

Development of a new monoclonal antibody to ovine chimeric IgE and its detection of systemic and local IgE antibody responses to the intestinal nematode *Trichostrongylus colubriformis*

T. Bendixsen^{a,1}, R.G. Windon^{a,*}, J.F. Huntley^b, A. MacKellar^b,
R.J. Davey^a, S.J. McClure^a, D.L. Emery^{a,2}

^a*F.D. McMaster Laboratory, CSIRO Livestock Industries, Locked Bag 1, Delivery Centre, Armidale, NSW 2350, Australia*

^b*Moredun Research Institute, International Research Centre, Pentland Science Park, Bush Loan, Penicuik EH26 0PZ, UK*

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Abstract

The J558L cell line, previously transfected with the ovine Cε gene, was induced to secrete a chimeric IgE protein composed of the ovine heavy chain and a mouse light chain with MW of approximately 80 and 26 kDa, respectively. After purification, the chimeric protein was used to immunise BALB-c mice and monoclonal antibodies (mAbs) were generated. The mAb 2F1, which had greatest anti-IgE activity in preliminary screens, was chosen for further characterisation and an examination of systemic and local IgE responses to the intestinal nematode, *Trichostrongylus colubriformis*.

The chimeric IgE protein was not recognised in enzyme linked immunosorbent assay (ELISA) by mAbs raised against ovine IgG1, IgG2, IgA or IgM. However, 2F1 was highly specific to the chimeric IgE protein, and did not cross-react with ovine IgG1, IgG2 or IgA. Western blot analysis also showed that 2F1 and secretory IgA (sIgA) did not cross-react, and that 2F1 and the anti-IgA mAb identified different MW bands from colostrum (approximately 200 and 400 kDa, respectively). 2F1 bound to mucosal mast cells (MMC) isolated from the intestines of lambs infected with *T. colubriformis*, but cultured bone marrow-derived mast cells (BMMC) required prior incubation with the chimeric IgE protein for this binding to occur. Distinctive staining of plasma cells and putative mast cells were observed using 2F1 on immunohistological sections of mesenteric lymph node and jejunum.

ELISA incorporating 2F1 was able to detect >0.4 ng chimeric protein. Total IgE in ovine colostrum and intestinal homogenates was quantified using a capture ELISA, with known amounts of chimeric protein used to produce a standard

Abbreviations: AP, alkaline phosphatase; BMMC, bone marrow-derived mast cells; Cε, epsilon C-terminal constant region of the heavy polypeptide chain of the immunoglobulin molecule; ES, excretory/secretory; FPLC, fast protein liquid chromatography; GIN, gastrointestinal nematode; HR lambs, lambs from a line of sheep (high responder) selectively bred for increased resistance to *T. colubriformis*; HRP, horse radish peroxidase; IFA, incomplete Freund's adjuvant; LR lambs, lambs from a line of sheep (low responder) selectively bred for decreased resistance to *T. colubriformis*; kDa, kilodalton; MLN, mesenteric lymph node; MMC, mucosal mast cells; MW, molecular weight; NPS, 4-nitrophenyl phosphate substrate; PBS/T80, 0.5% Tween 80 in PBS; PCA, passive cutaneous anaphylaxis; RB lambs, lambs from an unselected line of sheep that acts as a control for the HR and LR selection lines; SCF, stem cell factor; sIgA, secretory IgA; SP3, antigen consisting of soluble proteins prepared from TcL3; TcL3, *T. colubriformis* infective (third stage) larvae

* Corresponding author. Tel.: +61-2-6776-1446; fax: +61-2-6776-1333.

E-mail address: ross.windon@csiro.au (R.G. Windon).

¹ Present address: National Veterinary Diagnostic Centre, Phuong Mai, Dong Da, Hanoi, Vietnam.

² Present address: Faculty of Veterinary Science, University of Sydney, Private Bag 3, Camden, NSW 2570, Australia.

curve. Colostrum from outbred Merino ewes had 0.55–11.05 ng ml⁻¹ total IgE, and their lambs, at necropsy after infection with a total of 18,000 *T. colubriformis* infective larvae over a 9-week period, had 45–620 ng g⁻¹ total IgE in intestinal tissue. Compared to genetically susceptible lambs, antigen-specific levels of IgE were significantly higher in genetically resistant lambs after infection with 4500 *T. colubriformis* infective larvae (TcL3) per week for 9 weeks (161.4 versus 44.8 geometric mean titres; $P = 0.043$). In western blots, distinctive bands (19–21 and 27 kDa) from *T. colubriformis* larval antigen were differentially recognised by IgE, as identified by 2F1, in intestinal homogenates from genetically resistant animals.

These results have demonstrated the value of 2F1 for quantification of IgE responses in samples derived from ovine fluids and tissues using ELISA, western blots and immunohistology. In this respect, it recognises native ovine IgE and does not require pre-treatment of the sample with denaturing agents or ammonium sulphate.

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1. Introduction

The acquisition of immunity to gastrointestinal nematode (GIN) parasitism in laboratory rodents is associated with “allergic” phenomena such as mastocytosis, eosinophilia and elevated serum IgE (Jarrett and Miller, 1982; Miller, 1984). Although a proportion of this increased IgE is parasite-specific in man and rodents, a direct association between specific IgE and immunity to parasites has been, in general, difficult to obtain (Capron and Dessaint, 1975; Bell, 1996; Lobos, 1997). Associations, however, have been described between IgE levels and resistance to *Schistosoma japonicum* (Nara et al., 1994) and *Trichinella spiralis* (Ahmad et al., 1991). Recently, Clarke et al. (2001) identified three alleles of the bovine and ovine Cε gene, one of which was significantly associated with resistance to the intestinal nematode *Trichostrongylus colubriformis* in sheep.

In one of the earliest studies of ovine reagenic antibody (presumed to be IgE), Hogarth-Scott (1969) described elevated passive cutaneous anaphylaxis (PCA) activity during GIN infection. Yilmaz et al. (1993) have used the reagenic antibodies in hyperimmune serum generated against *Ascaris suum* antigens to raise and characterise anti-ovine IgE antibodies in rabbits. However, the development of a specific enzyme linked immunosorbent assay (ELISA) to measure total and antigen-specific IgE has proved to be technically challenging because of difficulties associated with the isolation of ovine IgE and the production of mAbs directed against this molecule. Consequently, a variety of innovative methods have been used to circumvent these problems, including the generation of IgE

reagents by immunisation of mice with purified mucosal mast cells (MMC) (Shaw et al., 1996), recombinant (*E. coli*) peptides based on the ovine Cε gene sequence (Kooyman et al., 1997), or chimeric myeloma proteins (Clarke et al., 1997).

