MUCIN CHANGES ASSOCIATED WITH ABOMASAL PARASITISM IN SHEEP

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ABSTRACT

Mucins play important roles in host-pathogen interactions, influencing host resistance, establishment of infection, as pathogen recognition sites and a source of nutrients. They are highly glycosylated molecules and changes in monosaccharide composition during parasitism have been reported. Effects of parasites on monosaccharide component of fundic and duodenal mucins of sheep were investigated in 3 age ranges (i) 4-4.5, (ii) 6 and (iii) 8-9 months old: (1) non-infected; (2) infected with 10,000 *Haemonchus contortus* and euthanased 21 days post infection (p.i.); (3) infected with 50,000 *Teladorsagia circumcincta* and euthanased 28 days p.i. Three days-old lambs and 9 weeks-old lambs: (a) milk-fed, (b) solid-fed and (c) solid-fed, infected with *T. circumcincta* were also included. The effects of *H. contortus* and *T. circumcincta* infection in mucin changes were significantly different in the fundus, however, both of them shared some similarities. Infected sheep showed lower proportion of fucose and sialic acids in fundic and duodenal mucins compared with non-infected animals, the level of sulphation varied depending on the age of infected sheep: decrease in young sheep but increase in older animals. *H. contortus* infection also caused increased proportions of GlcNAc and Gal in fundic mucins and duodenal mucins respectively at all ages, however, in *T. circumcincta* infection, it was shown that the alterations of mucins were age-dependent. *T. circumcincta* infected sheep showed the significant changes at young ages (4-6 months-old) while 8-9 months-old animals showed less change in fundic mucins compared with non-infected animals. Effects of *H. contortus* and *T. circumcincta* infection differed in the fundic mucins but were similar in the duodenum. The study showed that parasitism caused the modifications of monosaccharide composition in gastrointestinal mucins of sheep. These alterations may result from parasite species differences, causing different effects from the host's immune response. The changes in mucin profiles observed in the duodenum of sheep infected with abomasal nematodes suggested that the host may respond to parasitism. This would facilitate the use of mucins from accessible sources, without euthanasing the animals, to investigate the changes in mucin compositions which can be used to diagnose the susceptibility or resistance of sheep to parasites.

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LIST OF ABBREVIATIONS

AB	Alcian blue
AMPS	ammonium persulphate
µC	microcoulon
µg	microgram
µl	microlitre
10X	10 times
AAL	*Aleuria aurantia* lectin
AB/PAS	Alcian blue/ Periodic acid Schiff
ANOVA	Analysis of variance
Asp	Asparagine
BPL	*Bauhinia purpurea* lectin
BSA	Bovine serum albumin
BSM	Bovine submaxillary mucin
Ca^{2+}	Calcium ion
CD4+	Co-receptor
CF	Cystic fibrosis
ConA	Concanavalin A
CsCl	Cesium chloride
Da	Dalton
DAB	Diamino benzidine
DBA	*Dolichos biflorus* agglutinin
ddH_2O	Distilled water

DNA	Deoxyribonucleotide
DTT	Dithiothreitol
E. trivolvis	*Echinostoma trivolvis*
EDTA	Ethylene diamine tatra acetic acid
ELLA	Enzyme-linked lectin assay
ES	Excretory/ secretory products
F. hepatica	*Fasciola hepatica*
FEC	Faecal egg count
Fuc	Fucose
g	gram
Gal	Galactose
GalN	Galactosamine
GalNAc	N-acetyl galactosamine
GalNAcTs	N-acetyl galactosaminyl transferase
Glc	Glucose
GlcN	Glucosamine
GlcNAc	N-acetyl glucosamine
GSA-I	*Griffonia simplicifolia* lectin I
GSA-II	*Griffonia simplicifolia* lectin II
GuHCl	Guanidinium chloride
h	hour
H. contortus	*Haemonchus contortus*
H. pylori	*Helicobacter pylori*
H_2SO_4	Sulphuric acid

HCl	Hydrochloric acid
HID	High iron diamine
HPAEC	High performance anion exchange chromatography
HPLC	High performance liquid chromatography
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
kDa	kiloDalton
KOH	Potassium hydroxide
L_1	First stage larvae
L_2	Second stage larvae
L_3	Third stage larvae
L_4	Fourth stage larvae
LCA	*Lens culinaris* agglutinin
LPA	*Limulus polyphemus* agglutinin
LTA	*Lotus tetragonolobus* lectin
MAL	*Maackia amurensis* lectin
Man	Mannose
min	minute
ml	millilitre
mM	millimolar
MMC	Mucosal mast cells
MNC	Mucous neck cell
MPL	*Maclura pomifera* lectin

MQ-water	MilliQ water
MUC	Mucin type
MW	Molecular weight
MWCO	Molecular weight cut-off
n	number
N. brasiliensis	*Nippostrongylus brasiliensis*
NaCl	Sodium chloride
NANA	N-acetyl neuraminic acid
NaOH	Sodium hydroxide
ND	Non-diseased
NEM	N-ethylmaleimide
NGNA	N-glycolyl neuraminic acid
nm	nanometre
O. dentatum	*Oesophagostomum dentatum*
O. ostertagi	*Ostertagia ostertagi*
p	probability
P. aeruginosa	*Pseudomonas aeruginosa*
PAD	Pulse Amperometric Detection
PAGE	Polyacrylamide gel electrophoresis
PAS	Periodic acid Schiff
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PES membrane	Polyethersulfone membrane

PHA	*Phaseolus vulgaris* agglutinin
p.i.	post infection
PMSF	phenylmethylsulphonyl fluoride
PNA	Peanut agglutinin
PSA	*Pisum sativum* agglutinin
PSM	Porcine submaxillary mucin
PTL	*Psophocarpus tetragonolobus* lectin
PVDF	Polyvinylidene fluoride
RCA	*Ricinus communis* agglutinin
RNA	Ribonucleic acid
RO water	Reverse osmosis water
rpm	Revolution per minute
S. venezuelensis	*Strongyloides venezuelensis*
SBA	Soybean agglutinin
SD	Standard deviation
SDS	sodium dodecyl sulphate
sec	second
Ser	Serine
SMC	Surface mucous cell
SNA	*Sambucus nigra* agglutinin
T. axei	*Trichostrongylus axei*
T. circumcincta	*Telardosagia circumcincta*
T. colubriformis	*Trichostrongylus colubriformis*
T. muris	*Trichuris muris*

T. spiralis	*Trichinella spiralis*
T. taeniaformis	*Taenia taeniaformis*
TBS	Tris-buffered saline
TEMED	N, N, N', N'- tetramethylethylenediamine
TFF	Trefoil peptide family
Th	T-helper
Thr	Threonine
TLC	Thin layer chromatography
TNF	Tumour Necrosis Factor
UEA	*Ulex europaeus* agglutinin
UV	ultraviolet
V	violet
V_0	Void volume
V_e	Elution volume
V_t	Total volume
WGA	Wheat germ agglutinin
Y. enterocolitica	*Yersinia enterocolitica*

INTRODUCTION

For many years, the agricultural industry has played a key role in the New Zealand economy. The products obtained from sheep and cattle contribute a large part to the total national income. With 40 million sheep in the country (stated on the 30 June 2005 by Statistics New Zealand), products from sheep not only supply the domestic population, but they are exported to many other countries. Since sheep are living on pasture, they can easily become infected by helminths. Once these parasites infect the sheep, they may cause significant economic losses due to reduced wool production and acute disease and death in susceptible young lambs.

Gastrointestinal nematodes cause serious loss to farm production. In sheep, parasitism by *H. contortus* and *T. circumcincta* cause anemia, anorexia, reduction in body weight and wool growth and death. The emergence of multiple-drug resistant nematodes has increased the need for improved and sustainable methods of controlling nematodes, as well as selection of resistant or resilient lines of sheep. To develop novel therapies or select for hosts resistant to parasites, more needs to be learned about the host-parasite interaction.

Mucus and mucins are at the site of interaction of host and parasite. Many pathogens targeting epithelial surfaces recognize their niche for invasion and interact with the tissues through specific carbohydrate residues on the cell membranes and mucins. Parasitic nematodes may also identify their site of infection in this way. Many parasites degrade mucins to invade host tissues and for nutrition. Gastrointestinal mucus is involved in expulsion of parasites, yet little is known about the changes in mucin glycosylation during parasitism. Answers to these questions may open new opportunities to interfere with parasite establishment.

The present project aims to identify the changes in the mucins of the fundus and duodenum of sheep while the parasites *H. contortus* and *T. circumcinta* are present in the abomasum. Little is known about the changes in carbohydrate content of mucins that occur during the infection with these nematodes. It is hoped that increased knowledge of these carbohydrate residues may lead to a better

understanding of the host-parasite interactions and the role of these moieties in host protection or parasite establishment.

The project involves both chemical analysis of mucins, histochemistry and lectin binding to tissues collected from the abomasum and duodenum. Chemical analysis can be used to identify changes in the proportions of hexoses and hexosamines making up the mucins and histochemistry can be used to study post-translational modifications, such as sialylation and sulphation. Lectin histochemistry provides an insight into the presence and distribution of specific carbohydrate residues in different cell types which secrete mucins. The overall objective of the project is to gain more knowledge about which alterations occur in mucins in the gastrointestinal tract with age and infection with abomasal parasites.

Chapter 1

LITERATURE REVIEW

1.1. ABOMASAL NEMATODE PARASITES

Genera of nematode parasites that cause losses in the farming industry include *Haemonchus*, *Ostertagia*, *Trichostrongylus*, *Nematodirus* and *Cooperia*. Among these gastrointestinal parasites*, Haemonchus contortus* or the barber's pole worm and *Teladorsagia (Ostertagia) circumcincta,* the brown stomach worm, which both belong to the family Trichostrongylidae are two of the predominant nematodes that infect sheep in New Zealand.

1.1.1. Life cycle of abomasal nematode parasites

H. contortus and *T. circumcincta* have a similar life cycle but differ in the length of developmental stages. The life of a nematode begins when adults mate in the stomach of sheep and adult female worms start laying eggs. The number of eggs laid by female worms varies with the species and can be as many as 7000 eggs per day in *H. contortus*, depending on host factors and parasite density (Coyne et al., 1991; Coyne and Smith, 1992). Fertilised eggs are released with the faeces of the infected sheep onto pasture. Eggs hatch and become first stage larvae. The time needed for eggs to develop into larvae depends on the weather conditions, particularly the temperature and moisture and may take five days to several months. Larval development usually takes place in moist conditions and at a temperature between 10°-40°C for *H. contortus* and 1°-35°C for *T. circumcincta* (O'Connor et al., 2006). The optimum temperature ranges for larvae to develop are 16°-30°C and 25°-37°C respectively. The first stage larvae (L_1) develop into second stage larvae (L_2) and then develop into the third stage larvae (L_3), which is the infective stage.

After being ingested by sheep, the L_3 exsheath in the rumen and move to the abomasum with the food (Sommerville, 1957, 1964; Roger and Sommerville, 1960, 1963, 1968; Hertzberg et al. 2002). There, larvae penetrate the gastric pits and glands and undergo further development. *H. contortus* emerges after 2-4 days from ovine gastric glands (Stoll, 1943; Sommerville, 1963; Hunter and MacKenzie, 1982) while *T. circumcincta* remains there for 5-6 days (Sommerville, 1954; Dash, 1985) before completing their development into adult worms in the lumen. It takes *H. contortus* about 14 days and *T. circumcincta* approximately 21 days to develop to mature worms.

1.1.2. Clinical diseases

H. contortus and *T. circumcincta* infections cause many adverse effects in the animals including altered protein, carbohydrate, fat, energy and mineral metabolism, gastrointestinal function, diarrhea causing dehydration, anorexia and weight loss (reviewed by Holmes, 1987; Fox, 1993, 1997; McKellar, 1993). Oedema may also occur in severe cases. Prolonged nematode infection may cause impaired development of the skeleton (Sykes et al., 1977). Anemia also occurs in *H. contortus* infection (Rowe et al., 1988; Le Jambre, 1995). The blood intake of each worm can be up to 50 µl per day (Rowe et al., 1988). Excessive blood loss results in illness through the reduced number of oxygen-transporting erythrocytes and can be fatal.

Anorexia is an important cause of loss of production and reduced body weight in sheep infected with gastrointestinal parasites, including abomasal nematodes (Horak and Clark, 1964; Sykes et al., 1977; Abbott et al., 1986; Coop and Holmes, 1996). Loss of appetite may be caused by altered gut motility, cytokines or hormones such as gastrin, leptin or ghrelin (Bueno et al., 1982; Fox, 1997; Fox et al., 1989; Kyriazakis et al., 1998).

Because of the impaired metabolism, sheep infected by nematodes also suffer deficiencies in sulphur amino acids particularly cysteine (Holmes, 1987) which reduces wool production. Their wool also becomes easy to break. Wool quality is adversely affected in resistant sheep (Barger and Southcott, 1975). Orwin et al. (1984), Blair et al. (1985) and Simpson et al. (2009) reported a loss of uniformity, increasing crimp and loss of wool quality in infected sheep.

1.1.3. Pathophysiology

Sheep infected with *H. contortus* or *T. circumcincta* have elevated gastric pH, increased serum concentrations of pepsinogen and gastrin and reduced acid secretion (Anderson et al., 1965, 1976; Ritchie et al., 1966; Christie et al., 1967; Ross et al., 1968; Christie, 1970; Mapes and Coop, 1970, 1973; Blanchard and Wescott, 1985; Nicholls et al., 1985, Fox et al., 1988; Lawton et al., 1996; Simpson et al., 1997; Simcock et al., 1999). The pH of the abomasum of uninfected sheep is around 2.8, but in infected sheep, the pH may be 4 and above (Lawton et al., 1996; Simcock et al., 1999). Previously challenged sheep may be able to acidify their abomasal fluid and keep the pH low, while naive animals lose the ability to maintain abomasal pH (Anderson et al., 1976). Lawton et al. (1996) and Scott et al. (1998b) observed that, after infection, hypergastrinaemia began at the same time as abomasal pH became elevated, but remained high and continued to increase when pH had reached a maximum and had returned towards normal. Serum gastrin may be depressed when abomasal pH exceeds 5.5 (Lawton et al., 1996). Hyperpepsinogenaemia is commonly used in ruminants as an indicator of abomasal parasitism (McLeay et al., 1973; Hertzberg et al., 1995, 2000; Lawton et al., 1996; Simpson et al., 1997; Simcock et al., 1999). Serum pepsinogen usually increases at the time of parasite emergence from the glands or a little earlier and remains elevated for up to 30-60 days after a single infection while the parasites are still present in the abomasum (Armour et al., 1966; Lawton et al., 1996; Simpson et al., 1997, 2009; Scott et al., 2000). The magnitude of the increase varies between animals, from almost zero (Coop et al., 1977; Lawton et al., 1996; Simpson et al., 1997; Simcock et al., 1999; Hertzberg et al., 2000) to marked increase in immune animals, even with small worm burdens (Yakoob et al., 1983).

Abomasal pH, serum gastrin and pepsinogen concentrations all increase within the first day after transfer of adult *H. contortus* (Simpson et al., 1997), *Ostertagia ostertagi* (McKellar et al., 1986), or *T. circumcincta* (Anderson et al., 1985; Lawton et al., 1996; Simpson et al., 1999; Scott et al., 2000). These effects on abomasal secretion are suggested to be initiated by parasite chemicals (Anderson et al., 1985; Lawton et al., 1996; Scott et al., 1998b, 2000), but subsequently also to involve the host response. Worm excretory/secretory products (ES), which are released by gastrointestinal nematodes to their environment,

contain many bioactive components, including chemotaxins, metabolic end-products, enzymes, immunomodulators and growth factors. ES has been shown to inhibit acid secretion in vitro (Merkelbach et al., 2002), as well as inhibit the enterochromaffin-like cells, which may indirectly cause decreased acid secretion by parietal cells (Hertzberg et al., 1999). Nematode chemotaxins recruit granulocytes in vitro (Wildblood et al., 2005), which could be another way of damaging parietal cells in vivo (Scott et al., 1998).

1.1.4. Immune responses

In sheep infected with abomasal nematodes, there are increased numbers of neutrophils and eosinophils, lymphocytes, mucosal mast cells and the presence of globular leucocytes in the gastrointestinal mucosa (Pfeffer et al., 1996; Scott et al., 1998a, 2000). Mast cells produce pro-inflammatory cytokines including TNF-α, proteinases, histamine, leukotrienes and prostaglandins (Gazzola et al., 2001) and their levels are increased in serum and mucus (Huntley et al., 1987; Douch et al., 1996; Harrison et al., 1999). In immune animals, there is an increased production of parasite-specific antibodies particularly IgE, IgG_1 and mucosal IgA (Meeusen, 1999; Gasbarre et al., 2001; Balic et al., 2002; MacDonald et al., 2002). Whereas Th1 cells produce IL-2 and IFN-γ, in nematode infections, the dominant T helper cell response is the Th2-type, involving the cytokines IL-4, IL-5, IL-9, IL-10 and IL-13 (Gasbarre, 1997; Balic et al., 2000, Madden et al., 2004).

Th2 cytokines are believed to influence resistance to gastrointestinal nematodes and the ability to develop a Th2 response has been associated with worm rejection (Else et al., 1992; Shi et al., 1994a; Pritchard et al., 1997; Else and Finkelman, 1998). Cytokines secreted by Th2 cells promote mastocytosis, eosinophilia and the production of IgE and IgG_1, which are believed to play an important role in host protective immunity against extracellular parasites (Urban et al., 1992; Else and Finkelman, 1998; Bancroft et al., 2001; Gause et al., 2003), whereas Th1 type responses are necessary for protection against intracellular parasites. Mucosal mast cells (MMC) have been implicated in worm expulsion (Miller, 1984), although increased globule leucocytes (degranulated mast cells) correlate better with nematode expulsion than MMC numbers (Douch et al., 1986; Seaton et al., 1989; Stear et al., 1995). Both mast cells and eosinophils could also contribute to inflammatory responses which cause tissue damage. IgE has also

been associated with worm expulsion, although excessive production may have a different outcome (reviewed by Pritchard et al., 1997). Similarly, eosinophils are associated with killing *H. contortus* larvae (Balic et al., 2006) and neutrophils are also reported to be able to kill nematode larvae (Hayes et al., 2004), although other experiments do not support this, particularly in a primary infection (Winter et al., 1997).

The effect of the immune response on worm expulsion appears to depend on the host and parasite involved, the dose of parasites, host genetics, parasite species and the age of the host. Some breeds of sheep are naturally resistant to gastrointestinal nematodes (Courtney et al., 1984; Zajac et al., 1988; Mugambi et al., 1997; Amarante et al., 1999) and genetic selection can increase parasite resistance in a flock (Wedrychowicz et al., 1992; Douch et al., 1994; Schallig et al., 1994; McClure et al., 1998). Resistant lines produce a greater Th2 response, different cytokine profiles and tissues have more mast cells and eosinophils than random-bred sheep (Gill et al., 2000). A protective immune response requires repeated infection (Smith et al., 1985) and immunity develops in older animals, but can also be influenced by nutrition (Van Houtert et al., 1995; Coop and Kyriazakis, 1999; Datta et al., 1999; Sykes and Coop, 2001).

Immunity to parasitism develops in older animals while younger lambs are very susceptible. Lambs fed on milk replacer (Zeng et al., 2001) have low worm burdens. Maternal antibodies ingested in colostrum and milk protect unweaned animals to some extent against gastrointestinal parasites (Carlier and Truyens, 1995). Although newborn lambs can show a protective immune response to viruses and bacteria (Soulsby et al., 1981), ruminants younger than six months have a low resistance to helminths (Smith et al., 1985; Watson and Gill., 1991). Some immunological differences have been observed between young and old animals, e.g. young sheep have fewer CD4+ cells in blood, lymph and skin than mature sheep and lymphocytes from young sheep produce less IFN-γ than cells from older animals (Watson et al., 1994; Colditz et al., 1996). When young lambs consume a low protein diet, they develop significantly lower protective immunity to parasites than age-matched animals fed a high protein diet, or older animals irrespective of diet (reviewed by Sykes and Coop, 2001). Lambs 4.5 months-old were less

resistant to parasite challenge and had reduced local immune responses compared with 10 months-old sheep (Smith et al., 1985).

Nematode carbohydrates are strongly antigenic and recognised by the host immune system (Harrison et al., 2003; Maass et al., 2007). C-type lectins (calcium-dependent carbohydrate-binding lectins), which are specific carbohydrate recognition receptors in dendritic cells and macrophages, play key roles in initiating and controlling innate and adaptive immune system (Janeway and Medzhitov, 2002). Human C-type lectin, a macrophage galactose (Gal)-type lectin, can specifically recognise α/β-GalNAc present on tumour cells and helminth parasites (van Vliet et al., 2005). Intelectins (Gal-binding lectins), mucus-associated proteins which are abundantly expressed in mice infected with *Trichinella spiralis* (Pemberton et al., 2004) or *Trichuris muris* (Datta et al., 2005) and in sheep challenged with *T. circumcincta* (French et al., 2008), are suggested to be involved in parasite rejection. In sheep, another lectin, ovine galectin-11 (or galectin-15) was also found in large amounts after infection (Dunphy et al., 2000). In humans, intelectin can recognise bacterial cell walls by the presence of galactofuranosyl residues which are not present in mammals. This recognition facilitates the induction of host protective immune responses resulting in the elimination of pathogens (Tsuji et al., 2001). Although further studies are required to elucidate their functions, these lectins are suggested to play important role in changing the properties and activities of mucus (French et al., 2008).

1.1.5. Histopathology

Abomasal nematode larvae enter the gastric pits and glands, where they cause local lesions, the nodules, which have at their centre developing larvae in distended glands lined by many flat undifferentiated cells, but few secretory cells. These nodules are numerous on the abomasal folds (Armour et al., 1966; Scott et al., 1998). Adjacent parasitised glands show mucous cell hyperplasia with fewer parietal cells and chief cells. These changes become more widespread after the L_4 or immature adults emerge (Anderson et al., 1985; McKellar et al., 1986; Lawton et al., 1996; Scott et al., 1998a, b, 2000). Luminal stages remain close to and within the superficial mucus (Miller, 1984). The abomasa of infected sheep are much heavier than control animals and the thickness of the mucosa is much greater (Anderson et al., 1988; Fox, 1993). The pits are enlarged and there are fewer

parietal cells and chief cells but more mucous neck cells (MNCs) and undifferentiated cells (Armour et al., 1966). Similar generalised histological changes are seen after transfer of adult *T. circumcincta* (Scott et al., 1998a, 2000) or *H. contortus* (Simpson et al., 1997) although there are no damaged glands where larvae have developed.

There may be a link between the pathology, histopathology and the inflammatory responses to the presence of nematodes, particularly through the loss and inhibition of parietal cells. Infection stimulates the release of such cytokines as IL-1β, IFN-γ and TNF-α (Section 1.1.4) which are inhibitors of parietal cells (Robert et al., 1991; Prinz et al., 1997; Beales and Calam, 1998). Loss of parietal cells not only results in reduced acid secretion and raised abomasal pH (Section 1.1.3), but also fewer chief cells, because parietal cells produced growth factors required for MNCs to mature into chief cells (Simpson, 2000). This occurs in other pathologies and experiments where parietal cells are lost, such as transgenic mouse models (Li et al., 1995, Li and Mardh, 1996; Canfield et al., 1996; Karam et al., 1997; Franic et al., 2001). The increase in mucus-producing cells in general, and MNCs in particular, in consistent with the increased mucin secretion observed in sheep infected with *H. contortus* by Newlands et al. (1990).

1.2. GASTROINTESTINAL MUCUS

The mucus layer has a protective role for the underlying epithelium, which is enhanced by the surfactants, such as phospholipids and phosphatidylcholine, which are secreted, migrate to the luminal surface and contribute to the lubrication and hydrophobicity of the surface of the mucosa. Mucus traps bicarbonate ions secreted by the epithelial cells and maintains a pH gradient between the acidic gastric lumen and the neutral epithelial surface (Slomiany et al., 1984; Baczako et al., 1995; Beil et al., 2000). The mucosal barrier requires the regulation of mucus production and bicarbonate secretion, but also includes the maintenance of the blood supply, the integrity of the epithelial cell barrier and the immune barrier (Fromm, 1981; Guth and Ballard, 1981). The mucus layer at the epithelial surface is a mixing and diffusion barrier and remains unstirred; the resistance to proton diffusion is four times higher, and permeability to K^+, Na^+ and Cl^- about half, that in saline (Guth and von Engelhardt, 1989).

1.2.1. Mucus-producing cells in the gastrointestinal tract

The sheep abomasal fundic mucosa from the luminal surface to the basement membrane (Figure 1.1) consists of the surface epithelium, the gastric pits, the isthmus and the glands, which open into the gastric pits. The upper part of the glands contains a mixture of acid-secreting parietal cells and mucus-secreting neck cells, while the lower parts of the glands adjacent to the submucosa contain parietal, endocrine and chief cells. Mucus is secreted by the columnar surface mucous cells (SMCs) and the MNCs, both of which are generated from stem cells (undifferentiated cells) in the isthmus. Those undifferentiated cells that are destined to become SMCs move upward into the gastric pits to the abomasal surface, while MNCs are generated from precursors in the isthmus and migrate down to mature and become chief cells (reviewed by Karam, 1993, 1999; Karam and Leblond, 1993a, b).

In the sheep, the secretion from the fundic region is highly acidic and rich in enzymes (Harrison and Hill, 1962). In the human stomach, mucus produced by SMCs is thick, viscous, insoluble and cloudy, while that produced by MNCs is soluble (Baczako et al., 1995). The SMCs contain mainly neutral glycoproteins with vicinal diol groupings and terminal fucose (Fuc) residues. In contrast, MNCs contain acidic sulphated glycoproteins with terminal sialic acids (Suprasert et al., 1999). Secretions from different population of cells do not mix with each other and form separate layers over the epithelium.

In the pylorus (or antrum), the cell types are SMCs, pit cells and gland cells, including endocrine cells (Figure 1.2). All originate from stem cells in the base of the gland (reviewed by Karam, 1993, 1999; Karam and Leblond, 1993a, b). Mucus is secreted by SMCs, pit cells and gland cells. The secretion from sheep pyloric glands is alkaline and contains mucus (Harrison and Hill, 1962).

In the duodenum, mucus is secreted by goblet cells and Brunner's glands. Goblet cells are located amongst the enterocytes on the villi and the crypts of Lieberkuhn, while Brunner's glands are in the submucosa (Schumacher et al., 2004) (Figure 1.3). Brunner's glands are multi-cellular, have distinct peripheral

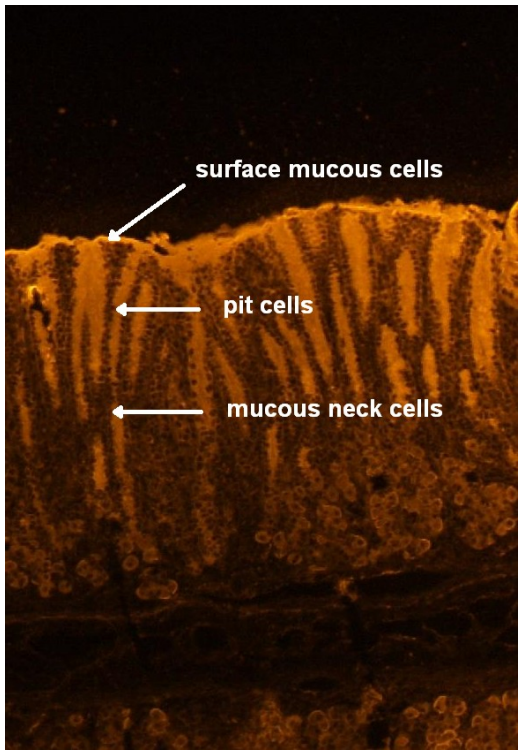

Figure 1.1. Sheep fundic tissue stained with lectin (SBA for α/β-linked Gal/GalNAc). Mucus producing cells are surface mucous cells and mucous neck cells. Original magnification 100x.

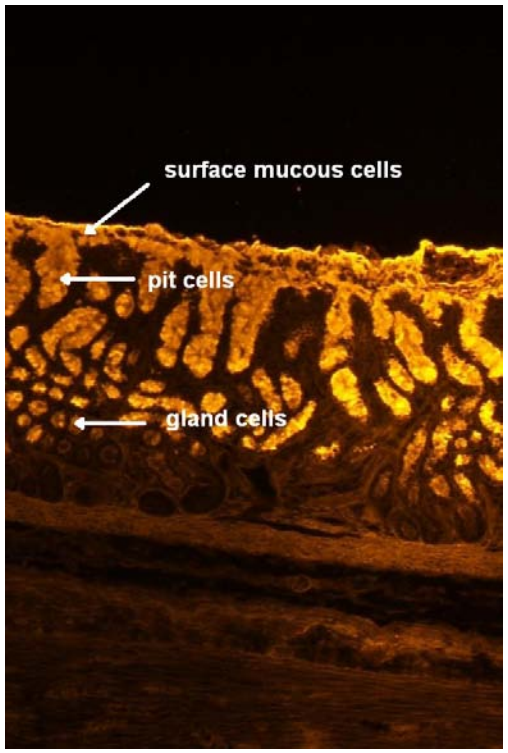

Figure 1.2. Sheep antral tissue stained with lectin (SBA for α/β-linked Gal/GalNAc). Mucus producing cells are surface mucous cells, pit cells and gland cells. Original magnification 100x.

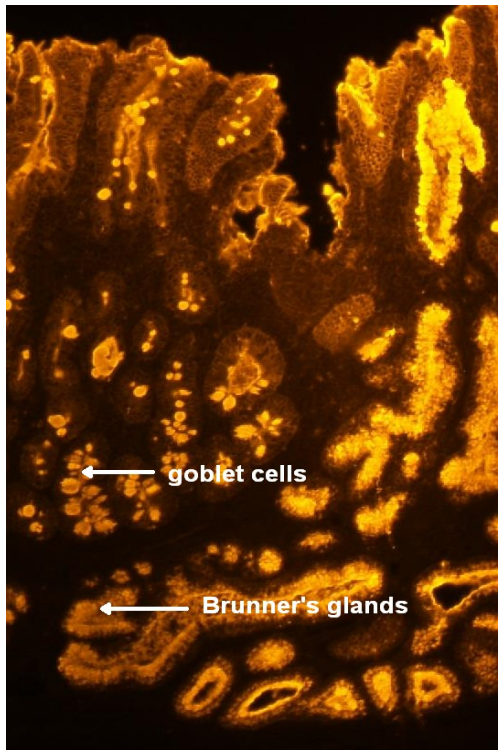

Figure 1.3. Sheep duodenal tissue stained with lectin (SBA for α/β-linked Gal/GalNAc). Mucus producing cells are goblet cells and Brunner's glands. Original magnification 100x.

areas and central lobules (Verdiglione et al., 2002) and open into the crypts of Lieberkuhn at the base of the mucosa. Brunner's glands originate from the duodenal epithelium and crypt of Lieberkuhn, or sometimes from both (Krause, 2000), while some also originate from the pyloric epithelium lining the antrum (Schumacher et al., 2004). Brunner's glands produce clear alkaline mucus (pH 8.2-9.3) with a high content of bicarbonate (Farkas and Gero, 1989). Secretion from Brunner's glands makes up a viscous and elastic layer due to the gel-forming properties of the glycoproteins which mainly consist of O-linked oligosaccharides. The secretions of goblet cells contain neutral and acidic sugar groups (reviewed by Verdugo, 1990). Mucus produced by Brunner's gland resembles the mucus of pyloric glands more than that of duodenal goblet cells (Schumacher et al., 2004).

1.2.2. Secretion of mucus

The mucus gel layer covering the gastrointestinal tract lubricates and protects the epithelium against potential pathogens, mutagens and physical and chemical damage (reviewed by Powell, 1981; Lichtenberger, 1995; Bansil and Turner, 2006). Mucus and the epithelial bicarbonate secretion, which is trapped in the mucus, are important components of the mucosal barrier which help neutralise luminal acid and maintain a pH gradient within the mucus gel. The phospholipids in mucus increase the hydrophobicity of mucus (reviewed by Hills et al., 1983), which also contributes to protecting the mucus from being degraded by gastric acid.

Mucus contains water, glycoprotein, ions, lipids, salts, DNA and cellular debris as the main components. Water accounts for 95%, mucins 5% and the rest are minor fractions includes secretory IgA, lactoferrin, lysozyme, uric acid, ascorbic acid, reduced glutathione and prostaglandin (reviewed by Clamp, 1997a, b; Allen, 1981; Neutra and Forstner, 1987; Van Klinken et al., 1998; Wiggins et al., 2001; Argueso et al., 2002). Other secreted products from mucous cells associate with the mucus layer including epidermal growth factor, the trefoil peptide family and proteinase inhibitors (Krause, 2000). Epidermal growth factor is important in healing of the damaged mucosa (Konturek et al., 1981), inhibiting gastric acid secretion (Konturek et al., 1984) and stimulating gastric mucus secretion (Kelly and Hunter, 1990; Ichikawa et al., 2000). The trefoil peptide family (TFF) are mucus-associated peptides; TFF1 is produced by gastric SMCs, TFF2 by gastric gland

mucous cells and TFF3 by intestinal goblet cells (Tomasetto et al., 1990; Hanby et al., 1993; Podolsky et al., 1993; Ruchaud-Sparagano et al., 2004). They are also involved in repairing and healing the gastric mucosa after injury (Hoffmann, 2004) and in mucus formation to protect the gastrointestinal mucosa (Tomasetto et al., 2000). Non-steroidal anti-inflammatory drugs can damage the gastric mucosa by reducing the synthesis of prostaglandins (Sharma and Schumacher, 1995b; Sharma et al., 1995), which stimulate mucus secretion (Nishizaki et al., 1994).

Mucus is not only the environment for survival of many pathogens, but is also where parasites can be eliminated. It is both a physical barrier and also contains antibodies and other molecules, such as alkaline phosphatase and histamine, which are associated with the host response to pathogens (Lee and Ogilvie, 1981; Miller et al., 1983). The anti-nematode properties of sheep intestinal mucus depend on chemical mediators (Douch et al., 1983; Jones et al., 1990) and antibody (Lee and Ogilvie, 1981; Miller, 1987; Carlisle et al., 1991). Mucus from resistant sheep has nematode-paralyzing activity (Douch et al., 1983) and mucus collected from sheep infected with *Trichostrongylus colubriformis* contains high levels of parasite-specific antibodies and histamine (Harrison et al., 1999). In mammals, intestinal parasites induce hyperplasia and hypertrophy of intestinal goblet cells, seen by histochemical staining (Manjili et al., 1998; Shekels et al., 2001; Theodoropoulos et al., 2005; Thomsen et al., 2006) and alteration of terminal sugars of goblet cell mucins associated with worm expulsion (Nawa et al., 1994). Some parasites secrete mucin-degrading enzymes to penetrate the protective mucus layer using specific proteolytic enzymes and glycosidases (Section 1.6). Mucus is also a nutrient source and binding site for microorganisms, including large numbers of endogenous and pathogenic bacteria (Satchithanandam et al., 1996).

After synthesis, mucus is stored in granules in mucus-producing cells (Neutra and Forstner, 1987; Specian and Oliver, 1991) and released in response to signals such as neurotransmitters (acetylcholine, calcitonin gene-related peptide, vasoactive intestinal peptide), hormones (gastrin) and biologically active peptides (reviewed by Forstner and Forstner, 1994).

Secreted mucus forms two layers: the adherent mucus, which is firmly attached to epithelial cells, while the other is mobile, loosely attached and easily removed by washing or digestion by acid and pepsin in the gastrointestinal tract

(Allen et al., 1993). After the loose mucus layer has been removed, the adherent layer remains continuous, sticky and strongly attached to the epithelium. It has been suggested that the firmly adherent mucus layer is important in protecting the mucosa from acid, while the loosely adherent layer binds luminal noxious agents (reviewed by Phillipson et al., 2008). The adherent mucous gel layer is produced by both the SMCs and the gland cells (Nordman et al., 1998; Reis et al., 2000; Ho et al., 2004; Taylor et al., 2004). The secretions of these two cell types do not mix, but form laminating layers which can be visualised histochemically (Ota and Katsuyama, 1992; Nordman et al., 1997; Hidaka et al., 2001). The relative contributions of mucus from surface and gland cells to the adherent and mobile mucus layers is unknown, although SMC secretion was found in both mucus phases (Phillipson et al., 2008).

The rate of synthesis of mucoproteins per day exceeds the amount present in the rat small and large intestine, indicating that intestinal mucoproteins are renewed every 24 hours (Faure et al., 2002). Measurements of the thickness of the surface mucous gel layer in the gastrointestinal tract are very variable and depend on species, health status and the location, as well as how much of the loose mucus layer is being eroded, degraded or renewed. All these factors have resulted in varying reports of the appearance and thickness of the gastrointestinal mucus layer, ranging from 50 μm up to 450 μm (Kerss et al., 1982; McQueen et al., 1983; Sellers et al., 1986). Atuma et al. (2001) observed in rats that the thickest mucus layer was in the colon (approximately 830 μm) and the thinnest in the jejunum (approximately 123 μm). The adherent mucus layer was approximately 80 μm thick in the gastric corpus, 154 μm in the antrum, 116 μm in the colon, but in the small intestine was very thin (approximately 20 μm) or absent.

1.3. MUCIN

Mucins, the major components of mucus, are high-molecular-weight O-glycosylated glycoproteins responsible for the physical and rheological properties of mucus. Mucin differs from proteoglycans, which are another class of high-molecular-weight glycoconjugates, in that mucins do not contain uronic acids and xylose (Toribara et al., 1993). Mucins consist of a limited number of monosaccharides, but a wide variety of oligosaccharides because of the various

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Table 1.1. The distribution of secreted and membrane-bound mucin types in different organs.

Organ	Secreted mucins	Membrane-bound mucins
Salivary glands	MUC5B, MUC7, MUC19	MUC1, MUC3, MUC4
Oesophagus	MUC5B	MUC1, MUC4, MUC20
Stomach	MUC5AC, MUC6,	MUC1, MUC3, MUC13, MUC20
Duodenum	MUC2, MUC6	MUC1, MUC3, MUC17, MUC20
Small intestine	MUC2	MUC1, MUC3, MUC3, MUC20
Colorectum	MUC2	MUC1, MUC3, MUC4, MUC12, MUC13, MUC17, MUC11, MUC20
Airways (upper& lower)	MUC2, MUC5AC, MUC5B, MUC7, MUC8	MUC1, MUC3, MUC4, MUC11, MUC13, MUC20
Hepatobiliary	MUC2, MUC5AC, MUC5B, MUC6	MUC1, MUC3,
Pancreas	MUC5AC, MUC5B, MUC6	MUC1, MUC11, MUC12, MUC20
Female reproductive tract	MUC5AC, MUC5B, MUC6, MUC8, MUC9	MUC1, MUC4, MUC12
Male reproductive tract	MUC8	MUC1

linkages to N-acetyl galactosamine (GalNAc) which is attached to the core peptide in α-conformation.

