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Original article

Proteomic approach to identify candidate effector molecules during the in vitro immune exclusion of infective *Teladorsagia circumcincta* in the abomasum of sheep

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Abstract – In the present study we have employed an in vitro organ challenge model to study the post-challenge responses in parasite naïve and immune gastric tissue of sheep, in an attempt to identify the host derived factors involved in immune exclusion of *Teladorsagia circumcincta* larvae. Proteins present in the epithelial cells and mucus from ovine abomasa following parasite challenge in previously naïve and immune animals were analysed through Matrix Assisted Laser Desorption/Ionization-Time of Flight (MALDI-Tof)-MS and shotgun proteomics. MALDI-ToF analysis of epithelial cell lysates revealed that a number of proteins identified were differentially expressed in naïve and immune cells. These included intelectin and lysozymes, which were present at higher levels in epithelial cell lysates derived from immune samples. A large number of proteins were identified in the mucosal wash from immune tissue which were not present in the mucosal wash of the naïve tissue. Some of these proteins were present in washes of immune tissue prior to the parasite challenge including immunoglobulin A, galectin 14 and 15 and sheep mast cell protease 1. However, other proteins, such as calcium activated chloride channel and intelectin were only detected in the washings from the challenged tissue. The latter may be related to an enhanced mucus release, which may result in entrapment of infective larvae and thus reduced establishment in tissue that has been previously challenged with the parasite. In conclusion, several proteins have been identified which may be involved, either directly or indirectly, in the exclusion and immune elimination of incoming infective larvae. In the present study, the usefulness of the in vitro model has been confirmed, and the global proteomic approach has identified proteins that had not previously been associated with parasite exclusion from abomasal mucosa, such as the calcium activated chloride channel.

immunity / mucus / proteomics / sheep / *Teladorsagia circumcincta*

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1. INTRODUCTION

Host immunity to gastrointestinal nematodes is expressed in many ways and is targeted against adult and larval stages of nematode parasites. Resistance against adult nematodes can be manifested as reduction in fecundity of females, changes in the morphology and or the expulsion of parasites whereas resistance against larval stages of nematodes is manifested as arrested development, reduced establishment and exclusion of immature nematodes [1, 21]. The mechanisms that are involved in these immune processes are complex, and probably involve both acquired and innate immune factors including antibodies, inflammatory cell mediators, changes in mucus quality and associated factors. However, despite the plethora of studies to define these immune mechanisms, the final effector molecules which elicit parasite expulsion remain unknown.

The phenomenon of immune exclusion of infective larvae in primed hosts is commonly observed in ruminant gastrointestinal nematode infections. Miller et al. [20] reported that immune sheep prevented the establishment and expelled the majority of *Haemonchus contortus* L3 within 48 h post infection, with more than 95% of the larval challenge failing to establish in the immune tissue. Similar expulsion rates were observed in immune sheep infected with a *Trichostrongylus colubriformis* larval challenge [17]. An in vitro method has recently been developed, in which abomasal tissue explants were challenged with exsheathed *Teladorsagia* (*T.*) *circumcincta* to investigate this phenomenon of immune exclusion of immature larvae [13]. In this study it was shown that: (i) in vitro establishment rates of L3 were similar to those reported in vivo, in naïve and immune tissue; and (ii) larval establishment was higher in previously naïve than in immune tissue. The epithelial tissue retained its histological integrity during the short term (up to 3 h) incubation, indicating that the exclusion of applied larvae to immune tissue was due to the functional activity of pre-existing or newly secreted factors.

In the present study we have therefore employed this in vitro model to study the induction of post-challenge responses in previously naïve and immune gastric tissue of sheep, in an attempt to identify the host derived factors involved in larval immune exclusion. Tryptic fragments of proteins present in the mucus from ovine abomasa were analysed through liquid chromatography electrospray ionisation (LC-ESI) in combination with tandem mass spectrometry (MS/MS), which enabled the simultaneous detection of multiple proteins [2], whereas those isolated from the epithelial cells of the gastric mucosa were individually isolated from two dimensional gels and identified with Matrix Assisted Laser Desorption/Ionization-Time Of Flight (MALDI-ToF).

2. MATERIALS AND METHODS

2.1. Animals and post mortem collection of abomasa

Gastric tissue from 6 parasite naïve and 8 immune sheep was used for this study. Naïve sheep were reared indoors under conditions that minimised parasite exposure. All naïve animals were screened for nematode eggs in their faeces and were negative prior to slaughter. The immune animals were ewes that had been exposed to natural parasite challenge, consisting mainly from *T. circumcincta*, grazing normal grass/clover pastures. All immune animals were positive for nematode egg counts (consistently lower than 10 eggs per gram). Sheep were killed by lethal injection (Pentobarbital). The abdomen was opened along the midline to allow the removal of the gastrointestinal tract. Abomasa of sheep were removed immediately from animals, opened and parts of the abomasal folds approximately 2×2 cm were removed for the in vitro challenge. Abomasal tissue was gently rinsed in saline (0.85%) to minimise contamination with abomasal digesta.

2.2. In vitro model and sample preparation

This method is a modification of the one described in detail by Jackson et al. [13]. In brief, three tissue sections from abomasal folds measuring approximately 2×2 cm were challenged with 2 500 L3 of *T. circumcincta* (in 0.5 mL of water)

into isolation chambers formed with the barrels of 5 mL syringes. The tissue sections were surrounded by media consisting of 20 mmol/L Hepes with 2 mL phenol red added to 1 L of sterile water (pH 7.6). Following the incubation of the tissue sections at 37 °C in high oxygen environment and darkness for 3 h, the syringe barrels were rinsed with saline, and tissues were washed vigorously by immersing the tissue sections 30 times in 25 mL of phosphate buffered saline (PBS). Subsequently, instead of progressing with the digestion of the tissue section in 1% pepsin/1% HCl solution as described by Jackson et al. [13], tissue sections were then placed into a tube containing 30 mL of digestion medium consisting of 5 mL of 1% Foetal Calf Serum (FCS, Sigma, Irvine, UK), 250 µg of gentamycin (25 µg/mL, Sigma), 5 mL of penicillin/streptomycin (1%) and 85 µL of each of the enzymes, collagenase (75 U/mL, Sigma) and dispase I (20 µg/mL, Sigma) in 500 mL of complete DMEM (D5671, Sigma). The aim of the enzymatic dispersion of the epithelial cells was two-fold: (i) to release the larvae that were closely associated to the gastric mucosa, and (ii) to achieve dispersion of epithelial cells for the proteomic analysis. The enzymatic dispersion was based on the method described by Dziva et al. [4].