The recent development of these specific ovine antibody reagents has allowed the characterisation of circulating total IgE (Shaw et al., 1997; Huntley et al., 1998) and worm-specific IgE in serum and gastric lymph (Huntley et al., 1998; Shaw et al., 1998, 1999) during GIN infection. The mAb produced against the chimeric protein by Clarke et al. (1997), named Y41, has been successfully used in immunohistological studies (Colditz et al., 1994; MacDiarmid et al., 1995), as well as ELISA using supernatants from cultured abomasal and mesenteric lymph node cells (Gill et al., 2000) but not serum (MacDiarmid et al., 1995; Windon, Clarke and Beh, unpublished data). However, this mAb now appears to have lost its original reactivity, perhaps due to mutation resulting from chromosomal reduction or resegmentation.

As part of ongoing research into the regulation of mucosal IgE responses during GIN infection, we describe here the production and characterisation of a new mAb, designated 2F1, generated against chimeric protein from the same transfected cell line as that used to produce Y41. 2F1 was then used to analyse systemic IgE responses in blood from outbred sheep and local *T. colubriformis*-specific IgE antibody in the intestines of sheep having genetically determined resistance and susceptibility to *T. colubriformis*. Our results demonstrate that 2F1 is a valuable research tool for quantification of IgE responses in samples

derived from ovine fluids and tissues using ELISA, western blots and immunohistology.

2. Materials and methods

2.1. Sheep, infection and processing of ovine tissues

Samples of colostrum and purified secretory IgA (sIgA) were obtained as described by Smith et al. (1975) from mature Blackface ewes maintained on pasture at Moredun Research Institute, Edinburgh, UK. These samples had relatively high levels of IgE and IgA using mAb IE7 (Kooyman et al., 1997) and anti-IgA mAb (Beh, 1988), respectively, when compared to those in serum (data not shown).

Twenty-six outbred flock Merino ewes, housed in pens under worm-free conditions from 2 weeks before lambing until weaning, produced a total of 30 lambs. The lambs, reared under these worm-free conditions from birth, were fed a standard pelleted ration. Samples of colostrum from ewes and serum from lambs were collected prior to suckling (pre-colostrum). Starting between 2 and 6 weeks of age, lambs were infected with a total of 18,000 *T. colubriformis* third-stage (infective) larvae (TcL3) over 9 weeks before necropsy for recovery of jejunum, mesenteric lymph nodes (MLN) and bone marrow cells. Samples of jejunum and MLN were stored in liquid nitrogen prior to cryosectioning of both tissues for immunohistology and homogenisation of jejunum for ELISA.

Lambs derived from lines of sheep selectively bred for increased (HR) or decreased (LR) immunological responsiveness to *T. colubriformis* (Windon, 1991) together with a contemporaneous random-bred (unselected; RB) line (Windon et al., 1980) were reared in pens from birth (R.G. Windon and C.W. Wong, unpublished). A total of 15 HR, 15 LR lambs and 10 RB lambs were used. At 18 weeks of age, 5 HR and 5 LR were necropsied (time 0 weeks) for collection of control (uninfected) samples, and the remaining 30 lambs were infected with 1500 TcL3, three times weekly. Five lambs of each genotype were necropsied for tissues at either 5 or 9 weeks after the commencement of the infection. At necropsy, jejunal tissue was recovered and stored in liquid nitrogen until homogenisation.

Samples for homogenisation were weighed while frozen, and placed in a volume (ml equivalents mg^{-1} ; w/v) of ice cold PBS containing protease inhibitors (Sigma, Australia). Each segment was processed on ice using a Pro 200 homogeniser (Proscientific, USA), centrifuged ($405 \times g$ for 5 min at 4°C), and the supernatant stored at -20°C until assayed.

All animal experiments were approved by the CSIRO Livestock Industries, Prospect or Moredun Research Institute Animal Ethics Committees.

2.2. Preparation of MMC and bone marrow-derived mast cells (BMMC)

MMC were prepared from the intestines of neonatally infected sheep as described previously (Jones et al., 1992). In addition, BMMC were cultivated in vitro from bone marrow cells using Biorich 1 (ICN, Australia) medium supplemented with 5% FBS (CSL, Australia), $100 \mu\text{g ml}^{-1}$ gentamycin (Sigma, Australia), and using ovine interleukin (IL)-3 and ovine stem cell factor (SCF) (Macaldowie et al., 1997). For 20 ml BMMC cultures in T75 flasks (75 cm^3), 0.2 ml of each of the IL-3 and SCF culture supernatants (1:100) were added three times a week, and the culture medium was replaced each week over a period of 8 weeks.

2.3. Histology and immunohistology

Cryostat sections from adjacent tissues, fixed in 4% paraformaldehyde, were stained for MMC with toluidine blue, pH 0.5, as previously described (Huntley et al., 1992). Immunostaining of cryostat sections from lambs was conducted as described by McClure et al. (1989) and counterstained with Harris haematoxylin (Fronine, Australia). Haematoxylin stock was diluted 1:5 with tap water and filtered (Whatman No. 1 filter) just prior to use. The slides were stained for 30 s, and then washed in water. Immunoperoxidase localisation of IgE was performed on cytospot preparations of cultured BMMC, as described previously by Macaldowie et al. (1997) but using the 2F1 mAb.

2.4. Preparation of ovine IgE chimeric protein

The J558L cell line transfected with the ovine C ϵ gene construct was obtained from Drs. R.A. Clarke and K.J. Beh (CSIRO Livestock Industries).

The transfected cells, which secreted a chimeric protein composed of the ovine heavy chain and a mouse light chain (Clarke and Beh, 1994), were cultured in Biorich 1 medium supplemented with 5% FBS, and 100 µg ml⁻¹ gentamycin. The ovine chimeric IgE protein was purified from the culture supernatant using a NIP-OVA-CnBr sepharose column (Clarke and Beh, 1994). PBS was used as loading and washing buffer. Ovine IgE protein was eluted from the column with 0.1 M diethanolamine (Sigma, Australia), pH 11, and neutralised with solid glycine. Protein concentrations were determined by the Bradford method using a Biorad Protein Assay kit (Biorad Laboratories, USA).