Mucins can be classified according to whether they are membrane-bound and secreted mucins (Biemer-Huttmann et al., 1999). The types of mucins and the organs where they are present are summarised in Table 1.1 (Andrianifahanana et al., 2006). Most secreted mucins have a higher molecular weight (MW) than membrane-bound mucin (Thornton et al., 1997; Escande et al., 2001), as secreted mucins generally consist of subunits linked through disulphide bridges, while membrane-bound mucins are monomeric (reviewed by Theodoropoulos et al., 2001). The large, secreted, gel-forming mucins are MUC2, MUC5AC, MUC6, MUC7, MUC8, MUC9 and MUC19. The membrane-bound mucins are either the large mucins MUC3A, MUC3B, MUC4, MUC11, MUC12, MUC16 and MUC17 or small mucins MUC1. Both groups share a similar O-glycans structure, but the membrane-bound mucins differ from the secreted mucins in that they use a hydrophobic trans-membrane series of amino acids to anchor to the membrane (Takehana et al., 2000). Figure 1.4 illustrates these features of typical secreted and membrane-bound mucin glycoprotein. Nineteen mucin genes have been identified, cloned and sequenced in humans and homologs to many of them have been found in the mouse and rat. Those membrane-bound mucins (MUC1-7) and secreted mucin genes (MUC2, 5AC, 5B and 6) are located in a cluster within ~500kb on the short arm of chromosome 11 (11p15) (reviewed by Bansil and Turner, 2006).

Mucins can also be distinguished on their charge properties (neutral or acidic subtypes). The neutrality or acidity of mucins is determined by their monosaccharide components of neutral, amino and acidic sugars. Neutral sugars do not contain either amino (NH_2-) group or carboxyl (COO-) groups. In an amino sugar, one or more nonglycosidic hydroxyl groups are replaced by an amino or substituted amino group (i.e., galactosamine, glucosamine). These amino sugars can be acetylated (GalNAc, N-acetyl glucosamine GlcNAc) or sulphated (glucosamine-6-sulphate). Sialic acids are amino sugars as they contain amino group but are also carboxylated sugars, as the hydroxyl groups are replaced by carboxyl groups. The chemical formulae of typical neutral (Gal), amino (GalNAc) and carboxylated sugars N-acetyl neuraminic acid (NANA) present in mucin are

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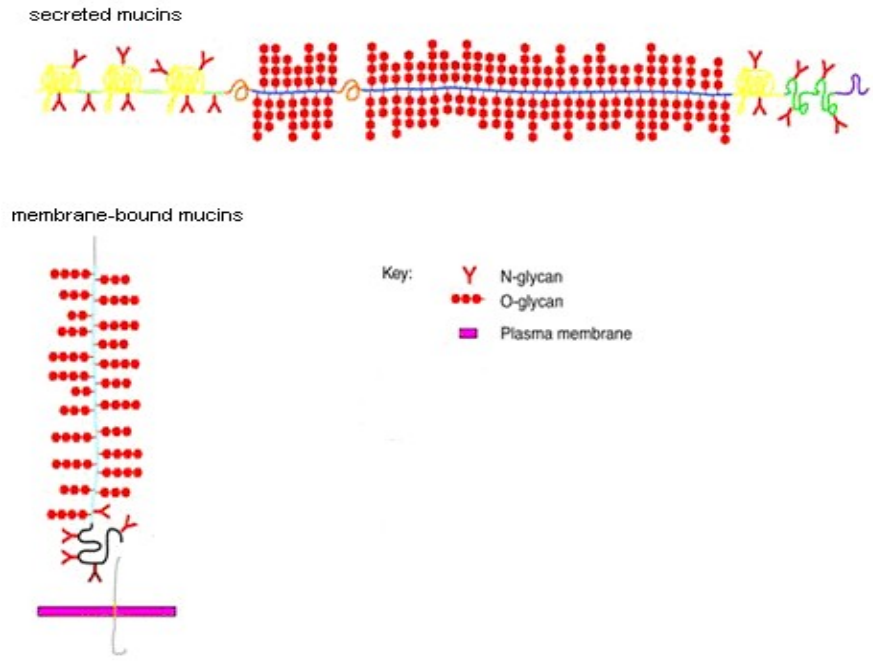
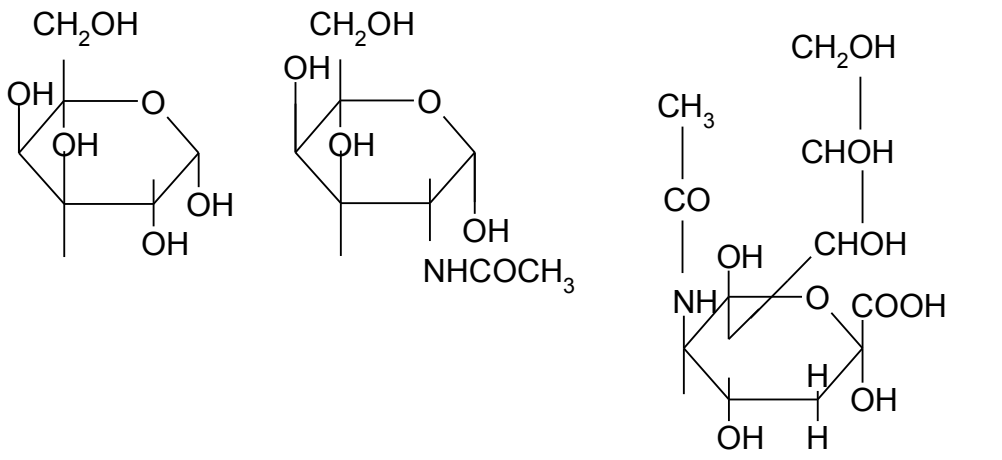


Figure 1.4. Two types of mucin. Secreted mucins (top) and membrane-bound mucins (bottom) are composed of O-glycans and N-glycans. Membrane-bound mucins anchor to the membrane through a series of amino acids (modified from Bansil and Turner, 2006)



Gal (Neutral sugar) GalNAc (Amino sugar) NANA (Amino/Carboxylated sugar)

Figure 1.5. The chemical formulae of typical neutral, amino and carboxylated sugars in mucins.

shown in Figure 1.5. Mucins containing sialic acids (sialomucins) or sulphate residues (sulphomucins) are acidic mucins (Sheahan and Jervis, 1976).

Histochemically, neutral mucins and acidic mucins can be distinguished by periodic acid Schiff (PAS) and Alcian blue (AB) staining. All mucins are stained by PAS, but only acidic mucins are stained by AB. Acidic mucins can be separated into sialylated and sulphated mucins by staining with AB/PAS at different pH.

The gel forming property of mucus depends on the formation of disulphide bridges between peptides in mucins (Allen, 1981). Mucin sol-gel transition is influenced mainly by covalent and non-covalent forces and the presence of protein and glycolipids (Forstner et al., 1982). For gastric mucins, sol-gel transition depends on pH: it is a solution at neutral pH and a soft viscoelastic gel in acidic pH, with the transition occurring at about pH 4 (Cao et al., 1999; Celli et al., 2005; Celli et al., 2007). The arrangement of side chains along the protein backbone of the mucin glycoprotein also contributes to its viscoelasticity, as ion interactions between carbohydrate residues and the peptide core produce a stiffened conformation. Each glycosylated subunit is covalently bound to a 70,000-100,000 Da peptide region (Forstner et al., 1982). Ionic interactions between sialomucins and calcium may contribute to mucus viscosity (Tabak et al., 1982). The presence of sialic acid and sulphate reduces ionic strength also affects the gel forming property of mucins.

1.3.1. Structure of mucins

Mucins have MW from $0.5x10^6$ to $25x10^6$ Da (reviewed by Wiggins et al., 2001; Moncada et al., 2003) and consist of a number of disulfide-linked subunits and covalently bound subunits with a MW range of $2\text{-}3x10^6$ Da. Mucins have a polypeptide core to which oligosaccharides are attached, creating a structure like test-tube brush. The oligosaccharides account for 80-90% of the glycoprotein mass (Mukkur et al., 1985). When observed under the microscope and in light-scattering studies, mucins appear to be long, straight-chain molecules (Slayter et al., 1974, 1984; Carlstedt and Sheehan, 1984; Sheehan et al., 1986), e.g. pig gastric mucins form a long fibre of about 400 nm (Fiebrig et al., 1995). MUC1, a small membrane-bound mucin, has very little alpha helix structure, a small amount of beta structure and mostly is a random coil (Fontenot et al., 1993).

1.3.1.1. Protein core

The protein core makes up about 20% of the molecular mass of the mucin molecule. This polypeptide consists of a central glycosylated region and less glycosylated regions. The former is comprised of a large number of tandem repeats rich in serine (Ser), threonine (Thr) and proline (STP repeats) making up about 60% of the total amino acids. The latter are located at the amino and carboxyl terminals or interspersed between STP repeats and have relatively fewer O- and N-glycosylation sites and a high proportion of cysteines. The sequence of the STP-tandem repeat region of each MUC gene is unique to each species, unlike the non-glycosylated regions, which share a large degree of similarity (reviewed by Bansil and Turner, 2006). The peptide core of each mucin subunit contains more than 5000 amino acids. Mucin glycoproteins have massive MW, due to subunits forming dimers and polymers via disulphide bridges at the cysteine rich region at nonglycosylated sites (reviewed by Bansil and Turner, 2006). This is seen in pig gastrointestinal mucins: gastric mucin, with a MW of $2x10^6$, consists of subunits of MW ~ $5x10^5$; small intestinal mucin, with a MW of $1.7x10^6$, is made up of subunits of MW ~ $2.4x10^5$; whereas colonic mucin, with a MW of $15x10^6$, consists of subunits of MW ~ $6x10^5$ or $7.6x10^5$ (Snary et al., 1970; Scawen and Allen, 1977; Marshall and Allen, 1978).

After apomucin production, oligosaccharide up to 20 monosaccharides in length are attached to the polypeptide core by O-linkage, in which GalNAc is linked to hydroxyl groups of Ser or Thr (Kobata, 1992; Karlsson et al., 2000) and N-linkage in which GlcNAc is linked to an amine group of asparagine (Kobata, 1992). The formation of sugar chains is carried out in the Golgi complex (Silberberg, 1988) by different glycosyltransferases. O-glycosylation is the main form of glycosylation in animal mucins and often called “mucin-type” glycosylation (Schulz et al., 2002). There is also a small amount of N-glycosylation, which is the predominant form in eukaryotic extracellular glycoproteins.

Table 1.2. Monosaccharide composition of secreted mucins. The percentages presented are of total glycoproteins.

	Fuc	GalNAc (GalN)	GlcNAc (GlcN)	Gal	NANA	Reference
Pig colonic mucin	10.4	8.3	23.9	20.7	9.9	(Marshall and Allen, 1978)
Dog gastric mucin	7.6	9.8	16.4	14.2	4.2	(Piotrowski et al., 1991)
Sheep colonic mucin	6.5	3.7	8.4	10.8	6.1	(Draper and Kent, 1963)
Ovine subma-xillary mucin		28.5			34.3	(Perez-Vilar and Hill, 1999)
Calf intestinal mucin	4.7	23.6	24.1	42.1	2.4	(Montagne et al., 2000)
Porcine stomach mucin	11.0	22.7	24.9	27.2	1.5	(Snary and Allen, 1971)
Rabbit ocular mucin	12.7	20.4	23.0	26.1	2.0	(Tseng et al., 1987)

Sheep small intestinal mucin had a MW of $5.0\pm0.1x10^6$ and contains 34% protein and 66% carbohydrate (Mukkur et al., 1985). The glycoprotein contained 7.1% (v/v) sulphate, neutral hexoses accounted for half the total monosaccharides, followed by galactosamine (GalN) and glucosamine (GlcN). There were small amounts of Fuc and sialic acids, but uronic acid was not detected. The monosaccharide composition of mucins from different parts of the gastrointestinal tract and other organs of several species are summarised in Table 1.2.

1.3.1.2. N-linked oligosaccharides

Mucins contain only a small proportion of N-linked oligosaccharides compared with O-linked oligosaccharides. N-linked oligosaccharides are attached to the amine group of an asparagine residue on the protein core through a GlcNAc residue at the reducing terminus. Attached to this saccharide is a core sequence, which extends the oligosaccharide chain. The N-linked oligosaccharides in mucins are poorly described, but are likely to be similar to other oligosaccharides, which have a pentasaccharide, Man α1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc, as the "trimannosyl core". According to the structures and the location of the extra sugar residues added to the trimannosyl core, N-linked sugar chains are further classified into three subgroups (Figure 1.6) (reviewed by Kobata, 1992).

In the first group, **the complex type** (Figure 1.6A), the outer chains consist of GlcNAc linked to the two α-mannose (α-Man) residues of the trimannosyl core. These outer chains contain GlcNAc, Gal, Fuc, sialic acids, GalNAc and sulphate. The complex type has the largest structural variation.

In the second group, **the high-mannose type** (Figure 1.6B), sugar chains contain only α-Man residues attached to the trimannosyl core. There is a high density of Man residues. The oligosaccharide that is commonly found is: Manα1-6(Manα1-3)Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc.

The third group, **the hybrid type** (Figure 1.6C), has the structural features of both the complex-type and high-mannose-type sugar chains. One of the two α-mannosyl residues is linked to the Manα1-6 of the trimannosyl core, as found in the high-mannose-type and the outer chains found in complex-type sugar chains are linked to the Manα1-3 of the core of this group. The presence or absence of Fuc

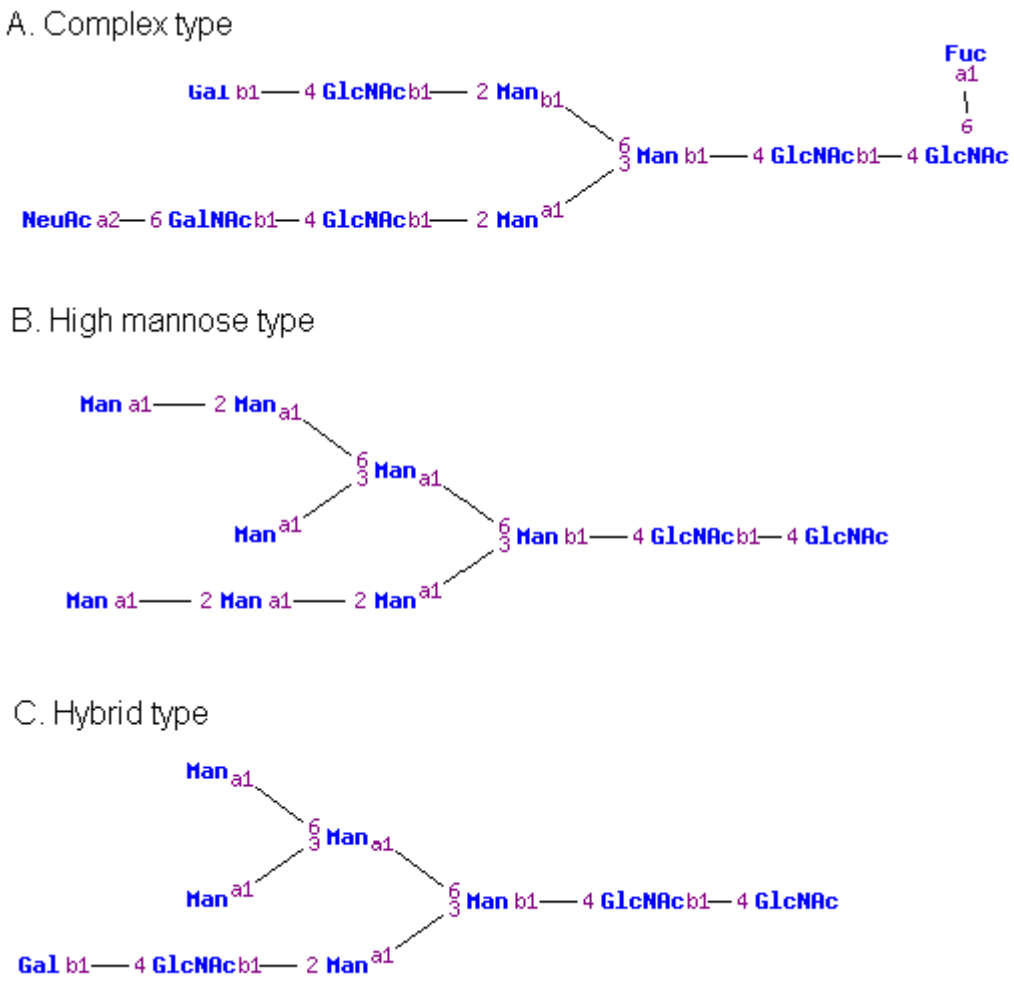


Figure 1.6. Common structures of N-linked oligosaccharides. The N-linked oligosaccharides can be divided into 3 subgroups (A) Complex type; (B) High mannose type and (C) Hybrid type.

Abbreviation: Fuc=fucose; Gal=galactose; GlcNAc=N-acetyl glucosamine; GalNAc=N-acetyl galactosamine; Man=mannose; a=α and b=β.

(adapted from http://www.expasy.ch/tools/glycomod/glycomod-doc.html)

and GlcNAc linked to the trimannosyl core also contributes to the variation in the sugar chains.

1.3.1.3. O-linked oligosaccharides

There are many O-glycosylation sites per mucin molecule at Ser and Thr rich sequences on the protein core, to which the oligosaccharides covalently bind, forming highly glycosylated regions (reviewed by Kobata, 1992). In gastric mucins, there are about 100-200 chains per glycoprotein molecule with MW of $5x10^5$. The side chains enable mucin molecules to form viscoelastic structures (Wiggins et al., 2001).

O-glycosylation is initiated in the Golgi complex when an N-acetylgalactosaminyl peptidyltransferase transfers GalNAc to a Ser or Thr (Rose and Voynow, 2006). Each O-glycan is then elongated by the stepwise addition of hexoses, hexosamines or sialic acids by at least 14 different polypeptide N-acetyl galactosaminyltransferases (ppGalNAc Ts) (Wang et al., 2003). Enormous variations in mucin glycosylation are created by the elongation of the side chains from both the C^3 and C^6 of GalNAc with glucose (Glc), Gal, GlcNAc, GalNAc, GlcN, GalN, sialic acid NANA and N-glycolyl neuraminic acid (NGNA), Fuc and sulphate groups (reviewed by Kobata, 1992).

A side chain consists of the core, backbone and periphery (Silberberg, 1988; Toribara et al., 1993). In a typical mucin, such as sheep small intestinal mucins, the side chain can be up to 10 monosaccharide residues long, depending on the presence of a specific glycosyltransferase enzyme. An individual sugar can be linked to the previous one in various ways, for example, a 1,2 linkage is between the number 1 carbon of the distal sugar and the number 2 carbon of the proximal sugar, similarly 1,3 and 1,6 linkage are between the number 1 carbon of the distal sugar and the number 3 and number 6 carbon of the proximal sugar respectively (Jefcoat et al., 2001). The side chains can be as short as a single monosaccharide GalNAc as in pig submaxillary mucins (Carlson, 1968), disaccharides in sheep submaxillary mucins (Gottschalk et al., 1972),

Table 1.3. Structural elements of mucin-type O-linked glycans (Gum et al., 1989)

	Core structures
Tn antigen, sialyl-Tn	GalNAcα1-, NeuAcα6GalNAc-Ser/Thr
Core 1	Galβ1-3GalNAcα1-Ser/Thr (TF antigen when unsubstituted)
Core 2	Galβ1-3(GlcNAcβ1-6)GalNacα1-Ser/Thr
Core 3	GlcNAcβ1-3GalNAcα1-Ser/Thr
Core 4	GlcNAcβ1-3(GlcNAcβ1-6)GalNAcα1-Ser/Thr
Core 5	GalNAcα1-3GalNAα1-Ser/Thr
Core 6,7,8	GlcNAcβ1-6GalNAc-, GalNAcα1-6GalNAc-, GalNAcα1-6GalNAc- Ser/Thr
	Backbone structures
Type 1	Galβ3GlcNAcβ -
Type 2	Galβ4GlcNAcβ-
Unbranched I antigen	GlcNAcβ3Galβ-
Branched I antigen	GlcNAcβ3(GlcNAcβ6)Galβ-
	Peripheral structures
A, B, H blood group	GalNAcα3(Fucα2)Galβ-, Galα3(Fucα2)Galβ-, Fucα2Galβ-
Le^a , Le^b	Galβ3(Fucα4)Galβ-, Fucα2Galβ3(Fucα4)Galβ-
Le^x, Le^y	Galβ4(Fucα3)Galβ-, Fucα2Galβ4(Fucα3)Galβ-
Sialyl-Le^a, Sialyl-Le^x	NeuAcα3Galβ3(Fucα4)Galβ-, NeuAcα3Galβ4(Fucα3)Galβ-
Sulphate	HSO_3-3Galβ-, HSO_3-6GlcNAcβ-
Sd^a/Cad	GalNAcβ4[NeuAcα3]Galβ-

pentasaccharides in pig submaxillary mucins (Carlson, 1968), 6-8 monosaccharides in pig small intestinal mucins (Mantle and Allen, 1981) and up to 19 monosaccharides in pig and human gastric mucins (Schrager and Oates, 1971; Slomiany and Meyer, 1972; Slomiany et al., 1984).

The structural components of mucin-type O-linked glycans (core, backbone and peripheral structures) are shown in Table 1.3. In addition, O-linked sugar chains with the GlcNAcβ1-6GalNAc core and the GalNAcβ1-3GalNAc core are found in a limited number of glycoproteins (Kobata, 1992). The core structure of these oligosaccharides in sheep gastric mucins can consist of either Galβ1-3GalNAc or Galβ1-3GlcNAcβ1-6GalNAc or GlcNAcβ1-3GalNAcβ1-6GlcNAc attached to the peptide through an O-glycosidic linkage (Hounsell et al., 1980). In addition, the cores [Galβ1-3(GlcNAcβ1-6)GalNAcα1-] and [GlcNAcβ1-3(GlcNAcβ-16)GalNAcα1] have also been isolated from the gastric mucins of sheep (Hounsell et al., 1981).

The locations where each mucin synthesis and secretion step occurs are described in Figure 1.7. The peptide core is synthesised in the rough endoplasmic reticulum, while the oligosaccharide side chains are added during the passage through the Golgi apparatus. The oligosaccharides are initiated by the transfer of GalNAc from UDP-GalNAc to Ser/Thr residues by the membrane-bound UDP-GalNAc: polypeptide α-GalNAc transferase enzyme. The cores of the oligosaccharides are then added by a series of glycosyltransferases, using such donors as UDP-Gal, UDP-GalNAc, UDP-GlcNAc, GDP-Fuc and CMP-sialic acids. The chain termination is carried out by α-sialyl-, fucosyl-, galactosyl- or N-acetyl galactosyl transferases (Schachter and Roseman, 1980; Schachter et al., 1982; Schachter and Williams, 1982). The biosynthetic pathway of O-glycan core structures is illustrated in Figure 1.8.

Mucin carbohydrate backbones are often made up of disaccharides consisting of Gal and GlcNAc linked through a β-linkage, with two types of linkages: Galβ1-3GlcNAc (type 1 disaccharide) or Galβ1-4GlcNAc (type 2 disaccharide or N-acetyllactosamine). The periphery is often made of Fuc and acid groups (sialic acids and sulphate) attached terminally to the oligosaccharides (Reid and Clamp, 1978). The negative charge of mucin oligosaccharides, created by sialic acid residues and sulphate groups that are terminally attached to GlcNAc and Gal,

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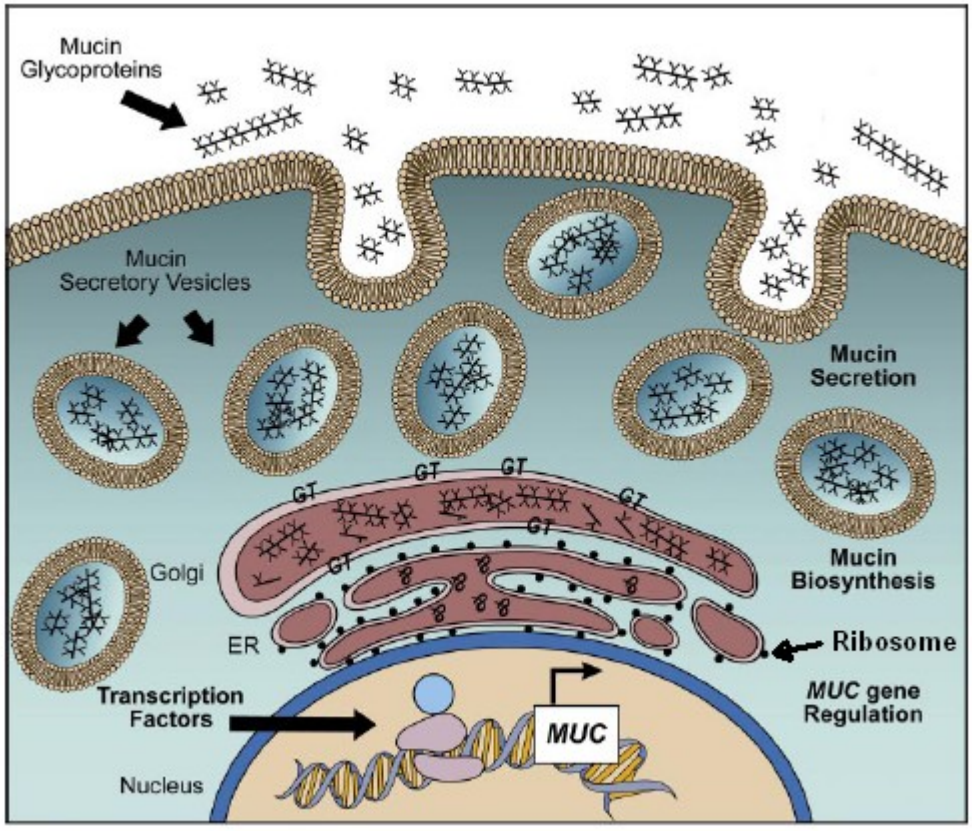

Figure 1.7. Mucin biosynthesis and secretion in mucus cells. In the nucleus, transcription factors upregulate MUC gene expression. MUC protein cores are transcribed in the ribosomes and inserted to the endoplasmic reticulum (ER). The mucin polypeptide cores then are transferred into the Golgi apparatus for glycosylation. O-glycosylation is initiated by GalNAc peptidyltransferase, which attaches GalNAc to Serine/Threonine residues on the protein. Subsequent monosaccharides are added by a series of glycosyltransferases such as fucosyl-, galactosyl-, (N-acetyl) galactosaminyl-, (N-acetyl) glucosaminyl- and sialyl-transferase. The fully glycosylated mucins are stored in mucin secretory vesicles and secreted after appropriate signals (adapted from Rose and Voynow, 2006).

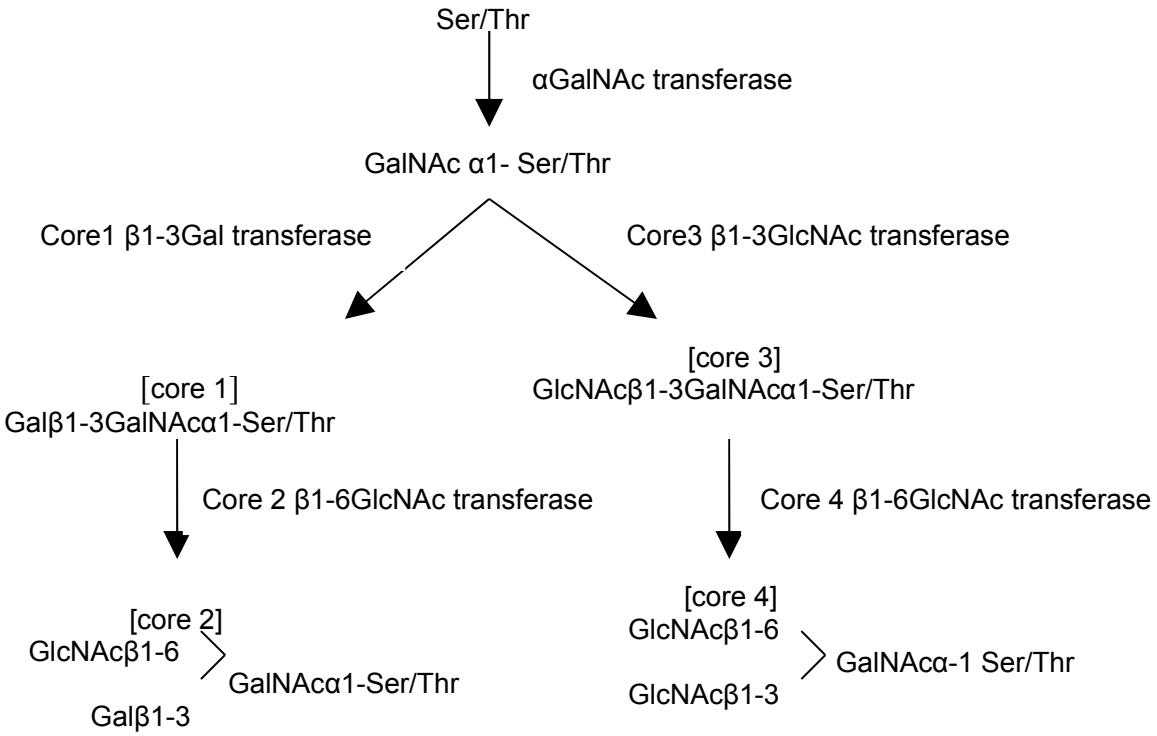

Figure 1.8. Biosynthetic pathway of O-glycan core structures. This process is initiated in the Golgi apparatus by the transfer of GalNAc from UDP-GalNAc to hydroxyl groups on Ser/Thr in the peptide backbone. After the initiation step, mucin carbohydrate side chains are extended by the addition of GalNAc, GlcNAc or Gal to generate the core (core 1, 2, 3 and 4).

contribute to the rigidity of the mucin molecules (Wiggins et al., 2001). The glycosylation of mucins can also be the target for adhesion of microbes (Gaudier et al., 2004). However, the glycosylation of host mucins can be altered by diseases (Section 1.6), perhaps as part of the defence against infection at mucosal surfaces, or alternatively, the pathogens can up-regulate glycosyltransferases, perhaps for their own benefit (Holmen et al., 2002).

1.3.1.4. Sialylation

In mammalian tissues, sialylation is a post-translational process which adds sialic acid at the terminals of the oligosaccharide chain in α2-3, α2-6 and α2-8 linkages (Becker and Lowe, 2003). Sialic acids are acidic nine carbon sugars, the most commonly found being NANA and NGNA (Figure 1.9) (reviewed by Reutter et al., 1997) Sialic acids are present in secretory, but not membrane-bound, mucins. They are added to short core structure, to oligosaccharide backbones or to blood group complexes (reviewed by Jass and Walsh, 2001). Much of the sialic acid is O-acetylated, which may function to protect oligosaccharides from being degraded by neuraminidases.

Sialic acids, being negatively charged, contribute to the cross-linkages between glycoprotein molecules (Gottschalk, 1960). Although they play an important role in associating glycoprotein molecules, there is no evidence to confirm that sialic acids determine the viscosity of mucus gel (Guslandi, 1981). Sialylated glycans are important parts of glycoproteins and glycolipids. Neuraminic acids may contribute to protecting mucin from degradation by bacteria, such as *Colostridium septicum,* which produces neuraminidase (sialidase) to release sialic acid molecules. This provides access for other enzymes which degrade the mucin molecule and enhancing the invasiveness of the organism by facilitating penetration of the mucus layer and then the gut mucosa (Macfarlane et al., 2001). Sialylation also determines the susceptibility of mucin glycoproteins to degradation by proteolytic enzymes (Nasir-ud-Din et al., 2003).

Sialic acids play an important role in mediating cellular recognition and adhesion processes (reviewed by Varki, 1997; Traving and Schauer, 1998). They can either be an attachment site or inhibit infection (Shen et al., 2000). Whereas sialylated glycoproteins can inhibit the binding of *Helicobacter pylori* (Simon et al.,

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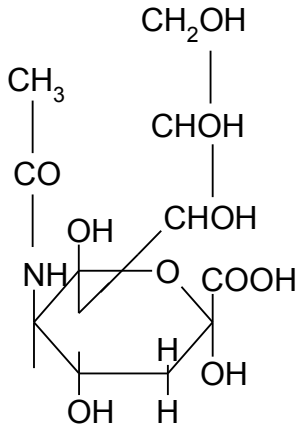
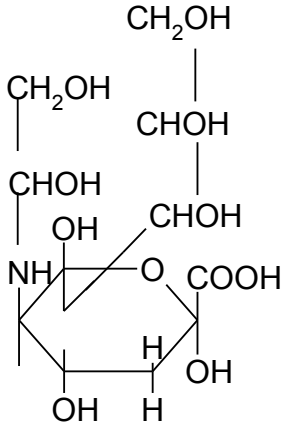


(a)



(b)

Figure 1.9. The chemical formulae of sialic acids commonly found in mucins. (a) N-acetyl neuraminic acid (NANA) and (b) N-glycolyl neuraminic acid (NGNA).

1997), they are involved in the attachment of *Pseudomonas aeruginosa* (Ramphal and Arora, 2001) and favour the survival of the human protozoan parasite *Trypasonoma cruzi* (Nasir-ud-Din et al., 2003).

1.3.1.5. Fucosylation

Fucosylation is the post-translational process which adds the Fuc residue to the side chain of mucin glycoprotein, but unlike sialylation, Fuc is not only added to the termini, but also can be attached to the core O-glycan on Ser and Thr residues and to N-glycan in α-1,6-linkage (Becker and Lowe, 2003). The typical α-1,6-linkage is between Fuc and GlcNAc residue of an N-glycan. Fuc may be present at the terminal chains of oligosaccharides of membrane-bound or secreted mucins (Staudacher et al., 1999). The degree of fucosylation, which depends on the activity of fucosyltransferases, can be used to diagnose some diseases and monitor the success of therapies. Various functions have been established for Fuc, including important roles in fertilisation (Johnston et al., 1998; Mori et al., 1998), development (Ruggiero-Lopez et al., 1991; Xiang and Bernstein, 1992; Bruckner et al., 2000; Moloney et al., 2000), cancer (Fukuda, 1996) and apoptosis (Hiraishi et al., 1993).

1.3.1.6. Sulphation

Sulphation, together with sialic acid, adds negative charges to mucins, which influences their chemical and physical properties, hydration and metal ion binding (Brockhausen, 1997; Nordman et al., 1998). Sulphation is an early event occurring when mucin subunits are assembled; sulphate is necessary for the formation of high MW mucus glycoproteins (Liau et al., 1991). On O-linked oligosaccharides, sulphation is most frequently found on Gal and GlcNAc residues, at the 3-position and 6-position respectively. On N-glycan chains, sulphate may be added to Man residues on the core GlcNAc, Gal or GalNAc (Bergwerff et al., 1995; Brockhausen, 1997). Sulphate groups have been identified in most secreted mucus, including salivary (Thomsson et al., 2005), tracheobronchial (Lo-Guidice et al., 1994) and intestinal mucus (Robbe et al., 2003). In gastric glands, the degree of mucin sulphation depends on their location: in the antrum, mucus cells lose their

ability to add sulphate to mucins when they migrate from the gland to the surface of gastrointestinal tract (Kramer et al., 1978).

The specific function of sulphation is still unknown and its significance in contributing to the rigidity and protective function of the mucus gel is still controversial (Tissot et al., 2006). The secretory cells and luminal secretions contain a high density of sulphate groups and the content of this terminal moiety seems to be a regulatory factor of mucus viscosity (Kasinathan et al., 1991; Slomiany et al., 1991). Sulphate esters may mask antigens or lectin binding sites and also regulate the biosynthesis, half-life and biological roles of glycans (Becker and Lowe, 2003). Sulphation is believed to inhibit peptic attack, because sulphate binds to proteins that are targets of pepsin and protects proteins from being degraded by pepsin (Ishihara et al., 1985). It has been suggested that sulphation increases the resistance of mucus to bacterial degradation (Rhodes et al., 1985; Amerongen et al., 1998). The sulphation of tracheobronchial mucin has been proposed to contribute to the resistance of mucin to infection by *P. aeruginosa* by limiting mucin as a source of nutrition, therefore limiting bacterial growth (Chance and Mawhinney, 2000).

Mast cell mucins contain highly sulphated acidic mucin glycoproteins and goblet cell mucins are frequently sulphated. Sulphomucins are reported to prevent the colonisation of the gastric mucosa by *H. pylori* (Piotrowski et al., 1991).The physicochemical characteristics of these sugars may also influence parasitism of the intestinal mucosa (Shi et al., 1994a). Sulphated mucins may contribute to expelling *N. brasiliensis* as Syrian golden hamsters which have higher sulphated mucins expelled worms faster than other breeds with less sulphated mucins (Ishikawa et al., 1994; Shi et al., 1994b). The distribution of *Strongyloides venezuelensis* along the intestinal tract was dependent on the level of sulphation. *S. venezuelensis* adult worms are not able to invade mucus where it is highly sulphated, so a large number of worm relocate to the large intestine where goblet cells produce less sulphated mucins (Maruyama et al., 2002).

Each post-translational modification plays a different role in the general functions of mucin. Removal of peripheral Fuc and GlcNAc reduced the viscoelasticity of mucins by 5%, removal of sialic acid resulted in an 18% reduction in viscosity and deglycosylation caused a 40% reduction. In contrast, hydrogen ion

permeability increased by 7%, 28% and 42% respectively (reviewed by Slomiany et al., 1987). The presence of charged groups in glycoproteins also affects their physiological functions. Sialic acid and sulphate groups in cervical mucus help maintain the structural and functional integrity of the mucus glycoprotein in its biological environment (Nasir-ud-Din et al., 2003).

1.3.2. Gastrointestinal mucin

In the gastrointestinal tract, there are many secreted and membrane-bound mucins (Toribara et al., 1993; Bayliss et al., 1995; Reis et al., 1997), of which MUC1, MUC5AC and MUC6 are the most prominent in the stomach and MUC2, MUC3 and MUC6 are predominant in the intestine (Table 1.4). MUC5AC and MUC6 are secreted mucins in the abomasum and MUC2 and MUC6 in the duodenum. In the stomach, MUC5AC is expressed by the SMCs of the fundus and antrum (Bayliss et al., 1995; Choo et al., 1999; de Bolos et al., 2001), while MUC6 is secreted by MNCs of the fundic and antral glands (de Bolos et al., 2001). The secretions of these two cell types do not mix, but form laminating layers (Nordman et al., 1997; Ota and Katsuyama, 1992; Hidaka et al., 2001).