Tissue was maintained in the digestion medium for 2 h and were vigorously rotated at 37 °C.

The percentage of the larval population closely associated with the mucosa was calculated using the standard formula:

$$\%\text{tissue association} = (\text{DL/TLP}) \times 100$$

where DL is the number of larvae recovered from the digested tissue and TPL the total larval population (larvae in the rinse, wash and digest). Student's *t*-test (Microsoft Excel) was used to statistically analyse the percentage of the larval population closely associated with the mucosa in previously immune and naïve tissue.

Mucosal washes were thoroughly mixed and a subsample was removed to count the number of larvae. The remaining material was centrifuged and the pellet, which included nematode larvae, was discarded. The supernatant was freeze dried to increase the concentration of soluble molecules, redissolved in a low volume of water, desalted by gel filtration in distilled water (PD-10, GE HealthCare, Bucks, UK) and further freeze dried. Mucosal wash samples were also prepared from unchallenged controls.

Gradient centrifugation was used to isolate epithelial cells from larvae and cell debris present in the cell suspension following incubation in the digestion medium. For this purpose a layer of the epithelial cell suspension was laid on top of Lymphoprep (Axis Shield Diagnostics, Dundee, UK) and the sample was centrifuged at 1 900 $g \times 15$ min. The epithelial cell layer was then removed and subjected to three washes in HSBB, to remove Lymphoprep and other debris. Cells were then assessed for their viability, by staining them with 0.1% nigrosin. Cytospins (Shandon cytospin 4, Thermo, Waltham, UK) were prepared for immunohistochemistry, to estimate the percentage of epithelial cells present in the purified suspension.

2.3. One-dimensional SDS-PAGE and shotgun proteomics

Individual mucosal wash samples, prepared as above, were pooled in a 1:1 ratio into four large samples characterised as either derived from naïve or immune tissue, and challenged or non-challenged in vitro with L3 larvae. Prior to creating the pooled samples, individual samples were run on a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel, to assess the degree of variability between the samples. Protein content of the pooled samples was calculated with a standard bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford USA). Samples were diluted with distilled water to ensure similar loadings of protein. 1-D gel electrophoresis (NuPage Novex Bis-Tris gels, 4–12% acrylamide; Invitrogen, Paisley, UK) was performed in the pooled samples and stained with Coomassie Blue (SimplyBlue Safestain, Invitrogen). Each of the gel lines was divided into 25 slices and each of the resulting 25 gel slices was then subjected to standard in-gel de-staining, reduction, alkylation and trypsinolysis procedures. Tryptic digests were transferred to sealed High Performance Liquid Chromatography (HPLC) sample vials and stored at 4 °C until required for analysis by an automated LC-ESI-MS/MS system. Liquid chromatography was performed using an Ultimate 3000 nano-HPLC system (Dionex, Sunnyvale, USA) comprising a WPS-3000 well-plate micro auto sampler, a FLM-3000 flow manager and column compartment, a UVD-3000 UV detector, an LPG-3600 dual-gradient micropump and an SRD-3600 solvent rack controlled by Chromeleon (Dionex) software.

A micropump flow rate of 246 µL/min was used in combination with a cap-flow splitter cartridge, affording a $^{1}/_{82}$ flow split and a final flow rate of 3 µL/min through a 200 µm ID monolithic reversed phase column (Dionex) maintained at a constant 50 °C. Samples of 4 µL were applied to the column by direct injection. Peptides were eluted by the application of a 15 min linear gradient from 8% to 45% solvent B (80% acetonitrile, 0.1% formic acid) and directed through a 3 nL UV detector flow cell. LC was interfaced with a 3D high capacity ion trap mass spectrometer (Esquire HCTplus™, Bruker Daltonics, Coventry, UK) via ESI utilising a low-volume (50 µL/min max) stainless steel nebuliser (Agilent, South Queensferry, UK). MS/MS analysis was initiated on a contact closure signal triggered by Chromeleon software. This method provides a global analysis of complex protein mixtures [2, 32].

Searches were performed using Mascot™ version 2.1 software (Matrix Science, London, UK) and the SwissProt and NCBInr databases. The interpretation of MS/MS data was performed in accordance with published guidelines [29]. The peptide and fragment mass tolerances applied were 1.5 and 0.5 daltons (Da) respectively. Individual MS/MS spectra for peptides with a Mascot Mowse score lower than 40 were inspected manually and only included in the statistics if a series of at least four continuous y or b ions were observed. Protein ID is based on the assignment of at least two peptides per protein, otherwise MS/MS spectra were verified manually.

2.4. Two-dimensional SDS-PAGE gel electrophoresis and MALDI-Tof-MS

Samples of epithelial cells were lysed in rehydration buffer (8 M urea, 2% CHAPS, 0.4% DTT, 0.2% Biolytes 3-10 NL) prior to electrophoresis. Samples were initially vortexed for 1 min, then agitated gently for 1 h at room temperature and centrifuged at 11 000 $g \times$ 15 min. Cell extracts from individual (rather than pooled) samples were used for the two dimensional gel electrophoresis. Protein content of the individual samples was calculated with a standard 2-D Quant kit (Amersham, Buckinghamshire, UK). Samples were diluted with rehydration buffer to ensure similar protein loadings across samples. First dimension isoelectric focusing was carried out on immobilised pH gradient strips (18 cm, pH 3-10 NL, Amersham). Focusing was achieved at 8 000 V for a minimum of 40 000 Vhs. After focusing, strips were equilibrated in 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS. Samples were initially reduced with the addition of DTT and then free cysteines were derivatised by the addition of iodoacetamide. Second dimension electrophoresis was performed on polyacrylamide 0.5 mm precast gels (ExcelGel XL SDS 12–14%, Amersham). Total protein loadings for gels were approximately 150 µg. Gels were stained with SimplyBlue Safestain (Invitrogen). Spots of interest were excised from the gel and subjected to standard in-gel de-staining, reduction, alkylation and trypsinolysis procedures. Spots of interest included abundant spots or spots that appeared to change in intensity. The resulting digests were analysed using an Ultraflex™ II MALDI-ToF tandem mass spectrometer (Bruker Daltonics), scanning the 600–5 000 Dalton region. The spectra generated were mass-calibrated using known standards and the peaks de-isotoped. Databases were searched with the masses obtained using the MASCOT search database and a 50 ppm mass tolerance window. Levels of significance of the proteins that were a positive match were also calculated.

