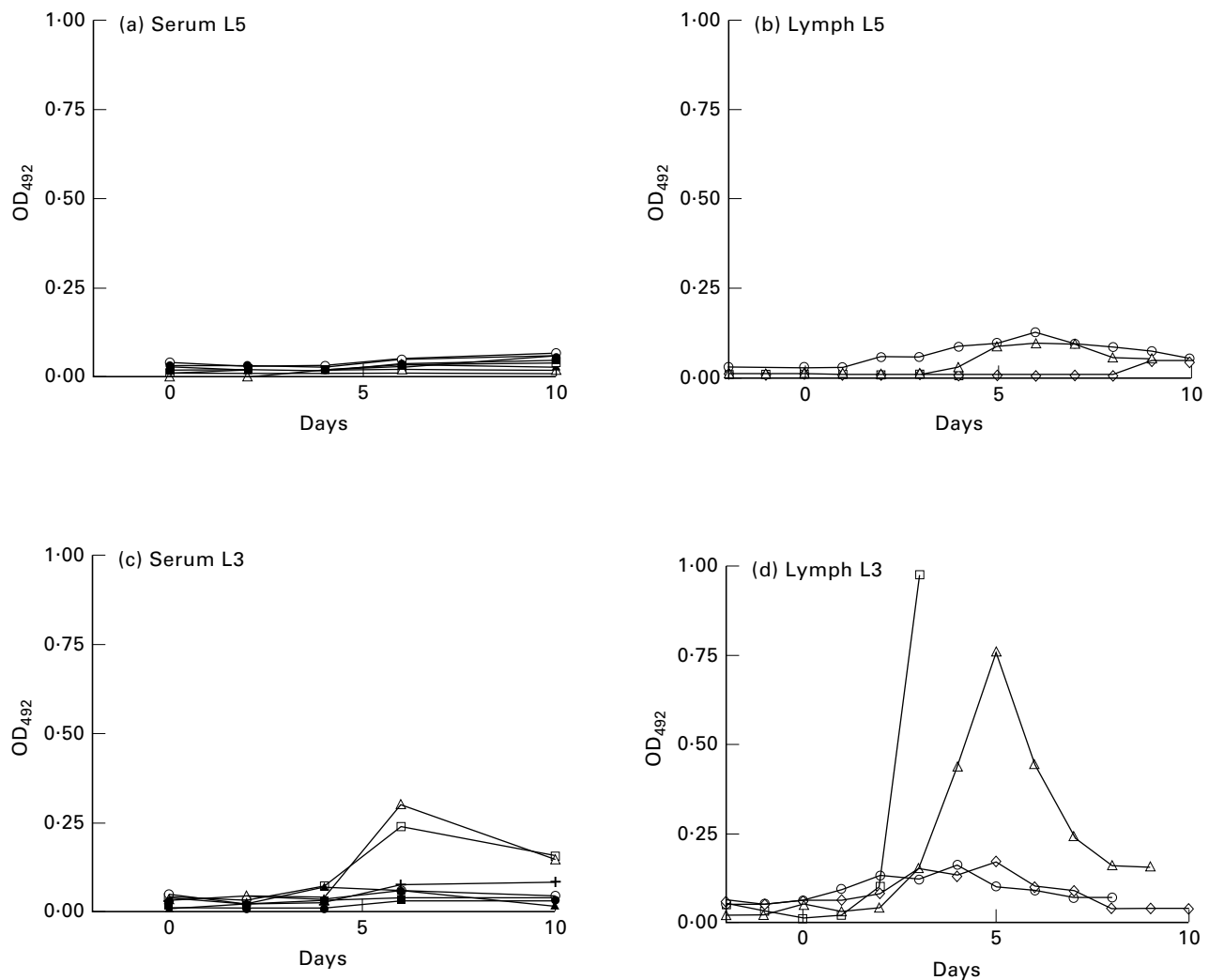


Figure 2 (a)-(d). The IgE antibody response following challenge with 50 000 L3 *Teladorsagia* to previously infected sheep from expt. 1. Figure 2 (a): Serum response to L5 antigen. Figure 2 (b): Lymph response to L5 antigen. Figure 2 (c): Serum response to L3 antigen. Figure 2 (d): Lymph response to L3 antigen. Each symbol represents the values from individual lambs, and shows eight and four animals for sera and lymph, respectively.