The normal gastric mucosa produces mainly neutral mucins, except the mucus-secreting cells of the neck glands that secrete acidic mucins (Filipe and Fenger, 1979). Surface-type mucins stain with galactose oxidase-cold thionine Schiff, while gland-type mucins stain with paradoxical concanavalin A (Irimura et al., 1991). In porcine gastric mucosa, MNCs produce more charged mucins than SMCs (Karlsson et al., 1997). MUC1 and MUC5AC are expressed in the foveolar epithelium and MUC6 in the glands. They differ in size and glycosylation. Lewis type-1 chain antigens (Le^a and Le^b) are expressed in foveolar epithelium, whereas Lewis type-2 chain antigens (Le^x and Le^y) are expressed in the glands. MUC5AC-producing cells also express Lewis type-1 antigen Le^a, whereas MUC6-producing cells express Lewis type-2 antigen Le^y (De Bolos et al., 1995). In gastrointestinal mucins, there are trace amounts of Man (Clamp, 1978), which is evidence of the presence of a small proportion of N-linked oligosaccharides. N-glycosylation of glycoproteins plays a key role in their folding, sorting and secretion (Parry et al., 2006).

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Table 1.4. Types and origins of gastrointestinal mucins

Mucin	Type	Location	References
MUC1	Transmembrane	Stomach Duodenum	(Gendler, 2001)
MUC2	Secretory	Small intestine	(Gum et al., 1992)
MUC3A/B	Transmembrane	Small intestine	(Ho et al., 1993; Pratt et al., 2000)
MUC4	Transmembrane	Stomach	(Moniaux et al., 2000)
MUC5AC	Secretory	Stomach Intestine	(Porchet et al., 1995)
MUC6	Secretory	Stomach Duodenum	(Toribara et al., 1993; De Bolos et al., 1995; Bartman et al., 1998)
MUC 12	Transmembrane	Stomach Small intestine	(Packer et al., 2004)
MUC 13	Transmembrane	Stomach Small intestine	(Packer et al., 2004)
MUC 17	Transmembrane	Small intestine	(Gum et al., 2002)

1.4. MODIFICATION OF MUCINS WITH AGE

There have been a small number of studies on the modifications of the carbohydrates in mucins with age in mammals, mainly in pigs and rats. These studies show that the monosaccharide compositions of mucin glycoproteins differ between younger and older animals within a species.

In rats, mucin glycoproteins were observed to be different chemically and physically between newborn and adult animals. In CsCl density gradient centrifugation, small intestinal glycoproteins have a higher buoyant density in newborn rats (1.55 g/ml) than in adult animals (1.47 g/ml) (Shub et al., 1983). The molar ratio of carbohydrate to protein is higher in adult rats because of the increase in Fuc and GalNAc (Biol-N'garagba and Louisot, 2003). A change from sialylation to fucosylation of intestinal mucins in rats occurs during postnatal development, along with more α1,6-Fuc than α1,2-Fuc (Torres-Pinedo and Mahmood, 1984). Shub et al. (1983) observed an increase in Fuc and GalNAc in adults compared with newborn rats, as well as a decrease in sulphate (0.9% versus 5.5%).

In the small and large intestine of pigs, total sialic acid was decreased in 2 weeks-old pigs compared with newborns, while there was no significant difference among groups of 2 weeks-old, 4 weeks-old and adult pigs (Malykh et al., 2003). The amount of sulphomucin increased from one day- to 3 weeks- and 10 weeks-of-age in the pig small intestine (Brown et al., 1988), and in the colon and caecum during the first three to four months (Brunsgaard, 1997). Turck et al. (1993) compared colonic mucins from day 0 to day 180: the amount of Fuc and GlcN increased from day 0 to 21 by 165 and 37%, respectively; there was a further increase in Fuc in mature pigs, while the amount of sulphate was less in mature pigs than in young animals to 21 days-of-age (Turck et al., 1993).

Changes in mucin glycoproteins with age are also observed in the duodenum of fallow deer. There was a significant decrease in sulphated

glycoconjugates in duodenal glands during development and an increase in the carboxylated oligosaccharides in mucin produced by goblet cells. Most of the secreted neutral and carboxylated glycoconjugates contained sialic acid (Scocco et al., 2001)

Ishihara et al. (1995) found that in the abomasum, the total glycoprotein per gm of tissue was greater in sheep than in lambs and that mucins were highly sulphated in lambs. Sulphated oligosaccharides were present in goblet cells, but not in the Brunner's gland cells of adult sheep (Ohwada and Suzuki, 1992), while a moderate amount of Fuc was found in epithelial cells and duodenal gland cells, supporting the suggestion that Fuc may be a function of maturation in sheep, as has been observed in pigs (Gelberg et al., 1992).

Modifications of mucin profiles with age mainly depend on activities of enzymes involved in glycosylation, which change during development. The general pattern is that sialyltransferase activity decreases and fucosyltransferase increases in older animals. However, changes with age in mucin sulphation were not consistently found in all animals, suggesting that there are species-specific and individual-specific mechanisms involved in the modulation of sulphomucins synthesis.

1.5. MODIFICATION OF MUCIN BY DISEASES

Many diseases such as cancer, ulcerative colitis and cystic fibrosis have been associated with changes in the type and glycosylation of mucins. Histochemical studies demonstrated alterations in the proportions of neutral, sialo- and sulphomucins (Filipe and Fenger, 1979; Ehsanullah et al., 1982a, 1982b; Morrissey et al., 1983; Reid et al., 1984; Reid et al., 1985). Lectin staining showed changes in the terminal sugars on the side chains (Ishikawa et al., 1993; Ishikawa, 1994).

1.5.1. Cancer

Many aspects of the changes in mucin in cancer have been extensively investigated, such as gene expression, mucin production, glycosylation and secretion. Numerous studies have shown that there is a correlation between changes in mucin and malignancy; these findings led to the suggestion that mucins

can be used as tumour-associated markers in diagnosing and treating cancer patients (Feller et al., 1990). Many cancers, such as pancreatic cancer, express large amounts of MUC1, which can be cleaved and released into the serum and other body fluids (Metzgar et al., 1984). In all malignant patients, mucins share the similarity of short, cancerous structures (Radziejewska et al., 2009) with increased acidic mucins, however, there are variations in mucin modification depending on the organ-specific mucin gene expression and stage of the disease.

In gastric cancer, there is loss of expression of MUC5AC (Ho et al., 1995; Reis et al., 1997), increased mucin heterogeneity (Ho et al., 1995) and glycosylation changes, with shorter and simpler mucin-type carbohydrates (David et al., 1992; Carneiro et al., 1994). Human malignant gastric epithelial mucins contained significantly increased sulphomucin and decreased neutral mucins compared with control patients (Cheah and Ramachandran, 1994). Mucus glycoproteins from the non-malignant antrum, from the uninvolved gastric mucosa and tumour site of patients with intestinal-type adenocarcinoma differ. Non-malignant mucins showed a high content of carbohydrate and low sulphated monosaccharides, while uninvolved gastric mucosal mucins showed a decrease in carbohydrate content and increased sulphated monosaccharides (Sidebotham et al., 1998).

In colonic cancer, mucin secretion halved from that in the normal colon and the oligosaccharide length was reduced from 10 to 6 sugars (Boland and Deshmukh, 1990). MUC1 is a highly O-glycosylated glycoprotein, which carries a wide variety of glycoform structures and is a well-known marker of many cancers (MacLean et al., 1997; Tissot et al., 2006), increases significantly. The glycosylation of MUC 1 alters: the length of oligosaccharides is shortened and the protein core is more densely glycosylated (Lloyd et al., 1996; Beatty et al., 2001; Taylor-Papadimitriou et al., 2002). MUC5AC, often absent in the normal colon, is present in colonic cancer. The core 3 structure GlcNAcβ3GalNAc with complex branches, is commonly seen in normal colonic mucin, but is not found in colon cancer mucin. Instead, it is replaced by such short O-glycans, such as the Tn antigen (GalNAcαThr/Ser), TF antigen (Galβ3GalNAc) and sialyl-Tn (NeuAcα6GalNAc) (Toribara et al., 1993). Normal colonic tissues produced both neutral and acidic mucins, while there were only acidic mucins expressed in colonic

cancer (Chirwa et al., 2007). Mucin sulphation decreased, but very often relatively higher amounts of sialic acids was observed in cancer (Filipe, 1979).

In many tumours, there are striking changes in glycosylation, particularly the presence of very short oligosacchardises. The O-glycans in MUC1 are predominantly core type 1 in cancer cells, while those from normal breast cells contain mainly MUC1 with core type 2 (Gawenis et al., 2004). Ovarian cancer expresses high proportion of sialyl-Tn and Tn: the former antigen can be detected in the blood (Inoue et al., 1991; Kobayashi et al., 1992). Breast cancer MUC1 contains less total carbohydrates and GlcNAc but more sialic acid (Brockhausen et al., 1995).

Lung cancer patients express high level of α-3-Fuc-tranferase, which synthesises Le^x and Le^y epitopes (Asao et al., 1989) and their sialylated derivatives and multimeric Le^x also accumulate (Nilsson, 1992). Pancreatic hyperplasia and cancer tissues also express Le^x antigen, while normal tissues express Le^y (Kim et al., 1988). Abnormal fucosylation is also found with the loss of Le^b antigens (Idikio and Manickavel, 1991). Sialyl-Le^x increased compared with controls (Matsuura et al., 1998). Higher activities of α-2,2, α-2,3, α-2,4 fucosyltransferase were also observed in ovarian cancer (Chandrasekaran et al., 1992; Thompson et al., 1992).

1.5.2. Inflammatory diseases

An increase in sulphated mucins was observed in abomasal mucins in calves with ulcerative lesions (Pearson et al., 1987). In duodenal ulceration in humans, acidic mucosubstances are reduced in goblet cells, which are also fewer in numbers, but at the same time, neutral mucosubstances appeared in the cells of the surface epithelium. As these changes were reversed during healing, it was suggested that changes in mucin might be a protective response during ulceration (Morrissey et al., 1983).

Similarly, in ulcerative colitis in humans, during the active stage, both glycosylation and sialylation increase, however, mucin sulphation decrease (Irimura et al., 1991; Corfield et al., 1992a; Raouf et al., 1992; Dekker and Tytgat, 1993; Vavasseur et al., 1994; Campbell et al., 2001). Normal colonic mucins are heavily sulphated, which contributes to the protection of mucins from being degraded by

bacterial enzymes. Therefore, any alterations in mucus sulphation could be important in the pathogenesis of ulcerative colitis (Raouf et al., 1992). Furthermore, faecal samples from patients with ulcerative colitis, Crohn's disease and ileal disease contain mucinase activity which may cause the abnormally thin mucus layer observed (Dwarakanath et al., 1995a). MUC2 levels were significantly decreased in ulcerative colitis patients with active inflammation compared with controls, however, during the remission stage, glycosylation and sulphation increased compared with controls (Morita et al., 1993).

Cystic fibrosis (CF) is chronic lung disease in which there is excessive secretion of mucus in the airways of patients, leading to airway obstruction and respiratory failure and often bacterial infections. Modifications of mucin glycoproteins have been observed in CF patients along with increased secreted mucins in response to infection or the inflammation (Henke et al., 2007). CF mucins had a higher sugar content and more O-glycans than non-diseased (ND) individuals (Xia et al., 2005). The level of sialic acids increased in CF mucins and tended to increase together with the severity of the disease (Chace et al., 1983; Davril et al., 1999; Lamblin et al., 2001). CF mucins contained a higher proportion of sulphated O-glycans (Davril et al., 1999; Lamblin et al., 2001; Xia et al., 2005). Both CF and ND mucins contained GlcNAc-6-sulphate, Gal-6-sulphate and Gal-3-sulphate but CF mucins contained a higher amount (Xia et al., 2005). Mucins in the small intestine of CF mice showed an increase in Fuc (Fucα-1,2 linkage) compared with wild-type mice, due to the induction of the fucosyltransferase Fut2 (Thomsson et al., 2002).

1.5.3. Infection with microorganisms

Alterations in the number of mucus-producing cells occur in animals infected with bacteria. Challenge with *Salmonella typhimurium* led to a decrease in the number of goblet cells; sulphated mucins produced by goblet cells in the villi increased, whereas they remained unchanged in the crypts (Arnold et al., 1993). The ability of microorganisms to degrade mucins may be partly responsible for alterations in the mucin structure. Bacteria have mucinolytic activity, including proteolysis and glycolysis (Berry et al., 2002). Small intestinal mucin of rats infected with *Vibrio cholera* showed poorly glycosylated areas in the mucin protein (Crowther et al., 1987). Human small intestine infected with *Candida albicans*

exhibited degraded terminal mucin glycopeptides (de Repentigny et al., 2000). *P. aeruginosa* not only depleted high MW mucins, but some strains also possessed glycosidases, such as galactosidase, N-acetyl glucosaminidase and N-acetyl galactosaminidase, which would allow further degradation of the molecule (Aristoteli and Willcox, 2003).

Bacteria, including the normal gut flora, can modulate some steps in the glycosylation process even without the presence of live bacteria, but only their soluble factors. The soluble product modulin, produced by *Bacteroides thetaiotaomicron*, interferes with fucosylation and galactosylation. The enzymes generating Fucα1,2Galβ1,4GalNAc and Galβ1,4GalNAc are switched-on and those producing NeuAcα2,6Gal are switched-off in goblet cells in the colon in infected mice (Freitas et al., 2005). Germ-free animals showed a higher ratio of neutral to acidic mucins in the colon and sulphomucins increased, while sialylated mucins were lower than in conventionally raised mice (Enss et al., 1992). However, rats infected with human microbiota had more sialomucins and sulphomucins in the small intestine, but less in the large intestine than in conventionally raised rats (Sharma and Schumacher, 1995). When *Escherichia coli* was administered to germ-free rats, there was an increase in neutral mucins in the colon (Enss et al., 1996).

H. pylori is a gastric bacterium which is a long-term parasite in the stomach, like gastric nematode parasites. The effects it produces in the host are not acute reactions. A significant 18% reduction in the proportion of polymeric gel forming mucin was observed in *H. pylori* positive subjects (Newton et al., 1998), due to disulphide bridge cleavage (Windle et al., 2000). *H. pylori* inhibited the synthesis of MUC1 and MUC5AC (Byrd et al., 2000; Kocer et al., 2004). In the antrum, AB/PAS-staining acidic mucosubstances decreased during infection (Kawano et al., 1990). Wadstrom et al. (1996) found that *H. pylori* produced sialidase and fucosidase, while a sulphatase activity toward sulphated human gastric mucins was demonstrated in the extracellular material secreted by *H. pylori* (Slomiany et al., 1992a, b). These enzymes are suggested to play important roles in allowing bacterial survival in the mucus layer in the gastrointestinal tract (Wadstrom et al., 1996). On the other hand, the patterns of glycosylation in gastric mucins are

reversibly altered by *H. pylori* infection (Ota et al., 1998), which may either increase or reduce the protective functions of mucins.

The changes in carbohydrates in mucus glycoproteins can be a protective mechanism that the host uses to cope with pathogenic Helicobacter. Kawakubo et al. (2004) found that when O-glycans terminating with α-1,4 linked GlcNAc were present, human gastric mucins were not influenced by infection with *H. pylori*. The role played by sialic acids in facilitating or inhibiting the adherence of *H. pylori* in the mucus gel layer is still in debate. Increased sialylation appears to be related to a high risk of *H. pylori* infection: when the amount of sialic acids is low, it is suggested that patients have some resistance to *H. pylori* (Ikezawa et al., 2005). The binding of *H. pylori* to gastrointestinal epithelial cells was found to be inhibited by sialic acid-containing oligosaccharides (Simon et al., 1997), whereas in other studies, this organism preferred to attach to the mucus gel through sialic acid-containing oligosaccharides (Evans et al., 1993) and Fuc-containing glycoproteins (Boren et al., 1993; Falk et al., 1995).

1.5.4. Protozoan infection

The glycoconjugate composition of the intestinal mucosa of piglets infected with *Isospora suis* exhibited significantly increased reactivity to Glc/Man-specific lectins, indicating altered monosaccharide composition (Choi et al., 2003).

Protozoan parasites are capable of degrading mucins. *Naegleria fowleri* attacks mucus glycoproteins through a variety of mechanisms, including cysteine proteases (Aldape et al., 1994; Cervantes-Sandoval et al., 2008), phospholipases (Aldape et al., 1994) and pore-forming polypeptides (Herbst et al., 2004). *Trichomonas vaginalis*, which infects the vaginal mucosa, can degrade mucins through their proteolytic enzymes (Lehker and Sweeney, 1999). *Entamoeba histolytica* also contains cysteine proteinases, which degrade human colonic mucins (Moncada et al., 2000) and an α-glucosidase, which possibly participates in N-glycan deglucosylation (Bravo-Torres et al., 2003).

1.5.5. Cestode infection

In the intestine of the brown trout *Salmo trutta* infected with *Cyathocephalus truncates*, there were more mucous cells which contained acidic glycoconjugates,

while the number of mucous cells containing neutral glycoconjugates decreased (Bosi et al., 2005).

Rats infected with *Taenia taeniaeformis* in the liver showed mucosal hyperplasia in the gastrointestinal tract: both the stomach and small intestine were increased in size, the stomach weight more than doubled compared with before infection and the gastric mucosa was thicker (Cook and Williams, 1981; Rikihisa et al., 1986; Abella et al., 1997; Konno et al., 1999). The hexosamine concentration per mg of protein in the hyperplastic gastric mucosa was twice that of the mucosa in the control stomachs and there were many fewer AB-positive cells than PAS-positive cells (Rikihisa and Lin, 1984). Other studies have reported gastric histopathology similar to that caused by gastric parasites: increased acidic mucin containing cells, depletion of parietal cells and chief cells (Lagapa et al., 2008; Konno et al., 1999), however, the number of PAS-positive cells increased in some studies (Konno et al., 1999), but decreased in other (Rikihisa and Lin, 1984), perhaps depending on the stage of the infection. While parasite products cannot be ruled out, it is likely that products of the host inflammatory response are causing this effect in these other organs, as a parabiotic experiment showed effects in both the donor and recipient (Cook and Williams, 1981).

1.5.6. Trematode infection

Infection of mice with two related species of Echinostoma, one which was able to cause parasitism and the other rejected by the host, suggested the host response played a significant part in the increased goblet cell numbers and associated changes in mucin composition. *Echinostoma caproni* established in the intestine and caused intestinal mucosal damage, whereas infection with *Echinostoma trivolvis* resulted in less damage to the intestine, but also increased goblet cell numbers, although the parasites were expelled. Using lectin histochemistry, it was shown that there was an increase in Fuc, GlcNAc, GalNAc and sialic acid in the intestinal mucosa in mice infected with either parasite (Fujino and Fried, 1993; Fujino et al., 1993).

A range of glycosidases was detected in the ES of *Fasciola hepatica*. These enzymes were able to degrade mucin in vitro (Irwin et al., 2004), as has been shown for bacteria, fungi and protozoan parasites.

1.5.7. Nematode infection

The small number of studies in rodents suggests that nematodes are capable of modifying the glycosylation of host mucins, however, it is not known whether this also occurs during parasitism in ruminants. The effects of gastrointestinal nematode parasites on the morphology of the mucosa and on the mucus-secreting cells have long been recognised from histopathological studies, but only a few more recent studies have involved chemical analyses of the mucins. Typical changes in the intestine are increased mucus secretion and numbers of goblet cells, mucosal hyperplasia and an increase in acidic mucins. In the stomach, mucosal hypertrophy is accompanied by expansion of the MNC population and expression of acidic mucins in the neck region containing the MNCs (Section 1.1.5).

In the large intestine of pigs, *Trichuris suis* and *Oesophagostomum dentatum* infection caused enlarged crypts, hyperplasia of the goblet cells and hypertrophy and thickening of the tunica muscularis (Petkevicius et al., 2003; Thomsen et al., 2006). Intestinal goblet cell hyperplasia also occurred in guinea pigs infected with *T. colubriformis* (Manjili et al., 1998). During the intestinal phase of *T. spiralis* infection in pigs, there was an increase in stored mucin in goblet cells and increased acidic mucins (Theodoropoulos et al., 2005). Mice infected with *T. spiralis* produced more intestinal mucus, along with goblet cell hyperplasia and increased mucin glycoprotein in the cells (Shekels et al., 2001).

Several chemical analyses of mucin glycosylation showed that there were modifications in monosaccharide composition during intestinal parasitism of the rat (Oinuma et al., 1995; Karlsson et al., 2000) and mouse with *Nippostrongylus brasiliensis* (Holmen et al., 2002), as also occurs in humans infected with *H. pylori* (Ota et al., 1998; Kocer et al., 2004; Kang et al., 2008; Kobayashi et al., 2009). Increased sialylation during infection of rats with *N. brasiliensis* was also found by Oinuma et al. (1995) and Karlsson et al. (2000). In contrast, Koninkx et al. (1988) found that, although the number of goblet cells containing acidic mucins increased significantly, sulphomucins were predominant, whereas sialomucins were dominant in control animals (Koninkx et al., 1988). As well as more sialic acids, more GalNAc was present in the mucus granules of goblet cells in the small intestine of Lewis rats (Oinuma et al., 1995). The terminal sugars of goblet cells also included

more GalNAc (Ishikawa et al., 1993; Ishikawa et al., 1994). In rats infected with *N. brasiliensis*, seven additional neutral oligosaccharide structures were expressed in addition to 14 present in uninfected animal (Olson et al., 2002).

There are also reports of reduced mucus secretion, which may depend on the stage of infection, particularly the expulsion phase or during recovery of the host (Ingham et al., 2008). Immediately after infection of rats with *T. spiralis*, there was reduced glycosylation, especially sialylation, in intestinal mucins, whereas 8-10 days post infection (p.i.), when the parasites were being expelled, there was decreased sulphation and increased GalNAc and sialic acids (Theodoropoulos et al., 2001). In rats infected with *T. spiralis*, there was reduced sialylation and sulphation of secreted mucins during a primary infection and increased GalNAc and sialic acid during the ejection response (Gardiner, 1976). After a few hours of challenge infection, sialic acid was reduced and 24 hours p.i. the glycosylation and sialylation increased more than in a primary infection (Theodoropoulos et al., 2001). In guinea pigs infected with *T. colubriformis*, the proportion of sialylated mucins was unchanged, but there were significant differences in sulphomucins between goblet cells in the crypts and those in the villi, depending on the day after infection, particularly on days 14, 21 and 28 (Manjili et al., 1998). A microarray study of MUC5AC expression in sheep infected with *H. contortus* showed that the gene encoding MUC5AC was down-regulated on day 22 compared with day 3 after infection (Rowe et al., 2009). Host genetics may also be important, as MUC5AC was down-regulated more in resistant sheep than in susceptible sheep, both in a primary infection and subsequent challenge with *H. contortus* (Ingham et al., 2008).

In sheep, reports differ on the effects of abomasal nematodes on mucin glycosylation, probably because of the breed of sheep or varying times of sampling during the infection. A loss of PAS-positive neutral mucosubstances was found in 8 months-old Suffolk Cross sheep three days after infection with *H. contortus* (Christie et al., 1975). Similarly, Newlands et al. (1990) observed a widespread decrease in neutral mucins produced by SMCs and sulphated mucins produced by cells in the isthmic region of the gastric glands in 9-10 months-old Greyface Suffolk cross sheep, 48 hours after infection with *H. contortus*. Scott et al. (1998a) infected Poll Dorset sheep with 150,000 larvae followed by a trickle infection of 10,000 larvae three times a week, to day 45 and killed 10 days later, the amount of neutral

mucins was increased in tissue sections and PAS-positive material was present even in cells at the gland base. Increased mucins were also present 8 days after transfer of 15,000 adult *T. circumcincta*.

The changes in mucin profile may be induced by parasites chemicals. In the stomach mucosa of rats, which was infused with *T. circumcincta* ES, there was an increased amount of both neutral and acidic mucus in the glandular cells 24 hours later. Three days after the last administration, mucus secretion decreased below that of the control stomach. These results suggest that small peptides from Teladorsagia ES may specifically enhance mucus production and secretion in rat stomach and that this action is reversible (Rikihisa and Hammerberg, 1982). Nematodes probably also secrete exoglycosidases, as has been found for many pathogenic microorganisms. *F. hepatica* ES contained at least 8 different glycosidases, the most abundant of which were beta-N-acetylhexosaminidase, beta-galactosidase and beta-glucosidase, while alpha-fucosidase, beta-glucuronidase, alpha-galactosidase, alpha-mannosidase and neuraminidase were also present (Irwin et al., 2004). The susceptibility and resistance of the host may depend on the presence or absence of an appropriatedly glycosylated recognition site (Mouricout, 1997). Such carbohydrate moieties can serve both as attachment sites and nutrient sources for the intestinal microflora (Carlstedt-Duke et al., 1986a, b; Mukai et al., 1998).

It is still unknown whether the changes in mucins are part of the immune response of the host to protect itself from infection with parasites or mechanisms parasites use to obtain favourable conditions for their survival inside the host. The involvement of mucins in expelling nematodes has been observed in sheep infected with *H. contortus* (Miller et al., 1983; Miller et al., 1985) and in rats infected with *N. brasiliensis* (Oinuma et al., 1995). Mucus-producing cell hyperplasia, increased mucin secretion and qualitative changes in mucin following nematode infections may contribute to the elimination of parasites (Ishikawa et al., 1994; Shi et al., 1994a; Harrison et al., 1999).

1.6. CONCLUSIONS

In many diseases, mucins have been shown to play important roles in both the host immune response and recognition of the site of infection and

establishment of the pathogen. Much less is known about these interactions between the host and helminth parasites than for microorganisms and protozoa. Observations in nematode infections of sheep mainly involve tissue histology and immunology and there appear to be no chemical analyses of sheep gastrointestinal mucins.

The present study investigated the effects of *H. contortus* and *T. circumcincta*, two common abomasal parasites in sheep, on the post-translational processes of mucins in the abomasum and duodenum. In the next chapter, the purification process and some characteristics of these mucins are described. Purified mucins from the fundus and duodenum of sheep of different ages, infected with *H. contortus* or *T. circumcincta,* have been collected to study the alterations in monosaccharide composition with age and infection (Chapter 3). Experiments reported in Chapter 4 provide an insight into the effect of age, weaning and infection at early age. Cell-specific mucin production and the differentiation between neutral and acidic mucins in sheep abomasum and duodenum have been investigated histochemically (Chapter 5). The presence of monosaccharides and their distribution, structure and linkages in different mucus-producing cells using specific lectins are reported in Chapter 6.

Chapter 2

PURIFICATION AND CHARACTERISATION OF HIGH-MOLECULAR-WEIGHT GLYCOPROTEINS IN SHEEP FUNDIC AND DUODENAL MUCINS

2.1. INTRODUCTION

Many studies of the modification of mucins associated with diseases and infection have used histological (Corfield et al., 1992b; Ferri et al., 2001) or immunological (Aebischer et al., 2000; Sutton, 2001; Backhed et al., 2003; Cohen et al., 2003), lectin histochemical (Alroy et al., 1984; Fischer et al., 1984; Abel et al., 1987; Chan and Wong, 1991; Fujino and Fried, 1993; Baczako et al., 1995; Choi et al., 2003; Lueth et al., 2005) or immunocytochemical (Burchell et al., 1987; Reis et al., 1999; Kubota et al., 2007; Tsubokawa et al., 2009) techniques rather than chemical methods. Mucins collected from tissues, especially from the gastrointestinal tract, are contaminated with non-mucin substances such as proteins, acid, pepsin, bile, DNA and RNA which must be removed for chemical analysis.

2.1.1. Purification of mucins

Various methods have been used to purify mucins including: (1) gel filtration chromatography; (2) affinity chromatography; (3) cesium chloride (CsCl) isopycnic density gradient centrifugation; (4) equilibrium density gradient centrifugation and (5) ion exchange chromatography (reviewed by Allen, 1981). These methods use differences in MW, density and charge of mucin molecules. Using a single method or a combination of two or three of the above methods, mucins have been isolated and characterised in pigs (Hashimoto et al., 1964; Neiderhiser et al., 1971; Pearson

et al., 1981; Minkiewicz-Radziejewska et al., 2000), rats (Keryer et al., 1973; Clark and Marchok, 1979; LaMont and Ventola, 1980; Malinowski and Herp, 1981; Bodner and Baum, 1984; Tabak et al., 1985; Ohara et al., 1997), monkeys (Herzberg et al., 1979; Devaraj et al., 1993), humans (Chao et al., 1988; Parker et al., 1993; Thornton et al., 1995), sheep and cattle (Tettamanti and Pigman, 1968) from tissues including the stomach, salivary, submandibular and submaxillary glands, the colon and intestine.

A frequently used protocol is a combination of gel filtration followed by CsCl density gradient centrifugation (Feste et al., 1990; Paszkiewicz-Gadek et al., 1995; Minkiewicz-Radziejewska et al., 2000). The advantage of this technique is that it has been used for the purification of mucus glycoprotein in a range of species from many different organs such as the gastrointestinal tract, submaxiliary, saliva gland and lung. In all instances, the gel elution profile has been entirely predictable, with mucin eluting in the void volume at a low flow rate. Gel filtration should be used before CsCl density gradient centrifugation, as Marshall and Allen (1978), who used them in the reverse order, found that a single ultracentrifugation did not succeed in separating glycoproteins from nucleic acids.

The removal of proteins and DNA/RNA using these methods can be validated at each step by monitoring the absorbance at 280 nm, assaying fractions with Periodic Acid Schiff (PAS) to detect neutral carbohydrates and an Enzyme-Linked Lectin Assay (ELLA) to detect sugars on glycoconjugates. The peak fractions can then be subjected to two SDS-PAGE, one stained with PAS and the other transferred by Western blot and probed with lectins to locate the glycoprotein peak. Monitoring by A_{280} alone is not adequate because mucin glycoproteins contain only a small amount of aromatic amino acids. Although PAS is a sensitive detection method for neutral sugars, these may also be present on non-mucin glycoconjugates. Thus, using only one assay to detect mucin-containing fractions is not reliable. The sensitivity and reliability of the ELLA method has been investigated and shown to be specific for detecting mucins (Kodaira et al., 2000; Gull et al., 2007). Western blot followed by lectin probing is also effective in detecting the carbohydrate epitopes.

2.1.2. Molecular weight of mucins

The MW of native mucin molecules are very high (approximately 10^6 or greater) because of subunit assembly (Caspar, 1966). Mucins are oligomeric structures consisting of many monomers linked by non-covalent bonds i.e., physical forces such as hydrophobic or ionic interactions; or covalent bonds i.e., linked end-to-end via disulfide bonds in some mucins (Gum et al., 1992; Meerzaman et al., 1994; Desseyn et al., 1997; Toribara et al., 1997). The MW of mucin depends on the source of the sample, method used for preparation and experimental conditions employed (Holden et al., 1971a). For example, freshly-prepared pig gastric mucin glycoproteins have MW of $2x10^6$ Da (Carlstedt and Sheehan, 1984; Carlstedt et al., 1985) whereas the MW of commercial pig gastric mucins is $1.25x10^6$ Da (Jumel et al., 1996).

Molecular weights of sheep mucins have been reported. Those were investigated including colonic mucin (Allen and Kent, 1968; Kent and Draper, 1968), submaxillary mucin (Gottschalk et al., 1972; Hill et al., 1977a, b), and small intestinal mucin (Mukkur et al., 1985). Ovine submaxillary mucins have a MW as low as 394,000 Da using 0.2M sodium chloride extraction or as high as $1.3x10^6$ Da (Gottschalk et al., 1972). Hill et al. (1977a) reported the MW of sheep submaxillary mucins to be between 550,000 and 650,000 Da. Mukkur et al. (1985) reported the MW of sheep intestinal mucins to be $5.0\pm0.1x10^6$ Da. Differences in MW of mucins can arise from the size and number of subunits per one native molecule (Bennett et al., 1998).

Native mucins have a very high MW and maintain their structure as secreted while reduced mucins exist in subunit form (the smallest unit is a monomer). During reduction, bonds or interactions that link subunits together to make up the native molecule are broken down, releasing reduced products. The size of mucin subunits varies according to the reducing conditions. Allen et al. (1989) studied the reduction of pig gastric mucin glycoprotein in different conditions. The glycoprotein had a MW of $2x10^6$ Da only when the extraction and isolation of mucins were implemented in the presence of guanidinium hydrochloride (GuHCl) and treated with dithiothreitol (DTT), while a $5x10^5$ Da subunit was observed when mucins were further treated with 0.2 M β-mercaptoethanol. This suggested that mild reduction would result in

larger MW glycoproteins and complete reduction would result in smaller MW subunits (Allen et al., 1988)

2.1.3. Characterisation of bonding and linkage in mucins

Protease inhibitors such as phenylmethylsulphonyl fluoride (PMSF) and N-ethylmaleimide (NEM) are generally required to protect the native mucin molecule from being attacked and broken down into small fragments by proteolytic enzymes. Urea acts as a reducing or physical deaggregating agent (Maxfield and Davis, 1963) by disrupting non-covalent bonds, increasing the solubility of proteins; SDS also disrupts non-covalent bonds in proteins and denatures and causes the molecules to lose their conformation (unfolding into a rod-like shape). Using urea and SDS, mucin complexes linked by non-covalent bonds would be broken. The presence of covalent bonds can be investigated by using DTT, which reduces the mucin molecule by cleaving -S-S- groups, unfolding the peptide chains or by β-mercaptoethanol, which is used to completely reduce disulphide bonds.

The sheep fundic and duodenal mucus glycoproteins (mucins) were purified from the solubilised mucus by the procedure of size exclusion chromatography on a Sepharose 4B (gel filtration) column, followed by density gradient centrifugation in GuHCl/CsCl (Figure 2.1). This method was shown to be effective in isolating mucin glycoproteins without contamination. During the gel filtration process of mucin purification, the MW of native and reduced mucins was estimated.

2.2. MATERIALS AND METHODS

The mucus was solubilised and the protease inhibitors PMSF and NEM were added. Reduced mucin was obtained after being denatured with urea 6 M. Native and reduced mucins were separated from low MW proteins, lipids, DNA by size exclusion chromatography and CsCl density gradient centrifugation in the presence of GuHCl 4M.

2.2.1. Mucus collection

A total of 12 mucus samples (6 from the abomasum and 6 from the duodenum from sheep non-infected, *H. contortus*-infected or *T. circumcincta*-infected at 4.5 and 6 months-of-age) were used to validate the methods and

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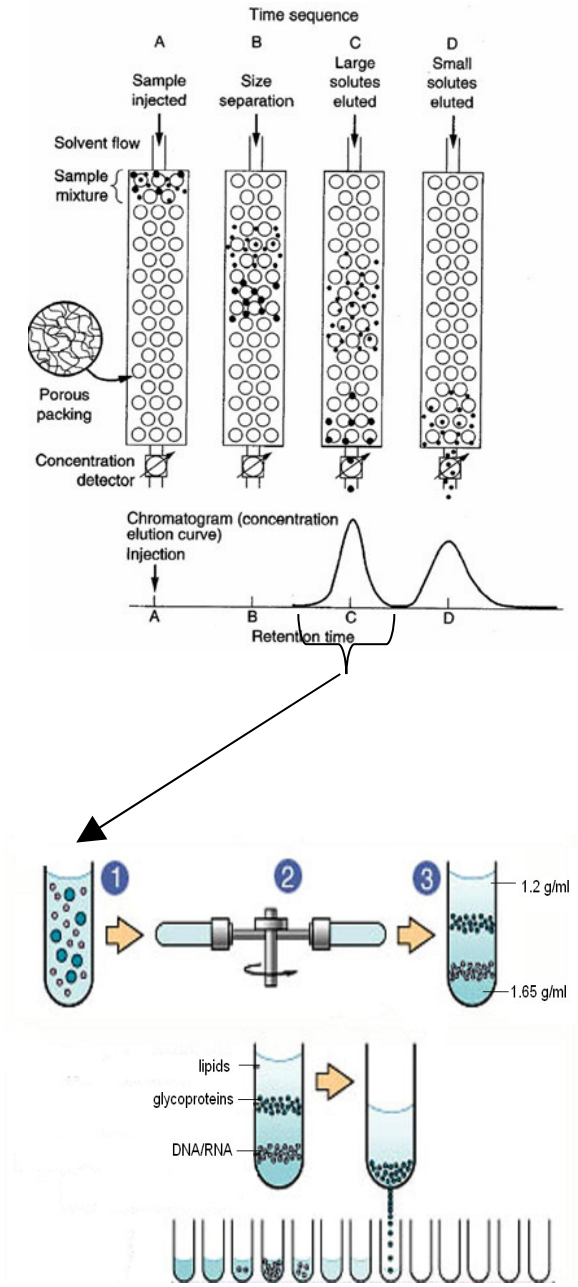

Figure 2.1. Two purification steps for sheep gastrointestinal (GI) mucins. Sheep GI mucins were purified by size exclusion chromatography (top section) and CsCl density gradient centrifugation (bottom section). Top section: Size exclusion chromatography separated proteins based on molecular weight into two populations (high and low MW). The high MW population was determined to be partially purified mucin glycoprotein eluted together with DNA. Low MW protein contaminants were removed from the mucin glycoproteins. Bottom section: Partially purified mucins were centrifuged in guanidinium chloride/CsCl to separate into lipid, glycoproteins, DNA and RNA fractions based on their density. Fractions contained glycoproteins were pooled and identified as purified mucins.

investigate some of the characteristics of sheep gastrointestinal mucins. Sheep were euthanased and the abomasum and duodenum were removed. Mucus was collected separately from the abomasum and duodenum of each sheep. The abomasum was cut open along its greater curvature and gently washed with saline four times to remove worms. A 20 cm length of the duodenum from the pyloric sphincter was removed, cut open along its length and rinsed with saline. Mucus was scraped using a microscope slide. In the stomach, mucus was scraped off the surface of the fundus and both sides of all folds, while in the duodenum, mucus was collected by scraping the inner surface of the first 20 cm of the intestine starting from the duodenal bulb. Samples were immediately frozen in liquid nitrogen and stored at -70^{o}C.

2.2.2. Purification of mucin glycoproteins

2.2.2.1. Preparation of native and reduced mucins

All chemicals were obtained from Sigma Aldrich unless indicated otherwise.