2.5. Image analysis of 2-D gels

Following SDS-PAGE, 2-D gels were imaged using a flat bed scanner (ImageScanner, Amersham). Image analysis and gel comparisons were performed using Progenesis PG200 software (Nonlinear dynamics, Newcastle, UK). This was done to allow comparison of spot intensity across the whole set of gels. Gels were automatically analysed using the spot detection feature of the software, with automated matching. Following matching of spots extensive checks were performed and mismatches were corrected manually. Spot volumes were corrected for background using the 'mode of non-spot method'. Spot volumes were normalised as a proportion of the total spot volume of the gel and results were multiplied by 1 000. Between gel comparisons for relevant spots were made by comparing relative spot volumes [10]. The latter were compared statistically between challenged naïve and immune tissue using *t*-test (Microsoft Excel).

2.6. Western blots

Western blots were performed for a selection of proteins, which may have an important role in immunity to parasites, in both mucosal wash samples and epithelial cell lysates. Preparations from

epithelial cell lysates were run on 4–12% Bis-Tris minigels (Invitrogen) and were then transferred to nitrocellulose membrane (Invitrogen). The membrane was incubated for 1 h at room temperature in a solution containing 0.5% Tween 80, 0.5 M NaCl and PBS, which aimed to increase the stringency and reduce non-specific binding of the membrane. Membranes were then probed with affinity purified chicken anti-intelectin (1:500) [5]. Membranes were then washed five times with the high salt solution and probed with the secondary antibody (donkey-anti-chicken IgY-horseradisch peroxidase (HRP) conjugate, 1:10 000; Jackson Laboratories; Maine, USA). After final washes, all blots were developed with chemiluminesence ECL (Amersham). Negative and positive controls were included.

Individual mucosal wash samples from in vitro challenged tissue were run on 12% SDS-PAGE minigels (Bio-rad, Hertfordshire, UK) and were then transferred to polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore, Hertfordshire, UK). Membranes were incubated for 1 h at room temperature in solution containing 2% Marvel in Tris buffered saline (TBS). Membranes were then probed with: (i) mouse anti-Immunoglobulin A (IgA, 1:1 000, Serotec, Kidlington, UK); (ii) chicken anti-intelectin (ITLN, 1:500) [5]); (iii) rat monoclonal anti-Sheep Mast Cell Proteinase (SMCP, 1:10, dilution of tissue culture supernatant) [24]; and (iv) rabbit polyclonal anti-CLCA (Cl channel Ca-activated) antibody (1:500 of an affinity purified antibody stock 1 mg/mL) that is directed against an amino-teminal epitope of mouse CLCA1/2, but also cross-reacts with mCLCA3 (alias gob-5) (Frank Thévenod, unpublished data). Following the washings, membranes were then probed with the secondary antibodies: (i) polyclonal rabbit anti-mouse HRP conjugate (1:1 000, Dako, Cambridgeshire, UK) for the IgA; (ii) donkey-antichicken IgY-HRP conjugate (1:10 000; Sigma) for intelectin; (iii) rabbit anti-rat HRP conjugate (1:1 000, Dako) for SMCP; and (iv) polyclonal goat anti-rabbit HRP (1:2 000, Dako) for CLCA. Antibody dilutions were made with the blocking solution. After final washes, all blots were developed with chemiluminesence ECL (Sigma).

2.7. Immunohistochemistry

In order to check the purity of the epithelial cell population isolated from the gastric mucosa, gastric samples were fixed in zinc salt solutions at slaughter [8] for embedding and sectioning. Staining of ovine epithelial cells was achieved with mouse antibodies to pancytokeratin (1:2 000, Sigma; clone PCK-26; broad spectrum antibody; reacts specifically with a variety of normal, reactive, and neoplastic epithelial tissues). Preliminary experiments showed that they were the most specific antibodies for ovine epithelial cells and thus were used for immunohistochemistry. Epithelial cell cytospins were also prepared to determine the purity of the epithelial cell suspension, and also stained for pancytokeratin. Cell counting was performed to determine the proportion of positively stained epithelial cells prior to preparation for the proteomic analysis. For sections and cytospins detection of mouse anti-pancytokeratin was performed using the Envision system (Dako).

3. RESULTS

3.1. In vitro larval counts

In previously naïve tissue, an average of 54% larvae was recovered from the tissue digests, compared to a 4% recovery from tissues deriving from previously exposed sheep ($P < 0.05$; Fig. 1).

3.2. Isolation of epithelial cells

All types of mucosal epithelial cells stained positively with the anti-pancytokeratin antibody, but not inflammatory cells (Fig. 2A). The use of the enzymatic cocktail resulted in isolation of epithelial cells as shown in Figure 2B. Quantification of stained cells showed that in the cell suspension, routinely more than 85% of cells present were epithelial cells. Staining for viability showed that more than 97% of cells were viable at the end of the incubation.

3.3. Identification of proteins of interest in epithelial cell lysates

Approximately 50 spots of proteins of interest were analysed and 26 were positively identified. The location of the identified spots is indicated in Figure 3 by the gene name. The reference map (Fig. 3) shows a typical epithelial cell lysate from immune gastric

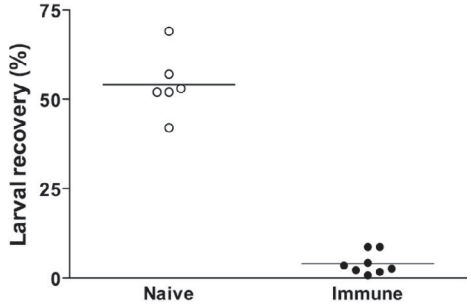

Figure 1. Percentage of infective larvae recovered from tissue digest of sheep either naïve (open circles) or immune (closed circles) to parasites, following the in vitro challenge with 2 500 *Teladorsagia circumcincta* L3. Tissue from each sheep was challenged in triplicate; each point represents the mean of three replicates. The horizontal line represents the mean for each group.

tissue. Mascot search data for all these proteins are presented in Table I. The level of significance of each hit is also shown in Table I. A small number of spots did not provide a significant ovine/bovine (or parasite) hit and thus were excluded from the reference map and Table I.