2.5. Production of mAbs

BALB-c mice, 6 weeks of age, were immunised at 0 and 3 weeks with 50 µg of the ovine chimeric IgE protein, emulsified with an equal volume of incomplete Freund's adjuvant (IFA; Difco, USA), with 0.2 ml administered by i.p. injection. At 5 weeks, mice were immunised i.p. with 50 µg ovine chimeric IgE protein in saline, and killed by cervical dislocation 7 days later. Spleen was removed and processed for fusion with NS1 myeloma cells as described by Galfre et al. (1977). Briefly, spleen cells were fused with NS1 cells at a 1:1 ratio (2×10^8 cells total) using polyethylene glycol (Roche, Switzerland). The fused cells (4×10^6), in 100 µl culture medium supplemented with 10% BIM conditioned medium (Roche, Switzerland), and 2% HAT medium (Roche, Switzerland), were aliquoted into 96-well plates (Nunc, Denmark) and incubated at 37 °C in a humidified atmosphere of 5% CO₂ in air. On day 7, cells were fed by removing half of the spent medium and replacing with fresh culture medium. On day 10, 50 µl of the culture supernatant fluid was removed from each well and assayed for anti-IgE activity using ELISA procedures. Clones that showed activity greater than 0.5 OD units in ELISA were sub-cultured into 24-well plates containing 1 ml of culture medium supplemented with 2% HT medium (Roche, Switzerland) instead of the HAT medium. After 5 days, supernatant fluids were removed and assayed for anti-IgE activity against chimeric protein in ELISA. Limiting dilutions of the clones showing anti-IgE activity were carried out in HT medium using 96-well plates. Clones were

fed at day 7 as described above, and wells containing colonies derived from a single cell were tested for anti-IgE activity. Further limiting dilutions of two positive clones, 2F1 and 6F4, that gave the highest anti-IgE OD readings were carried out as described above. Following assessment in ELISA, these clones were grown in culture medium in T75 flasks. Culture supernatants were harvested by centrifugation 5 days later and stored at -20 °C until used. As 2F1 showed greater anti-IgE activity, this clone was used in all subsequent assays and experiments.

2.6. Isotyping of anti-IgE antibodies

The isotype of the antibody produced by the 2F1 clone was determined using the Sigma isotyping kit (Sigma, Australia) and was of the IgG1 isotype.

2.7. Production of rabbit antisera to ovine IgE

Two adult New Zealand white rabbits were inoculated subcutaneously at five separate sites with 100 µl vaccine containing 10 µg chimeric protein in PBS emulsified 1:1 in IFA. Each rabbit was boosted with a similar inoculum after 4 weeks before being bled 7 and 10 days later. The IgG fraction was prepared from serum using T-gel chromatography (Pierce, USA), according to the manufacturer's directions. The T-gel derived IgG was further purified using a chimeric IgE protein affinity column. This column was prepared by coupling 1 mg of chimeric IgE protein to 5 ml of cyanogen bromide activated Sepharose 4B (Amersham Biosciences, UK). The coupling and antibody eluting methods were as described by the manufacturer. Antibodies were concentrated using a Centricon C10 (Amicon, USA), the concentration of affinity purified IgG was determined (Biorad Protein Assay kit), and the product was aliquotted and stored at -20 °C until used.

2.8. ELISA

ELISA was used in five separate assay formats to determine the specificity of anti-ovine IgE antibodies in hybridoma culture supernatant fluids, and the IgE concentrations and antigen-specific IgE antibody levels in serum and intestinal homogenates from sheep infected with *T. colubriformis*.

2.8.1. Determination of the immunoreactivity of the chimeric protein

These assays were carried out essentially as described by Emery et al. (1990). Microtitre plates (96-well Nunc-Immuno Plates, Nunc, Denmark) were coated with 50 μ l of dilutions from 0.1 μ g of the purified chimeric ovine IgE protein in 0.2 M carbonate buffer pH 9.6 (coating buffer), overnight at room temperature. Plates were then blocked for 1 h at room temperature with 2% Coffee Mate (Carnation Foods, Australia) in PBS (ELISA buffer), before a washing step in PBS. Fifty microliters of 1:3 dilutions of culture supernatant fluid containing mAbs reacting with IgG1, IgG2, IgA (Beh, 1987, 1988), and 2F1, prepared in ELISA buffer, was added to the appropriate wells. These plates were allowed to incubate for 1 h at room temperature, followed by five washes in PBS. Fifty microliters of anti-mouse IgG conjugated with alkaline phosphatase (AP; Sigma, Australia) was added to plates and allowed to incubate for a further 1 h at room temperature. Following washing, the plates were developed using 4-nitrophenyl phosphate substrate (NPS; 1 mg ml⁻¹ in 10% diethanolamine; Sigma, Australia). ODs were measured at 405 nm.

2.8.2. Assay of hybridoma fluids for anti-IgE activity

Fifty microliters of supernatant fluid was added, and the assay conducted as described above.

2.8.3. Determination of the Ig specificity of 2F1

Wells were coated with 0.1 μ g purified ovine IgM, IgG1 or IgG2, and wells of M129B plates (Dynex Technologies, USA) were coated with 0.2 μ g sIgA as for the chimeric protein as described above. The reactivity of 2F1 culture supernatant was assayed as above and compared with that of IE7.

2.8.4. Quantitation of total IgE in ovine fluids

A capture ELISA was developed using a checkerboard design to determine the optimal methodology and concentration of reagents. Plates were coated overnight with 10 ng 2F1 mAb in 50 μ l coating buffer. Following blocking with ELISA buffer, serial twofold dilutions of purified chimeric protein (400 ng ml⁻¹ in PBS or pre-colostral newborn lamb serum) or test fluids (e.g. serum, gut homogenate or lymphoid

supernatant fluids) were added and allowed to react for 1.5 h at room temperature. After five washes with PBS, bound IgE was detected sequentially with rabbit anti-IgE (1:10,000) and goat anti-rabbit IgG-AP (Sigma, Australia), each incubated for 1.5 h at room temperature. The colour product was developed with NPS. Total IgE in the test fluids was determined by extrapolation from linear regions of the standard curve (on a log–log scale) for the chimeric protein.