Native mucin (with protease inhibitors): Each frozen mucus sample was thawed and slowly stirred in 20 ml 0.05 M borate buffer pH 7.0, containing 0.01 M disodium-EDTA and 0.02% sodium azide, overnight at 4^{o}C. PMSF and NEM were added to the mucus solution to inhibit proteases at final concentrations of 1 mM and 2 mM, respectively and stirred for 10 min. Samples were centrifuged at 30,000 g for 30 min at 4^{o}C (Sorvall Ultracentrifuge) to remove insoluble materials and cellular debris. Supernatants were collected and dialysed exhaustively against MilliQ (MQ)-water (MilliQ Plus system, Milipore, Bedford, MA). Samples were freeze-dried.

Native mucin (without protease inhibitors): Mucus was extracted as above except the addition of PMSF and NEM was omitted.

Reduced mucin: Mucus with added protease inhibitors was reduced with 6 M urea. Before being centrifuged, solid urea was added to a final concentration of 6 M, the mixture was homogenised and stirred at 4^{o}C for 6 h. The mixture was then centrifuged. Supernatants were collected, dialysed exhaustively against MQ-water and freeze-dried.

2.2.2.2. Size exclusion chromatography to remove low-molecular-weight proteins

The freeze-dried samples were re-solubilised in 5 ml 1% SDS. The samples were centrifuged to remove particulate material if needed. If the sample were too viscous, it was diluted with 1% SDS. The sample was filtered with a 0.45 µm filter (Sartorius) before injection into the column. Sample injection was carried out via a 5-ml loop.

Gel filtration of mucins was performed on a Sepharose 4B column (2.5x100cm) (Pharmacia, GE Healthcare, Sweden) on a Biologic DuoFlow™ chromatography system (Biorad) coupled to the associated software version 5.0. The elution buffer contained 0.05 M borate buffer, 0.01 M disodium EDTA, 0.02% sodium azide and 1% SDS. The buffer was filtered through a Supor®-200 0.2µm filter (Gelman Sciences) and degassed under vacuum. The flow rate was 0.15 ml/min and 4 ml fractions were collected.

For all fractions, glycoprotein was detected by the modified PAS method of Mantle and Allen (1978) and ELLA (Huet et al., 2000). Protein and nucleic acids were measured at A_{280} and A_{260} respectively.

The void volume and the included volume were determined with Blue dextran and glycine respectively.

2.2.2.3. Periodic acid Schiff (PAS) assay to identify neutral sugars

A colorimetric assay for glycoproteins was based on the PAS method used by Mantle and Allen (1978).

Schiff's reagent (Appendix 6.1.7) was warmed to 37°C. 500 µl aliquots of each fraction from the Sepharose column were diluted in 1.5 ml MQ-water. 0.2 ml periodic acid (1 ml 0.5% periodic acid: 10 ml 7% acetic acid) was added to samples and vortexed. The mixtures were incubated for 2 h at 37°C. 0.2 ml Schiff's reagent was added and the mixtures were left at room temperature for 30 min. Absorbance at 555 nm was measured.

2.2.2.4. Enzyme-linked lectin assay (ELLA) to detect sugars on glycoconjugates

An ELLA was used to determine the mucin-containing fractions from the column. A modification of the method of Huet et al. (2000) was used.

Aliquots of 100ul of each fraction from the Sepharose 4B column were subjected to ELLA for glycoprotein identification. The volume of each reagent applied to the plate was 100 µl/well and all incubations, except for blocking (200 µl of blocking solution/well was used), were performed at room temperature (22^{o}C). The reagents, if not indicated otherwise, were diluted with phosphate buffered saline (PBS) containing 5 mM sodium phosphate buffer, 0.05% (v/v) Tween 20, pH 7.4 and 0.15 M NaCl which was also used for washing the plate between incubations.

Fractions from the Sepharose 4B column were used to coat the 96-well flat-bottom microtiter plates (Nunc-immuno plate, F96, Thermo Fisher Scientific, Maxisorp, Denmark) and incubated overnight at room temperature in a humidified chamber. After washing the plate (0.15 M NaCl, 5 mM sodium phosphate buffer, pH 7.4, 0.05% (v/v) Tween 20), unbound sites were blocked with blocking solution (0.15 M NaCl, 0.05 M Tris-HCl buffer, pH 7.6, 0.05% (v/v) Tween 20 and 1% (w/v) bovine serum albumin (BSA)) 200 µl/ well for 1 h. Biotinylated lectins (2 µg/ml diluted in blocking solution containing 0.1 mM $CaCl_2$) were added to each well and incubated for 1 h. The plate was washed to remove unabsorbed lectins and the alkaline phosphatase-conjugated streptavidin solution (Sigma, diluted 1:2,000) was added. After 1 h, the plates were washed at least four times and incubated with p-nitrophenylphosphate at 37^{o}C in 1 h. The absorbance was read at 405 nm in a microtiter plate reader (VERSA max, software SOFTmax® PRO 4.0).

A preliminary experiment was carried out to study the specificity of ELLA in identifying mucin glycoproteins using 6 different lectins which specifically bind to 6 different monosaccharides. Those lectins were SBA (specific for GalNAc), WGA (GlcNAc), UEA (Fuc), ConA (Man), SNA (sialic acid) and RCA (GalNAc/Gal). The binding patterns of those 6 lectins were observed to determine which were suitable for detecting mucins. Subsequently, UEA, SBA, RCA, WGA and ConA were used to detect mucin-containing fractions.

2.2.2.5. Molecular weight estimation for proteins

The column, chromatography system and eluent buffer described in Section 2.2.2.2 were used. 10 mg of each standard protein was dissolved in 5 ml 1% SDS and filtered through a 0.45 µm filter. The standards used were: blue dextran (MW 2,000,000 Da); porcine stomach mucin (1,250,000 Da); alcohol dehydrogenase (150,000 Da); BSA (66,430 Da); carbonic anhydrase (29,000 Da) and glycine (75 Da).

The column was equilibrated with borate buffer until the A_{280} was constant. Blue dextran was injected to determine the void volume (V_o) and glycine to determine total volume (V_t). Protein or glycoprotein samples eluted at V_e.

2.2.3. Validation of mucin purification

The purity of mucins after each purification step was investigated by separation on 3 gradient SDS-PAGE gels, one was stained with SimplyBlue for proteins, one was stained with PAS for carbohydrates and one was stained by Western blot probed with lectins for glycoproteins. The solutions were made up and gels were prepared as described in Appendix 3.

To investigate the bonding characteristics of mucin molecules, β-mercaptoethanol was added to the loading buffer to break the bonds completely and release the subunits.

2.2.3.1. Gradient SDS polyacrylamide gel electrophoresis

Fractionated mucins were analysed on a gradient SDS-PAGE 4-15% resolving gel (prepared in 1.5 M Tris-HCl pH 8.8, 0.1% (w/v) SDS) and a 3% stacking gel (prepared in 1.5 M Tris-HCl pH 6.8, 0.1% (w/v) SDS). The electrophoresis buffer contained 25 mM Tris, 250 mM glycine pH 8.3 and 0.1% (w/v) SDS. The sample buffer used was SDS gel loading buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol).

45 µl of each fraction eluted from the Sepharose 4B column was mixed with 15 µl of loading buffer (with or without 5% (v/v) β-mercaptoethanol) and incubated in boiling water for 3 min before applying to the gel. Each well can handle up to 50 µl samples.

Electrophoresis was carried out on gel electrophoresis apparatus (Model 73.1010V, Class II, Continental lab products, Inc., APOLLO instrument) at 80V for 20-40 min, followed by 100V for about 2 h until the tracking dye reached the bottom edge of the gel.

Prestained molecular weight marker (Novex Sharp Protein standard, Invitrogen) and normal marker (Sigma marker wide range MW 65,000-205,000) were both used in the gel. 15 µl of protein marker was loaded into the well.

Once the electrophoresis was finished, each of the 3 gels was stained with one of the following: SimplyBlue™ Safestain (Invitrogen) for proteins, PAS for carbohydrates and Western blot followed by lectin probe for glycoproteins.

a. SimplyBlue staining:

After finishing the run, the gel was washed with MQ-water and then washed with 100 ml MQ-water in a microwave for 1 min. It was shaken on an orbital shaker for 1 min. These washing steps were repeated 3 times. 30 ml SimplyBlue staining solution (Invitrogen) was added to the gel. The gel was then microwaved for 90 sec then shaken on an orbital shaker until bands were seen. The gel was destained with MQ-water until the background was clear, then photographed.

b. PAS staining: The protocol was according to Thornton et al. (1996)

Solutions A: 1% (v/v) periodic acid in 3% (v/v) acetic acid (fresh)

B: 0.1% (w/v) sodium metabisulfite in 10 mM HCl (fresh)

C: 50% (v/v) ethanol

D: 0.5% (w/v) sodium metabisulfite in 10 mM HCl (fresh)

E: 7.5% (v/v) acetic acid/5% (v/v) methanol in MQ-water

The gel was shaken in solution C in 30 min, washed with MQ-water for 10 min then shaken in solution A for 30 min and washed again with MQ-water 6 times for 5 min each. The gel was incubated with solution B for 10 min twice and then Schiff's reagent in the dark for 1 h. It was shaken in solution B again in the dark for 1 h. Finally, it was washed with solution D 4 times, each for 30 min in the dark and stored in solution E. The gel was then photographed.

2.2.3.2. Western blot followed by lectin probe

After electrophoresis, mucins were transferred to a nitrocellulose membrane (Amersham, Life Science) by using transfer apparatus (Mini Trans-blot Module, Biorad). The transfer buffer used was 39 mM glycine, 48 mM Tris-base and 20% (v/v) methanol.

Gels were soaked in transfer buffer prior to being sandwiched with nitrocellulose between six layers of filter paper soaked in the same transfer buffer. Transfer was carried out at a constant current of 100V for 1 h at 4°C prior to detection of glycoproteins using lectins.

The membrane staining procedure was described by Thornton et al. (1996). The membrane was incubated with blocking solution (0.01 M PBS, pH 6.8 containing 0.1% (v/v) Tween 20 and 1% (w/v) BSA) overnight at 4°C. The membrane was incubated with biotinylated lectin (10 µg/ml in PBS 0.01 M pH 6.8, 0.1% (v/v) Tween 20) for 30 min on a rocker. Unbound substances were removed by washing 3 times for 5 min each, then the membrane was incubated with peroxidase-labeled avidin (5 µg/ml in PBS-Tween 20). After washing, substrate solution (10 mg diaminobenzidine in 40 ml 0.01M PBS, pH 6.8 with 16 µl 30% hydrogen peroxide added immediately before use) was added for 30 min to develop the colour. The reaction was stopped by immersing the membrane in stop solution (1% (w/v) sodium azide) for 15 min. Finally, the membrane was washed in MQ-water 3 times for 5 min each.

The developed blot was photographed while still wet to obtain the best contrast. The nitrocellulose membrane was dried between filter papers and stored protected from light.

2.2.3.3. DNA removal by CsCl density gradient centrifugation

The mucin-containing fractions from the Sepharose column were pooled, dialysed exhaustively against MQ-water and freeze-dried. The mucin precipitate was dissolved in 4M GuHCl in 10 mM phosphate buffer, pH 7.0. Solid CsCl was added to an initial density of 1.39 g/ml. Samples were subjected to CsCl density gradient centrifugation in a Sorvall ultracentrifuge (T865 rotor; polyallomer tube;

tube capacity 35 ml were used) at 130,000 g at 4°C, the centrifugation time was varied from 72 to 89 h.

After centrifugation, 1 ml fractions were collected by piercing the bottom of the tube. The flow rate was 1ml/min. The absorbances at 260 and 280 nm of the fractions were recorded using an UV detector.

The density of each fraction was determined by weighing 1 ml aliquots. The fractions were analysed for the concentration of protein (absorbance 280 nm), DNA (absorbance 260 nm) and glycoproteins (PAS and ELLA).

Mucin-containing fractions were pooled, dialysed exhaustively against MQ-water and freeze-dried. The final solution was considered to be purified mucin.

2.3. RESULTS

2.3.1. Separation of native and reduced gastrointestinal mucins

Proteins were separated by gel filtration depending on their MW. Absorbance at 280 nm identified the presence of proteins. PAS was used to detect sugars and ELLA to detect the presence of carbohydrate on glycoproteins.

Absorbance at 280 nm showed that native mucin separated into two populations: a high MW population which eluted in the void volume (fractions 15-19) and a low MW population which eluted in the included volume (fractions 45-55) (Figure 2.2A). In native fundic mucin, the concentration of glycoprotein in fractions measured by PAS assay was high in 2 populations: fractions 14-20 (Peak 1) and fractions 48-56 (Peak 2). The hexose to protein ratio of peak 1 (2.08) was higher than that of peak 2 (0.365). Similarly, in duodenal mucin, glycoproteins were observed first in a high MW population in the void volume and secondly in a low MW population in the included volume.

In native fundic mucins, the ELLA reactivity was quite high in fractions corresponding to the high MW population eluted in the void volume while the second population (low MW) did not show binding to lectin (Figure 2.3A).

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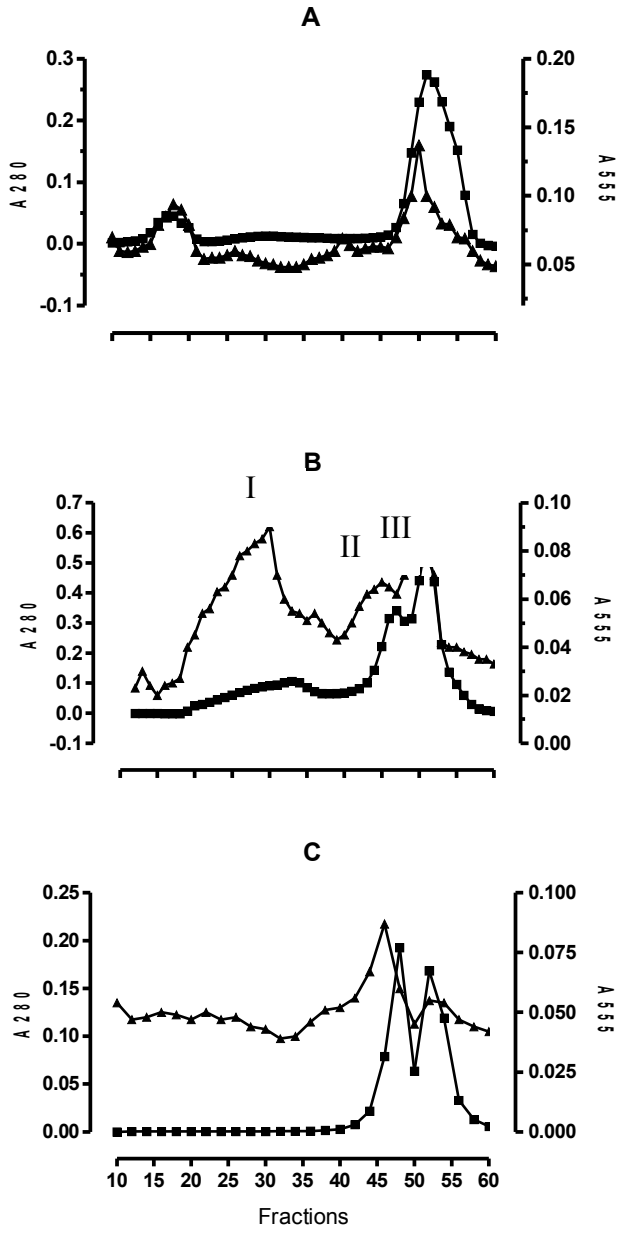

Figure 2.2. Elution profile of fractions separated from sheep gastrointestinal mucus on a Sepharose 4B column monitored by PAS assay to detect carbohydrates. The mucus was extracted in borate buffer and chromatographed on a Sepharose 4B column (2.6x100 cm) with the same buffer. Five milliliters of mucus solution in 1% SDS was applied to the column and 4-ml fractions were collected. Fractions were measured for protein (■) at absorbance 280 nm and neutral sugars (▲) using a PAS assay at absorbance 555 nm: (A) native fundic mucus (sample 37); (B) reduced fundic mucus (sample 39); (C) reduced duodenal mucus (sample 40).

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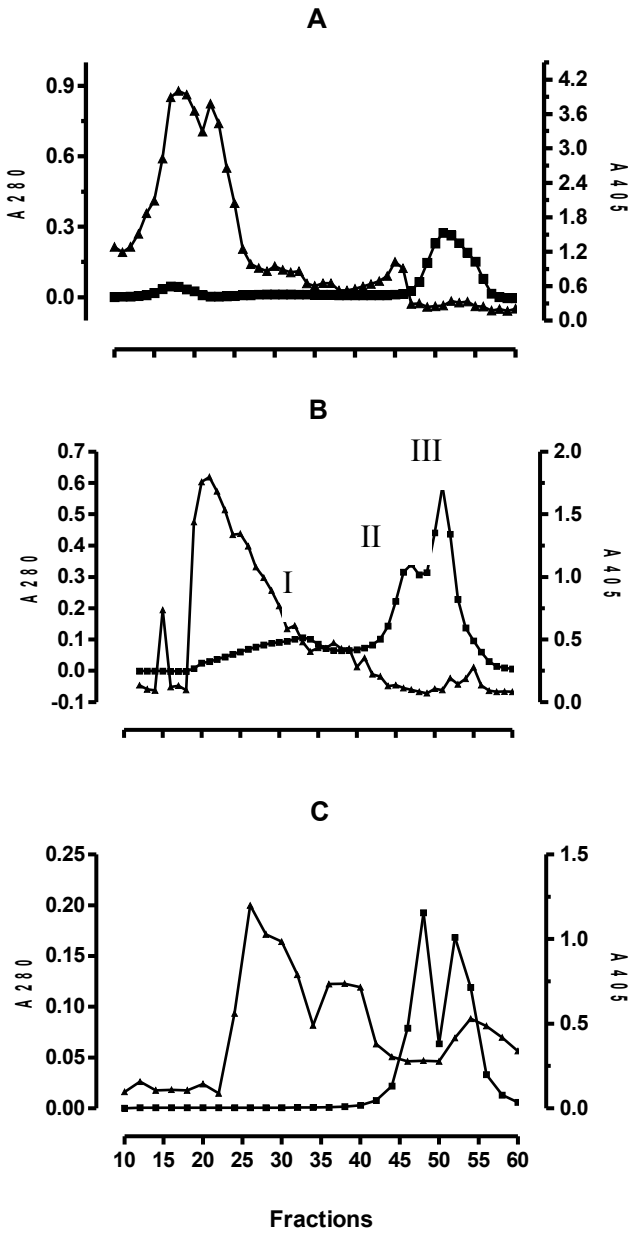

Figure 2.3. Elution profile of fractions separated from sheep gastrointestinal mucus on a Sepharose 4B column monitored by ELLA to detect sugars on glycoproteins. The mucus was extracted in borate buffer and chromatographed on a Sepharose 4B column (2.6x100 cm) with the same buffer. Five milliliters of mucus solution in 1% SDS was applied to the column and 4-ml fractions were collected. Fractions were measured for protein (■) at absorbance 280 nm and sugars (▲) using ELLA at absorbance 405 nm: (A) native fundic mucus (sample 37); (B) reduced fundic mucus (sample 39); (C) reduced duodenal mucus (sample 40).

After reduction, mucins separated into three populations for fundic mucins (Figure 2.2B) and two populations for duodenal mucins (Figure 2.2C). The peak at fraction 17 in native mucins was not seen in reduced fundic mucin; instead there were two other peaks at fractions 34 and 46 (Figure 2.2B), suggesting that the high MW population which eluted at fraction 17 was reduced to two smaller MW populations eluting at fractions 34 and 46. The PAS assay showed that there were glycoproteins in all 3 populations, but of lower concentrations than in native mucins. Reduced duodenal mucins eluted into two populations at fractions 45-50 and 50-55. The glycoprotein concentration was higher in the first population which eluted at fraction 47 than the second population which eluted at fraction 54 (Figure 2.2C). Subunits of reduced fundic mucin were not detected specifically by lectins (Figure 2.3B). Similarly, subunits of duodenal mucin did not show specific detection by lectins (Figure 2.3C).

ELLA, using one of the 6 lectins, performed on fractions separated from gastrointestinal mucins on the Sepharose column showed that WGA, RCA and SBA had similar patterns of detection (Appendix 4). UEA and ConA also gave positive results for these fractions, but with different intensity. Only SNA did not discriminate between mucin-containing and non-mucin-containing fractions. Except for SNA, the other five lectins gave the same result for mucin-containing fractions (see Table 6.1 for the binding specificity of each lectin).

The elution positions of each standard protein are summarised in Table 2.1. The separation range of Sepharose 4B is 70-200 kDa and sheep native fundic mucin eluted in the void volume of the column, suggesting that the molecular weight of sheep native mucin was greater than 200 kDa. Alcohol dehydrogenase (150,000 Da) and BSA (66,430 Da) eluted in the included volume of the column at 155 and 176 ml respectively. Reduced fundic mucin eluted in the volume at 136 and 184 ml, indicating that the approximate MW of fundic mucin subunits with one greater than 150 kDa and the other less than 66 kDa. Reduced/alkylated duodenal mucins eluted at 188 ml, indicating the MW of the subunits to be less than 66 kDa.

Table 2.1. Elution position of sheep fundic and duodenal mucin on Sepharose 4B column.
(A) Elution volume and molecular weight (MW) of standard proteins
(B) Elution volume of subunits in sheep fundic and duodenal mucin

(A)

Standard protein	Elution	Volume (ml)	MW (Da)
Blue dextran	V_0	52	2,000,000
Porcine stomach mucin		59	1,250,000
Alcohol dehydrogenase		155	150,000
Bovine serum albumin		176	66,430
Carbonic anhydrase		240	29,000
Glycine	V_t	240	75

(B)

		Volume (ml)
Native mucin		60
Reduced fundic mucin	First population	136
	Second population	184
Reduced duodenal mucin		188

2.3.2. Validation of the mucin purification method

2.3.2.1. Size exclusion chromatography

The purification of mucins was investigated after each step by gradient SDS-PAGE. The native fundic or duodenal mucin was subjected to SDS-PAGE with a gradient of 4-15%, before gel filtration (Figure 2.4).

Before being separated on a Sepharose column, native mucin stained with SimplyBlue showed a smeared band from the top of the stacking gel to the end of the separating gel, with a band staining strongly at around 100-110 kDa. When stained with PAS, glycoprotein was only detected in the stacking gel and in the edge between the stacking and separating gels. Western blotting also showed positive binding at the top of the gel and a smeared band in the separating gel. The bands in duodenal mucins were more weakly stained than those in fundic mucins.

Native mucins extracted without protease inhibitors showed a ladder-like pattern containing a series of proteins with MW from 10 to 70 kDa confirmed by SDS-PAGE followed by Western blot. Figure 2.5 illustrates very clearly the activities of proteolytic enzymes in breaking high MW mucin molecules into a series of low MW protein bands.

After being purified and separated on a gel filtration column, native mucins treated with protease inhibitors separated into 2 main populations: a high MW population which eluted in the void volume and a low MW population which eluted in the included volume of the column. The high MW fractions were determined to be mucin glycoprotein. Staining with SimplyBlue (Figure 2.6) revealed two bands which stained strongly at the top of the stacking and separating gels and a smeared band was observed in the separating gel without any band at 110 kDa, which was seen in native mucin before gel filtration. Fraction 17 of the fundic mucin sample stained pink with PAS, showing the presence of glycoprotein (Figure 2.7) and was also detected by Western blot (Figure 2.8). It suggested that the low MW proteins had been separated from the high MW glycoproteins and mucin had been partially purified. Therefore, the mucin glycoprotein-containing high MW fractions were confirmed by SDS-PAGE and staining with PAS and Western blot.

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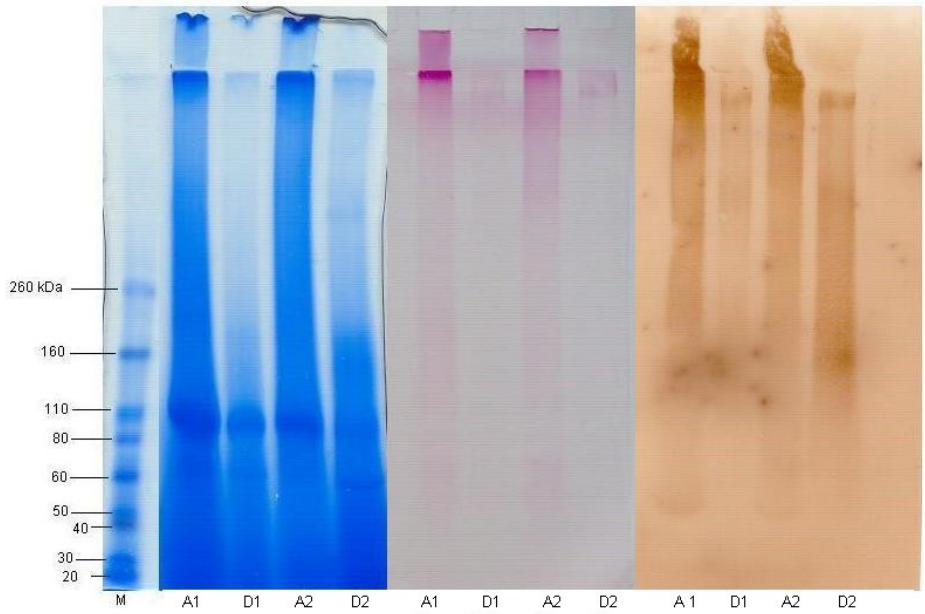

Figure 2.4. SDS-PAGE of native mucus before gel filtration. Following gel electrophoresis, the gels were stained with SimplyBlue for protein (left); PAS for glycoprotein (middle) and probed with lectin by Western blot (right). A1, A2- Abomasal mucins; D1, D2- Duodenal mucins. Protein marker (left lane).

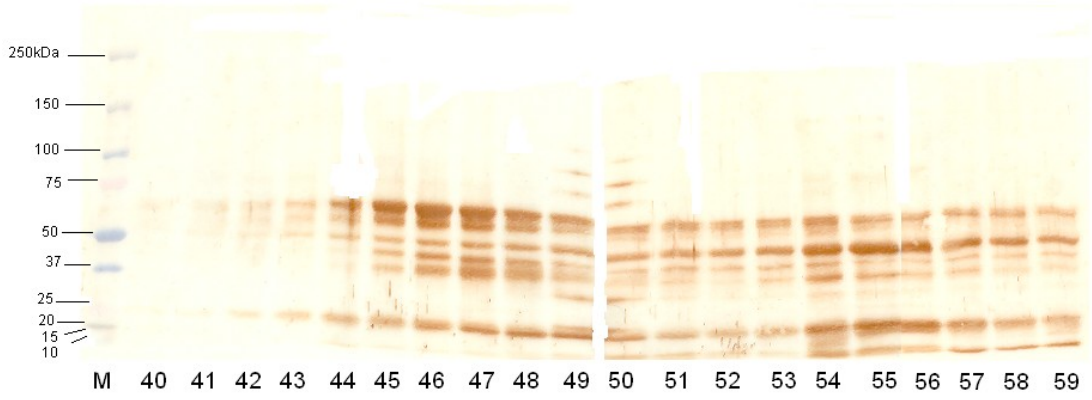

Figure 2.5. SDS-PAGE of native fundic mucus extracted without protease inhibitors. Fractions 40-59 from size exclusion chromatography, which showed high absorbance at 280 nm, were subjected to gradient SDS-PAGE to investigate the effects of proteases on mucin glycoproteins breakdown. Without protease inhibitors, mucin molecule was broken down into a series of small MW proteins by proteolytic enzymes in the mucin.

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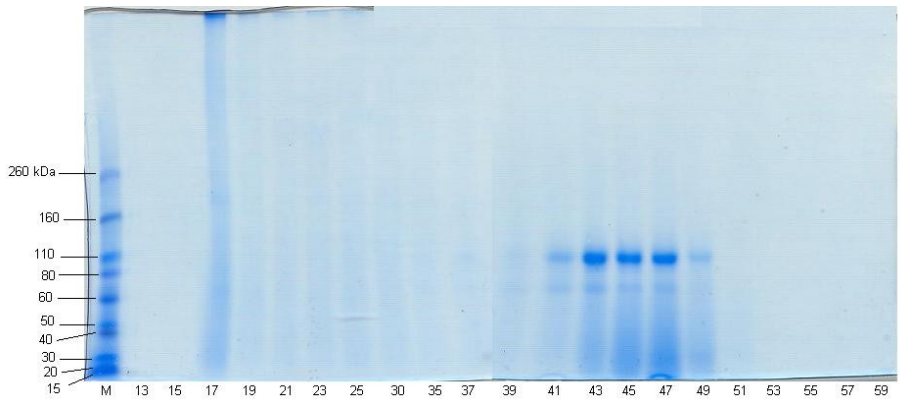

Figure 2.6. Separation by SDS-PAGE of fractions of sheep native fundic mucins following separation on a Sepharose 4B column. The gel was stained with SimplyBlue for protein detection. The loading buffer did not contain β-mercaptoethanol.

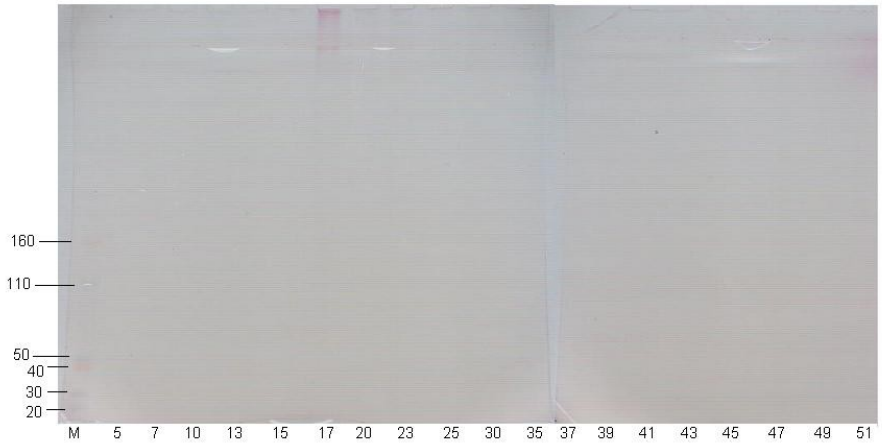

Figure 2.7. Separation by SDS-PAGE of fractions of sheep native fundic mucins following separation on a Sepharose 4B column. The gel was stained with PAS for glycoprotein detection. Fraction 17 stained pink with PAS, indicating that it contained mucin glycoproteins.

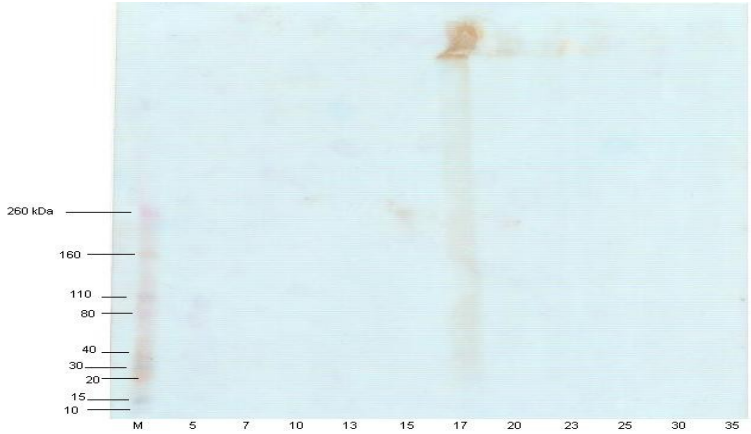

Figure 2.8. Separation by SDS-PAGE of fractions of sheep native fundic mucins following separation on a Sepharose 4B column. The gel was subjected to Western blot followed by lectin probe. After SDS-PAGE, native fundic mucins were transferred to the nitrocellulose membrane and probed with lectin (SBA). Fraction 17 showed positive binding to lectin in Western blot.

The reduced mucin samples were also subjected to Sepharose gel filtration and SDS-PAGE (Figure 2.9 and 2.10). For both abomasal and duodenal mucins, no band was observed in the void volume. The band at 100-110 kDa in fundic mucins spread from fractions 34 to 52 and stained with SimplyBlue but not PAS, suggesting that they were proteins without sugar residues.

The addition of β-mercaptoethanol to the loading buffer to break completely the native mucins into subunits made no difference compared with mucins sample without β-mercaptoethanol treatment (Figure 2.11)

2.3.2.2. CsCl density gradient centrifugation

During separation on size exclusion chromatography, DNA (monitored at A_{260}) eluted together with mucin glycoproteins (Figure 2.12). The second purification step, CsCl density gradient centrifugation, was carried out to remove DNA contamination.

Fractions which showed PAS reactivity and stained with a lectin probe on Western blot had a density of 1.36-1.48 g/ml (Figure 2.13). A mucin peak, located at the above density, was collected, dialysed and concentrated. Protein and nucleic acids distributed in the higher-density fractions (above 1.48 g/ml) were well separated from mucin glycoproteins.

Centrifugation time was varied from 72 to 89 h and no difference was observed in the lipid, glycoprotein, DNA and RNA separation.

2.4. DISCUSSION

2.4.1. Validation of mucin purification method

The purification method of gel filtration followed by CsCl density gradient centrifugation used in this study was shown to be effective in isolating mucin glycoproteins free from contamination with low MW proteins and DNA.

Gel filtration is reported to be the most rapid and convenient method to obtain mucin-enriched fractions from native mucus. The purification of native mucins by this procedure was effective in isolating mucin glycoproteins without contamination (Corfield et al., 1992b; Probert et al., 1995). After gel filtration,

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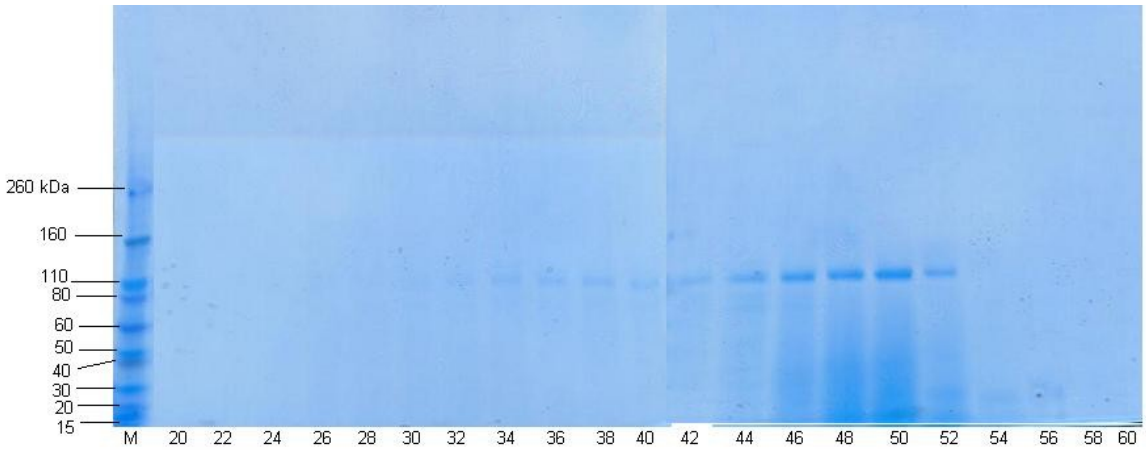

Figure 2.9. Separation by SDS-PAGE of fractions of sheep reduced/alkylated fundic mucin following separation on a Sepharose 4B column. The gel was stained with SimplyBlue for protein detection.

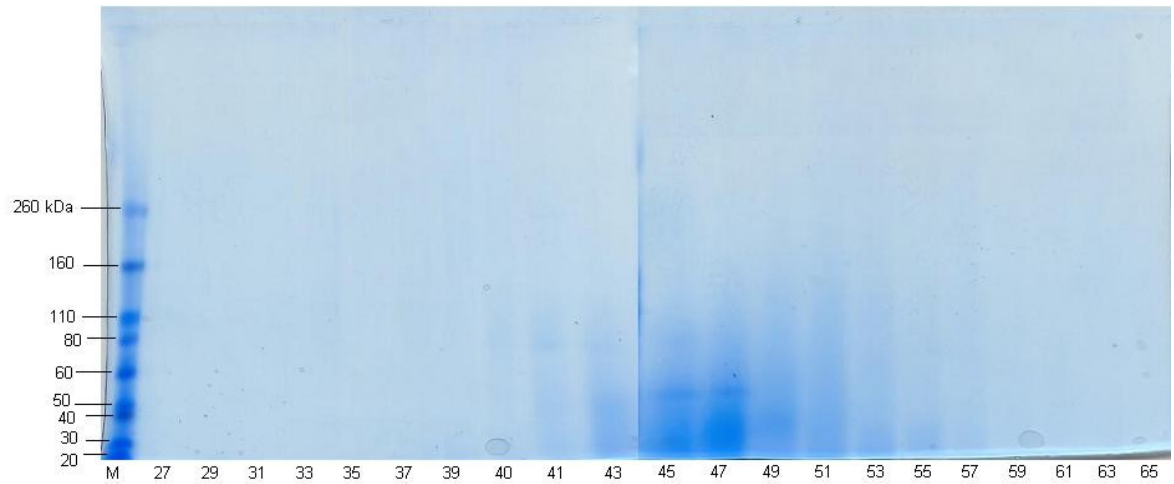

Figure 2.10. Separation by SDS-PAGE of fractions of sheep reduced/alkylated duodenal mucin following separation on a Sepharose 4B column. The gel was stained with SimplyBlue for protein detection.

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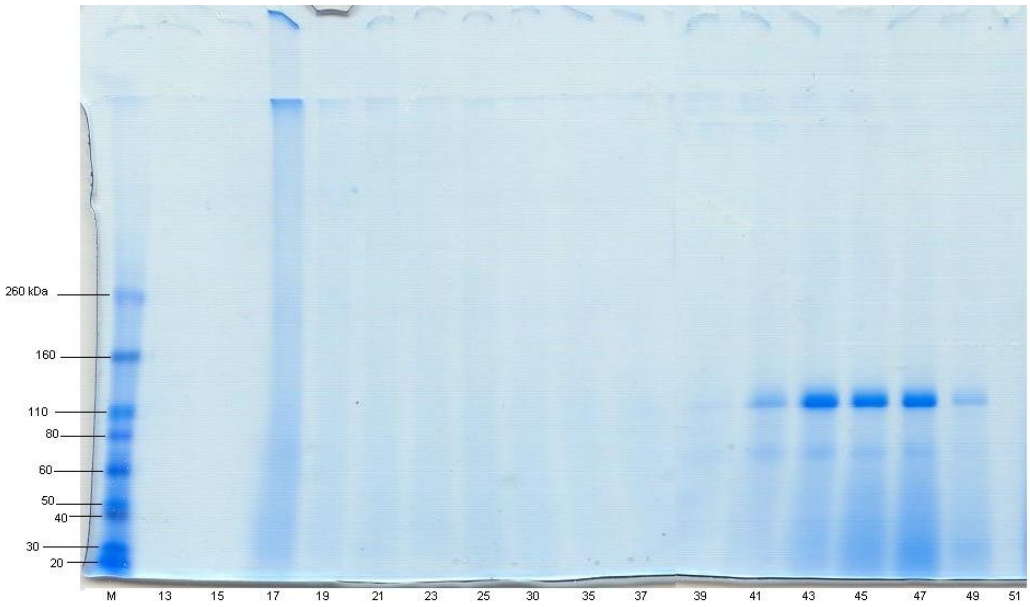

Figure 2.11. Separation by SDS-PAGE of fractions of sheep native fundic mucins following separation on a Sepharose 4B column. The gel was stained with SimplyBlue. Fractions were treated with β-mercaptoethanol and subjected to SDS-PAGE to study the effect of disulphide bond cleavage.