The study focused on proteins that were differentially expressed, in the epithelial cell lysates from naïve and immune gastric mucosa. Spots of interest included abundant spots or spots that appeared to change in intensity between immune and naïve mucosa. Figure 4 shows the total spot volume of two proteins that were statistically shown to be differentially expressed between naïve and immune tissue following the in vitro challenge. Lysozyme 1 (LYZ1) was up regulated in immune samples (Fig. 4); similarly, intelectin 2 (ITLN2) was up-regulated in immune tissue ($P < 0.05$). ITLN2 spots in particular were positively identified from 6 out of 8 immune animals, whereas they were identified in one of the naïve samples. Other proteins, such as endoplasmin (HSP90B1), were more highly expressed in naïve tissue, although the difference observed between naïve and immune tissue was not significant. No other proteins, whether identified or not, differed

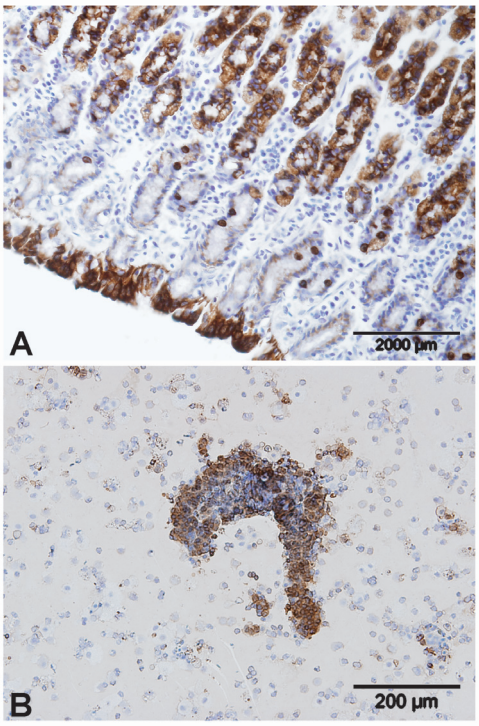

Figure 2. (**A**) Gastric epithelium fixed with Zinc salts and stained with pancytokeratin antibody. The staining was specific for surface mucus cells, mucus neck cells and gastric chief cells, whereas no inflammatory cells are stained. (**B**) Epithelial cell cytospin following the enzymatic dispersion of epithelial cells from the gastric mucosa. Cytospin was fixed in cold methanol and stained with pancytokeratin antibody. Note the intact gastric pit.

significantly between immune and naïve samples.

3.4. Analysis of mucosal wash by LC-ESI-MS/MS

Mucosal washings deriving from previously naïve or immune gastric tissue, with or without in vitro larval challenge, were analysed by LC-ESI-MS/MS in combination with database searching against NCBI and Swiss-Prot databases. Numerous proteins were identified from each sample. A total of 74 proteins with significant identity were

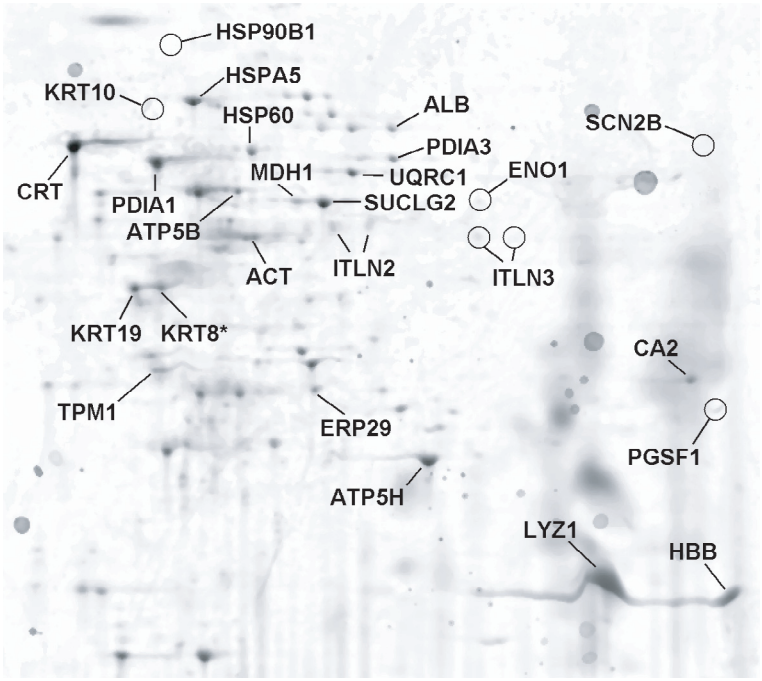

Figure 3. Two-dimensional electrophoretic reference map of epithelial cells derived from immune gastric mucosa following in vitro challenge with 2 500 *T. circumcincta* L3. Proteins were identified by tryptic peptide mapping from Coomassie-blue stained gels. The locations of the spots identified in other gels, but which were not present in this specific gel, are shown with open circle.

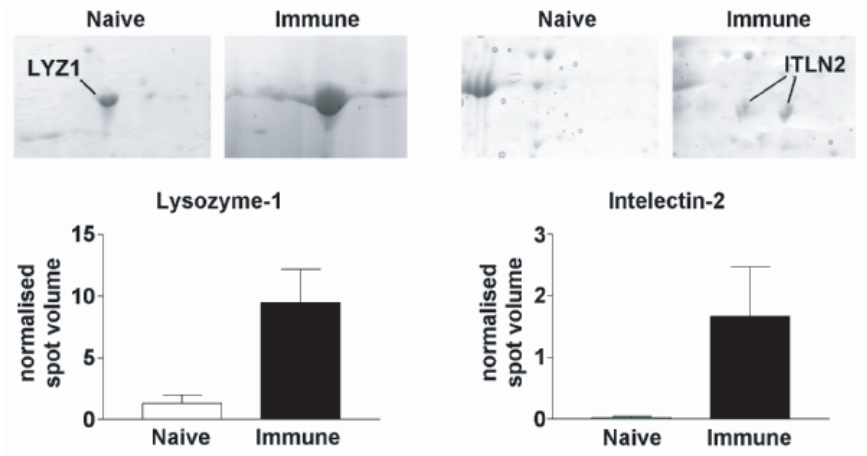

Figure 4. Total spot volume of lysozyme (LYZ1) and intelectin 2 (ITLN2) spots as detected by 2-DE of epithelial cell lysates from naïve or immune gastric tissue following in vitro challenge with 2 500 *T. circumcincta* L3. Vertical bars indicate standard errors. Examples from typical 2-DE gels are shown.