2.8.5. Detection of antigen-specific IgE antibodies in gut homogenates

The parasite antigen used, SP3, consisted of soluble proteins prepared from TcL3 as described by Emery et al. (1991). This ELISA was carried out using procedures adapted from those described by Windon et al. (2002). Plates were coated with 1 μ g of SP3 in coating buffer. Following the blocking and washing steps, 100 μ l of intestinal homogenates, serially diluted in ELISA buffer (ranging from 1:1 to 1:2048), was added to plates and incubated for 1 h at room temperature. The reaction was successively developed with 2F1 mAb culture supernatant diluted 1:2 with ELISA buffer, followed by biotin conjugated sheep anti-mouse IgG and Extravidin-AP (Sigma, Australia) at optimal dilutions. The NPS substrate and OD measurement were as described above. Results from HR, LR and RB intestinal homogenates were expressed as titres. Individual titres were determined by calculating the midpoint, on a log–log scale, of the straight line section of the OD curve using linear regression. The titre represents the inverse of the dilution at this point.

2.9. Western blotting

SDS-PAGE analysis was performed using pre-formed commercial gels (7.5% polyacrylamide NuPAGE TA gels; Novex, USA), and samples of colostrum, IgA and SP3 were separated under non-reducing conditions at 150 V for 1 h 10 min. Prior to separation, samples of colostrum were precipitated with saturated ammonium sulphate, as previously described for serum (Kooyman et al., 1997). Protein molecular markers were from Novex (MK12Novex). Separated proteins were transferred using semi-dry apparatus onto nitrocellulose membranes (Schleicher and Schuell, Germany) at 0.9 mA/cm² of gel for 1 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 1 h to block non-specific absorption. To determine the specificity of the IgE mAbs, the blots were then treated in a similar manner to that described previously (Huntley et al., 1998), and incubated with mAb 2F1 or IE7 (diluted 1:100 in PBS/T80), anti-IgA (1:200 in PBS/T80) or PBS/T80 alone, followed by goat anti-mouse with conjugated horse radish peroxidase (HRP) (Dako, Denmark) diluted 1:1000 in PBS/T80. Peroxidase activity was identified with diamino-benzidine/urea/H₂O₂ substrate (Sigma, UK). To detect parasite allergens recognised by host IgE, blots of SP3 were incubated in intestinal homogenate diluted 1:2 in PBS/T80 prior to their sequential incubation in 2F1 and goat anti-mouse HRP, and the substrate as described above.

2.10. Statistical analyses

Anti-SP3 IgE antibody titres from HR, LR and RB lambs were transformed $\log_{10}(x + 10)$ to normalise data. Consequently, results are presented as geometric means. Because of the unbalanced design (there were no random-bred uninfected lambs), two analyses were carried out. The first analysis of variance included data from the three mating types (HR, RB and LR) slaughtered at 5 and 9 weeks after the commencement of the infection. The second analysis of variance was carried out on data from uninfected HR and LR lambs and HR and LR lambs slaughtered at 5 and 9 weeks after the commencement of the infection. The significance of differences between group means was estimated by Tukey's HSD for multiple comparisons using the statistical analysis software Systat Version 9.01 (SPSS Inc., USA). *P*-values less than 0.05 were considered to be significant.

3. Results

3.1. SDS-PAGE analysis of the ovine chimeric IgE protein

Fig. 1 shows the SDS-PAGE analysis of the ovine chimeric IgE protein eluted from NIP-OVA-CnBr sepharose column. The chimeric protein possessed

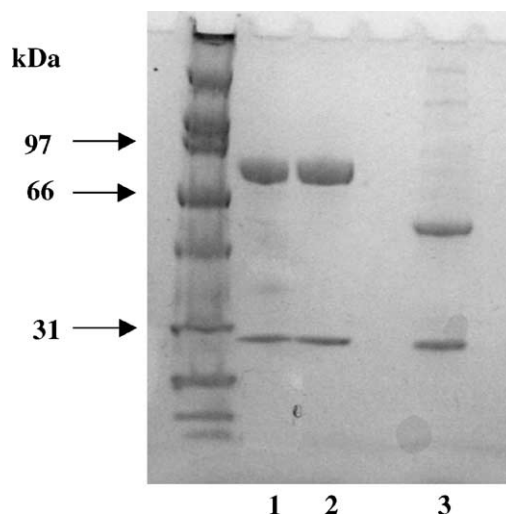


Fig. 1. SDS-PAGE analysis of ovine chimeric IgE protein eluted from NIP-OVA-CnBr sepharose column (lanes 1 and 2, 5 μ g loaded). For comparison, 5 μ g of sheep Ig (lane 3) purified from a T-Gel column is included. The arrows show the position of the molecular weight markers indicated.

heavy and light chains with MW of approximately 80 and 26 kDa, respectively.

3.2. Immunoreactivity of the chimeric IgE protein and 2F1 mAb

The chimeric IgE protein was not recognised in ELISA by mAbs raised against ovine IgG1, IgG2, IgA or IgM. 2F1 reacted with the chimeric protein in a dose-dependent fashion up to 1:1000 dilution of mAb culture supernatant fluid (Fig. 2A). The protein was also recognised by specific rabbit antiserum diluted up to 1:50,000 (data not shown). In an analogous situation, 2F1 did not bind to wells coated with dilutions of purified ovine IgG1 or IgG2, but reacted with chimeric ovine IgE protein to concentrations between 1 and 0.1 ng ml⁻¹ (Fig. 2B). The relevant homologous mAb produced a rapid reaction product (data not shown). In wells coated with sIgA, a strong colour reaction occurred in all wells that received anti-IgA mAb, whereas no colour was observed in wells that received 2F1 or IE7, confirming the non-reactivity of the anti-IgE mAbs with ovine sIgA (data not shown).

In western blots, several high MW bands were observed in lanes that received colostrum and sIgA