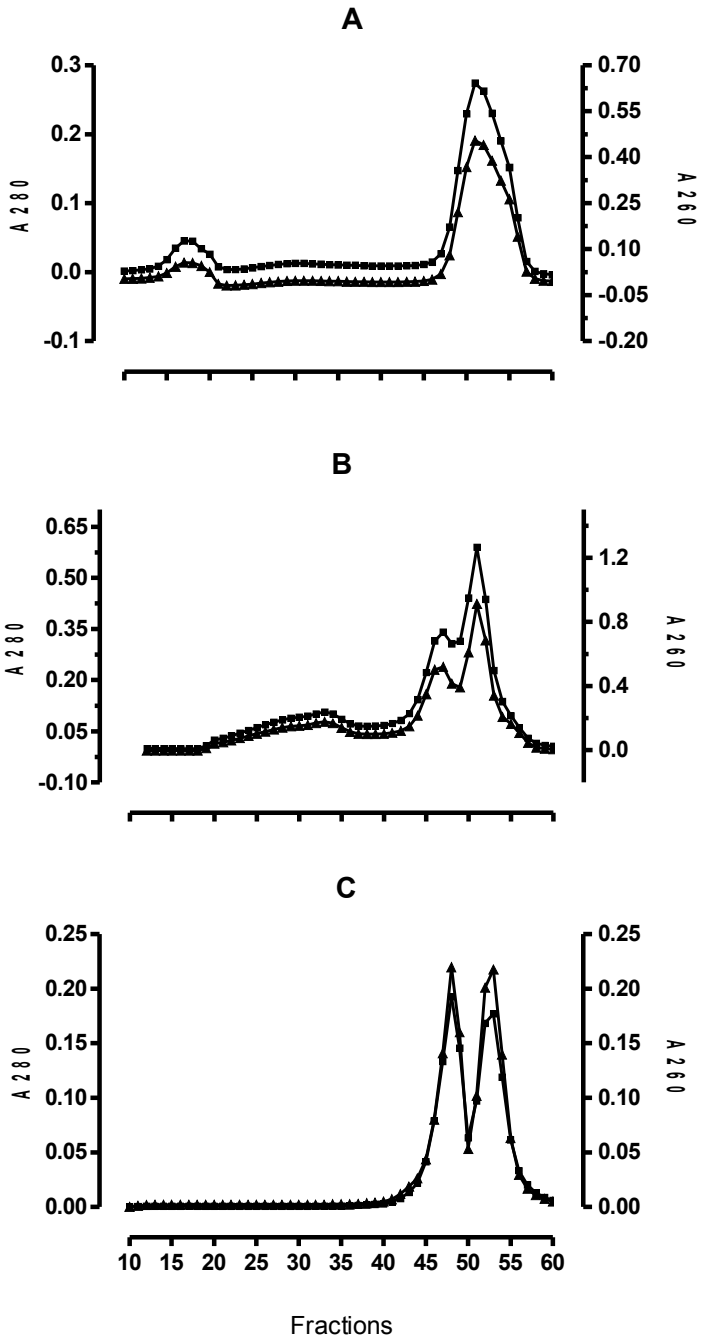

Figure 2.12. Elution profiles of DNA and mucin glycoproteins in fractions collected from a Sepharose 4B gel filtration column. Protein concentration was measured at A_{280} (■) and DNA at A_{260} (▲): (A) native fundic mucus (sample 37); (B) reduced fundic mucus (sample 39); (C) reduced duodenal mucus (sample 40).

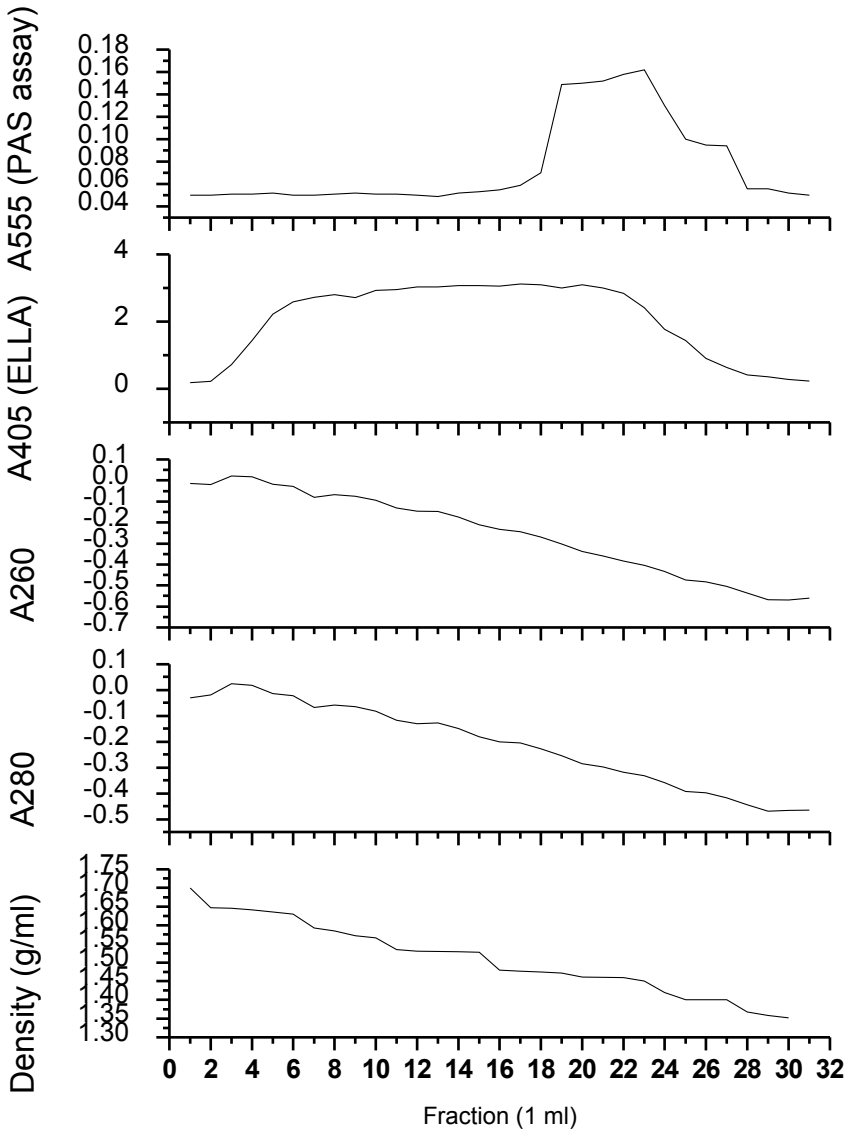

Figure 2.13. Fractionation of mucins which had been purified by density gradient centrifugation in GuHCl 4M/CsCl. The density of each 1 ml fraction was calculated from the weight. ELLA and PAS were used to detect mucin glycoprotein. Protein and nucleic acids were determined by A_{280} and A_{260}, respectively.

contaminating low MW proteins had been removed, as shown by PAS, ELLA and SDS-PAGE. CsCl density gradient centrifugation completely removed DNA and RNA from the partially purified mucins, resulting in purified mucins for further chemical analysis.

For native mucin, gel filtration separated high MW glycoproteins (void volume V_o) from low MW proteins, however, occasionally a small amount of non-mucin substances migrated with the mucin glycoprotein. The high MW fractions eluted from the Sepharose column were pooled and shown to be free of proteins, as demonstrated by SDS-PAGE; the absence of the low MW components in the separating gel verified the purity of the mucins. Mucin polymers appeared at the top of the separating gel when subjected to SDS-PAGE analysis, in which no lower MW glycoproteins were seen. ELLA using different lectin probes resulted in positive binding to the void volume fractions corresponding to the high MW glycoprotein population. Duodenal mucins stained less strongly than fundic mucins, due to the lower concentration of glycoprotein in duodenal than abomasal samples. There were no small peaks appearing between the high MW and low MW populations, which contain smaller mucus glycoproteins or mucin breakdown products.

The glycoprotein separation profile of the sheep gastrointestinal mucin is in good agreement with studies of mucin isolated from the gastrointestinal tract of pig, rat and human (Scawen and Allen, 1977; Pearson et al., 1981; Goso and Hotta, 1994; Mall et al., 1999) and other sources such as the human ovary (Mall et al., 2007). Any of the five lectins WGA, SBA, UEA, ConA and RCA could be used for detecting mucin glycoproteins in sheep mucin samples. Only SNA did not show specific binding to mucin-containing fractions. This can be explained by the very low amount of sialic acid in mucin glycoproteins or it might be due to the fact that the amount diluted in elution buffer was under the detection limit.

The mucin fraction was excluded from the gel but was contaminated with DNA and other non-mucin substances, as described by Allen and Snary (1972). A further purification step was carried out by subjecting the material eluted in the void volume from the Sepharose 4B column to CsCl density gradient centrifugation, which separates components based on their density (Creeth and Denborough, 1970). CsCl density gradient centrifugation separated the mucin into fractions with a density of 1.36-1.48 g/ml. This is consistent with other studies in which

glycoproteins were separated from low-density lipid and from high-density nucleic acids (Creeth and Denborough, 1970; Creeth and Cooper, 1974; Starkey et al., 1974), e.g. human mucins were found between the buoyant density of 1.40 to 1.45 g/ml whereas DNA was at a density of 1.62 g/ml (Feller et al., 1990), pig gastric mucins appeared at a density of 1.4-1.5 g/ml close to the bottom of the tubes, well-separated from material at the top of the gradient (Nordman et al., 1997).

2.4.2. Molecular weight and bonding of sheep mucins

During purification, the MW and linkage properties of these glycoproteins were investigated. Native mucin in sheep eluted exclusively in the void volume of the column, indicating that the MW of mucin is very large, probably similar to that of porcine stomach mucin (greater than $1x10^6$ Da). Alternative methods, such as light scattering, viscosity measurement, creep compliance, spinability, magnetic rheogoniometry, nuclear magnetic resonance or cone and plate mechanical spectroscopy could determine the properties and MW more accurately. Using different methods to determine the MW of mucin glycoproteins has given variable results. MW of submaxillary mucin from cattle were reported to be $4x10^6$, sheep 10^6 and pig $0.8x10^6$ as determined by sedimentation and light scattering (Pigman and Gottschalk, 1966) whereas Pigman and Tettamanti (1968) using chemical measurements reported the MW of bovine submaxillary mucin to be $0.375x10^6$ and ovine submaxillary mucin $0.394x10^6$. In addition, the MW of native mucin is usually difficult to determine due to a heterogeneity in chain length or the numbers of subunits making up the molecule.

Both the abomasal and duodenal native mucins, after being reduced and alkylated, had subunits with lower MW, which eluted in the included volume of the gel filtration column. There is considerable variation in the reported MW range of subunits of gastrointestinal mucins, even from the same species. In human gastric, pig gastric, pig small intestinal and pig colonic mucin, the glycoproteins released subunits with molecular weights ranging from 250,000 to 720,000 Da (Pearson et al., 1980; Mantle and Allen, 1981).

The use of gel electrophoresis to determine the MW of mucin glycoproteins has also been reported to be ineffective because their charge to mass ratio may vary over a considerable range. The MW estimated by gel electrophoresis did not

agree with the weight determined by light scattering and sedimentation studies. Concentrated glycoproteins contain intertwined high MW molecules making separation on porous gels impossible. Therefore, gel electrophoresis only can be used for identification but not for assessment of actual mass (Tytgat et al., 1995; Van Klinken et al., 1998).

The mucin subunits appeared to be linked via non-covalent bonds such as hydrophobic or ionic interactions rather than by disulfide bonds in sheep fundic mucins. Native mucins treated with urea and SDS produced subunits with much lower MW than native molecule. The large MW native mucin in the void volume was no longer present and was reduced into subunits which eluted in the included volume. Using a high concentration of urea (6 M), native mucin molecules were reduced completely, releasing the subunits. In contrast, neither DTT nor β-mercaptoethanol had any effect on sheep gastrointestinal mucins (Figure 2.11). This is similar to the results for ovine and bovine submaxillary mucins whereas porcine and canine submaxillary mucins, canine tracheal mucins and bovine cervical mucins were released by β-mercaptoethanol (Holden et al., 1971b).

The purification process, validated for mucin samples, was subsequently used to prepare mucins for further investigation of the changes in carbohydrate composition with age and parasitism by *H. contortus* and *T. circumcincta*.

Chapter 3

MONOSACCHARIDE COMPOSITIONS OF FUNDIC AND DUODENAL MUCINS IN 4-9 MONTHS-OLD SHEEP

3.1. INTRODUCTION

Mucin plays an important role in maintaining normal gastric functions and protecting the host from the attack by pathogens. The protective ability of gastrointestinal mucins depends largely on their oligosaccharide chains. Alterations in mucin carbohydrate composition can result in the failure of this protective barrier (Mall et al., 1997). Many pathogens need a specific cell surface carbohydrate structure for attachment to occur (Hakansson et al., 1996), which are often the terminal oligosaccharides of mucins. The host provides receptors, commonly carbohydrate residues of glycoconjugates (glycoproteins or glycolipids). Those carbohydrates are species-specific and tissue-specific (Freitas et al., 2002). This specificity plays a vital role, as the availability of suitable receptors may be determined by the age of the host and which site is infected. Therefore, the oligosaccharides on the host cell surface may be a key genetic susceptibility factor in infection.

Mucins, which are high-molecular-weight glycoproteins consisting mainly of O-glycans, are suggested to greatly contribute to determining the susceptibility or resistance of the host to pathogens such as *H. pylori* (Wadstrom et al., 1996; Ota et al., 1998). The array of oligosaccharides expressed on the mucins of an individual may play a key role in governing the susceptibility to infection (Thornton and Sheehan, 2004). Mucins display tissue-specific patterns of O-glycosylation (Corfield et al., 2000). Alterations in the glycosylation of mucins commonly occur in many mucosal diseases. Alteration of terminal sugars of goblet cell mucins has

been proposed to be associated with the expulsion of nematodes (Ishikawa et al., 1994).

Carbohydrates, which account for 70-90% of the total mass of mucins, consist of neutral, amino and acidic sugars. Several studies on chemical analysis of mucin glycosylation showed that there were modifications in monosaccharide composition during infections, such as in the rat (Oinuma et al., 1995; Karlsson et al., 2000) and mouse infected with *N. brasiliensis* (Holmen et al., 2002) and humans infected with *H. pylori* (Ota et al., 1998; Kocer et al., 2004; Kang et al., 2008; Kobayashi et al., 2009). It is not known whether changes in mucin composition are caused by the host inflammatory response or by the pathogens, many of which are capable of degrading mucins. Porcine gastric mucin can be degraded by glycosidase enzymes released by some strains of *P. aeruginosa* (Aristoteli and Willcox, 2003).

The small number of studies in rodents suggests nematodes are capable of modifying the glycosylation of host mucins, however, it is not known whether this also occurs during parasitism in ruminants. For this study, abomasal and duodenal mucins were collected from sheep aged between 4 and 9 months, when immunity to nematodes is developing (Manton et al., 1962; Urquhart et al., 1966; Dineen et al., 1978). Lambs are still susceptible to nematodes from 3-6 months-of-age, although immunity is developing, but at different rates in individual animals (Stear et al., 1996). By 10 months-of-age, sheep have developed their acquired immunity (Smith et al., 1985). The sheep were assigned to three age groups: 4-4.5 months-, 6 months- and 8-9 months-of-age. Sheep were mainly Romney or Romney cross, but other breeds were included, rather than studying sheep from selected lines. Mucus samples were collected on day 21 and 28 p.i. from sheep infected with *H. contortus* or *T. circumcincta* respectively, when adult parasites were present. Purified mucins, obtained as described in Chapter 2, Sections 2.2.1 to 2.2.3, were hydrolysed in highly acidic conditions, which convert GalNac and GlcNac into GalN and GlcN respectively.

Although there are several ways of hydrolyzing mucin glycoproteins (Takeuchi et al., 1987; Fu and O'Neill, 1995; Soga and Heiger, 1998), the method of Soga and Heiger (1998) has been shown to be effective in releasing total neutral and amino sugars. Neutral and amino sugars in hydrolysed mucins of sheep can

be separated and quantified using high performance anion exchange chromatography (HPAEC). The HPAEC is able to separate neutral and amino sugars under the same chromatographic conditions, with a low detection limit, high sensitivity, reproducibility and good correlation between the concentration of sample and pulse amperometry detection (PAD) response. Acidic sugars (sialic acids) need to be assayed using different conditions or by the thiobarbiturate method. The later method was used in the present study.

Sulphation also plays an important role in the host and parasite interaction. A mucin sample sent to the Campbell Microanalytical Laboratory (University of Otago, Dunedin, New Zealand) to be analysed for C, H, N, S proportions showed that they accounted for 43.52%, 6.73%, 12.36% and 0.68% respectively. As the sulfur content of the mucin samples was below 1%, the absolute amount could not be determined. Therefore, sulphation was studied histochemically (Chapter 5) and the chemical analysis was restricted to studying the neutral, amino and carboxylated sugar composition (sialic acids).

3.2. MATERIALS AND METHODS

3.2.1. Experimental design

A total of 27 male sheep of various breeds, mainly Romney or Romney cross, were included in the study. Nine sheep were assigned to each group: control (non-infected), *H. contortus*-infected or *T. circumcincta*-infected. Within each of three infection groups, there were three sheep of age 4-4.5 months-old, three of 6 months-old and three of 8-9 months-old. After infection, sheep were euthanased when adult worms were present: day 21 for *H. contortus* infection and day 28 for *T. circumcincta* infection.

3.2.2. Sheep

Sheep at 2-3 months-of-age were obtained from pasture, where they had been exposed to field infection. When brought indoors, they were drenched with a double dose (1ml/5kg) of Matrix (Ancare, New Zealand) followed by a single dose (1ml/10kg) of Matrix 2 days later. Faecal egg counts (FEC) were carried out a week later and a few days before infection to confirm they were nematode-free (Appendix

1.3). After being drenched, sheep were housed indoors for at least 4 weeks before infection. They were fed with lucerne chaff and water ad libitum.

3.2.3. Infection

Pure cultures of *H. contortus* and *T. circumcincta* were maintained (Appendix 1.5) for infection of sheep (Appendix 1.4). Sheep were infected orally with either 10,000 *H. contortus* L_3 and euthanased 21 days p.i. or 50,000 *T. circumcincta* L_3 and euthanased 28 days p.i.

Nine sheep were left uninfected as control animals. Non-parasitised sheep were euthanased at least 4 weeks after being drenched; FEC showed no eggs were present to confirm the absence of nematodes. Three non-infected sheep were assigned to each age group and euthanased during the designated age period.

3.2.4. Sample collection after euthanasia

Sheep, fasted overnight but with free access to water, were euthanased by captive bolt and exsanguination. The abomasum and duodenum were removed for sample collection. The abomasum was opened along the greater curvature and gently washed four times with saline. Tissues for histology were removed from one fundic fold, the antrum, duodenal bulb and duodenum 20 cm from the pyloric sphincter (reported in Chapter 5, Section 5.2.1). Mucus was scraped from the mucosa as described in Chapter 2, Section 2.2.1. Mucus was immediately frozen in liquid nitrogen and stored at -70^{o}C.

The 8-9 months-old sheep had few worms in the fundus and more in the antrum, but these were small, immature and not well-developed.

3.2.5. Mucus purification

Mucus was thawed, extracted and purified on a Sepharose 4B gel filtration column and by CsCl density gradient centrifugation, as described in Chapter 2 (Sections 2.2.2 and 2.2.3). Purified native mucins, with added protease inhibitors, were used for neutral and amino sugar determination by HPAEC and carboxylated sugar determination by the thiobarbiturate method.

3.2.6. Total hydrolysis of purified mucins

The hydrolysis procedure of Soga and Heiger (1998) was used. The purified mucin was dissolved in 2 ml of 2 M HCl in a hydrolysis tube fitted with a screw cap and heated at 95°C for 6 h in a heating block to release all monosaccharides.

After cooling down to room temperature, the mixture was transferred to a Vivaspin concentrator MWCO 5,000 Dalton-PES membrane (Vivascience, Sartorius). 2 ml MQ-water was added and centrifuged at 3000 rpm (Centrifuge 5810R, Eppendorf) for 1 h at 20°C to remove the protein core. The retentate was washed with 1 ml MQ-water, centrifuged again, and the supernatant was collected and stored at 4°C.

The supernatant was evaporated with a rotary-evaporator (Rotavapor, Buchi, Switzerland) and dissolved in 500 µl MQ-water.

3.2.7. Thin layer chromatography (TLC)

Thin-layer chromatography was carried out on 2 fundic mucins (No. 7 and 19, Appendix 5.3) and 2 duodenal mucins (No 8 and 20) to identify the monosaccharides present in hydrolysed fundic and duodenal mucins and determine their purity. 5 µl aliquots were placed on 10 cm TLC plates (Silica gel 60 F_{254}, Merck, Germany) at 1 cm intervals. The solvent used for development was isopropanol: water: ammonia (7: 2: 1). The plate was developed in about 2 h.

Solutions of 10 mM each of Fuc, GalN, GlcN, Gal, GalNAc, GlcNAc, NANA and NGNA were run on the same plate as references.

The monosaccharides were visualised with a mixture of anisaldehyde (9.5 ml), acetic acid (3.75 ml), 95% ethanol (338 ml) and concentrated sulphuric acid (12.5 ml). After the plate had been developed, it was dried with a heat gun and dipped into visualisation reagent and dried again. Monosaccharides were seen as spots of different colours and locations on the plate.

3.2.8. Quantification of neutral and amino sugars

3.2.8.1. High Performance Anion Exchange Chromatography (HPAEC)

The HPAEC system used for monosaccharide quantification consisted of a DX-300 gradient chromatography system, Dionex Advanced Gradient Pump Module and a Dionex ED40 electro-chemical detector with an amperometric cell (Sunnyvale, CA, USA) and an Advanced Computer Interface. The Dionex Eluent Degas Module (using Helium) was employed to sparge and pressurise the eluents with helium. The eluents were prepared by diluting 50% NaOH solution in MQ-water. Samples were injected with a 100 µl Hamilton syringe (Hamilton, Nevada, USA) via a valve equipped with 100 µl loop. The system was monitored with a PeakNet™ Chromatography Workstation (Dionex, Sunnyvale, CA).

The detector was operated in the pulse amperometric detection (PAD) mode and was equipped with a gold working electrode and a Ag/AgCl reference electrode.

3.2.8.2. Solutions

NaOH 50% solution (Sigma), sodium acetate (Sigma) and MQ-water were used to make up the eluents:

Eluent 1: MQ-water

Eluent 2: 25 mM NaOH and 0.25 mM sodium acetate

Eluent 3: 200 mM NaOH and 300 mM sodium acetate

Eluent 4: 125 mM NaOH and 10 mM sodium acetate

All the eluents were filtered through 0.22 µm filter membranes and degassed before use.

3.2.8.3. Protocol

Monosaccharides were separated by the method of Michalski and Capon (2000). The column used was a CarboPac™ PA-20 (3 mmx150 mm) coupled to an

Amino trap column (4 mmx50 mm). The following pulse potentials and durations were used for detection:

t (s)	E (V)	Integration period:	
0.00	0.10	Begin (s)	End (s)
0.50	0.10	0.20	0.40
0.51	0.60		
0.61	0.60		
0.62	-0.60		
0.72	-0.60		
0.73	0.10		

The flow rate was 0.4 ml/min. The high back pressure limit was maintained below 3000 psi. The following elution gradient was used for the separation of neutral and amino monosaccharides

Time (min)	Eluent 1 (%)	Eluent 2 (%)	Eluent 3 (%)	Eluent 4 (%)
0	92	8	0	0
20	92	8	0	0
50	92	8	50	0
60	42	8	50	0
63	0	0	0	100
70	0	0	0	100
80	92	8	0	0
95	92	8	0	0

The column was regenerated after each run by washing with 100% Eluent 4 for 7 min (minutes 63-70) and then equilibrated again with 8% (v/v) of Eluent 2 for 15 min (minutes 80-95) before the next sample was injected.

Both neutral and amino sugars were separated as sharp and symmetrical peaks in about 20 min. No interfering peaks were observed in the area in which the four monosaccharides Fuc, GalN, GlcN and Gal eluted (Appendix 5.1).

The resulting chromatographic data were integrated and plotted using Origin software (Origin 7.5 SR2 v7.5817 (B817) OriginLab Corp., Northampton MA 01060 USA).

Standard curves were constructed for the monosaccharides identified by TLC. Mixtures of four sugars (Fuc, GalN, GlcN and Gal) were made up at a concentration of 25 µg/ml each, then diluted to make a concentration range from 0.5 to 25 µg/ml.

There were two standard curves for GalN and Gal: one which was used in the range of 2-25 µg/ml monosaccharides and the other, in the range of 0-2 µg/ml, was used when the concentration was calculated to be negative, not detected or below the detection limit of the first standard curve (Appendix 5.2).

3.2.8.4. Data analysis

To determine the absolute amount of each monosaccharide in the sample, each peak was plotted with Origin software and fitted with a standard Gaussian curve (Appendix 5.4). Baselines were subtracted and adjusted until the distribution curve fitted satisfactorily to the chromatogram. Peak area and peak height were calculated.

Based on the peak area, the amount of each monosaccharide was calculated from the standard curves constructed from standard monosaccharides injected into the same column to obtain corresponding elution and peak area and peak height values. The total neutral and amino sugars were calculated as the sum of the four components and the percentage of each was calculated by dividing the amount of each by the amount of total monosaccharides. Data were expressed as percentage of each sugar (Mean ± SD).

3.2.9. Quantification of sialic acids

Sialic acids were determined by the method of Matsuno and Suzuki (2008), which is a modification of the thiobarbiturate method of Warren (1959).

Freeze-dried purified mucins were weighed, hydrolyzed in 2 ml 50 mM sulphuric acid (H_2SO_4) at 80°C for 60 min to release sialic acids, then neutralised with 45 ul 1 M sodium hydroxide. The solutions were incubated with 250 ul periodic acid solution (25 mM in 62.5 mM sulphuric acid) at 37°C for 30 min. The reaction was terminated by the addition of 200 ul sodium arsenite (2% in 0.5 M HCl) and left for 3 min. 2.0 ml thiobarbituric acid (0.1 M, pH 9.0) was added to the solution and the resultant solutions were heated in a boiling water bath for 7.5 min. The solutions were then cooled in ice water and mixed with 5 ml n-butanol/concentrated HCl solution (95:5, v/v).

The solutions were shaken vigorously and the absorbance of the butanol layer was measured at 550 nm. The concentration of sialic acids was read from a standard curve constructed with NANA from 1-500 µg/ml dissolved in MQ-water (Appendix 5.5). The weight of sialic acids was divided by the weight of freeze-dried mucin used for hydrolysis in H_2SO_4 to obtain the percentage by weight of glycoprotein.

3.2.10. Statistical analysis

The effect of age was determined by fitting linear or higher order regression to a graph of percentage of monosaccharides vs age.

The effect of parasitism was determined by comparing the monosaccharide profiles of gastrointestinal mucins from *H. contortus*- and *T. circumcincta*-infected sheep with those of the control group (non-infected animals). Effects of age, infection and their interaction were determined by two-way ANOVA. Bonferroni post-hoc tests were carried out to determine the differences between age groups. $P<0.05$ was considered significant. Data from individual sheep were used, except when there was a missing value, mean and standard deviations were compared.

Data were analysed using Prism software (version 4.0, GraphPad software, Inc.).

3.3. RESULTS

3.3.1. Monosaccharide components of fundic and duodenal mucins

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Table 3.1. Percentage of fucose (Mean ± SD, n=3) in fundic mucin of sheep aged 4-9 months old, infected with *H. contortus* or *T. circumcincta* and euthanased on day 21 or 28 p.i. respectively. The results of two-way ANOVA are shown. Means with the same superscript are significantly different (Bonferroni post-test)

Age (months)	Percentage of fucose (Mean ± SD)		
	Non-infected	*H. contortus* infected	*T. circumcincta* infected
4-4.5	12.6 ± 1.2	6.3 ± 4.2	8.3 ± 1.9
6	16.3 ± 10.3	10.1 ± 6.4	5.2 ± 3.4
8-9	22.7 ± 7.3 [a]	5.0 ± 3.3 [a]	23.4 ± 0.5*
	Analyses of variance		
Age		P= 0.561	P= 0.005
Infection		P= 0.0005	P= 0.106
Interaction		P= 0.353	P= 0.26

a= p< 0.01; * n=2

Four mucin samples (No. 7,8,19 and 20) that were developed on TLC plates showed the presence of Fuc, GlcN, GalN, Gal and sialic acids (Figure 3.1).

3.3.2. Quantification of monosaccharide composition

3.3.2.1. Fundic mucins

3.3.2.1.1. Fucose

The percentages of Fuc in sheep fundic mucins at different ages and with infection are reported in Figure 3.2A and Table 3.1.

The percentages of Fuc were plotted against age for all non-infected sheep. A linear relationship had the greatest significance (P=0.105). This was considered to be approaching a significant effect of age. The standard deviation of the mean differed for the 3 age groups, being small for the 4-4.5 months-old group and much higher for the other groups.

In *H. contortus* infection, there was no age effect to the percentages of Fuc in sheep fundic mucins whereas *T. circumcincta* infection showed significant increase in the fucosylation with age (P=0.005). Inspection of the group means showed a trend of increasing fucosylation in non-infected sheep from 12.6-22.7% and in *T. circumcincta*-infected animals from 8.3% and 5.2% to 23.4%.

Two-way ANOVA showed that *T. circumcinta* infection did not significantly affect the percentage of Fuc (P=0.106), however, *H. contortus* infection significantly decreased fucosylation (P=0.0005). The decrease was significant at 8-9 months old (P<0.01), while group means were also lower at the other two age ranges.

3.3.2.1.2. Galactosamine

The percentages of GalN in sheep fundic mucins at different ages and with infection are reported in Figure 3.2B and Table 3.2.

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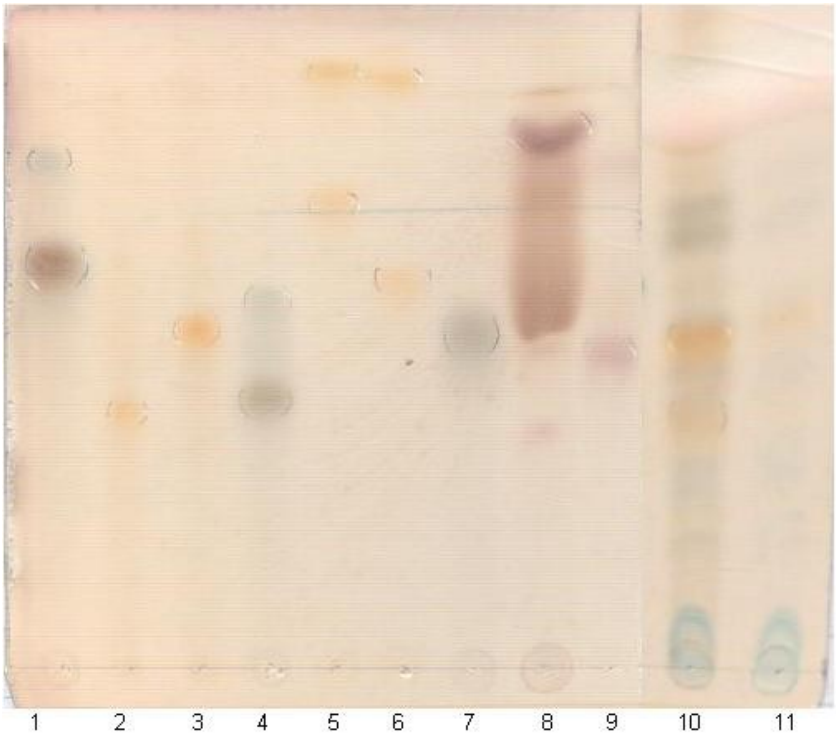

Figure 3.1. Thin layer chromatography of hydrolysed fundic and duodenal mucins showing the presence of Fuc, GalN, GlcN, Gal and sialic acids. The plate was developed with isopropanol:water:ammonia (7:2:1) and visualised with mixture of anisaldehyde, acetic acid, ethanol and sulphuric acid.

Lanes are 1- Fuc; 2- GalN; 3- GlcN; 4- Gal; 5- GlcNAc; 6- GalNAc; 7- Man; 8- NANA; 9- NGNA; 10- Fundic mucin (No. 7); 11- Duodenal mucin (No. 8).

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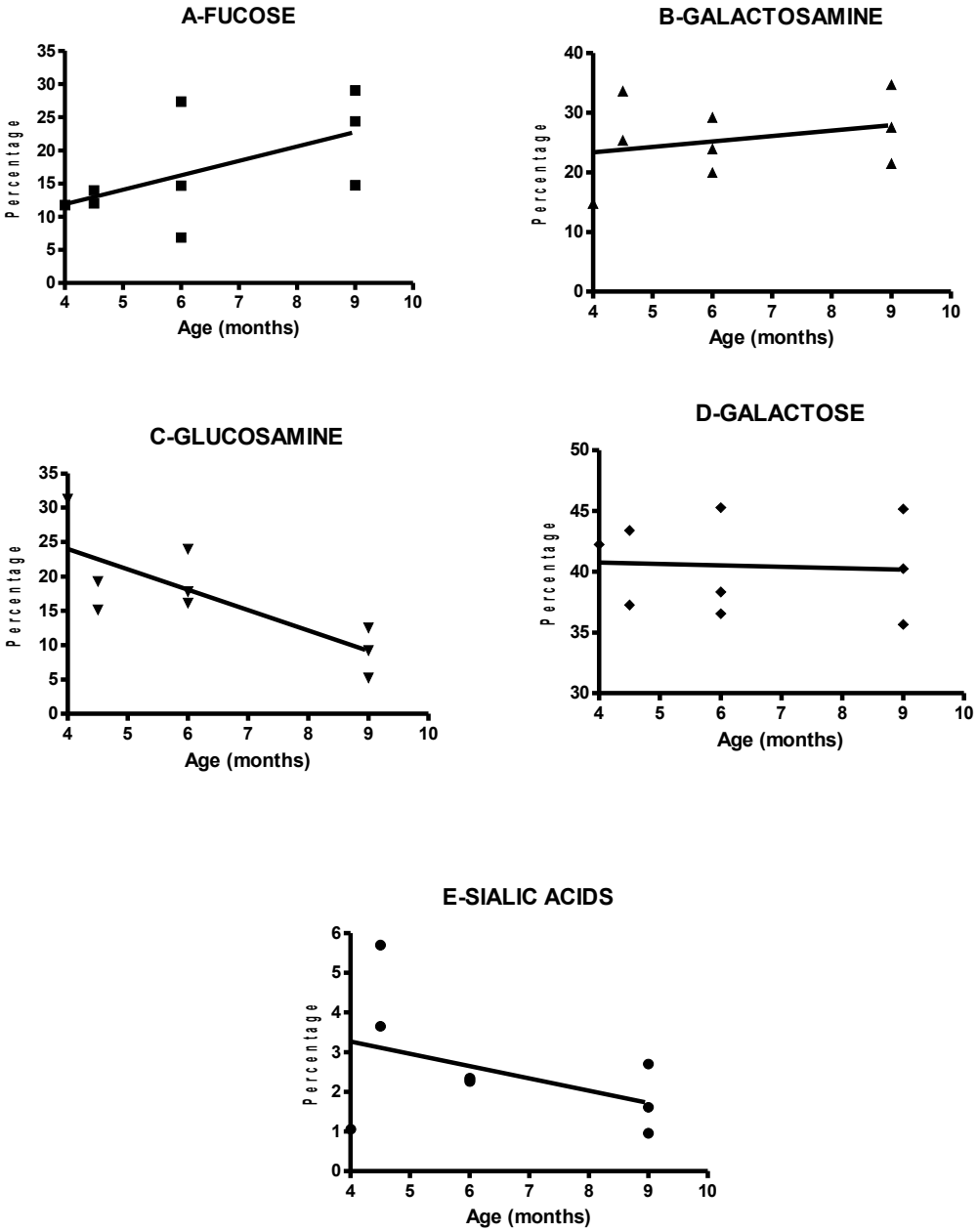

Figure 3.2. The relationship between the age of the sheep and the percentage of each monosaccharide present in fundic mucins in individual non-infected sheep: (A) fucose, P=0.105; (B) galactosamine, P=0.45; (C) glucosamine, P=0.012; (D) galactose. P=0.866 and (E) sialic acids, P=0.243.

There was no significant effect of age in non-infected sheep (P=0.45). Two-way ANOVA for the *H. contortus*-infected sheep showed no age effect (P=0.423), but did for the *T. circumcincta*-infected sheep (P=0.043). However, there was a significant age-infection interaction (P=0.039) and there was a significant decrease in *T. circumcincta*-infected lambs compared with controls at 4-4.5 months-of-age (P<0.05), which probably caused the age effect.

H. contortus infection had no significant effect on the percentage of GalN.

T. circumcincta infection decreased the proportion of GalN in the 4-4.5 months-old group (P<0.05) and overall was approaching significance (P=0.072). There was a significant age-infection interaction (P=0.039).

3.3.2.1.3. Glucosamine

The percentages of GlcN in sheep fundic mucins at different ages and with infection are reported in Figure 3.2C and Table 3.3.

There was a significant decrease with age (P=0.012) in non-infected sheep. Two-way ANOVA also showed a significant decrease in the percentage of GlcN in *H. contortus* infection (P=0.053) and *T. circumcincta* infection (P=0.049).

H. contortus infection increased the percentage of GlcN (P=0.029). The age-infection interaction was approaching significance (P=0.113), the mean percentage showing that there appeared to be no effect at 6 months old, unlike the other ages.