Table I. List of proteins recovered from 2-DE gels from epithelial cell lysates, following the in vitro challenge of previously naïve or immune tissue with 2 500 *T. circumcincta* L3. Peptide sequences were searched against NCBI and Swiss-Prot databases. The Mascot score, number of peptides matched and sequence coverage are shown, as well as theoretical expected MW and pI.

Gene name (Swiss-Prot)	Identity MASCOT	Accession No.	Mascot score	Level of significance	Peptides matched	Coverage (%)	exp MW kDa	exp pI
ACT	Actin (*Bos taurus*)	gi|54036676	99	y	17	50%	41.7	5.31
ALB	Serum albumin (*Ovis aries*)	gi|57164373	122	y	14	31%	71	5.8
ATP5B	ATP synthase beta chain (*Bos taurus*)	gi|28461221	200	y	37	78%	56.2	5.15
ATP5H	ATP synthase d chain (*Bos taurus*)	gi|27807305	83	y	9	60%	18.7	6
CA2	Carbonic anhydrase 2 (*Ovis aries*)	gi|118582300	68	y	8	44%	29.2	6.4
CRT	Calreticulin (*Bos taurus*)	gi|27806723	193	y	21	52%	48.1	4.31
ENO1	Enolase 1 (*Bos taurus*)	gi|87196501	101	y	13	37%	47.6	6.4
ERP29	Endoplasmic reticulum protein 29 (*Bos taurus*)	gi|115495555	116	y	12	43%	28.8	5.6
HBB	Hemoglobin beta subunit (*Ovis aries*)	gi|122546	128	y	10	77%	16.1	6.75
HSP60	60 kDa heat shock protein (*Bos taurus*)	gi|76644268	144	y	17	34%	61.1	5.7
HSP90B1	Endoplasmin (*Bos taurus*)	gi|33301108	108	y	19	27%	92.3	4.76
HSPA5	78 kDa glucose regulated protein (*Bos taurus*)	gi|122144501	153	y	30	46%	72.2	5.07
ITLN2	Intelectin 2 (*Ovis aries*)	gi|145308887	124	y	12	41%	36.4	5.8
ITLN3	Intelectin-3 (*Ovis aries*)	gi|157886697	130	y	11	39%	36.6	8.8
KRT10	Keratin 10 (*Bos taurus*)	gi|27805977	82	y	14	21%	55	5
KRT19	Keratin type 1, cytoskeletal 19 (*Bos taurus*)	gi|62751472	184	y	20	51%	43.8	4.9
KRT8*	Keratin type ii cytoskeletal 8 (*Bos taurus*)- fragment	gi|75812916	133	y	15	30%	53.6	5.7
LYZ1	Lysozyme 1 (*Ovis aries*)	gi|5802272	141	y	8	66%	14.3	6.08
MDH1	Cytosolic malate dehydrogenase (*Bos taurus*)	gi|77736203	64	n (0.072)	9	31%	36.7	6.2
PDIA1	Protein disulfide isomerase A1 (*Bos taurus*)	gi|148878430	274	y	25	53%	57.6	4.8
PDIA3	Protein disulfide-isomerase A3 (*Bos taurus*)	gi|729433	91	y	17	36%	57.3	6.2
PGFSI	Prostaglandin-F synthase 1 (*Bos taurus*)	gi|119905464	63	n (0.077)	8	17%	29.7	6.8
SCN2B	Sodium channel beta-2 subunit (*Canis familiaris*)	gi|73955080	52	n (0.081)	9	16%	56.5	7.9
SUCLG2	Succinyl-CoA ligase (GDP-forming) beta-chain (*Bos taurus*)	gi|146231894	120	y	12	34%	47	7.5
TPM1	Tropomyosin 3 isoform 2 isoform 18 (*Canis familiaris*)	gi|73961101	104	y	15	40%	26.6	4.8
UQCRC1	Ubiquinol-cytochrome-c reductase complex core protein 1 (*Bos taurus*)	gi|4139392	202	y	18	56%	50	5.5

n: Non significant hit; y: significant hit.

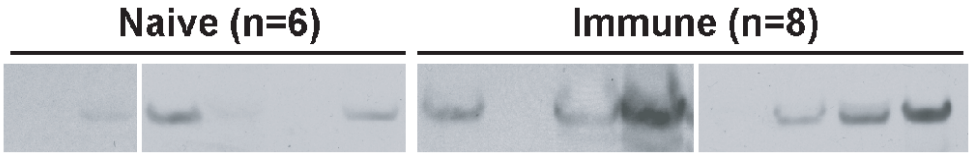

Figure 5. Western blots of intelectin in individual samples of epithelial cell lysates, following the in vitro challenge with 2 500 *T. circumcincta* L3. ITLN was detected in 3 out of 6 samples that were naïve prior to the in vitro challenge, whereas it was highly expressed in 6 out of 8 samples that were immune prior to the in vitro challenge.

recovered from the mucosal washes. The minimum score that was considered significant was 50 with a minimum of two matched peptides. Within each individual sample, a cumulative score was calculated for each identified protein, to account for cases where a protein was present in more than one gel slice. Although this score gives a suggestion as to the relative amount of a particular protein in a particular sample, it should be stressed that this is not quantitative, and should not be over interpreted. Although the results give reliable evidence for the existence of specific proteins in mucosal washes, any apparent differences between samples must be treated with caution. The proteins identified in each sample are shown in Table II. In cases where there were multiple hits for particular proteins, hits deriving from ovine or bovine databases are presented. Proteins with the highest Mascot score, and likely to be among the most highly abundant proteins in mucosal washes included serum albumin, complement component 3, heat shock cognate 71 kDa protein, creatine kinase, gelsolin and lysozymes. The identified proteins were categorised as those involved in the cytoskeleton, plasma proteins, or indicators of innate and adaptive immune responses. Proteins that were identified in the mucosal wash of immune, but not naïve tissue, included SMCP, IgA, and galectins 14 and 15. ITLN and CLCA were only present in the wash deriving from immune tissue following the parasite challenge. Conversely, fatty acid binding protein 5 was observed only in naïve washes. In addition to those proteins, nuclear proteins, such as histones, and a variety of enzymes were present in all samples, including carbonic anhydrase, alpha enolase, glutathione-s transferase, creatine kinase and proteinase inhibitors.