through an alcohol series immunolocalization. Sections were preincubated in PBS containing 1% bovine serum albumin (PBS/BSA) for one h, and then sequentially incubated with naive or immune gastric lymph (pool samples as described for Western blotting and pretreated as described for ELISA but diluted 1/4 in PBS/BSA), followed by monoclonal IE7 (1/50 in PBS/BSA) and goat antimouse-HRP (1/100 in PBS/BSA).

Sections were given 6×5min washes in PBS between incubation steps. Peroxidase activity was revealed with diaminobenzidine/urea/H₂O₂ substrate. Control sections consisted of replacing the gastric lymph with PBS/BSA diluent alone.

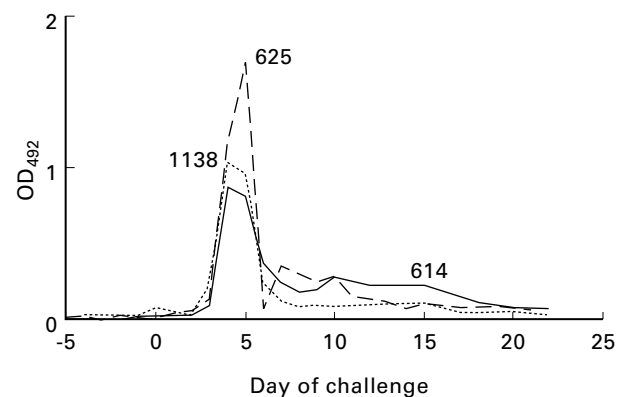


Figure 3 The lymph IgE antibody response to L3 antigen of three previously infected sheep from a second experiment, following challenge with 50 000 *Teladorsagia* L3.

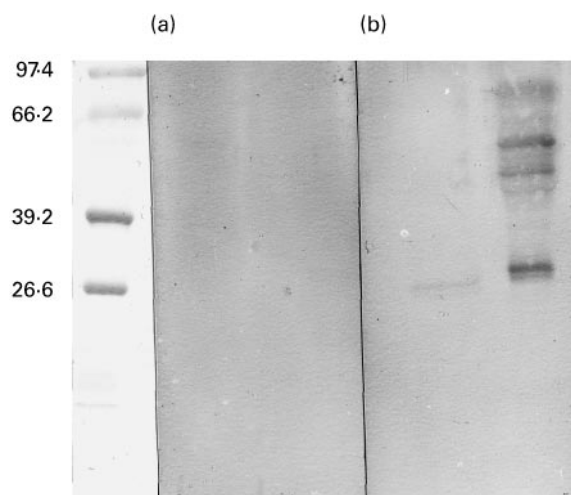


Figure 4 (a, b). Western blot analysis following SDS-PAGE of worm proteins, showing immunoreactivity of IgE antibody in gastric lymph from (a), noninfected sheep and (b), previously infected animals five days after challenge. For each blot, left and right hand lanes consist of 5 µg of either adult (L5) or larval (L3) antigen. Molecular weight markers are (from top), Phosphorylase B (97.4KD), Bovine serum albumin (66.2KD), Aldolase (39.2KD) and Triose Phosphate isomerase (26.6KD).

RESULTS

Primary IgE antibody responses

The IgE specific antibody responses to L5 or L3 antigens measured in expt. 1 after a primary infection with 50 000 *T.*

circumcincta are shown in Figure 1. Only trace amounts of IgE antibody to either antigen were detected in serum from 14 to 21 days post primary infection in 6/7 animals, although a more pronounced response was observed in the seventh sheep (Figure 1a,c). In lymph, a clear primary IgE antibody response to either antigen was observed in one of the two sheep successfully sampled for the full duration of the experiment (Figure 1b,d). Curiously, this sheep did not show a corresponding serum response.