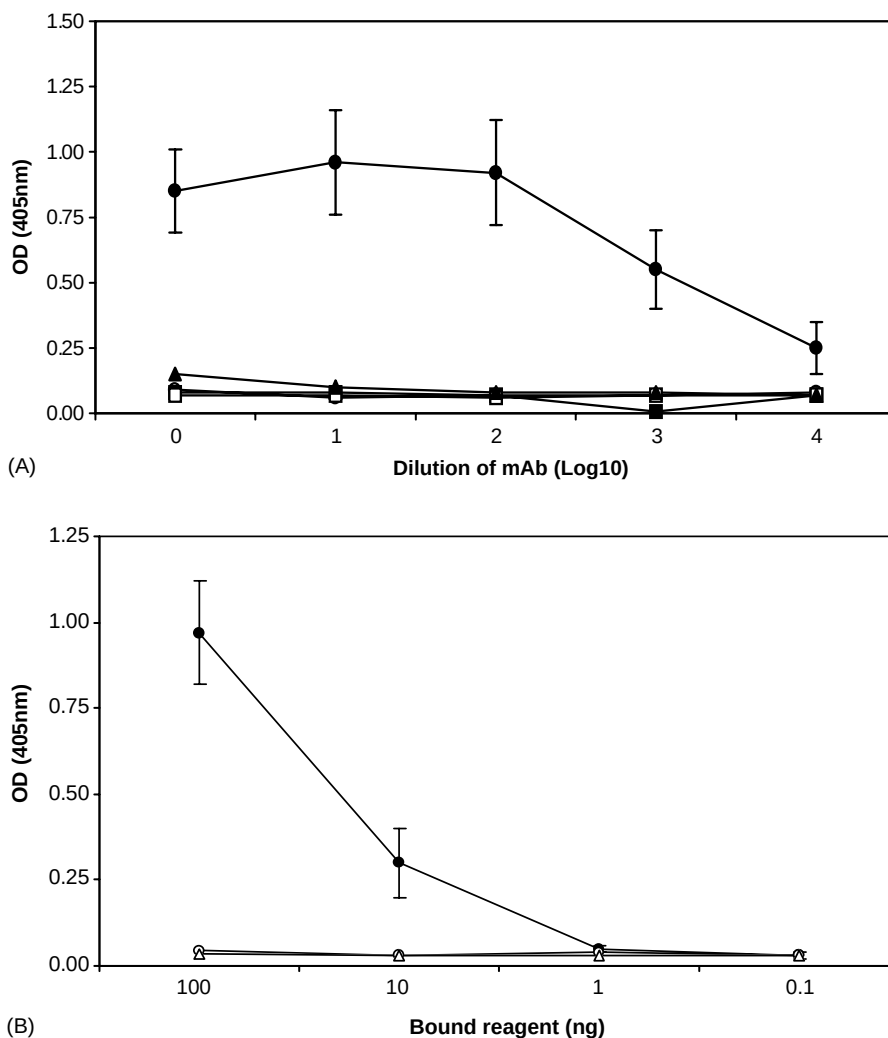


Fig. 2. Immunoreactivity and specificity of the chimeric IgE protein and 2F1 mAb: (A) reactivity of mAbs to ovine IgE (2F1, ●), IgG1 (○), IgG2 (■), IgA (□) and IgM (▲) against bound ovine chimeric IgE protein (0.1 µl) in ELISA; (B) reactivity of 2F1 (culture supernatant diluted 1:3) against bound ovine IgG1 (○), ovine IgG2 (■) or chimeric IgE protein (●) as well as no bound protein (△) in ELISA.

and reacted with anti-IgA mAb. The pattern was identical for both lanes, with the most prominent bands having an estimated MW of approximately 400 kDa (Fig. 3). However, when lanes containing colostrum were probed with either 2F1 or IE7, the main reactive bands had a MW of approximately 200 kDa (data not shown). For both 2F1 and IE7, there was no reaction in lanes that received sIgA or in control blots where PBS/T80 replaced the mAbs. In contrast to IE7, 2F1 did not react in reducing western blots.

3.3. Quantitation of ovine IgE

The ELISA which used 10 ng of 2F1 to “capture” IgE and a 1:10,000 dilution of rabbit anti-IgE to detect, was able to measure >0.4 ng of chimeric protein diluted in PBS or pre-colostral newborn lamb serum on the standard dilution curve (Fig. 4). Quantities of IgE in the colostrum of the 26 Merino ewes at lambing ranged from 0.55 to 11.05 ng ml⁻¹, whereas the levels in intestinal homogenates from the 30 lambs

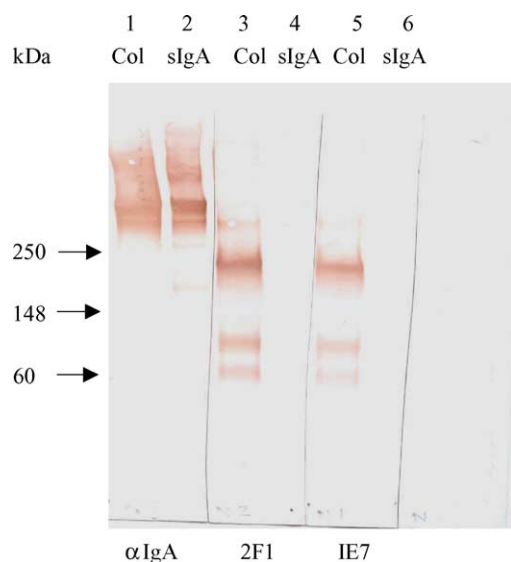


Fig. 3. Western blot analysis of colostrum (lanes 1, 3, 5) and purified sIgA (lanes 2, 4, 6), probed with anti-IgA (lanes 1, 2), 2F1 (lanes 3, 4), or IE7 (lanes 5, 6). The arrows show the position of the molecular weight markers indicated.

infected with TcL3 for 9 weeks ranged from 45 to 620 ng IgE g⁻¹ tissue (data not presented).

3.4. Immunohistology

Indirect immunoperoxidase staining of MMC cyto-spot preparations using 2F1 revealed strong reactivity with MMC (not shown). In contrast, there was no reactivity with BMMC unless these cultured cells were initially incubated with 200 ng ml⁻¹ chimeric IgE protein for 30 min at room temperature (Fig. 5). On cryostat sections of MLN from neonatal lambs, diffuse reticular staining of the follicular areas of the germinal centres was accompanied by staining of putative mast cells and plasma cells in the lymph node trabeculae and medulla (Fig. 6). In the jejunum, 2F1 identified putative sub-epithelial MMC and plasma cells in the lamina propria (Fig. 6) and in areas adjacent to submucosal blood vessels. Adjacent jejunal tissues fixed with 4% paraformaldehyde and stained with toluidine blue (pH 0.5) revealed a similar distribution and number of