T. circumcincta infection showed no effect of infection (P=0.391).

3.3.2.1.4. Galactose

The percentages of Gal in sheep fundic mucins at different ages and with infection are reported in Figure 3.2D and Table 3.4.

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Table 3.2. Percentage of galactosamine (Mean ± SD, n=3) in fundic mucin of sheep aged 4-9 months old, infected with *H. contortus* or *T. circumcincta* and euthanased on day 21 or 28 p.i. respectively. The results of two-way ANOVA are shown. Means with the same superscript are significantly different (Bonferroni post-test)

Age (months)	Percentage of galactosamine (Mean ± SD)		
	Non-infected	*H. contortus* infected	*T. circumcincta* infected
4-4.5	24.6 ± 9.5 [a]	18.8 ± 2.7	9.4 ± 3.0 [a]
6	24.3 ± 4.6	30.0 ± 5.4	30.0 ± 5.6
8-9	27.9 ± 6.6	19.7 ± 6.2	20.0 ± 0.1*
	Analyses of variance		
Age		P= 0.423	P= 0.043
Infection		P= 0.258	P= 0.072
Interaction		P= 0.238	P= 0.039

a= p< 0.05; * n=2

Table 3.3. Percentage of glucosamine (Mean ± SD, n=3) in fundic mucin of sheep aged 4-9 months old, infected with *H. contortus* or *T. circumcincta* and euthanased on day 21 or 28 p.i. respectively. The results of two-way ANOVA are shown. Means with the same superscript are significantly different (Bonferroni post-test)

Age (months)	Percentage of glucosamine (Mean ± SD)		
	Non-infected	*H. contortus* infected	*T. circumcincta* infected
4-4.5	21.9 ± 8.4	34.7 ± 9.0	19.4 ± 7.5
6	19.3 ± 4.2	16.7 ± 5.0	10.4 ± 4.7
8-9	9.00 ± 3.7	25.4 ± 8.3	12.9 ± 1.3*
	Analyses of variance		
Age		P= 0.053	P= 0.049
Infection		P= 0.029	P= 0.391
Interaction		P= 0.113	P= 0.224

* n=2

There was no age effect in non-infected sheep (P=0.866) or *H. contortus* infected sheep (P=0.327). Two-way ANOVA showed that *T. circumcincta* infected sheep showed a difference with age (P=0.016). The percentage of Gal increased more in younger animals.

H. contortus infection did not affect the percentage of Gal (P=0.267).

T. circumcincta infection increased Gal in both the 4-4.5 months-old group (P<0.001) and 6 months-old group (P<0.05). Two-way ANOVA showed that *T. circumcincta* infection significantly increased the percentage of Gal (P=0.0001) and the age-infection interaction was also significant (P=0.023).

3.3.2.1.5. Sialic acids

The percentages of sialiac acids/glycoproteins in sheep fundic mucins at different ages and with infection are reported in Figure 3.2E and Table 3.5.

The relationship between age and percentage of sialic acids was best fitted by a linear regression. This was not significant (P=0.243), however group means of non-infected sheep decreased progressively from 3.4-1.7%. One value for a 4 months-old lamb was 1.06%, considerably lower than for any other animals of this age. If this data point were omitted, the line of best fit now was significant (P=0.042). There was no age effect on the percentage of sialic acids in fundic mucins with either *H. contortus* (P=0.485) or *T. circumcincta* infection (P=0.6).

H. contortus and *T. circumcincta* infection significantly decreased the percentage of sialic acids (P=0.004 and 0.008 respectively).

3.3.2.2. Duodenal mucins

3.3.2.2.1. Fucose

Table 3.4. Percentage of galactose (Mean ± SD, n=3) in fundic mucin of sheep aged 4-9 months old, infected with *H. contortus* or *T. circumcincta* and euthanased on day 21 or 28 p.i. respectively. The results of two-way ANOVA are shown. Means with the same superscript are significantly different (Bonferroni post-test)

Age (months)	Percentage of galactose (Mean ± SD)		
	Non-infected	*H. contortus* infected	*T. circumcincta* infected
4-4.5	41.0 ± 3.3 [a]	40.2 ± 4.7	62.9 ± 7.8 [a]
6	40.1 ± 4.6 [b]	43.2 ± 7.4	54.4 ± 2.6 [b]
8-9	40.4 ± 4.8	49.9 ± 7.6	43.7 ± 1.0*
	Analyses of variance		
Age		P= 0.327	P= 0.016
Infection		P= 0.267	P= 0.0001
Interaction		P= 0.272	P= 0.023

a= p< 0.001; b= p< 0.05; * n=2

Table 3.5. Percentage of sialic acid/glycoprotein (Mean ± SD, n=3) in fundic mucin of sheep aged 4-9 months old, infected with *H. contortus* or *T. circumcincta* and euthanased on day 21 or 28 p.i. respectively. The results of two-way ANOVA are shown. Means with the same superscript are significantly different (Bonferroni post-test)

Age (months)	Fundic mucin (Mean ± SD)		
	Non-infected	*H. contortus* infected	*T. circumcincta* infected
4-4.5	3.47 ± 2.33 [a]	0.51 ± 0.13 [a]	0.33 ± 0.05*
6	2.31 ± 0.05	0.54 ± 0.28	0.28 ± 0.16*
8-9	1.76 ± 0.88	0.63 ± 0.02*	0.57 ± 0.31*
	Analyses of variance		
Age		P= 0.485	P= 0.600
Infection		P= 0.004	P= 0.008
Interaction		P= 0.399	P= 0.469

a= p< 0.05, *n=2

Table 3.6. Percentage of fucose (Mean ± SD, n=3) in duodenal mucin of sheep aged 4-9 months old, infected with *H. contortus* or *T. circumcincta* and euthanased on day 21 or 28 p.i. respectively. The results of two-way ANOVA are shown. Means with the same superscript are significantly different (Bonferroni post-test)

Age (months)	Percentage of fucose (Mean ± SD)		
	Non-infected	*H. contortus* infected	*T. circumcincta* infected
4-4.5	11.7 ± 3.7*	14.1 ± 0.9	12.4 ± 3.1*
6	26.9 ± 6.3 [a] *	11.9 ± 4.4 [a]	21.0 ± 8.3
8-9	30.7 ± 7.8 [b, c]	4.9 ± 4.8 [b]	6.8 ± 1.4 [c] *
Analyses of variance			
Age		P= 0.16	P= 0.064
Infection		P= 0.0006	P=0.023
Interaction		P= 0.004	P=0.049

a, c= p< 0.05; b= p< 0.001; * n=2

Table 3.7. Percentage of galactosamine (Mean ± SD, n=3) in duodenal mucin of sheep aged 4-9 months old, infected with *H. contortus* or *T. circumcincta* and euthanased on day 21 or 28 p.i. respectively. The results of two-way ANOVA are shown. Means with the same superscript are significantly different (Bonferroni post-test)

Age (months)	Percentage of galactosamine (Mean ± SD)		
	Non-infected	*H. contortus* infected	*T. circumcincta* infected
4-4.5	43.4 ± 9.5*	26.9 ± 2.3	23.5 ± 8.9*
6	31.2 ± 18.2 [a] *	20.4 ± 3.3	6.9 ± 0.7 [a]
8-9	21.6 ± 5.0	5.7 ± 3.0	27.6 ± 4.1*
Analyses of variance			
Age		P= 0.003	P= 0.088
Infection		P= 0.003	P= 0.024
Interaction		P= 0.791	P= 0.055

a= p< 0.05; * n=2

The percentages of Fuc in sheep duodenal mucins at different ages and with infection are reported in Figure 3.3A and Table 3.6.

The increase in the percentage of Fuc in non-infected sheep duodenal mucins with age was approaching the significant level of 5% (P=0.053). Two-way ANOVA showed that neither *H. contortus* infection (P=0.16) nor *T. circumcincta* infection (P=0.064) changed the proportion of Fuc significantly, but were also approaching the significant level.

H. contortus infection significantly decreased the percentage of Fuc (P=0.0006). The effect of infection was significant in the 6 months-old group (P<0.05) and 8-9 months-old group (P<0.001). The age-infection interaction was also significant (P=0.004).

T. circumcincta infection also had a significant effect on the percentage of Fuc (P=0.023). The proportion of Fuc decreased significantly in the 8-9 months-old group (P<0.001) compared with non-infected sheep at the same age. The age-infection interaction was also statistically significant (P=0.049).

3.3.2.2.2. Galactosamine

The percentages of GalN in sheep duodenal mucins at different ages and with infection are reported in Figure 3.3B and Table 3.7.

In non-infected sheep, the decrease in the proportion of GalN with age approached the significant level of 5% (P=0.056). In *H. contortus* infection, there was a significant decrease in the percentage of GalN with age (P=0.003). However, in *T. circumcincta* infection, age did not change the percentage of GalN significantly (P=0.088), the significant decrease was found only in 6 months-old group (P<0.05).

H. contortus infection significantly decreased the percentage of GalN (P=0.003). The group means were lower than in non-infected groups at all ages.

T. circumcincta infection also significantly decreased the proportion of GalN (P=0.024). A significant decrease was found in the 6 months-old group (P<0.05). The age-infection interaction was approaching significance (P=0.055).

3.3.2.2.3. Glucosamine

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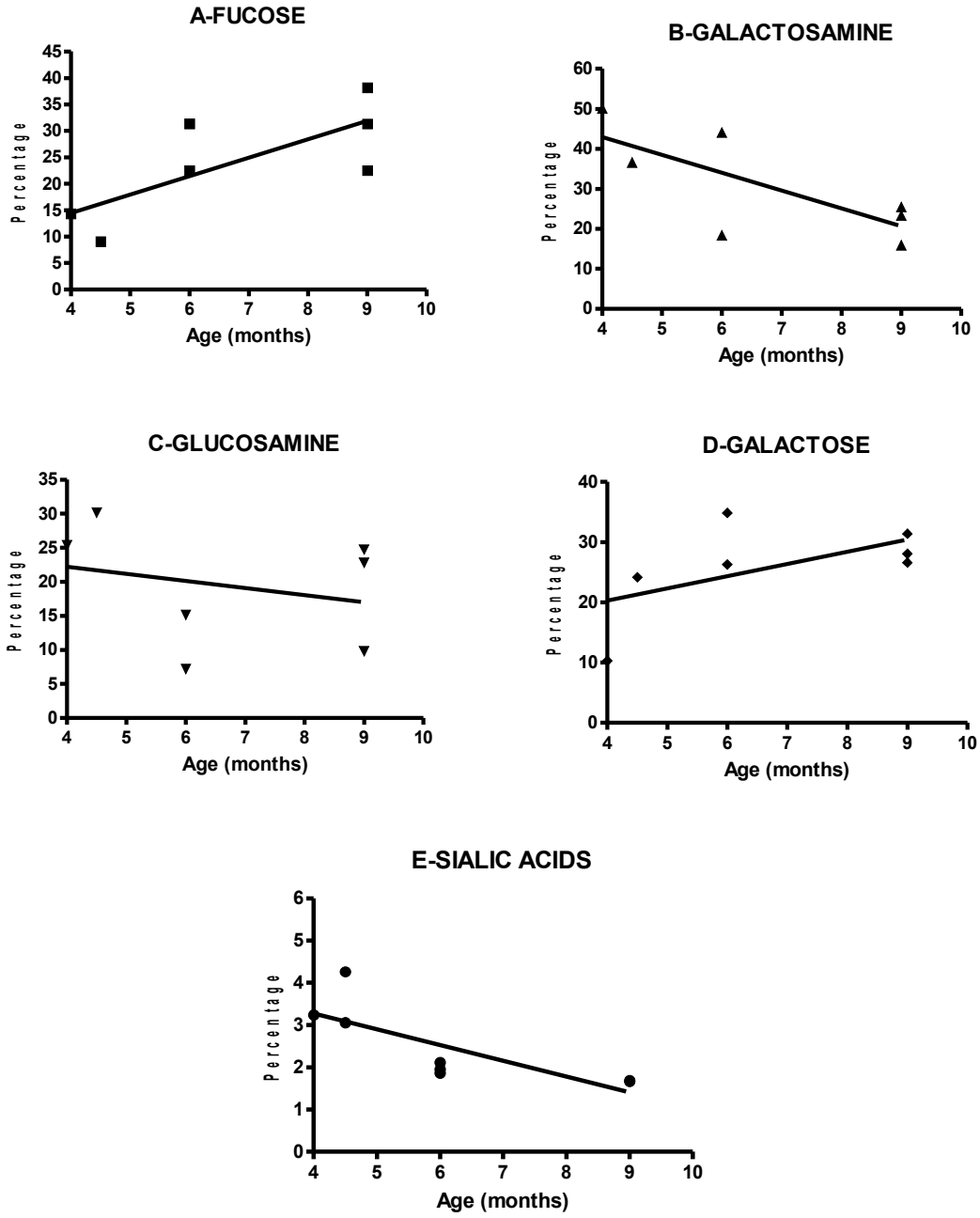

Figure 3.3. The relationship between the age of the sheep and the percentage of each monosaccharide present in duodenal mucins in individual non-infected sheep: (A) fucose, P=0.053; (B) galactosamine, P=0.056; (C) glucosamine, P=0.566; (D) galactose, P=0.18 and (E) sialic acids, P=0.024.

The percentages of GlcN in sheep duodenal mucins at different ages and with infection are reported in Figure 3.3C and Table 3.8.

There was no age effect on the percentage of GlcN in non-infected sheep (P=0.566). Neither *H. contortus* nor *T. circumcincta* infection caused a significant change in the percentage of GlcN with age (P=0.145 and 0.177 respectively).

Neither *H. contortus* nor *T. circumcincta* infection caused a significant change in the proportion of GlcN. However, interaction between age and *T. circumcincta* infection caused the significant change in the percentage of GlcN (P=0.018). The considerable high percentage of GlcN in *T. circumcincta* infected sheep at 6 months old could cause this significance (P<0.05).

3.3.2.2.4. Galactose

The percentage of Gal in sheep duodenal mucins at different ages and with infection are reported in Figure 3.3D and Table 3.9

There was no age effect in non-infected sheep (P=0.18). Two-way ANOVA showed that in *H. contortus* infection, there was a significant increase in the proportion of Gal with age (P=0.007). However, in *T. circumcincta* infection, age did not cause a significant change to the percentage of Gal (P=0.065).

Both infection with *H. contortus* and *T. circumcincta* caused a significant change in the percentage of Gal. In *H. contortus* infection, P=0.0003, a significant increase was observed in the 8-9 months-old group (P<0.001), whereas in *T. circumcincta* infection, P=0.009, a significant increase in the percentage of Gal was found in the 4-4.5 and 8-9 months-old groups.

3.3.2.2.5. Sialic acids

The percentages of sialic acids/glycoproteins in sheep duodenal mucins at different ages and with infection are reported in Figure 3.3E and Table 3.10.

Table 3.8. Percentage of glucosamine (Mean ± SD, n=3) in duodenal mucin of sheep aged 4-9 months old, infected with *H. contortus* or *T. circumcincta* and euthanased on day 21 or 28 p.i. respectively. The results of two-way ANOVA are shown. Means with the same superscript are significantly different (Bonferroni post-test)

Age (months)	Percentage of glucosamine (Mean ± SD)		
	Non-infected	*H. contortus* infected	*T. circumcincta* infected
4-4.5	27.8 ± 3.4*	24.7 ± 9.5	17.1 ± 11.8*
6	11.2 ± 5.6 [a] *	24.0 ± 4.3	50.3 ± 18.5 [a]
8-9	19.1 ± 8.1	25.6 ± 3.1	10.9 ± 6.1*
	Analyses of variance		
Age		P= 0.145	P= 0.177
Infection		P= 0.126	P= 0.305
Interaction		P= 0.186	P= 0.018

a= p< 0.05; * n=2

Table 3.9. Percentage of galactose (Mean ± SD, n=3) in duodenal mucin of sheep aged 4-9 months old, infected with *H. contortus* or *T. circumcincta* and euthanased on day 21 or 28 p.i. respectively. The results of two-way ANOVA are shown. Means with the same superscript are significantly different (Bonferroni post-test)

Age (months)	Percentage of galactose (Mean ± SD)		
	Non-infected	*H. contortus* infected	*T. circumcincta* infected
4-4.5	17.2 ± 9.8 [a] *	34.3 ± 9.8	46.9 ± 6.0 [a] *
6	30.6 ± 6.1*	43.7 ± 11.9	21.9 ± 13.6
8-9	28.7 ± 2.5 [b, c]	63.8 ± 2.9 [b]	54.8 ± 3.4 [c] *
	Analyses of variance		
Age		P= 0.007	P= 0.065
Infection		P= 0.0003	P= 0.009
Interaction		P= 0.109	P= 0.016

a, c= p< 0.05; b=p< 0.001; * n=2

There was a significant decrease in the proportion of sialic acids with age in non-infected sheep (P=0.024). Two-way ANOVA showed that in *H. contortus* infection, proportion of sialic acids did not change significantly with age (P=0.366), whereas in *T. circumcincta* infection, sialic acids significantly decreased with age (P=0.015).

H. contortus infection did not have any effect on the proportion of sialic acid (P=0.193). *T. circumcincta* infection caused a significant decrease in the percentage of sialic acids (P=0.002). The decrease was significant in the 4-4.5 months-old group (P<0.01).

3.4. DISCUSSION

3.4.1. Monosaccharide components of mucins

In sheep fundic and duodenal mucins, five monosaccharides were identified by TLC: Fuc, GalN, GlcN, Gal and sialic acid. During acid hydrolysis, GalNAc and GlcNAc undergo loss of the acetyl group to become GalN and GlcN (Fu and O'Neill, 1995). N-acetyl groups are replaced by acetic anhydride so that amino sugars hydrolysed in high-concentration acid solution will be de-acetylated. In sheep abomasal and small intestinal mucins, the monosaccharide constituents would be Fuc, Gal, GlcNAc, GalNAc and sialic acids as found by Ishihara et al. (1985) and Mukkur et al. (1985). These are the typical constituents of O-linked oligosaccharides in mucins: in frog (Ferri et al., 2001), in pig gastric mucosa (Karlsson et al., 1997), in rat sublingual mucus glycoproteins (Yu et al., 2008) and in cervical mucus (Nasir ud et al., 2003).

Man, an N-linked sugar is also present in mucins (Clamp, 1978), however, N-linked oligosaccharides account for only a small portion of total sugars and only trace amounts of Man are detected. In this study, no Man was found, indicating the absence or a small proportion of N-linked oligosaccharides.

The four sugars Fuc, GlcN, GalN and Gal were quantified by HPAEC using a modification of the method of Michalski and Capon (2000). Sialic acids were determined separately, because they are extremely acid-labile and free sialic acids are very unstable in acidic solution. Conditions that result in a maximum yield of neutral hexoses will cause complete destruction of sialic acids. The thiobarbiturate

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Table 3.10. Percentage of sialic acid/glycoprotein (Mean ± SD) in duodenal mucin of sheep aged 4-9 months old, infected with *H. contortus* or *T. circumcincta* and euthanased on day 21 or 28 p.i. respectively. The results of two-way ANOVA are shown. Means with the same superscript are significantly different (Bonferroni post-test)

Age (months)	Duodenal mucin (Mean ± SD)		
	Non-infected	*H. contortus* infected	*T. circumcincta* infected
4-4.5	3.52 ± 0.65 [a]	1.39 ± 1.0*	1.47 ± 0.47 [a] *
6	1.98 ± 0.13	2.3 ± 1.76*	1.18 ± 0.73*
8-9	1.68 ± 0.01*	1.65 ± 0.12*	1.02 ± 0.38*
	Analyses of variance		
Age		P= 0.366	P= 0.015
Infection		P= 0.193	P= 0.002
Interaction		P= 0.097	P= 0.109

a= p< 0.01, *n=2

method is effective in determining the proportion of sialic acid to glycoprotein, although it does not discriminate between NANA and NGNA. The overall percentage of sialic acids by weight of glycoprotein in both abomasal and duodenal mucins of sheep was lower than those reported in the colon. Kent and Allen (1968) found in sheep colonic mucosa that NGNA and NANA were 8.5 and 5.2% by weight of glycoprotein respectively and NGNA was the predominant sialic acid.

3.4.2. Modification of the neutral and amino sugar compositions of fundic and duodenal mucins with age

Over the age range of 4-9 months-old, the principal change in non-infected sheep was the trend of increasing fucosylation and decreasing sialylation. In the duodenal mucins, the increase in Fuc and decrease in sialylation were significant (P=0.053 and 0.024 respectively). In the fundic mucins, a similar trend was observed. The increase in fucosylation and decrease in sialylation observed are consistent with reports in rats and pigs (Shub et al., 1983; Torres-Pinedo and Mahmood, 1984; Turck et al., 1993; Malykh et al., 2003).

Other changes in hexoses were decreased GlcNAc in fundic mucin and GalNAc in duodenal mucin. GlcNAc is a component of core 3 and 4 structures, so this may represent a change in the core structures of the mucin in the fundus. In contrast, an increase of GalNAc was found in adult rat small intestine during postnatal development (Biol et al., 1987). Overall, there was a higher carbohydrate:protein ratio caused by the increase in Fuc and Gal with age was also found in rats (Biol-N'garagba et al., 2003).

There was more individual variation in fundic mucin, perhaps caused by different rate of maturation of animals of different genetic backgrounds or previous exposure to different environmental factors in the field. There was no attempt to select animals of the same genetic background. This variation was more apparent in the fundic mucins than duodenal mucins, which showed a significant increase in fucosylation and decrease in sialylation and GalNAc (Figure 3.3). In fundic mucin, there was a significant decrease in the proportion of GlcNAc, but increases or decreases in the proportion of Fuc and sialic acids respectively were less clearcut (Figure 3.2). Statistically, these changes were approaching significance and may

represent real age effects in groups of three animals with high individual variations. The greatest variation was in the 6 months-old group, suggesting the rate of maturation is more variable in the abomasum.

3.4.3. Modification of the neutral and amino sugar compositions of fundic and duodenal mucins with parasitism

It is unclear whether the different mucin profiles in non-infected sheep as they grow older result in part from previous exposure to parasites which is never reversed. All sheep are infected in the field, so programmed development is inseparable from previous infection. The two parasites produced different changes in mucin glycosylation, but share some common features of reduced fucosylation and sialylation with infection in both fundic and duodenal mucins.

3.4.3.1. *H. contortus* infection

H. contortus infection caused significant alterations in the monosaccharide profiles of both fundic and duodenal mucins. There were less pronounced differences with age, unlike the variation following *T. circumcincta* infection. *H. contortus* is not prevalent in cooler parts of New Zealand, therefore few of these sheep may have been previously exposed in the field to this species. The infection may be a primary rather than a challenge infection.

In the fundus, there was consistent reduction in terminal sugars at all ages ($P=0.0005$ for Fuc and 0.004 for sialic acids). As this is the site of infection, the parasites may be responsible through secretion of glycosidases, as has been shown for mucin dwelling pathogens including bacteria, fungi and protozoan parasites. Wadstrom et al. (1996) found that the gastric bacterium *H. pylori* produced sialidase and fucosidase. A range of glycosidases have also been detected in *F. hepatica* ES products, which were able to degrade mucin in vitro (Irwin et al., 2004). There were at least 8 different glycosidase activities: beta-N-acetylhexosaminidase, beta-galactosidase and beta-glucosidase, alpha-fucosidase, beta-glucuronidase, alpha-galactosidase, alpha-mannosidase and neuraminidase (Irwin et al., 2004).

The only other change in the percentage of a monosaccharide in the fundic mucins of sheep infected with *H. contortus* was in GlcNAc, which increased

significantly (P=0.029), whereas there were no changes in the proportions of GalNAc or Gal (Tables 3.2 and 3.4). As this was associated with a reduced proportion of Fuc, it could be a percentage effect, but this is not likely, as the three sugars would be expected to increase proportionally. The increased percentage of GlcNAc may reflect increased glycosylation, as it is both a core and backbone sugar. Rikihisa and Lin (1984) reported an increased concentration of hexosamines in the hyperplastic gastric mucosa of rats during parasitism of the liver with *T. taeniaformis*.

In the duodenal mucins, there were significant decreases in fucosylation (P=0.0006) and in the proportion of GalNAc (P=0.003), but an increase in Gal (P=0.0003) at all ages. Sialylation was not significantly altered overall, but the interaction was approaching significance (P=0.097). The group means for the youngest sheep were 3.5% in the control and 1.4% in the infected sheep. In all groups of infected sheep, the proportion of GlcNAc was 24-26% of the total. The percentage of Gal was at least double that in uninfected sheep (Table 3.9), along with a decrease in GalNAc, to as low as 6% in the 8-9 months-old sheep. Some of these changes may be secondary percentage changes when other monosaccharides are increased or decreased. The effects in the duodenum are interesting, as the parasites were present in the abomasum and not in the duodenum. It could result from the stimulation of the host response having an effect beyond the site of infection or by worm chemicals released into the gastric lumen stimulating changes in the duodenal mucins.

3.4.3.2. *T. circumcincta* infection

T. circumcincta infection had more marked effects in sheep at particular ages, probably because of different levels of exposure to the parasites and development of immunity in older animals. At 8-9 months-of-age, the only significant change in monosaccharides in fundic mucins was decreased sialylation (Table 3.5). Unlike the younger animals, there was no change in the proportion of Fuc or Gal. The 8-9 months-old sheep had few worms in the fundus and more in the antrum, but these were small, immature and not well-developed.

The most significant changes in monosaccharides were seen in younger lambs (4-6 months old). In the 4-4.5 months-old sheep, there was a significant

decrease in the proportions of sialic acid and GalNAc and an increase in the percentage of Gal in fundic mucins. In the 6 months-old animals, there was a significant decrease in the proportions of sialic acid and an increase in Gal, but no change in GalNAc. In both groups, there was a trend for reduced fucosylation: the means were 8% and 5% in infected animals, compared with 13% and 16% in uninfected sheep at the same ages.

A surprising finding was the greater effect of *T. circumcincta* on duodenal than fundic mucins in the 8-9 months-old sheep. There was a significant decrease in fucosylation (down to 7% from 31%) and an increase in the proportion of Gal from 29% to 55%. There was no change in GalNAc, unlike the decrease in younger animals.

As for fundic mucins, there were marked changes in younger lambs in the composition of duodenal mucins. Both GalNAc and sialic acids were reduced in infected animals. GalNAc decreased by half in the 4-4.5 months-old group and was reduced by 80% in the 6 months-old group compared with non-infected animals. The proportion of GlcNAc showed a positive interaction between age and infection, with a decrease in the 4-4.5 months-old group, but a significant increase in 6 months-old group (from 11.2%-50.3%). Unlike GlcNAc, Gal increased significantly and slightly decreased at 4-4.5 and 6 months old respectively.

The greatest difference in monosaccharide compositions in abomasal and duodenal mucins between infected and non-infected sheep were observed in younger animals (4-6 months-of-age) and those differences were less significant at 8-9 months old. The differences with age are most likely caused by developing immunity to *T. circumcincta.* As most sheep were brought in from the field at about 3 months-of-age, it was likely that they had been exposed to *T. circumcincta* before they were drenched and housed indoors.

3.4.3.3. Proposed mechanisms for mucin changes observed during parasitism

The content of most mucins changes in response to infection (reviewed by Linden et al., 2008). Little is known about whether the changes in mucins during nematode infection are caused by the host immune response to the physical presence or chemicals of the parasites or by the parasites themselves to enable

their survival. However, extensive studies on the effects of other pathogens such as bacteria have been carried out and which may suggest mechanisms. Care is needed when interpreting the observations and comparing with bacterial effects, as each has its own characteristics.

The significant changes seen in both abomasal and duodenal mucins at one time point in a single infection are probably due to a combination of host immune responses and direct effects of the parasites on gene expression or degradation of mucins. Samples were collected at a time when adult parasites were present and laying eggs. In individual sheep, this may not represent the same stages of the immune response.

The effects on the duodenal mucins were similar for the two infections (decreased sialic acids, Fuc and GalNAc and increased Gal). Only the 8-9 months-old sheep infected with *T. circumcincta* differed in some features (no decreased GalNAc). Sialylation was higher in young uninfected animals, therefore there is more likelihood of a significant decrease with infection. This may represent host immunity expressing itself in other tissues, as the parasites are not in physical contact with the mucins. An example of a parasite in one organ causing effects in another is the finding that *T. taeniaformis* infects the liver, however, effects were also seen in the gastrointestinal tract (Cook and Williams, 1981; Rikihisa et al., 1986; Abella et al., 1997; Konno et al., 1999). Parasite ES released into gut contents also could play a part in the changes in the duodenum. Teladorsagia ES may specifically enhance mucus production and secretion in the rat stomach and this action is reversible (Rikihisa and Hammerberg, 1982). This suggests that there were chemicals released in the ES by the parasites which were the signal for initiating the host-parasite interaction.

The changes in the fundic mucins differed from those in the duodenum in both infections and also differed from each other. *H. contortus* infected sheep showed similar profiles at all ages, but this was not the case for *T. circumcincta*. 8-9 months-old sheep showed less effect on mucins but there still was decreased sialylation. Nematodes may secrete exoglycosidases to degrade mucin, as was found for *H. pylori* (Wadstrom et al., 1996) and *F. hepatica* (Irwin et al., 2004). Both released sialidases, which explained the decreased sialylation in mucins. The differences in mucins of sheep infected with the two nematodes also indicate that

worm species plays an important role. It is still unknown whether the significance of nematode species depends on the degree of damage or variation in the enzymes or antigens in their ES or on immune status of the sheep. Carbohydrate moieties can serve as nutrient sources for the intestinal microflora and pathogens colonising mucus (Carlstedt-Duke et al., 1986a, b; Mukai et al., 1998). *H. contortus* is a blood-feeder, but may also feed on mucin, as could *T. circumcincta*, for which the food sources have not been positively identified. The decrease in terminal sugars during infection raises the question whether gastrointestinal nematodes use sugars as their food source.

Nematodes could also chemically influence gene expression in the host. Postnatal colonisation of the gut by microflora changes gene expression, including fucosylation and galactosylation; the genes encoding enzymes generating Fucα1,2Galβ1,4GalNAc and Galβ1,4GalNAc are switched-on and those producing NeuAcα2,6Gal are switched-off in goblet cells in the colon in infected mice (Freitas et al., 2005). The gene encoding MUC5AC was down-regulated on day 22 compared with day 3 after infection with *H. contortus* (Rowe et al., 2009). Host genetics may also be important, as MUC5AC was down-regulated more in resistant sheep than in susceptible sheep, both in a primary infection and subsequent challenge with *H. contortus* (Ingham et al., 2008).

In these experiments, sheep were studied over a wide age range from 4-9 months-of-age, during which time immunity was likely to have developed, at least to *T. circumcincta*. Some of the variations in the proportions of each monosaccharide in the mucins could be due to different sheep breeds or whether it was a primary or challenge infection. Most of the developmental changes in mucins in the abomasum appear to have been completed by 4 month-of-age, so it was of interest to study younger animals. Experiments reported in Chapter 4 examined the effects of weaning on mucin composition and the effects of a trickle infection with *T. circumcincta* up to 9 weeks-of-age.

Chapter 4

MONOSACCHARIDE COMPOSITIONS OF FUNDIC AND DUODENAL MUCINS IN LAMBS

4.1. INTRODUCTION

In the experiments reported in the previous chapter, there were significant effects of infection on the monosaccharide compositions in mucins. Mucins were collected from sheep 4 to 9 months-of-age, a period when immunity is developing. Young lambs are more susceptible to parasites than older animals. This characteristic can be explained by young animals having a relative immune deficiency compared with mature sheep (Watson et al., 1994; Colditz et al., 1996; Stear et al., 1999a). Most lambs aged 6 months or less which have been previously infected with *H. contortus* or *T. colubriformis* were not resistant to the subsequent challenge, whereas the same procedure produced a higher degree of protection in older animals (Manton et al., 1962; Urquhart et al., 1966; Dineen et al., 1978). Courtney et al. (1985) found that Dorset, Suffolk and Finn sheep became more resistant to parasites when they reached 8-9 months-of-age and Smith et al. (1985) observed that 10-months-old sheep showed a greater resistance to *T. circumcincta* than did 4.5-months-old lambs. Lambs appear to express resistance to parasites by controlling the growth of the worms rather than the number of worms, while adult sheep control the size, development and number of worms (Stear et al., 1999b).

Although young sheep have greater susceptibility to parasites than older sheep, it is not true in milk-fed lambs. Bovine milk had an anthelmintic effect on Strongyle infections in foals (Lesse, 1943) and on nematode infections in pigs (Spindler and Zimmerman, 1944; Spindler et al., 1944; Shorb and Spindler, 1947). Unweaned calves have fewer, smaller *H. contortus* (Porter, 1941) and *Haemonchus placei*, *Cooperia* spp and *Oesophagostomum radiatum* (Rohrbacher

et al., 1958) than weaned calves. Zeng et al. (2001) studied the resistance or susceptibility of milk-fed and solid-fed lambs to *T. circumcincta*. They observed reduced *T. circumcincta* burdens in milk-fed lambs. The total worm burden and faecal egg count in milk-fed lambs were significantly lower than in solid-fed lambs. Satrija et al. (1991) concluded that the degree of ruminal development was the important factor in reducing the worm burden in milk-fed calves. This was not confirmed in milk-fed lambs as infection with exsheathed L_3 increased the number of worms in infected animals (Zeng et al., 2001). It was suggested that milk could have adverse effects on parasites, preventing their development in the abomasum. This was supported by the inhibition of motility in vitro of both sheathed and exsheathed *T. circumcincta* L_3 (Zeng et al., 2003). The reduced worm burdens may reflect an environment in the underdeveloped gastrointestinal tract which is unfavourable for parasite development as well as a possible anthelmintic effect of milk.

From age 4 to 9 months, fundic mucins showed the trend of decreasing sialylation and increasing fucosylation (Figure 3.2), but these changes in the duodenal mucins were significant (Figure 3.3). The effects of age in fundic mucins were smaller than in the duodenum, perhaps reflecting earlier maturation of the abomasum. Investigations of the effects of parasitism were extended to lambs younger than 4 months-of-age. The effect of diet at early age was also taken into account. Therefore, the experiment was designed to compare the monosaccharide component in gastrointestinal mucins of lambs euthanased at 3 days old, lambs fed entirely with milk replacer for 9 weeks, lambs weaned on to solid food beginning at 3 weeks-of-age and weaned lambs trickle infected with *T. circumcincta*. To reduce the effect of genetic variation among individual sheep, the lambs were all Romney breed.

4.2. MATERIALS AND METHODS

4.2.1. Lambs

At 3 days-of-age, twelve lambs were removed from their mothers and brought indoors. Three lambs were euthanased at 3 days-of-age. The other nine were assigned to one of 3 groups defined by the diet and infection: (1) 9 weeks-old, fed entirely on milk replacer (milk-fed); (2) 9 weeks-old, weaned on to solid food at

3 weeks-of-age and not infected (solid-fed); (3) 9 weeks-old, fed with milk and weaned on to solid food at 3 weeks-of-age and infected with 1,000 *T. circumcincta* larvae twice weekly for 5 weeks from week 4 to 9 after birth (solid-fed + *T. circumcincta* infection).

4.2.2. Feeding

From 3 days-of-age, the lambs were bottle-fed with commercial milk replacer (Anlamb, New Zealand Dairy, NZ for 3 weeks). Diarrhea occurred in two lambs when first brought indoors; milk was replaced with electrolyte solution for a day.

For the first three weeks, lambs in all groups were fed entirely with milk replacer (see Appendix 2.1 for detailed feeding time and volume per feed). Groups (2) and (3) were weaned over week 4, first on high protein cereal-based sheep pellets and then lucerne chaff was introduced; by week 5, they were fed entirely on solid food, ad libitum water was freely available for all lambs. For group (1), the lambs were fed entirely with milk replacer to 9 weeks-old.

4.2.3. Infection with *T. circumcincta*

After lambs in group 3 had been completely weaned and fed with chaff and pellets for 1 week, they were infected orally with 1,000 *T. circumcincta* larvae in a small volume of water through a syringe, twice weekly for 5 weeks (from weeks 5-9).

In 2 of the 3 lambs, there were few worms seen at post-mortem but the other had moderate number of worms.

4.2.4. Mucus collection

Three lambs were euthanased at 3 days-old and the others at 9 weeks-old (groups 1, 2 and 3), as described in Chapter 2, Section 2.2.1. Mucus was scraped off the fundus and duodenum, purified and analysed chemically as described in Chapter 2, Section 2.2.2 and Chapter 3, Section 3.2.3. The amount of mucus collected from the 3 days-old lambs was small, therefore, the 3 samples were pooled for further purification and chemical analysis.

4.2.5. Statistical analysis

Data were expressed as Mean ± SD, n=3. The percentages of each of Fuc, GalN, GlcN, Gal and sialic acids in milk-fed, solid-fed and solid-fed lambs infected with *T. circumcincta* were compared by one-way ANOVA. Bonferroni post-tests were carried out to compare milk-fed vs solid-fed and solid-fed vs solid-fed + infection.

4.3. RESULTS

4.3.1. Three days-old lambs

Mucus collected from the three 3 days-old lambs was pooled before purification and hydrolysis. Only the means of groups can be compared, no statistics are possible. The proportion of Fuc in 3 days-old lambs was very small: 2.5% in fundic (Table 4.1) and 1.9% in duodenal mucins (Table 4.2) compared with those of older lambs.

The percentage of sialic acids was very high in 3 days-old lambs (12% in fundic and 8.2% in duodenal mucin) (Table 4.3) while in 9 weeks-old lambs, that proportion was 3-4% irrespective of diet.

4.3.2. Milk-fed vs solid-fed lambs

There were significant reductions in the proportion of Gal ($P<0.05$) in fundic mucin (Table 4.1) and GalN ($P<0.01$) in duodenal mucins (Table 4.2) in solid-fed compared with milk-fed lambs. Fucosylation was not different between the two groups. There was a tendency for sialic acids to decrease in solid-fed compared with milk-fed lambs. In the fundic mucins, the proportion of GalN remained unchanged but the decrease in the proportion of Gal in solid-fed lambs was associated with the increase in Fuc and GlcN.

4.3.3. Solid-fed vs solid-fed lambs infected with *T. circumcincta*

The percentage of Gal increased from 23.6% in solid-fed, non-infection to 40.6% in solid-fed lambs infected with *T. circumcincta* ($P<0.01$).

Table 4.1. Percentage of neutral and amino sugars (Mean ± SD, n=3) in fundic mucins from 3 lambs 3 days-of-age; 9 lambs at 9 weeks-of-age which were milk-fed, weaned or weaned and infected with *T. circumcincta* twice weekly for 35 days. One-way ANOVA was carried out to compare milk-fed vs solid-fed and solid-fed vs solid-fed + infection. Means with the same superscript are significantly different.