Secondary IgE antibody responses

IgE antibody responses to L3 and L5 antigens following challenge of previously infected sheep are shown in Figure 2. The response to L5 antigen was weak but more pronounced in lymph than serum with 2/4 animals showing peaks between days 5–7 after challenge (Figure 2a,b). The antibody responses to L3 antigen were also variable. Increases in OD₄₉₂ were only observed in the sera of 2/8 sheep, by 6 days after challenge. However, the lymph response of these two sheep was markedly more pronounced (Figure 2d), although those of the further two sheep which had been sampled continuously were much lower.

Since the IgE antibody response appeared to be more prominent in previously infected sheep and to be directed mainly towards L3 antigens, gastric lymph samples from expt. 2 were also tested. A pronounced, short lived response was detected in all three animals between days 3–6, with maximum values on days 4 or 5 (Figure 3).

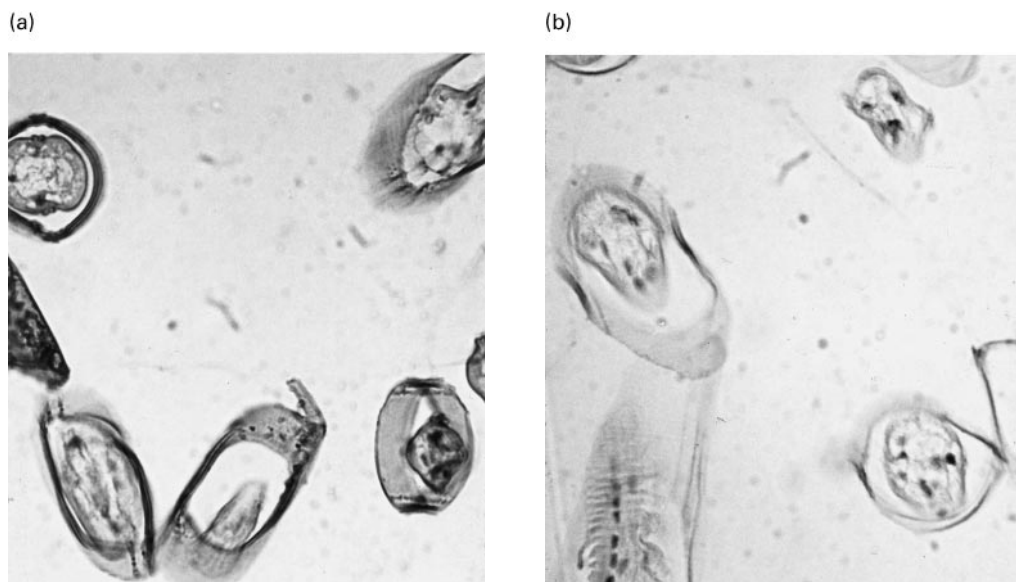


Figure 5 (a, b) Photomicrograph of sections of *T. circumcincta* L3 following incubation with gastric lymph from (a), previously infected sheep five days after challenge, or (b), noninfected sheep, demonstrating the immunolocalization of IgE antibody. Bouins fixation, × 500.

Correlation between IgE antibody responses and worm counts

In expt. 1, considerable individual variation was observed in the worm counts between animals, both in primary infected (worm burdens of 844–17 554) and in previously infected (worm burdens of 71–34 500) sheep. No significant correlation was observed between worm numbers and serum IgE antibody responses. Too few time points were available for this comparison to be performed for lymph.

Western blots

The result of the Western blots showing IgE antibody to *T. circumcincta* antigens are shown (Figure 4). No bands were detected with blots incubated with lymph from uninfected animals. However, several bands of varying intensity and molecular weight were detected when the L3 antigen preparation was incubated with lymph from previously infected sheep. Two weakly reactive bands of ≈ 26 KD were also observed in the L5 antigen preparation.