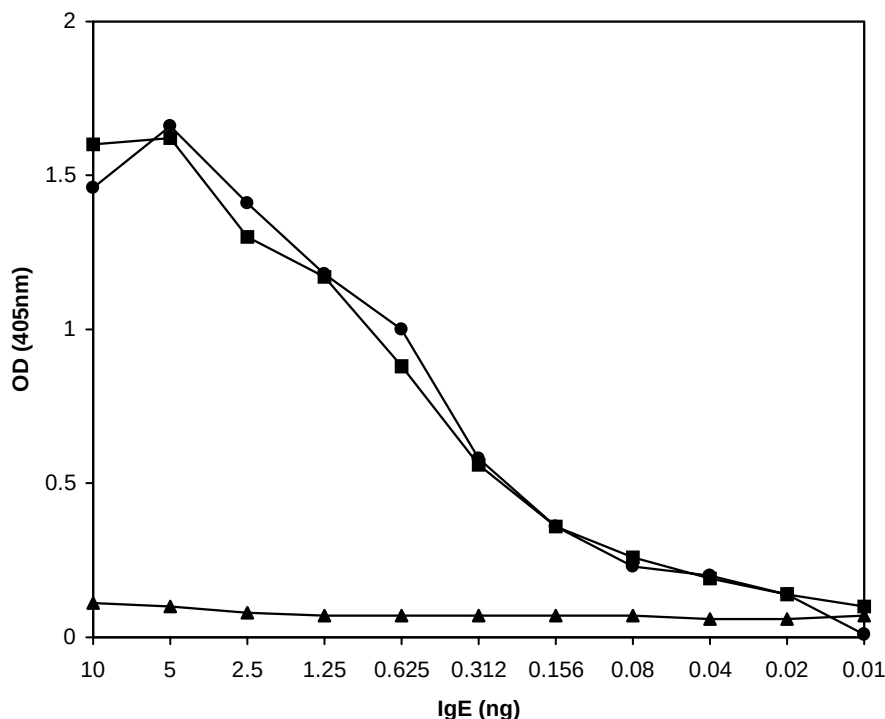


Fig. 4. A "capture" ELISA for ovine IgE. Ovine chimeric IgE protein in either PBS (●) or newborn lamb serum (NLS) (■) was "captured" by bound 2F1 (10 ng), and detected by rabbit anti-chimeric IgE protein (1:10,000). NLS alone (▲) was used as a control.

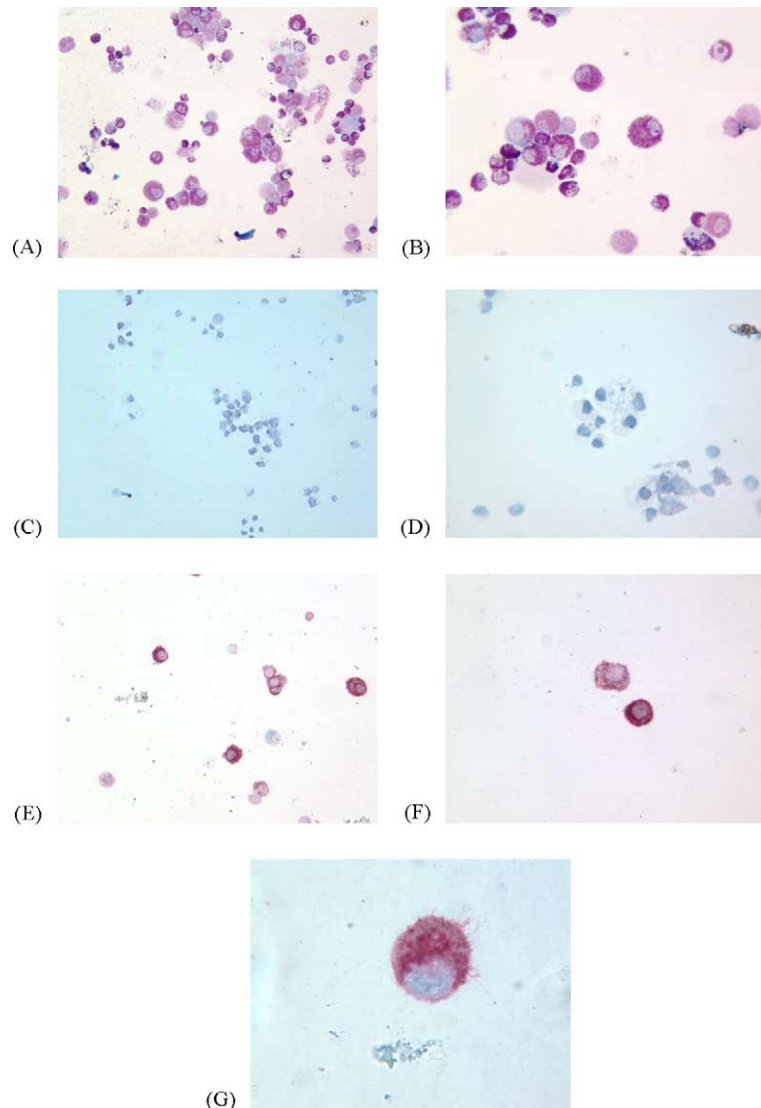


Fig. 5. Cytospots of BMMC, cultured for 8 weeks with ovine SCF and IL-3, were stained with Leishman's (magnification 200 $\times$ (A) and 400 $\times$ (B)) or subjected to immunoperoxidase localisation of IgE incorporating 2F1. The latter procedure was carried out on BMMC without chimeric IgE protein (200 $\times$ (C) and 400 $\times$ (D)) or after incubation with 200 ng ml⁻¹ of chimeric IgE protein for 30 min at room temperature (200 $\times$ (E), 400 $\times$ (F) and 1000 $\times$ (G)). Cytospots C–G were developed with Fast Red TR/Naphthol AS-MX, counterstained with haematoxylin.

stained MMC to that identified following immunoreaction with 2F1. Staining with 2F1 was more intense than that shown by IE7 on cryostat sections.

3.5. Detection of SP3-specific IgE antibodies

Table 1 shows geometric mean SP3-specific IgE titres in homogenised small intestine from uninfected

HR and LR lambs (Slaughter 1) and HR, RB and LR lambs necropsied at either 5 weeks (Slaughter 2) or 9 weeks (Slaughter 3) after the commencement of a trickle infection with *T. colubriformis*. IgE antibody levels increased in all groups during the course of infection. However, this increase was marginal in LR lambs, but in HR lambs titres were significantly higher at week 9 compared to week 0 (161.4 versus 39.3