3.5. Western blots of proteins of interest in epithelial cells lysates and mucosal washes

Representative Western blots were performed to validate the proteomic findings and in general they achieved to do so. In the individual epithelial cell lysates samples, ITLN was highly visible with strong reactive bands in 6 out of 8 immune samples; in only 3 out of 6 naïve samples there was a weak reactivity, whereas no reactive bands were observed in the remaining naïve samples (Fig. 5). Western blots on individual mucosal wash samples were also performed for IgA, ITLN, SMCP and CLCA (Fig. 6); as seen in Figure 6, strong bands for all these proteins were observed in the majority of immune challenged samples, but not in naïve challenged samples. This was in accordance with the proteomic analysis of the mucosal washes.

4. DISCUSSION

The present study utilized an in vitro organ challenge system and employed a global approach to identify effector molecules that may be responsible for the exclusion of infective *T. circumcincta* from the gastric mucosa, in the mucus and epithelial cell extracts. This in vitro system was used to examine the critical first phase of the establishment process, when larvae come in close association with the tissue [13]. The pattern observed i.e. fewer larvae being closely

Table II. List of proteins recovered from gastric mucosal wash, following the in vitro challenge of previously naïve or immune tissue with 2 500 *T. circumcincta* L3 (+) or the unchallenged controls (−). Peptide sequences were searched against NCBI database (Taxonomy: Other mammalia) through Mascot. For each identified protein the most significant hit and the number of peptides matched is shown. In addition, cumulative MASCOT scores are shown for each sample, since many proteins were spread over several gel slices. Mascot scores above 50 with two peptides matched were considered significant.

					Naïve		Immune	
Accession No. NCBI	Species	Protein name	MW (Da)	Identity hit	−	+	−	+
				MASCOT score (No. of peptides matched)	Cumulative MASCOT score			
Heat shock proteins and chaperones								
gi|66356310	*Ovis aries*	78 kDa glucose-regulated protein (GRP 78)	45241	113 (3)				113
gi|148887198	*Bos taurus*	Heat shock cognate 71 kDa protein	71424	325 (16)	249	447	301	543
Cytoskeleton								
gi|114051526	*Bos taurus*	Coactosin	16114	104 (3)	104	85	74	95
gi|2665740	*Ovis aries*	Beta actin	28096	109 (9)	275	280	111	159
gi|61553131	*Bos taurus*	Alpha 2 actin	45705	81 (6)		81		
gi|78369242	*Bos taurus*	Actinin alpha-1	103486	101 (5)		101		52
gi|119912356	*Bos taurus*	Keratin 13	50660	85 (3)			85	
gi|134085706	*Bos taurus*	Keratin 6	61182	199 (10)			199	
gi114051856	*Bos taurus*	Keratin 7	51603	148 (6)		52	231	137
gi|75812916	*Bos taurus*	Keratin 8	53552	143 (5)		218	189	
gi|27806351	*Bos taurus*	Villin 2	68832	291 (15)	87	51	117	291
gi|75775321	*Bos taurus*	Vitamin D-binding protein precursor	54904	53 (4)	69		53	100
gi|114050715	*Bos taurus*	Moesin	68047	70 (6)	70		76	
gi|77736201	*Bos taurus*	Gelsolin b	80966	233 (10)	189	138	230	277
gi|61888874	*Bos taurus*	Transgelin 2	22583	176 (9)	331	358	138	246
gi|28603774	*Bos taurus*	Rho GDP dissociation inhibitor (GDI) alpha	23464	67 (5)	57		67	
gi|62751593	*Bos taurus*	Profilin 1	15219	272 (16)	346	169	205	352
gi|115496125	*Bos taurus*	Radixin	68639	81 (5)				81
gi|543113	*Bos taurus*	Smooth muscle protein SM22	19326	139 (8)	139			
Nucleus								
gi|118151208	*Bos taurus*	Histone H2B	13867	108 (10)	52	74	185	313
gi|61845671	*Bos taurus*	Histone 4	11388	114 (5)	179	107	108	56

Plasma proteins								
gi|57164373	*Ovis aries*	Serum Albumin	71139	2106 (99)	5349	3566	4522	5477
gi|83764016	*Bos taurus*	Complement component 3	188715	122 (5)	120	50	396	432
gi|157954061	*Bos taurus*	A-2 macroglobulin precursor	168953	211 (13)	85		301	245
gi|57164383	*Ovis aries*	Antithrombin III	52979	116 (5)	116			56
gi|75831056	*Bos taurus*	Apolipoprotein A1	30258	82 (2)	82	56		
gi|164448674	*Ovis aries*	Beta globin	16050	140 (10)	39	228	53	63
gi|94966763	*Bos taurus*	Haptoglobin	45629	59 (3)		59		
gi|29135265	*Bos taurus*	Transferrin	79856	183 (11)	283	304	338	223
gi|57526651	*Ovis aries*	Transthyretin	15875	57 (2)	57			
gi|1787	*Ovis aries*	Alpha globin chain	15250	66 (3)		106	114	63
gi|57526674	*Ovis aries*	Fetuin	39511	50 (4)		50		
gi|31340900	*Bos taurus*	Serine proteinase inhibitor (SERPINA 3)	46289	95 (2)	145	78	66	169
gi|165839	*Ovis aries*	Beta lactoglobulin	20334	304 (10)		541		
Cellular proteins								
gi|76669648	*Bos taurus*	Annexin 10	37521	253 (10)	63	140	135	253
gi|27807289	*Bos taurus*	Annexin A2	38873	139 (8)		48	71	139
gi|120474983	*Bos taurus*	Annexin A5	36109	189 (9)	104	78	99	283
gi|74	*Bos taurus*	Annexin I	39158	195 (10)	56	175	264	216
gi|74354599	*Bos taurus*	Phosphatidylethanolamine binding protein	21106	63 (2)	145	50	59	75
gi|62751970	*Bos taurus*	Chloride intracellular channel 1	27317	67 (4)		67		
gi|27805805	*Bos taurus*	Fatty acid binding protein 5	15350	80 (3)	80	75		
gi|32189340	*Bos taurus*	Adenine nucleotide translocator	33174	55 (2)	55			
Enzymes								
gi|57526379	*Ovis aries*	Aldehyde dehydrogenase	55417	159 (9)	159			
gi|156120479	*Bos taurus*	Aldolase A	39925	68 (9)				68
gi|4927286	*Bos taurus*	Alpha enolase	47589	198 (9)	188	219	179	252
gi|119910440	*Bos taurus*	ATPase, H+/K+ exchanging, alpha polypeptide variant	115873	57 (2)	57			
gi|118582300	*Ovis aries*	Carbonic anhydrase 2	29193	69 (8)		119	68	56
gi|13096153	*Bos taurus*	Creatine Kinase	42836	209 (11)	49	322	374	285
gi|57164307	*Ovis aries*	Cytosolic NADP-isocitrate dehydrogenase	47153	101 (4)	78	101		