Immunolocalization of IgE antibody

Sections of L3 *T. circumcincta* following immunoreaction with lymph from naive or previously infected lymph are shown in Figure 5. No reaction product was observed with naive lymph. However, sections incubated with immune lymph showed peroxidase reaction product which was particularly evident on both the sheath and cuticle of the parasites. There appeared to be little reaction with the subcuticular tissues and organs.

DISCUSSION

The present study measured the IgE antibody responses following primary and secondary infection with *T. circumcincta*. While the response to L5 antigens appeared to be negligible, a marked IgE antibody response to L3 antigens was observed in the gastric lymph of five out of seven previously infected sheep which were responding to a challenge infection. The finding that IgE antibodies were more concentrated in lymph than serum supports the conclusion that IgE is generated in the mucosa or draining nodes (Mayrhofer *et al.* 1976, Huntley *et al.* 1998).

Studies investigating ovine IgE antibody responses have been hampered in the past by the lack of specific antibodies to ovine IgE. The production of monoclonal antibodies against recombinant protein (Kooyman *et al.* 1997), or to native IgE (Shaw *et al.* 1996), has finally allowed analysis of these responses in sheep. Until recently, measurements of IgE antibody responses had been confined to preliminary studies involving *Trichostrongylus axei* (Shaw *et al.* 1997)

and *Haemonchus contortus* (Kooyman *et al.* 1997). However, during the preparation of the present study, systemic ovine IgE responses to primary and challenge infections with *Trichostrongylus colubriformis* have been described (Shaw *et al.* 1998). Peak specific IgE responses to adult antigens occurred at day 20–27 post infection and a secondary IgE response to both adult and L3 excretory/secretory antigens. Differences between the two studies include the relative lack of specific IgE responses to adult *Teladorsagia* antigens following a secondary challenge in the present study, compared to those reported for *Trichostrongylus*. Whether this reflects a genuine difference in the response to the two parasites, or was a reflection of differences in challenge regime, breed of sheep or sensitivity of assay, requires further investigation.

Total IgE levels in the serum and lymph samples used in the present study have been estimated previously (Huntley *et al.* 1998), and it is interesting to compare those responses. While there was a marked elevation in the total IgE concentrations in serum and lymph following a single infection, the contribution which was detectable antibody would appear to be minimal. This phenomenon has been recognized in a number of parasite infections in rodents and man (Jarrett & Miller 1982), and the present results suggests that a similar response occurs in sheep. It has been proposed that the preponderance of the IgE produced in helminth infections in rodents and man consists of a polyclonal mixture of IgE responses to miscellaneous unknown antigens (Jarrett & Bazin 1974), involving an up regulation in the production of IL-4 (King *et al.* 1993). However, the present results would suggest that the proportion of total to specific IgE may alter during the development of immunity, since relatively high levels of IgE antibody were observed in lymph following challenge of immune sheep despite the presence of total IgE levels similar to those during a primary infection. Since mast cells and IgE have been implicated in the expression of immunity to gastrointestinal parasites (Miller 1996), the generation of relatively high levels of specific IgE may be of particular relevance in the activation of a mast cell mediated response.

IgE antibody in lymph from immune animals recognized several immunoreactive bands on Western blots of L3, but not L5 antigens. Together with the ELISA data, it is apparent that the IgE response was directed predominantly to the L3 stage. Preliminary immunolocalization studies indicated that these reactive allergens were located on the surface of the infective larvae including its sheath, which is normally discarded within the rumen (Armour *et al.* 1966). Work with other parasites, including *Schistosoma* and *Plasmodium* species, have identified surface allergens (Alyaman *et al.* 1994, Li *et al.* 1995), and cDNA analyses of nematode allergens have revealed sequence homology

between *Ascaris* major allergen, ABA-1, and surface-associated glycoproteins of several filarial parasites (Spence *et al.* 1993). The IgE-reactive *Teladorsagia* antigens may be structural or integral components of the parasite cuticle, alternatively they may be secreted proteins and enzymes associated with surface structure, such as those involved in the process of exsheathment. In view of their possible importance in the development of immunity, further studies are required to confirm the localization and establish the identity of these allergens.

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