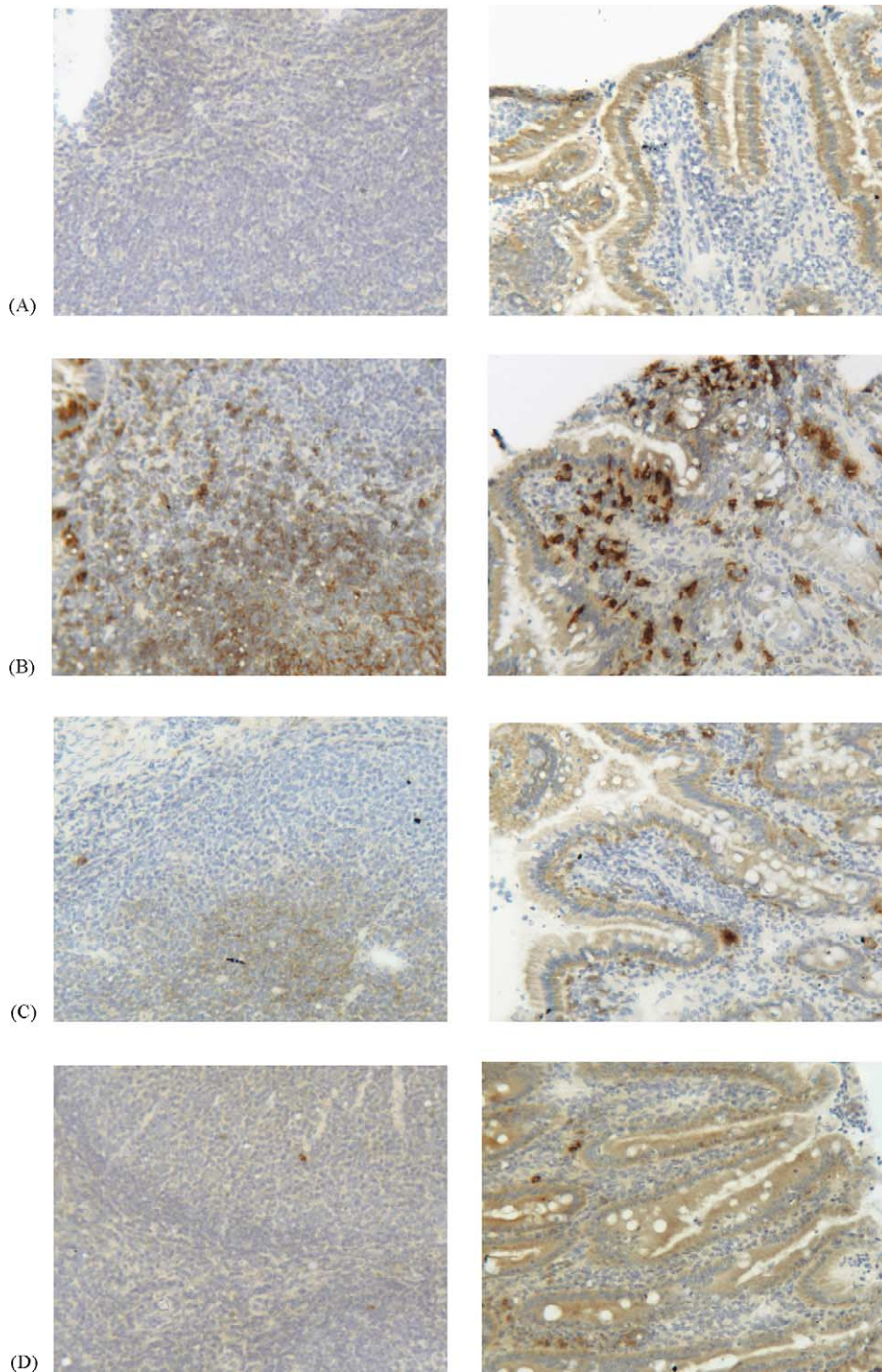


Fig. 6. Immunohistological staining of ovine mesenteric lymph node (left column) and jejunum (right column) with no anti-IgE reagent (control; A), 2F1 (B), IE7 (C), and Y41 (D), each image magnified 200 $\times$.

Table 1

Geometric mean SP3-specific IgE titres in homogenised small intestine from uninfected HR and LR lambs slaughtered at week 0, and HR, RB and LR lambs slaughtered at either 5 or 9 weeks after the commencement of a trickle infection of 4500 *T. colubriformis* infective larvae a week (1500 infective larvae three times a week)^{a,b}

Mating type	SP3-specific IgE titres (geometric means)		
	Slaughter 1 (0 week)	Slaughter 2 (5 weeks)	Slaughter 3 (9 weeks)
HR	39.3 ac	72.3 abc	161.4 bc
RB	ND ^c	47.0 ac	74.3 acd
LR	32.8 ac	28.9 ac	44.8 ad

^a Means with the same letters (a or b) within rows (mating type) are not significantly different at the 5% level, and means with the same letters (c or d) within columns (slaughter time) are not significantly different at the 5% level. This data has been transformed $\log_{10}(x + 10)$ for analysis and significance was determined by Tukey's HSD for multiple comparisons.

^b There are five animals per group.

^c Not done.

geometric mean titres, respectively; $P = 0.021$). HR lambs also produced significantly more IgE than LR lambs at 9 weeks after the commencement of the trickle infection (161.4 versus 44.8 geometric mean

titres, respectively; $P = 0.043$). IgE levels in RB lambs were intermediate between those of HR and LR lambs.

The intestinal homogenate from an HR lamb that had high IgE levels in ELISA was chosen for western blot analysis using SP3 antigen. For comparison, similar blots were performed with an intestinal homogenate from a non-infected 'naive' lamb, and a diluent control (PBS/T80). These western blots therefore identified parasite bands recognised by host IgE in the intestinal homogenates. Whereas a non-specific band of approximately 50 kDa was evident in lanes probed with both intestinal homogenates and control diluent, a similarly located band of 46.5 kDa and two additional clusters of bands of approximately 19–21 and 27 kDa were observed in the blot reacted with the HR sample (Fig. 7). Similar reactivity was demonstrated in replicated blots from three different sheep.