Table II. Continued.

Accession No. NCBI	Species	Protein name	MW (Da)	Identity hit	Naïve		Immune	
					−	+	−	+
gi|83035079	*Bos taurus*	Glutaredoxin or Thioltransferase 1	12003	53 (2)	53			61
gi|28461273	*Bos taurus*	Glutathione S-transferase Pi	25789	130 (6)	141	112	130	126
gi|10505253	*Bos taurus*	Hypoxanthine phosphoribosyltransferase	21151	71 (2)	79	71		53
gi|27807469	*Bos taurus*	Peroxiredoxin 2	22217	64 (3)	55	48	76	144
gi|57526769	*Ovis aries*	Preprochymosin	42447	177 (11)		356		
gi|119903961	*Bos taurus*	Protein disulfide-isomerase	64930	80 (4)				80
gi|47606778	*Bos taurus*	Ribonuclease UK114	14347	79 (6)				79
gi|57164261	*Ovis aries*	Thioredoxin	12090	57 (2)	54		57	
gi|61888856	*Bos taurus*	Triose phosphate isomerase	26901	63 (2)		63		
gi|530049	*Ovis aries*	Tyrosine 3 monooxygenase tryprophane 5	26450	98 (5)	56	168	81	165
Adaptive immune responses								
gi|119926764	*Bos taurus*	Fc fragment of IgG binding protein	340837	58 (3)				58
gi|109029	*Ovis aries*	Ig heavy chain C region	34327	292 (25)	212	301	492	417
gi|109030	*Ovis aries*	Ig lambda chain C region - sheep	11476	427 (15)	548	129	81	451
gi|52366986	*Ovis aries*	Ig lamda light chain region segment 1	11493	621 (22)	540	394	838	995
gi|165945	*Ovis aries*	IgM chain	54197	147 (6)	66		147	56
gi|2582411	*Ovis aries*	IgA heavy chain	51292	387 (12)			75	387
gi|7547266	*Bos taurus*	IgG1 heavy chain constant region	36510	67 (6)				67
Innate immune responses								
gi|119919506	*Bos taurus*	Mucin 5	407449	83 (8)	83			
gi|57163983	*Ovis aries*	Galectin 14	18354	61 (3)			61	
gi|57163975	*Ovis aries*	Galectin 15	15621	167 (8)			180	256
gi|145308887	*Ovis aries*	Intelectin 2	36386	56 (3)				56
gi|165970	*Ovis aries*	Lysozyme 2a	14833	255 (24)		447	147	391
gi|27923983	*Ovis aries*	Lysozyme C-1	16698	416 (30)	471	271	53	528
gi|57164171	*Ovis aries*	Mast cell proteinase 1	27202	74 (3)			74	93
gi|76613482	*Bos taurus*	Calcium activated chloride channel 1	101004	76 (3)				76

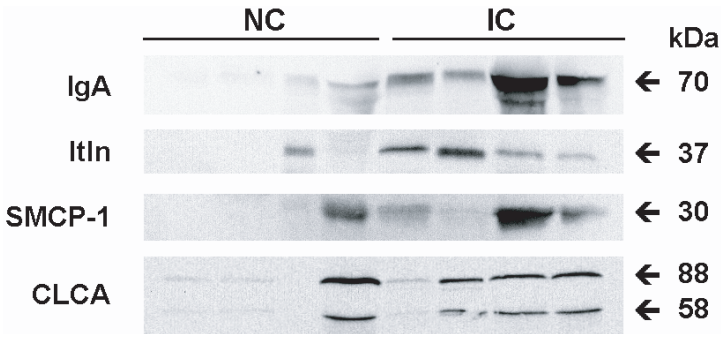

Figure 6. Western blots of individual mucosal wash samples either naïve (N) or immune (I) prior to the in vitro challenge with 2 500 L3 of *T. circumcincta* probed with antibodies to (a) IgA, (b) ITLN, (c) SMCP1, (d) CLCA. See material and methods for details.

associated with previously immune tissue than previously naïve tissue, is in agreement with previous studies, where both in vitro tissue association [13] and in vivo larval establishment in naïve tissue were shown to be between 30–60% [27]. The present study showed that the tissue explants can maintain immunological function in vitro, at least for the time tested in this study. From the changes observed in the protein content of epithelium and mucus following larval challenge it is apparent that the in vitro model provided a technique which can be usefully deployed in investigations into recently established or immediate tissue responses to larval challenge.