	Fuc	GalN	GlcN	Gal
3 days-of-age	2.5	24.9	25.4	47.2
Milk-fed	13.6 ± 5.3	38.4 ± 5.9	9.6 ± 0.3	38.4 ± 6.9 [a]
Solid-fed	19.8 ± 3.2	40.2 ± 3.6	16.4 ± 2.8	23.6 ± 1.7 [a, b]
Solid-fed + Infection	10.2 ± 6.6	35.4 ± 5.3	13.8 ± 6.7	40.6 ± 2.9 [b]

a= $p< 0.05$; b= $p< 0.01$

Table 4.2. Percentage of neutral and amino sugars (Mean ± SD, n=3) in duodenal mucins from 3 lambs 3 days-of-age; 9 lambs at 9 weeks-of-age which were milk-fed, weaned or weaned and infected with *T. circumcincta* twice weekly for 35 days. One-way ANOVA was carried out to compare milk-fed vs solid-fed and solid-fed vs solid-fed + infection. Means with the same superscript are significantly different.

	Fuc	GalN	GlcN	Gal
3 days-of-age	1.9	26.8	32.7	38.6
Milk-fed	22.1 ± 12.6*	51.8 ± 2.0 [a*]	7.9 ± 2.9*	18.2 ± 17.5*
Solid-fed	20.8 ± 2.9	34.6 ± 2.5 [a]	18.0 ± 2.2	26.6 ± 2.6
Solid-fed + Infection	19.4 ± 6.4	42.4 ± 3.9	10.2 ± 8.0	28.1 ± 13.6

a= $p< 0.01$, *n=2

Table 4.3. The proportion of sialic acid in the glycoproteins (Mean ± SD, n=3) in fundic and duodenal mucins from 3 lambs 3 days-of-age; 9 lambs at 9 weeks-of-age which were milk-fed, weaned or weaned and infected with *T. circumcincta* twice weekly for 35 days. One-way ANOVA was carried out to compare milk-fed vs solid-fed and solid-fed vs solid-fed + infection. Means with the same superscript are significantly different.

	Abomasal mucin	Duodenal mucin
3 days-of-age	12.0	8.2
Milk-fed	3.9 ± 2.5*	3.3 ± 0.07*
Solid-fed	3.0 ± 1.3	2.3 ± 1.5
Solid-fed + Infection	0.9 ± 0.6*	0.6 ± 0.4

* n=2

T. circumcincta infected lambs showed decreased sialylation (0.9% in fundic and 0.6% in duodenal mucin) compared with uninfected animals at the same age (Table 4.3).

4.4. DISCUSSION

In the experiments reported in the previous chapter, the variations in the proportions of each monosaccharide in the mucins may have been due to different sheep breeds or whether it was a primary or challenge infection. To reduce individual animal variation, the lambs used in this experiment were the same breed, housed together and raised on milk replacer for 3 weeks. The relative importance on mucin profiles of programmed development and the change of diet at weaning were evaluated. The infection protocol used followed that of Zeng et al. (2001), which had identified the inability to infect milk-fed lambs with a trickle infection with *T. circumcincta*, but susceptibility of early weaned lambs.

4.4.1. Modifications in monosaccharide profiles in mucins with age

The monosaccharide profiles of abomasal and duodenal mucins in the pooled sample from lambs at 3-days-of-age were very different from those of lambs aged 9 weeks- and 4-9 months-of-age. A change from high sialylation and low fucosylation during the postnatal period is very apparent from 3 days to 9 weeks, while further changes from 4-9 months were still significant in the duodenum, but not so clearcut in fundic mucins. In the small and large intestine of pigs, sialylation decreased during the first 2 weeks of life (Malykh et al., 2003). Similar changes from a predominance of sialyation to fucosylation in the gastrointestinal tract are seen in rodents (Srivastava et al., 1987; Taatjes and Roth, 1990; Dai et al., 2002); and pigs (King et al., 1995). Fucosylation is generally considered to be a marker of maturation in many species (Shub et al., 1983; Torres-Pinedo and Mahmood, 1984; Turck et al., 1993). The terminal sugars in N-linked and O-linked oligosaccharide chains of the lactase family change from sialic acid during the suckling period to Fuc in adulthood in rat small intestine (Buller et al., 1990).

Fucosyltransferase activity increases with age (Bry et al., 1996; Dai and Walker, 1999; Hooper et al., 2001; Dai et al., 2002; Nanthakumar et al., 2003). In

mammalian tissues, Fuc residues are terminal sugars which can have the linkages α-1,2 to a terminal Gal, α-1,3 or α-1,4 to subterminal GlcNAc residues of the antennae and α-1,6 to the innermost GlcNAc. Various functions of Fuc residues are discussed in Section 1.3.1.5. They play an important role in several cell recognition processes ranging from fertilisation and development through to pathological events and cell death.

During the postnatal development of the rat small intestine, it was found that activities of sialyltransferases decrease between the newborn and weaning periods and that they remain low during adulthood (Chu and Walker, 1986; Biol et al., 1987; Biol et al., 1991). The increase in fucosyltransferase activity is accompanied by a decrease in α-2,3- and α-2,6-sialyltransferase activity (Bry et al., 1996; Dai and Walker, 1999; Hooper et al., 2001; Dai et al., 2002; Nanthakumar et al., 2003). In the colon of the adult rat, α-2,6 sialyltransferase activity predominates over α-2,3 sialyltransferase, however the activity of the former decreases and the latter almost disappears during development from birth to weaning in the rat (Dall'Olio et al., 1990).

The degree of maturation of the gastrointestinal tract at birth varies with the species of mammal, so that direct comparisons of the rate of maturation of mucin profiles cannot be made. The rat, mouse and rabbit are born with an immature digestive system and the rat small intestine remains immature until the end of the third week after birth, whereas the horse and humans are born with a relatively developed gastrointestinal tract (Weaver, 1986). At birth, lambs are intermediate between rats and more mature species like humans and foals (Trahair and Robinson, 1986). Although the rate of maturation of different individuals and species varies, they still showed a similar trend of increasing fucosylation and decreasing sialylation during their development. The hormones which mediate intestinal maturation, cortisone and hydrocortisone, caused a significant rise in fucosyltransferase enzyme activity in suckling rats (Biol et al., 1992a), suggesting these hormones are involved in the increasing fucosylation of mucins.

The proportions of Gal, GalNAc and GlcNAc in both abomasal and duodenal mucins in the 3 days-old lambs differed from those in both the 9 weeks-old milk-fed and solid-fed lambs, although there were also some differences between the latter animals. At 3 days-of-age, Gal made up approximately 50% and GalNAc and

GlcNAc each 25% of the monosaccharides in fundic mucins. By 9 weeks-of-age, the proportion of GalNAc increased to around 40% irrespective of diet, while Gal and GlcNAc both decreased overall, although the ratio of Gal to GlcNAc in milk and solid-fed lambs was quite different (Table 4.1). The proportions of Gal, GalNAc and GlcNAc in the 3 days-old animals were virtually the same as at 4 months, allowing for a smaller Fuc in the former. It would appear that the 9 weeks-old animals which were artificially weaned early or kept on milk showed the different profiles from other sheep. In duodenal mucins, the striking difference in the 3 days-old lambs was the very low proportion of Fuc. As a result, it would be expected that the proportions of the other three monosaccharides would increase. Allowing for this, the profile was similar to that in 4 months-old sheep.

4.4.2. Monosaccharide composition after weaning

At 3 days-of-age, lambs were brought indoors and fed with bovine milk replacer (commercial lamb milk powder), not on ewe milk. Although the diet was changed from milk-fed to solid-fed, they could be considered to be artificially "weaned".

There were differences in the proportions of monosaccharides between weaned and milk-fed lambs. In common, there was a trend for doubling the proportion of GlcNAc. There was no difference in the sialylation or fucosylation of either fundic or duodenal mucins with diet. In solid-fed lambs, there was a significantly lower proportion of Gal in fundic mucin ($P<0.05$) and GalNAc in duodenal mucin ($P<0.01$). In the fundus, the decreasing proportion of Gal ($P<0.05$) was associated with the increased proportion of Fuc and GlcN. By 9 weeks-of-age, the proportion of GalNAc increased to around 40% irrespective of diet, while Gal and GlcNAc both decreased overall, although the ratio of Gal to GlcNAc was higher in the milk-fed than solid-fed lambs (Table 4.1). In the duodenum, the decrease in percentage of GalNAc was associated with increased proportion of GlcNAc and perhaps the proportion of Gal as well.

Both the 9 weeks-old animals which were artificially weaned early and fed on milk showed some differences in the monosaccharide profiles from those in older sheep. There was no difference in either sialic acid or Fuc and GalNAc was approximately 40% in both groups, much higher than the 25% at 4 months-of-age.

The sialylation of fundic mucins (3.9% in milk-fed lambs and 3.0% in solid-fed lambs) compared to that in 4-4.5 months-old sheep (3.5%). The milk-fed lambs and 4-4.5 months-old lambs had 40% Gal, but the weaned lambs had a much lower proportion (24%). Both solid-fed and 4-4.5 months-old lambs had 20% GlcNAc in fundic mucin, compared with 10% in the milk-fed lambs. In duodenal mucin, there were no difference in the proportion of Gal, but GlcNAc was lower in both milk-fed and solid-fed lambs than in 4-4.5 months-old lambs. The percentage of GalNAc in 4-4.5 months-old lambs was 44%, intermediate between the values for milk-fed and solid-fed lambs.

This experiment gives information on programmed development rather than change of diet that usually accompanies it. Species mature at different rates, therefore, studies in monogastric animals may not be relevant to ruminants. Many studies relating to weaning do not distinguish the effects of diet and aging. Intestinal glycoproteins of suckling rats expressed low Fuc and high Man. The amount of neutral sugars, which included Fuc, Gal and Man, were higher in fibre-fed rats than those fed a fibre-free diet or with prolonged nursing (Tardy et al., 1994). In the rat small intestine, the developmental variations in fucosyl- and sialyl-transferases were sensitive to the composition of the diet at weaning (Srivastava et al., 1987; Biol et al., 1992b; Tardy et al., 1994; Lenoir et al., 1995). It was reported that fucosyl- and galactosyl-transferase activities were low in young rats but largely enhanced at and after weaning (Shub et al., 1983; Chu and Walker, 1986; Ozaki et al., 1989; Biol et al., 1991). The change in diet from fat-rich milk to carbohydrate-rich solid food may be an important factor. In contrast, Hedemann et al. (2007) used lectin staining to show that there was no change in glycoconjugates in the apical membrane of porcine small intestine between 3 days prior to weaning to 9 days post-weaning.

The similar trend of increasing Fuc after weaning in rat small intestine is also found (Biol-N'garagba and Louisot, 2003) suggesting that the sialylation was maintained in animals of the same age irrespective of diet. This observation was similar to that in pig small intestinal mucins, in which total sialic acids in newborn pigs were higher than in 2 weeks-old pigs and there was no difference between 2 weeks-old, 4 weeks-old and adult pigs (Malykh et al., 2003).

4.4.3. Effects of infection with *T. circumcincta*

The similarities between the mucin profiles in the fundus after a single infection with 50,000 *T. circumcincta* larvae and after a trickle infection with 1,000 larvae twice weekly for 5 weeks suggests the infection protocol is not important. The trickle infection in the young lambs caused changes in the fundic mucin profile similar to that seen after a single infection in 4-6 months-old lambs. In the fundic mucins, there was a decrease in the proportions of sialic acid and GalNAc and an increase in Gal. The effect on Fuc was not clearcut in the fundic mucin and was unchanged in the duodenal mucin. The changes in the duodenum were different from those in sheep 4-9 months-of-age in that only decreased sialylation occurred. In older animals, there were significant decreases in fucosylation (P=0.0006) and in the proportion of GalNAc (P=0.003), but an increase in Gal (P=0.0003) in the duodenal mucins.

Previous experiments by Zeng et al. (2001) showed that milk-fed lambs were not able to be infected with *T. circumcincta* using an identical experimental design to the present one. Therefore, parasites were not administered to milk-fed lambs. Either a different mucin profile in the unweaned lambs or the milk itself is responsible for the failure of parasites to establish in the abomasum. Satrija et al. (1991) suggested that the less developed rumen in calves influences the establishment of gastrointestinal nematodes (Satrija et al., 1991). Zeng et al. (2001) included a milk-fed group infected with exsheathed larvae to eliminate this possibility.

The differences in gastrointestinal mucin profiles between the milk-fed and early weaned lambs (Table 4.1) would not appear to explain the failure of parasites to establish in milk-fed lambs. The higher proportion of Gal would not be expected to contribute to the susceptibility or resistance of the sheep to parasites, as this is not a terminal sugar. Fucosylation and sialylation were similar in both fundic and duodenal mucin in both groups, suggesting that the terminal sugar residues of the glycoprotein side chains did not change. Susceptibility to microorganisms frequently depends on the presence of specific sugar residues on cell membranes and mucins. These pathogens need a specific cell surface carbohydrate structure for attachment to occur (Hakansson et al., 1996), which is often the terminal sugar

on mucin oligosaccharides. Alteration of terminal sugars of goblet cell mucins is said to be associated with the expulsion of nematodes (Ishikawa et al., 1994).

The milk itself may have an anti-parasite effect or provide an unfavourable environment in the pre-ruminant stomach. The milk replacer and other milk products have been shown to inhibit the motility of *T. circumcincta* larvae (Zeng et al., 2003). The high pH of milk was suggested to protect against trichostrongylid nematodes in milk-fed calves (Rohrbacher et al., 1958). Milk can limit bacterial and viral infections of the gastrointestinal tract (Yolken et al., 1992; Dekker and Tytgat, 1993; Backhed et al., 2005). Milk from humans, cows, goats, sheep, pigs, horses, dromedary camels and rabbits were found to have therapeutic effects by inhibiting pathogen colonisation and infection (Gustafsson et al., 2005). Oligosaccharides and glycoconjugates in milk can protect the host from being infected in the gastrointestinal tract during the first year of life. The presence of oligosaccharides and particularly their anti-adhesive properties contribute to against pathogens (Coppa et al., 1990; Kunz and Rudloff, 1993; Newburg, 1996). Fucosyloligosaccharides in human milk can inhibit the binding and infection of *Campylobacter jejuni* (Ruiz-Palacios et al., 2003). The lactoferrin component in milk was reported to protect the neonatal rats from gut infections (Edde et al., 2001).

In conclusion, in this and the previous experiment, it was shown that both age and infection play significant roles in determining the carbohydrate composition of mucins. Sulphation of mucins is known to be important for host-pathogen interactions, but could not be determined chemically. This has been determined histochemically and is reported in Chapter 5. In addition, different mucins are produced by different mucus-producing cells in the gastrointestinal tract (Section 1.2.1). These have been investigated histochemically (Chapter 5) and by lectin staining (Chapter 6).

Chapter 5

HISTOCHEMISTRY OF ABOMASAL AND DUODENAL MUCINS

5.1. INTRODUCTION

Chemical analysis of mucins (Chapters 3 and 4) showed that infection of sheep with either *H. contortus* or *T. circumcincta* changed the monosaccharide composition of both fundic and duodenal mucins. There were differences in younger and older sheep, both infected and uninfected. Change in mucin sulphation could not be determined chemically due to the very low content of sulphate in these sheep mucins, but it can be investigated by histochemistry. The acidic mucins consist of sulphomucins and sialomucins, the latter decreasing with both age and infection in sheep (Chapters 3 and 4). Both sulphation and sialylation of mucins can be studied by staining sections from abomasal and duodenal tissues.

Histochemistry is an effective method of studying the mucus-producing cells which synthesise the mucin glycoproteins which consist of neutral, amino and acidic sugars (Spicer and Schulte, 1992; Madrid et al., 1997). Gastrointestinal mucosubstances have been extensively studied in eleven animals: the mouse, rat, hamster, gerbil, guinea pig, rabbit, cat, dog, Rhesus monkey, baboon and humans (Sheahan and Jervis, 1976). Other studies include human colon (Matsuo et al., 1997), bat stomach (Forman, 1971), camel duodenal glands (Takehana et al., 2000) and rat gastrointestinal tract (Koninkx et al., 1988; Kodaira et al., 1999; Soga et al., 2008). Sheahan and Jervis (1976) showed that in the stomach of all eleven species, neutral mucins were predominantly found in surface and pit cells and less was present in mucous neck cells (MNCs) in the fundus and gland cells in the antrum. However, there were large variations in the amount of sialomucins and

sulphomucins in different species and there were distinctly different distributions. A common feature was the presence of acidic mucosubstances in the intestines. Comparisons between species are therefore not of great significance.

Ishihara et al. (1995) found that in the abomasum, the total glycoprotein per gm of tissue was greater in sheep than in lambs and mucins were highly sulphated in lambs. Ohwada et al. (1992) reported the absence of sulphated mucins in the Brunner's glands of 5 female crossbred sheep of unknown age. In contrast, Pedini et al. (2001) demonstrated sulphomucins in both goblet cells and Brunner's glands in the duodenum, at least in some 2 months-old lambs. They reported age-dependence of sulphated mucins in the duodenum in sheep, apparently from a comparison of their observations with those in the literature. However, there appear to be no comparative studies in sheep of progressive changes in mucin profiles with age and infection.

The abomasa of infected sheep are much heavier than control animals and the thickness of the mucosa is much greater (Anderson et al., 1988; Fox et al., 1993; Scott et al., 1998a). Numerous histopathological studies of parasitised ruminants have identified that abomasal parasites cause enlargement of the fundic pits, depletion of parietal cells and chief cells, but more MNCs and undifferentiated cells (Anderson et al., 1965, 1966; Ritchie et al., 1966; Murray, 1970; Murray et al., 1970; Christie et al., 1975; Snider et al., 1983, 1988; Scott et al., 1998a). Similar generalised histological changes are seen after adult *T. circumcincta* transfer (Scott et al., 1998a, 2000).

The most detailed studies of changes in mucin type with infection are those of Miller et al. (1983) and Newlands et al. (1990). Included in their experiments were groups of 9-10 months-old, worm-free or immune Greyface cross Suffolk sheep which were infected with *H. contortus*. They measured the area of the tissue producing neutral and acidic mucins in four zones from the lumen to the submucosa: (1) the surface, pits and isthmus; (2) neck region; (3) lower neck and (4) area adjacent to the submucosa. In control sheep, the SMCs contained only neutral mucins and the pits contained both neutral and acidic mucins. The quantity of acidic mucins increased towards the base of the pits. The isthmus mainly contained acidic mucin, which was also present in the neck. Forty-eight hours after a primary infection, neutral mucin in the surface zone was significantly decreased,

but the amount of sulphated mucin was unchanged. In zone 2, the neck region, there was a decrease in both neutral and acidic mucins, but not in zone 3. In immune sheep, the SMCs contained mainly neutral mucins with a few sulphated mucin granules, the pit cells had neutral mucins and substantial amounts of sulphomucins, the zones 2-4 contained mucins all three types of mucin and sulphated mucins in zone 4. Infected immune sheep had a significantly increased sulphomucins compared with unchallenged animals in the isthmus mucous cells. The mucin profile of immune animals did not change from that in freshly immunised animals for 6 weeks, but by 12 weeks it resembled that in naive animals. At 6 weeks, the sheep were resistant to re-infection, but became susceptible by 12 weeks along with the changing mucin profiles.

The histochemical techniques used in the studies described above involved staining with PAS, AB/PAS pH 2.5 and HID. Inclusion of AB/PAS pH 1.0 in the protocol allows distinction of sialomucins and sulphomucins. These four techniques and staining protocols are described in Myers et al. (2008).

The PAS reaction is used to indicate the presence of carbohydrates in tissue. The principle of the reaction is that periodic acid oxidatively cleaves the carbon-to-carbon bond in 1,2-glycols or their amino or alkylamino derivatives. This cleavage results in the formation of di-aldehydes which will react with fuchsin-sulfurous acid and basic pararosaniline to form a magenta compound.

Staining with AB/PAS pH 2.5 is used routinely to demonstrate the presence of mucopolysaccharides and clearly distinguish acidic and neutral mucins. The principle is that by first staining all acidic mucins with Alcian blue at pH 2.5, those acidic mucins will not subsequently react with PAS, only the neutral mucins. In this way, acidic and neutral moieties will show distinct colours. AB/PAS at pH 2.5 and 1.0 can be used to differentiate the two subtypes of acidic mucins. AB/PAS pH 2.5 stains both sialomucins and sulfomucins, while AB/PAS pH 1.0 stains only sulphated mucins. By comparing the staining intensity of these pairs, together with HID staining, a distinction between sialylated and sulphated mucins can be made.

High iron diamine (HID) is used to demonstrate the presence of sulphated mucins. Ferric chloride acts as an oxidant as well as lowering the pH of the reacting

solution to 1.4. At this low pH, carboxyl groups remain unreacted and thus only sulphate groups will react with staining reagents.

In this study, these four techniques were used to investigate the occurrence, distribution and differentiation of neutral, sialylated and sulphated mucins in the abomasum and duodenum of sheep at different ages (3 days-old to 9 months-old) and after infection with *H. contortus* or *T. circumcincta*.

5.2. MATERIALS AND METHODS

5.2.1. Tissue collection

Tissues were collected at euthanasia from all animals described in Chapter 3 (Sections 3.2.1 to 3.2.4) and Chapter 4 (Sections 4.2.1 to 4.2.4).

The abomasum and duodenum of sheep were removed, opened and gently washed with saline four times to remove worms and adherent debris. A 2x2 cm piece of tissue was collected from the tip of a fold, base of the same fold, the centre of the antrum, duodenal bulb and duodenum 20 cm from the pyloric sphincter. Positions where tissue samples were collected are illustrated in Figure 5.1. A sample was not collected from the duodenum 20 cm from the pyloric sphincter in 3 days-old lambs.

Tissues were selected from one sheep of each age (4-9 months-old) from non-infected, *H. contortus* infected and *T. circumcincta* infected sheep. For lambs 3 days-old to 9 week-old, sections from two lambs were selected for staining. Sheep chosen are marked with asterisk (*) in Appendix 5.3.

5.2.2. Tissue processing

Tissues were fixed overnight in Carnoy's fluid (Appendix 6.1.1) and then in a solution of 4% paraformaldehyde: 2% calcium acetate (1:1 v/v) (Appendix 6.1.2. and 6.1.3) for 3 h. Tissues were then placed in 70% ethanol until they were routinely processed and embedded in paraffin wax. Sections were cut 5 µm thick.

5.2.3. Staining methods

5.2.3.1. Reagents

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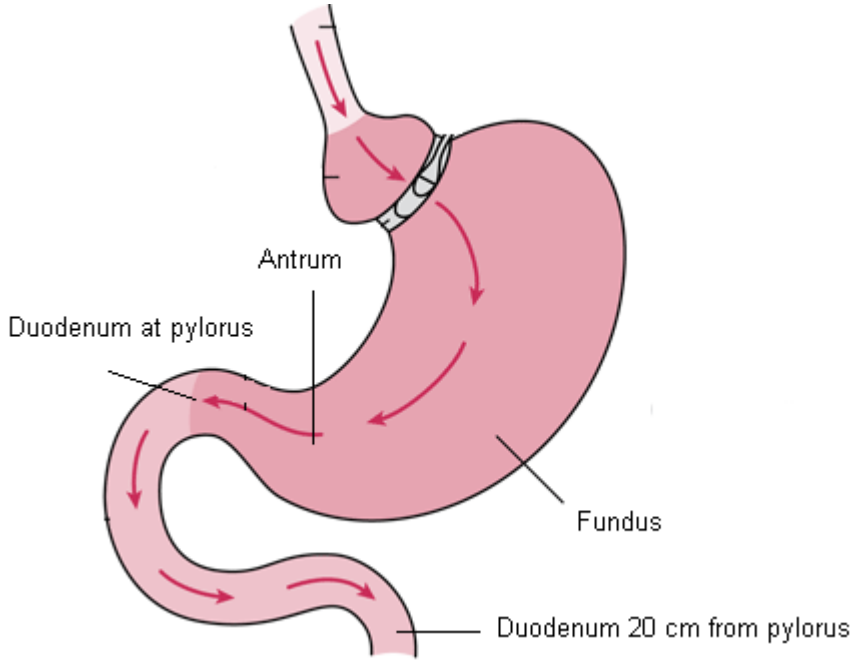

Figure 5.1. Tissue collection sites in the sheep gastrointestinal tract.

Reagents were obtained from Sigma except Alcian blue (Fluka), sodium metabisulfite (Riedel-de Haen), N,N-dimethyl-m-phenylendiamine dihydrochloride and N,N-dimethyl-p-phenylendiamine monohydrochloride (Fluka).

Preparation of Schiff's reagent, Alcian blue pH 1.0 and 2.5, HID is described in Appendix 6.1.7 to 6.1.9.

5.2.3.2. Staining of sections

Four stains were used to investigate neutral, amino and acidic sugars (Appendix 6.2.1 to 6.2.3): (1) PAS stained carbohydrate residues; (2) AB/PAS pH 2.5 stained both neutral and acidic sugars including sialylated and sulphated mucins; (3) AB/PAS pH 1.0 stained neutral and sulphated mucins and (4) HID stained only sulphated mucins.

Sections were photographed under bright field illumination with an Olympus Motorised system microscope Model BX61 with a coupled digital camera (Olympus DP71) and monitored by AnalySIS Professional software (LifeScience). The magnification used was 100x.

5.2.4. Interpretation of staining patterns

The staining of the mucus-producing cells, SMCs, pit cells and MNCs in the fundus, SMCs, pit cells and gland cells in the antrum and goblet cells and Brunner's glands in the duodenum is reported but not the background staining of other cell types.

PAS stained all glycoproteins, including mucins. Strong staining of mucus-producing cells was interpreted as indicating the presence of mucins. Mucins stained magenta, while other carbohydrates were a pale pink background.

Sulphomucins stained grey-brown with HID, whereas non-sulphated mucins remained unstained. These brown cells could be differentiated from nuclei which occasionally stained black.

Alcian blue pH 1.0 stained blue glycoproteins containing sulphated groups. Strong blue staining of mucus-producing cells indicated the presence of sulphomucins, while neutral and/or sialylated mucins stained magenta. Cells that

produced neutral, sialylated and sulphated mucins stained purple. The brown staining with HID correlated with blue or purple staining with AB/PAS pH 1.0.

Comparison of the staining patterns with AB/PAS pH 2.5 and AB/PAS pH 1.0 allows the identification of sialylated mucins, as Alcian blue pH 2.5 stains blue all acidic sugars, both sialylated and sulphated. Cells producing only acidic mucins stain blue. Other cells which produce only neutral, but not acidic, mucins stain magenta, while cells that produce both neutral and acidic mucins stain purple. Therefore, sialylated mucin is assigned to the mucus-producing cells that stain blue to AB/PAS pH 2.5 and magenta to AB/PAS pH 1.0.

5.3. RESULTS

Histochemistry was carried out on sections collected from both the tip and base of a fold in the fundus. As there were no significant differences in staining between these two locations, in Figures 5.2-5.14, only one section of the two from the base of a fold is shown.

5.3.1. Mucin types produced by mucus-producing cells

In sheep at all ages except in 3 days-old animals, in the fundus, SMCs contained only neutral mucins. Pit cells and MNCs contained both neutral and acidic mucins (sialylated and sulphated) in the fundus as did pit cells and gland cells in the antrum. Goblet cells in the duodenum produced all three mucins types, while Brunner's glands contained mostly neutral mucins, a small amount of sialylated mucins, but no sulphated mucins.

5.3.2. Effects of age and weaning on mucins

5.3.2.1. Sulphation

SMCs in the abomasum and Brunner's glands in the duodenum did not produce sulphated mucins. Sulphated mucins were found only in pit cells and MNCs in the fundus, pit cells and gland cells in the antrum and goblet cells in the duodenum.

The sulphation of mucins decreased with age. In the duodenum, goblet cells in the duodenum of young lambs 3 days-old to 9 weeks-old (Figures 5.2-5.4)

produced more sulphomucins than in older lambs 4-9 months-of-age (Figures 5.5-5.7). In the fundus, the staining of pit cells and MNCs for sulphated mucins decreased from 3 days-old to 9 months-old. A similar decrease with age was observed in the pit cells and gland cells of the antrum.

Weaning decreased the sulphation of mucins in the pit cells and MNCs in the fundus, but not in the antrum or duodenum (Figures 5.3 and 5.4).

5.3.2.2. Sialylation

Sialylated mucins were not found in the SMCs of the abomasum, except in the 3 days-old lambs (Figure 5.2). At all ages, sialomucins were produced abundantly in the pit cells and MNCs in the fundus, as well as in pit cells and gland cells in the antrum. There was a relatively large proportion of sialylated mucins compared with neutral mucins in the goblet cells in the duodenum. Overall, the total mucin stained by PAS in goblet cells was small compared with the abomasum. In the Brunner's glands, sialomucins were abundant at 3 days-of-age, but in older animals sialylation decreased with age and was absent by 6 months-of-age (Figure 5.6). The limitation in this study was not in detecting the absence or presence of sialylation but in comparing the relative amounts of acidic and neutral mucins using staining with AB/PAS at pH 2.5 and pH 1.0.

Any effects of weaning on sialylation were not clear, mainly because the total mucins were greater in the milk-fed lambs, making comparison of intensity of staining difficult (Figures 5.3-5.4).

5.3.2.3. Total mucins

The total mucins produced at the two different sites in the abomasum were different: PAS stained extensively in the antrum at all ages, whereas in the fundus, total mucins decreased with age. At 3 days-of-age, a large amount of glycoproteins were present in mucus-producing cells of the fundus (Figure 5.2). It remained high in the milk-fed lambs, but was less in the solid-fed group and further decreased from 4-9 months-of-age (Figures 5.3-5.7).

The total mucin stained with PAS in the goblet cells was small compared with the large amount found in the Brunner's glands at all ages. In addition, the

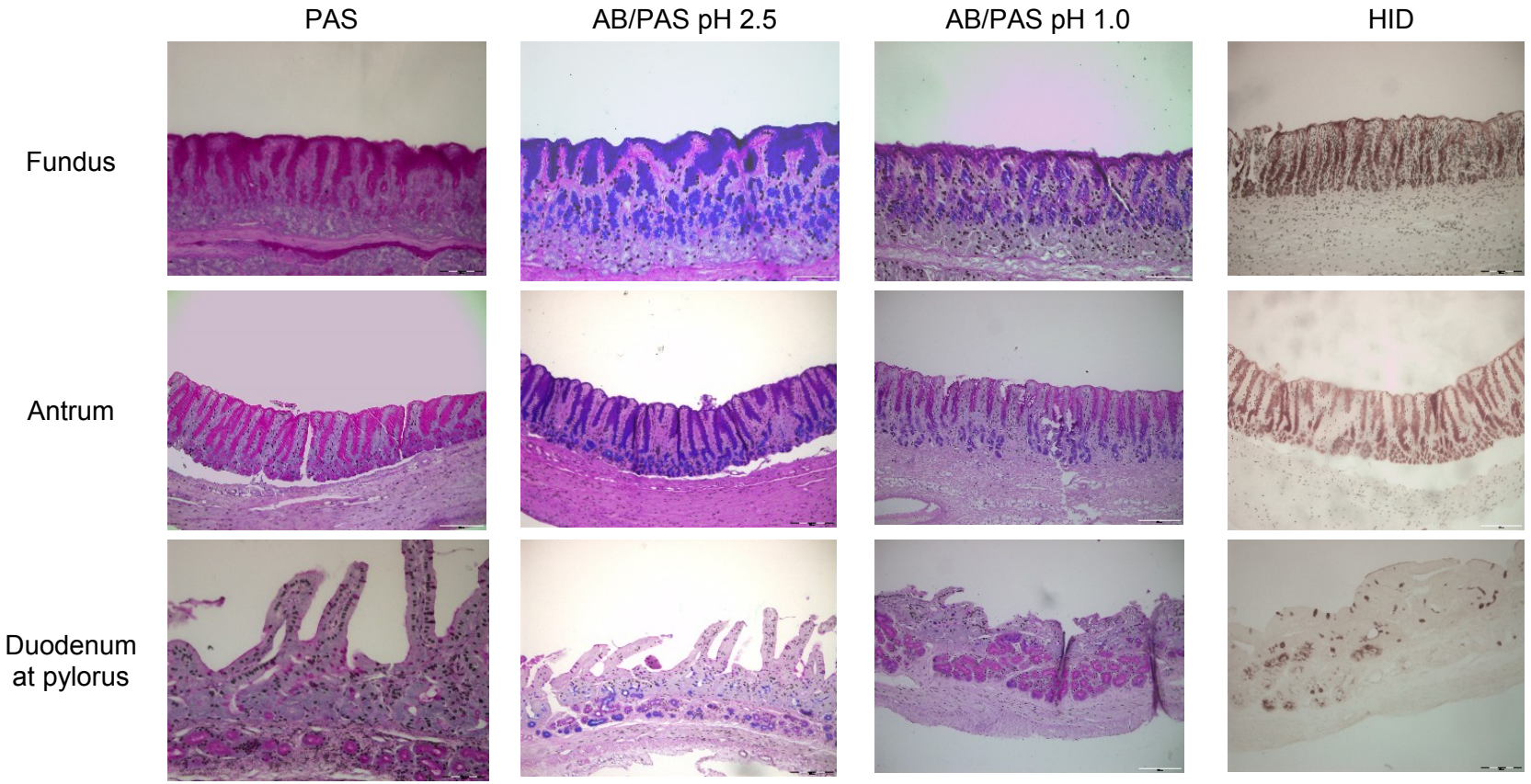

Figure 5.2. Histochemical staining of sections from a non-infected, 3 days-old lamb. PAS stains total mucins; AB/PAS pH 2.5 stains acidic mucins blue, neutral mucins magenta; AB/PAS pH 1.0 stains sulphated mucins blue, neutral and sialylated mucins magenta; HID stains sulphated mucins brown. Original magnification 100x.

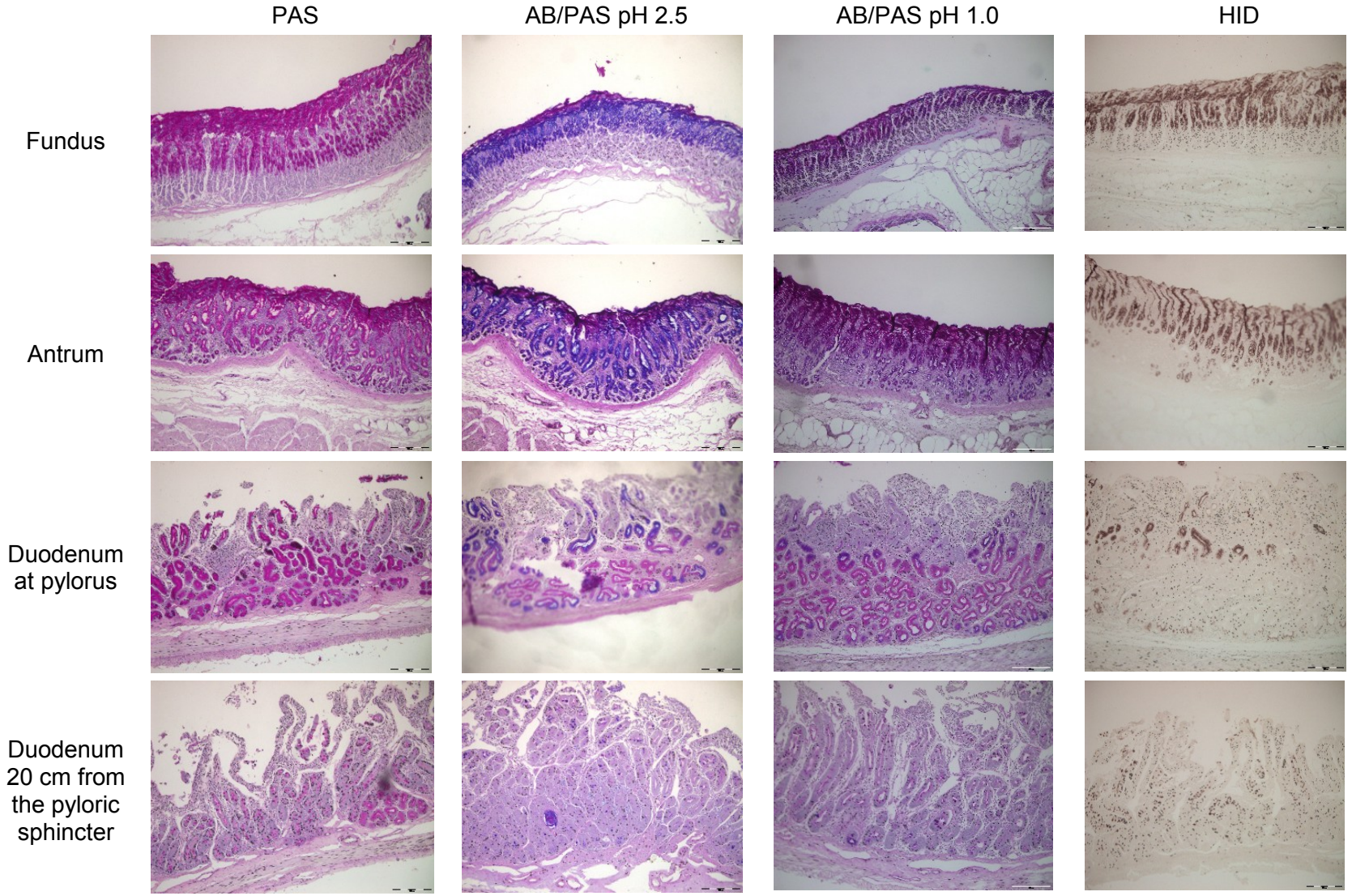

Figure 5.3. Histochemical staining of sections from a 9 weeks-old, milk-fed lamb. Original magnification 100x.