4. Discussion

The recombinant chimeric mouse/ovine IgE protein (Clarke and Beh, 1994; Clarke et al., 1997) was

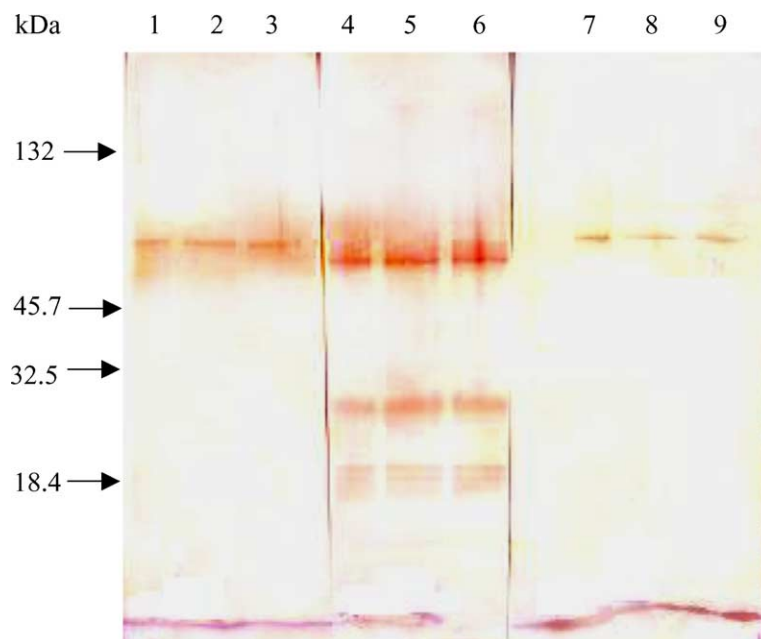


Fig. 7. Western blot analysis of SP3 probed with PBS/T80 (lanes 1–3) or intestinal homogenates from immune HR sheep (lanes 4–6) and helminthologically-naïve sheep (lanes 7–9). The arrows show the position of the molecular weight markers indicated.

successfully used to regenerate anti-ovine IgE mAbs. Additional studies on the protein revealed that it was not recognised in ELISA by mAbs reactive with ovine IgG1, IgG2, IgM or IgA, and possessed heavy and light chains with MW of approximately 80 and 26 kDa, respectively. The MW of the heavy chain was similar to those estimated previously after affinity purification of serum IgE using reagents generated against ovine MMC (Shaw et al., 1996), IgE heavy chain eluted from SDS-PAGE (Yilmaz et al., 1993), or recombinant C ϵ chain fragments (Kooyman et al., 1997). The chimeric protein bound to antigenically naïve BMMC cultivated from bone marrow, as evidenced by positive staining with 2F1 and polyclonal rabbit antisera.

The anti-IgE mAb used in these studies, 2F1, was chosen because it exhibited the greatest anti-IgE activity in preliminary ELISA screens. This mAb did not react with purified ovine IgG1, IgG2 or IgA in ELISA, confirming its specificity for ovine IgE. Furthermore, it produced similar patterns of immunoperoxidase staining on cryostat sections of lymph nodes and intestine to those described previously for the mAbs XB6 and YD3 (Shaw et al., 1996). The intensity of staining was greater than that observed with the mAb IE7 (Kooyman et al., 1997), which detects a conformationally altered form of IgE. Like many mAbs (excluding IE7), 2F1 did not react in reducing western blots, confirming that it recognised native IgE.

A capture (sandwich) ELISA was developed to calculate total IgE in ovine fluids including serum, colostrum and intestinal homogenates. The use of the chimeric protein as a standard on each ELISA plate provided a simple and reliable means to quantitate IgE without pre-treatment of the sample with denaturing agents (Kooyman et al., 1997). In this respect, the detection of chimeric IgE was not inhibited by the presence of serum proteins in newborn lamb serum (Fig. 4) or worm-free lamb serum with IgE levels $<4.5 \text{ ng ml}^{-1}$ (data not shown). For sheep colostrum, to our knowledge this is the first report of the detection of IgE, although Hudson et al. (1971) have previously reported homocytotropic antibody activity.

Reagents detecting ovine IgE have been used to demonstrate the kinetics and magnitude of IgE responses in serum and/or abomasal lymph, after infection with *Haemonchus contortus*, *Teladorsagia* (*Ostertagia*)

circumcincta and *T. colubriformis* (Huntley et al., 1998; Kooyman et al., 1997; Shaw et al., 1996, 1997). The close association of IgE responses with worm infection in both man and animals (Pritchard et al., 1997; King et al., 1997; Urban et al., 1984) was supported in the current study by immunohistological demonstrations of IgE⁺ cells in MLN and intestinal mucosa, and the significantly higher titres in intestinal tissue from HR lambs.

We have focussed on the local mucosal response, demonstrating for the first time a significant rise in *T. colubriformis* (SP3)-specific IgE in intestinal homogenates between 5 and 9 weeks after a trickle infection with TcL3. In contrast to the reports by Shaw et al. (1998, 1999) with serum, pre-treatment of the homogenates with ammonium sulphate was not required to detect antigen-specific IgE. The HR sheep used in our studies had commensurately lower worm-egg counts in faeces and worm burdens at necropsy than LR and RB animals (R.G. Windon and C.W. Wong, unpublished data). Clarke et al. (2001), also using animals from the same HR and LR selection lines, found a highly significant association between an IgE allele and resistance to *T. colubriformis*. As the association did not occur in animals from other unrelated flocks that were investigated, the authors suggested that the importance of this polymorphism remained equivocal until corroborating evidence could be obtained. The present report provides preliminary functional evidence, subject to more extensive verification, that supports the genetic association in this flock.

It was pertinent to examine SP3 for those antigens differentially recognised by IgE from HR (that is, genetically resistant) hosts. Previously, such antigens had been sought after fractionation of extracts by FPLC and testing for degranulation of IgE⁺ MMC from immune sheep (T. Bendixsen and D.L. Emery, unpublished data). Several recombinant excretory–secretory (ES) antigens have been active in this assay (Emery et al., 1997). The potential of the approach using 2F1 was the demonstration of specific, unique antigens in SP3 recognised in western blots using intestinal homogenates from HR lambs (Fig. 7). The technique has obvious application in the identification of “potential allergens” from extracts of ovine parasites.

The relationship and interactions between immunity to parasites, allergens, and specific IgE responses

remain unclear. Whether parasite antigens recognised by IgE can, in turn, induce IgE when inoculated in purified form is problematic (Lobos, 1997), and the most appropriate formulation and means of delivery are still being sought. It has been difficult to generalise on the “characteristics” of antigens likely to elicit antigen-specific IgE responses in vivo, and discriminate those from antigens recognised non-specifically by polyclonal IgE responses (Jarrett and Miller, 1982) generated by virtue of the host–parasite interaction. The means by which parasite infection induces IgE responses is also unclear (Kelso, 1998; Pritchard et al., 1997). A definitive protective role for IgE in ruminant helminthiasis awaits unequivocal demonstration, although the associations and kinetics are tantalisingly suggestive (Baker and Gershwin, 1993; Shaw et al., 1999).

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