The use of the described enzymatic methodology for the isolation of epithelial cells from the gastric mucosa was based on the previously described method for the isolation of gastric crypts of bovine mucosa described by Dziva et al. [4]. Cytospins confirmed that there was minimal contamination from non-epithelial lamina propria derived cells, compared to other methods used to isolate epithelial cells, such as mucosal scrapings and the use of EDTA (preliminary data, not included here), and only minimal contamination with albumin was observed in 2-D gels. The epithelial cells retained much of their viability during their isolation, although some dead cells (3%) were also observed. The analysis of the two-dimensional gel electrophoresis of epithelial cell lysates revealed that a number of proteins identified were differentially expressed in naïve and immune cells. For example, intelectin and lysozymes were more abundant in epithelial cell lysates from immune samples. ITLN is a calcium dependent lectin, which can recognise galactofuranose containing components of bacterial cell walls. It has been shown to be expressed by the Paneth cells in the small intestine of mouse [15] and the mucus neck cells in the abomasum of sheep [5]. Of particular interest is the finding that high ITLN expression was related to nematode expulsion in *T. spiralis* rodent models [25]. Furthermore, it has recently been shown to be highly expressed in the gastric mucosa of sheep infected with *T. circumcincta*, during the phase of parasite expulsion [6]. The authors suggest that this protein plays an important role in the exclusion of infective larvae from the gastric mucosa, and the present finding of increased levels of intelectin in epithelial cells from immune tissue, compared to those from naïve tissue, is supportive of this hypothesis. Although ITLN was detected at a low level in cell lysates from naïve epithelium, the increased level of expression in immune epithelium and its presence in mucus following larval challenge provides further supportive evidence of a possible role for ITLN in the host response to nematodes. Increased levels of both intracellular and mucus-related ITLN supports increased expression and release of this protein.

Although previous work has investigated histological and immunohistological changes in mucus from excised immune gastrointestinal tissue [19,20], the in vitro challenge model enabled a comparative study of the mucus contents within 2 h following larval challenge. This immediacy allowed a comparative analysis of proteins actively secreted into the mucus, in response to the larval challenge, and should reflect the functional activity of these tissues. The methodology used to attain the mucus for the proteomic analysis from the gastric mucosa, was the least invasive one as evaluated with histological sections of the gastric mucosa following the procedure. Alternative procedures tested included the use of swabs and gentle mucosal scraping to remove mucus (preliminary data not showed here). Although a small degree of contamination with epithelial cells can not be excluded, we are confident that such contamination was minimal and most of the proteins identified in the mucosal wash were derived from the mucus layer itself. As cytosolic and nuclear proteins were identified during the shotgun analysis, it is possible that the presence of some proteins identified in the mucosal wash, such as the nuclear histones H4 and H2B, may have been due to cell lysis rather than secretion. The products from lysed epithelial cells constitute a well documented component of mucus [22].

A number of proteins were identified in the mucosal wash from previously immune tissue, which were not present in the mucosal wash of the naïve tissue and thus may constitute proteins which are associated with the development of the adaptive immune response to nematodes. Examples of these were IgA, Galectin 14 and 15 and SMCP1, all of which have previously been shown to be up-regulated in gastric tissues in association with immunity to nematodes. IgA mediated suppression of nematode growth and fecundity appears to be one of the key mechanisms of resistance to *T. circumcincta* in sheep [28]. Furthermore, levels of local IgA in the lymph of sheep parasitised with *T. circumcincta* were higher in immune than in naïve sheep [9]. Galectins are carbohydrate-binding proteins that have been increasingly implicated in adaptive immune responses to nematodes. Galectin 14 in particular, is expressed by eosinophils which characteristically accumulate at the site of infection [18]. Similarly, galectin 15 (OVGAL11) expression was shown to be greatly up-regulated in helminth larval infected gastrointestinal tissue subject to inflammation and eosinophil infiltration [3]. Immunohistological analysis has showed that the protein is localized in the cytoplasm and nucleus of upper epithelial cells of the gastrointestinal tract and it has also been detected in mucus samples collected from infected abomasum but not from uninfected tissue [3]. Gastrointestinal infection is characterised by the presence of increased numbers of mucosal mast cells, and these are pivotal in the immediate hypersensitivity reaction associated with immune rejection of nematode parasites in rodents [12, 21]. During the process of immune expulsion, mast cells actively secrete a range of effector molecules, including histamine, monoamines, leukotrienes and proteases. The latter includes the secretion of SMCP1 [11] following experimental larval challenge in immune sheep. In mice, lack of the mast cell protease MMCP-1 resulted in delayed expulsion of the nematode *Trichinella spiralis* [14]. Thus, although the release and identification of these compounds was of no surprise, it does indicate that the in vitro challenged tissue responded in a manner consistent with previous in vivo studies, and is consistent with retention of functional activity within the tissue following challenge.

Of particular interest in the present study was the demonstration of increased levels of intelectin and CLCA in challenged mucus. While intelectin has been previously implicated in immunity to nematodes [6], the demonstration of a secreted CLCA protein in response to parasite challenge is novel. CLCA proteins are putative Ca^{2+}-activated Cl^{-} channels or channel regulators but have also been shown to affect epithelial secretion, cell-cell adhesion, apoptosis, cell cycle control, and mucus production (for review, see [16, 30]). hCLCA1 and mCLCA3 (alias gob-5) are distributed in mucin producing goblet cells [16]. Recent studies have shown that all subunits of hCLCA1 and mCLCA3 are

secreted soluble proteins rather than being trans-membrane proteins [7, 23]. Expression of hCLCA1 or mCLCA3 was found to be up-regulated following exposure to respiratory tract irritants that are responsible for a Th2 local inflammation [26, 34]. It has been shown that at transcript and protein level, expression of hCLCA1 was up-regulated by IL-13 in normal human bronchial epithelial cells in vitro [33]. Whether the secretion of the ovine CLCA protein has a direct or indirect function in worm expulsion remains unknown, although hCLCA1 has been associated with an increase in mucus production following a Th2 response of the respiratory tract [34]. Furthermore, increased expression of IL-9 resulted in increased expression of hCLCA1 mRNA by epithelial cells in patients suffering from asthma [31] suggesting that hCLCA1 may be responsible at least in part for the overproduction of mucus by the asthmatic patients. Increased volume or viscosity of mucus may result in entrapment of infective larvae and thus reduced establishment in tissues that have been previously challenged with the parasite. A similar mechanism of action of mucus has been hypothesised during the phase of expulsion of nematodes [20].

In conclusion, the exclusion of infective larvae from the gastric mucosa is associated with altered levels of a variety of proteins, most of which are directly related to the acquired Th2 pro-inflammatory response, as well as to innate immunity. The usefulness of this in vitro model has been confirmed, and the global proteomic approach has identified proteins having a potential role in the parasite exclusion from abomasal mucosa. Further attempts should be concentrated in quantifying their expression at transcript and protein level and identifying the role of these proteins in the exclusion of infective larvae.

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