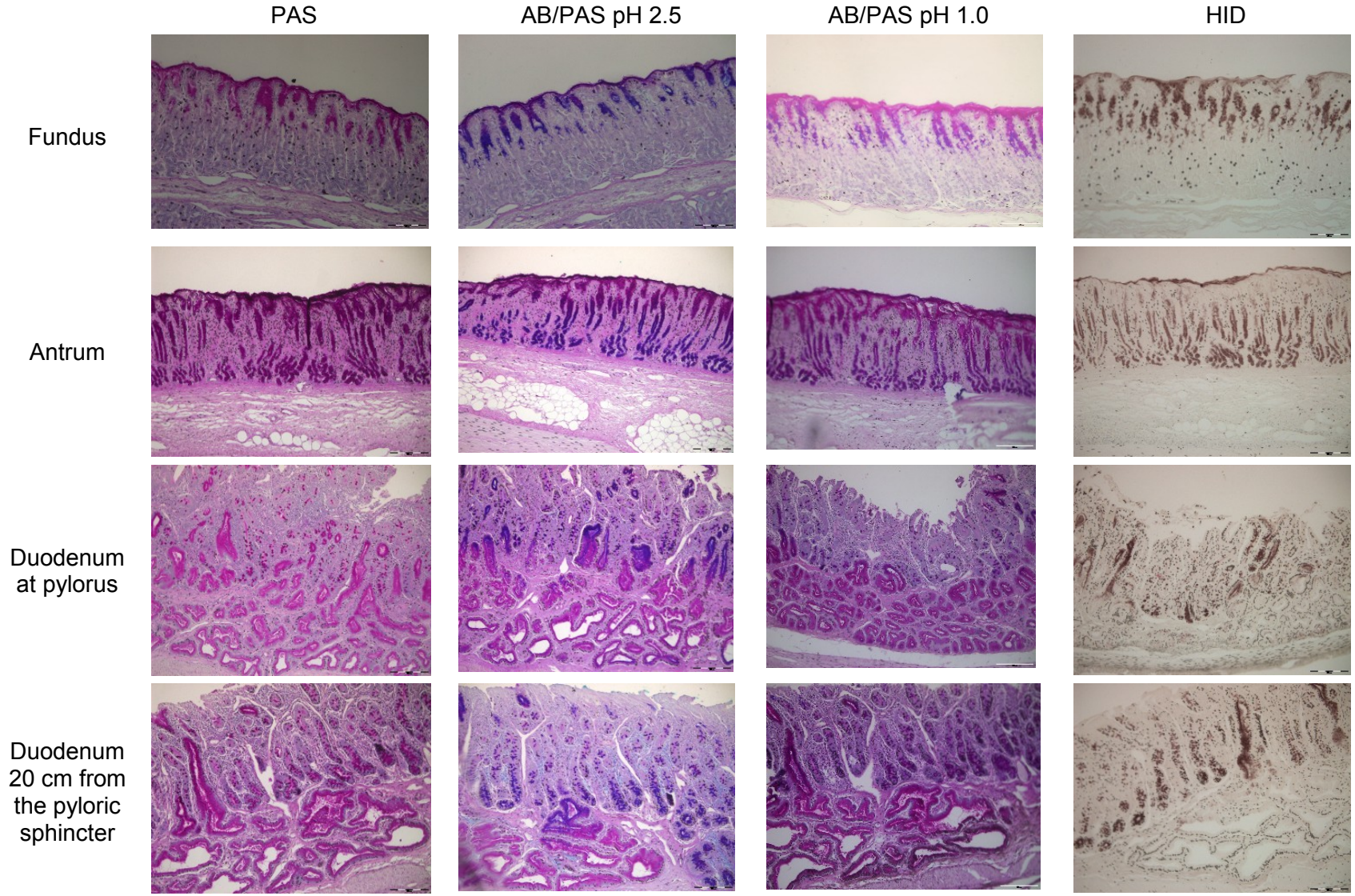

Figure 5.4. Histochemical staining of sections from a 9 weeks-old, solid-fed lamb. Original magnification 100x.

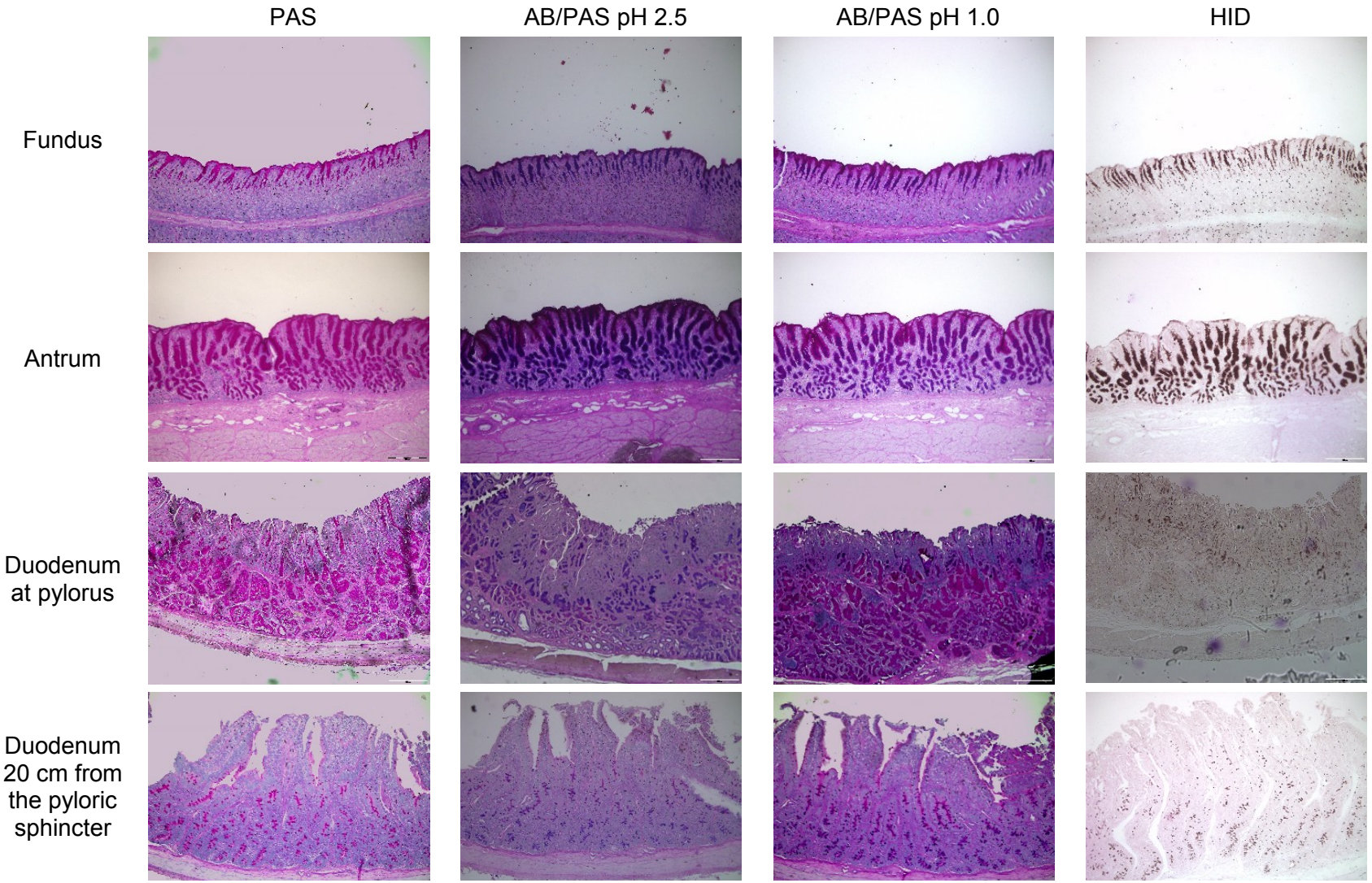

Figure 5.5. Histochemical staining of sections from a non-infected, 4 months-old sheep. Original magnification 100x.

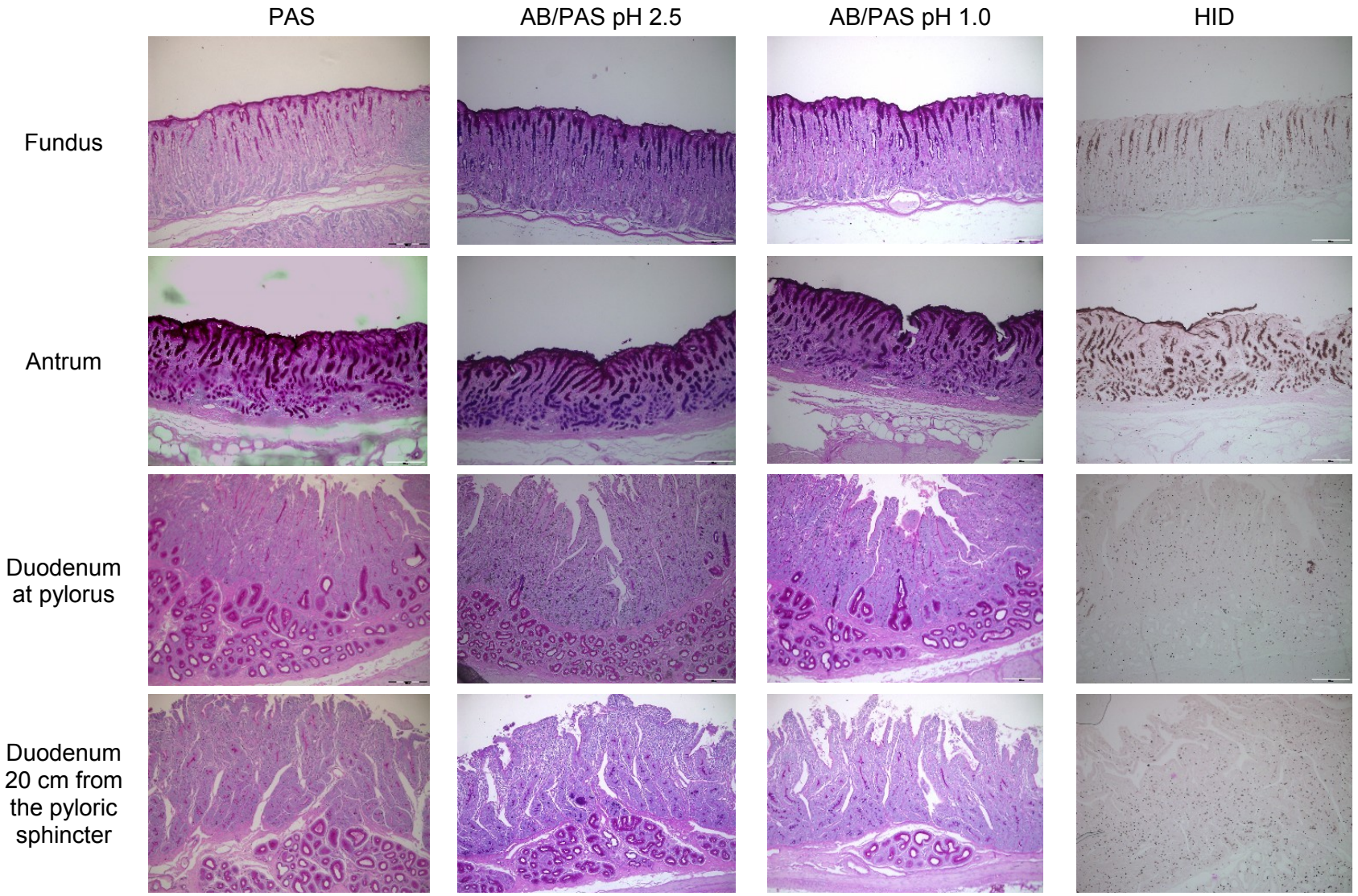

Figure 5.6. Histochemical staining of sections from a non-infected, 6 months-old sheep. Original magnification 100x.

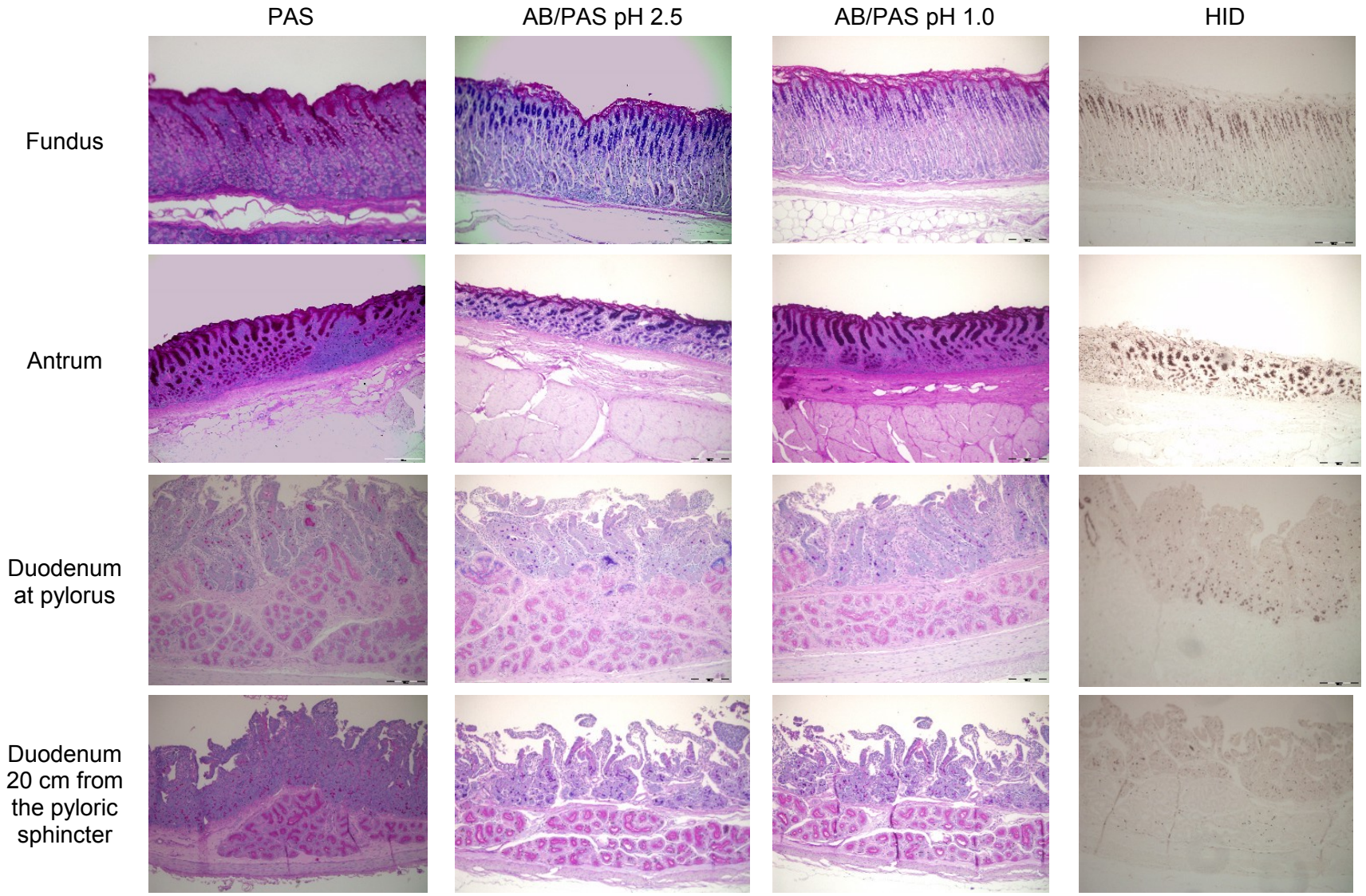

Figure 5.7. Histochemical staining of sections from a non-infected, 9 months-old sheep. Original magnification 100x.

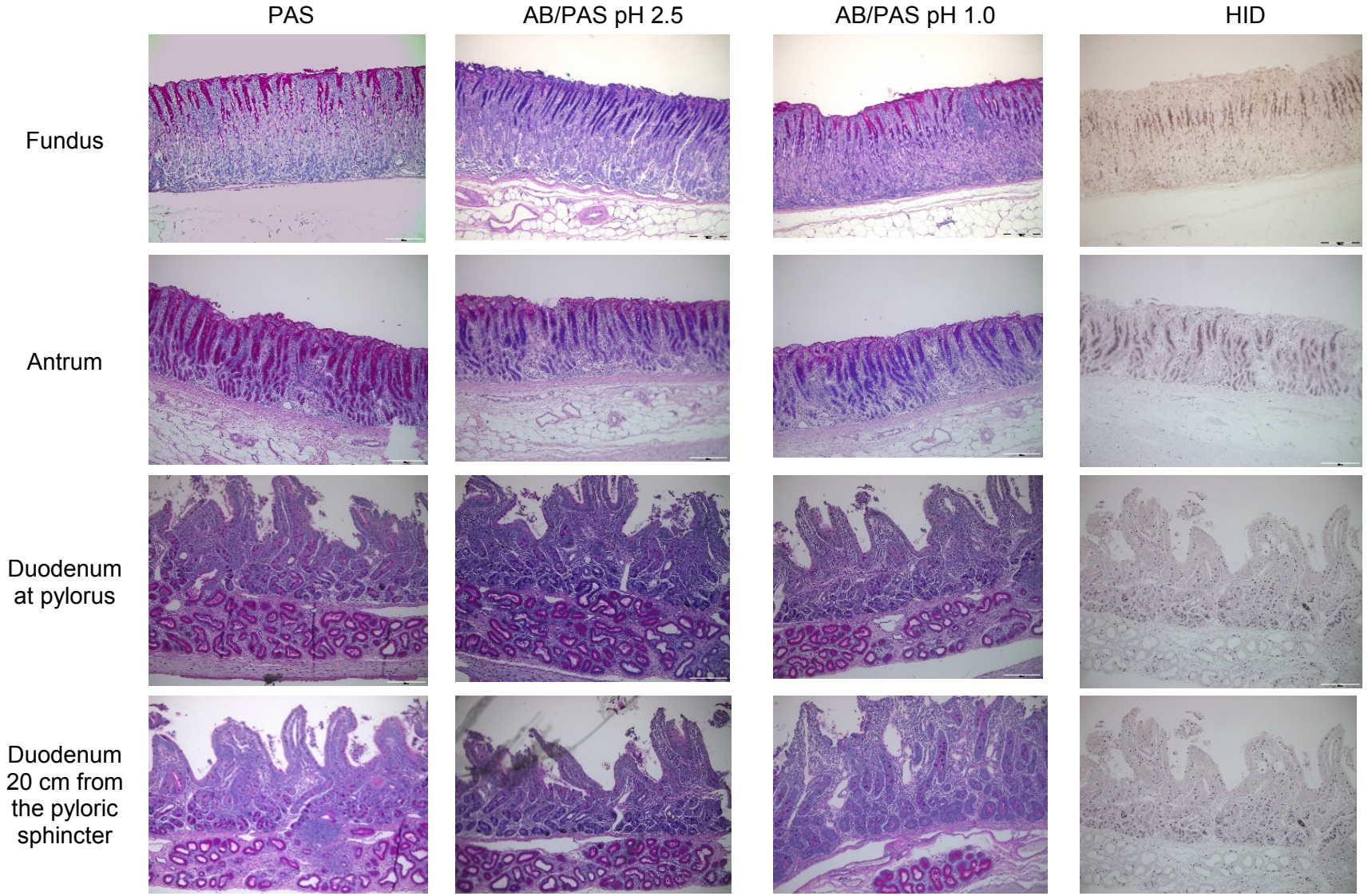

Figure 5.8. Histochemical staining of sections from a *H. contortus* infected, 4.5 months-old sheep, euthanased at day 21 p.i. Original magnification 100x.

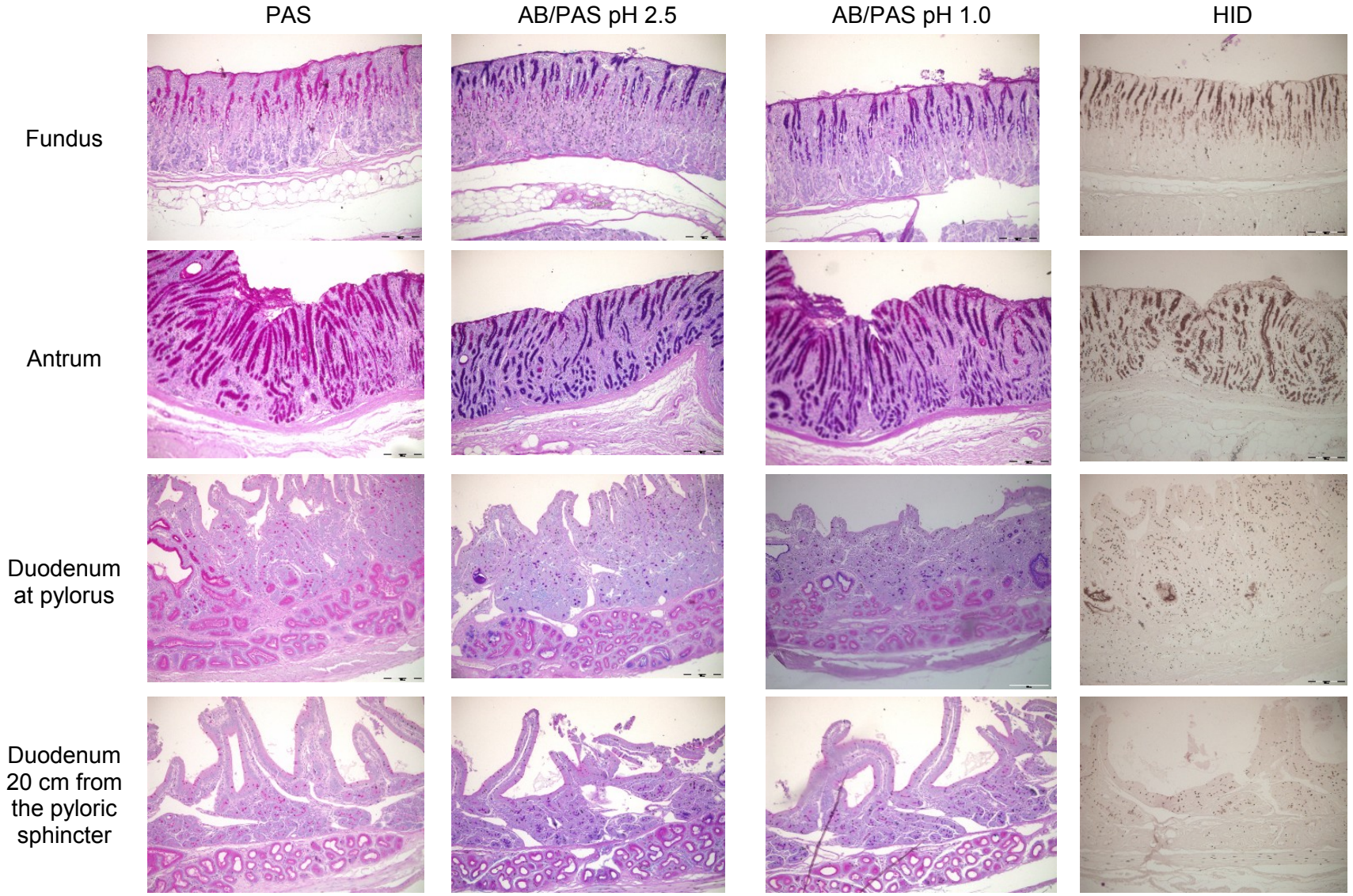

Figure 5.9. Histochemical staining of sections from a *H. contortus* infected, 6 months-old sheep, euthanased at day 21 p.i. Original magnification 100x.

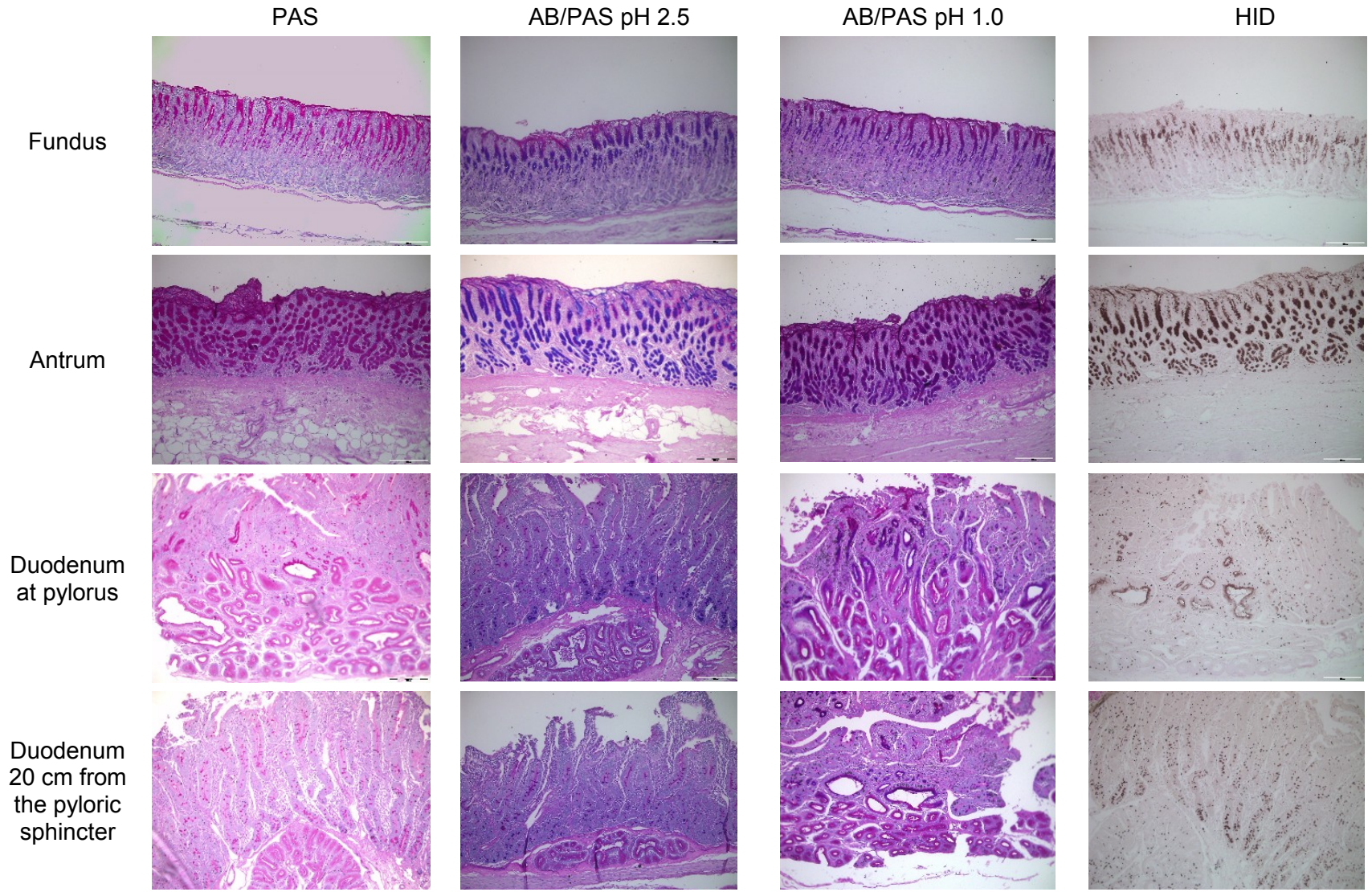

Figure 5.10. Histochemical staining of sections from a *H. contortus* infected, 9 months-old sheep, euthanased at day 21 p.i. Original magnification 100x.

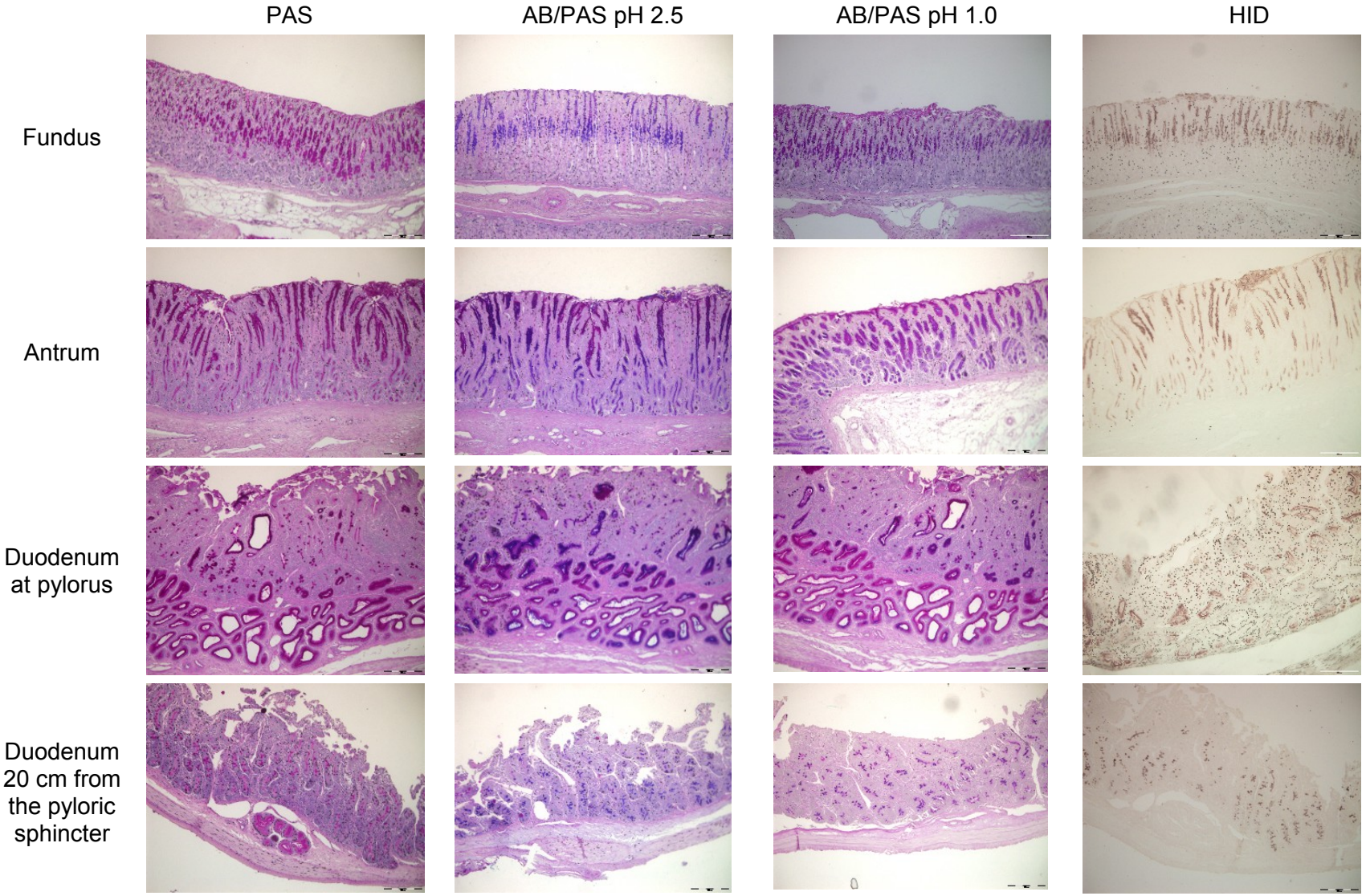

Figure 5.11. Histochemical staining of sections from a 9 weeks-old, solid-fed lambs infected with *T. circumcincta* twice weekly, euthanased at day 35 p.i. Original magnification 100x.

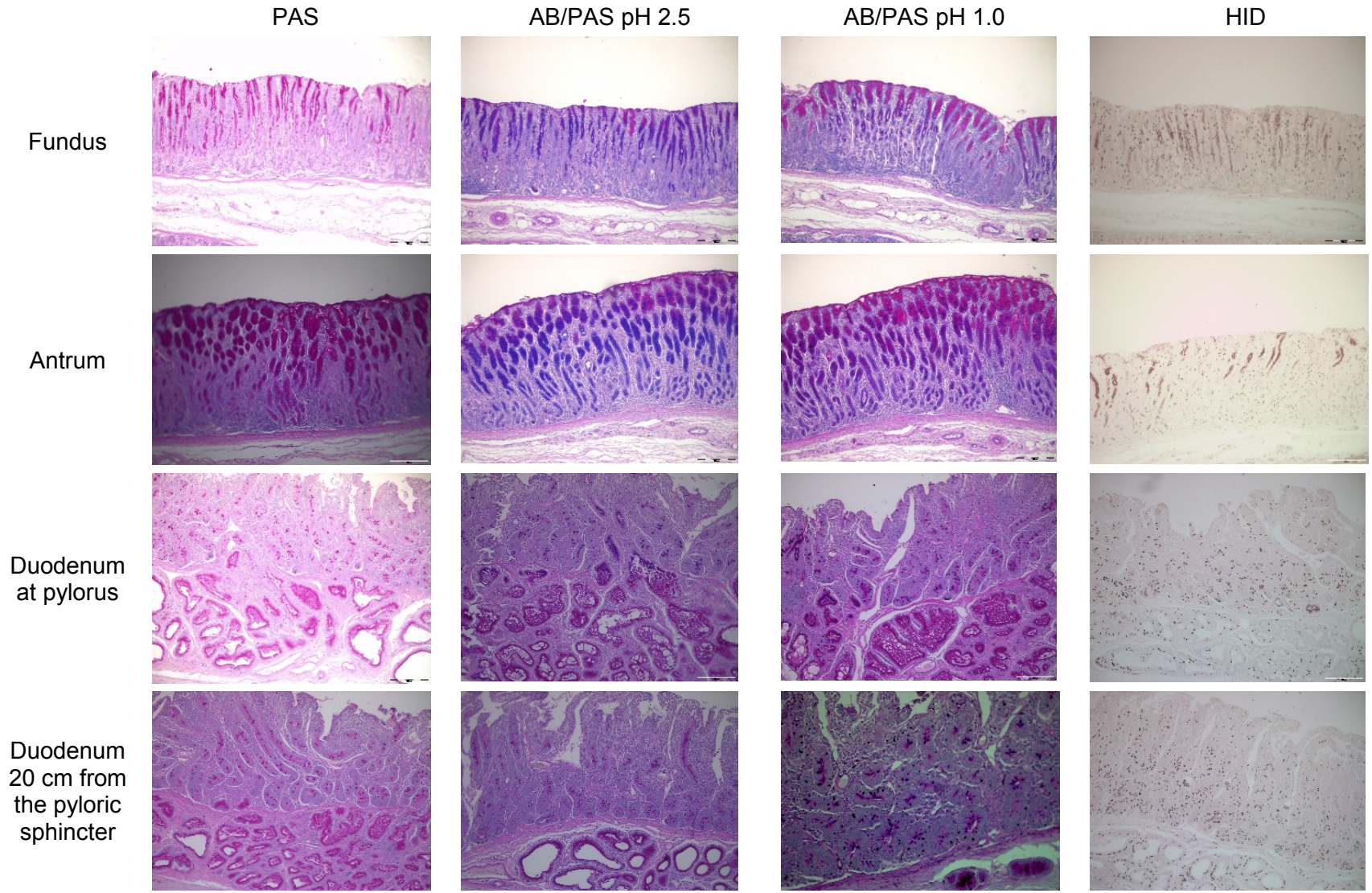

Figure 5.12. Histochemical staining of sections from a *T. circumcincta* infected, 4.5 months-old sheep, euthanased at day 28 p.i. Original magnification 100x.

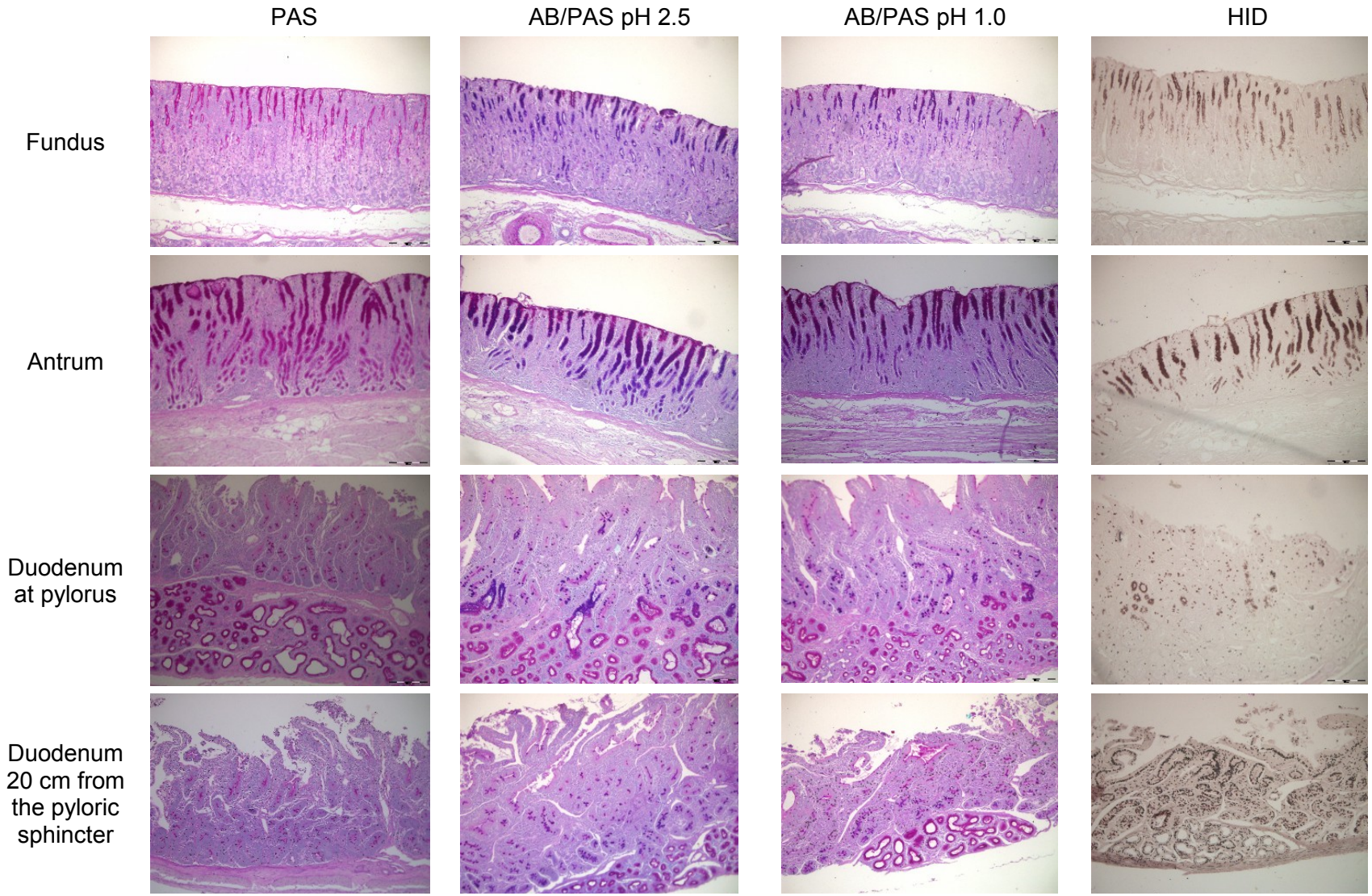

Figure 5.13. Histochemical staining of sections from a *T. circumcincta* infected, 6 months-old sheep, euthanased at day 28 p.i. Original magnification 100x.

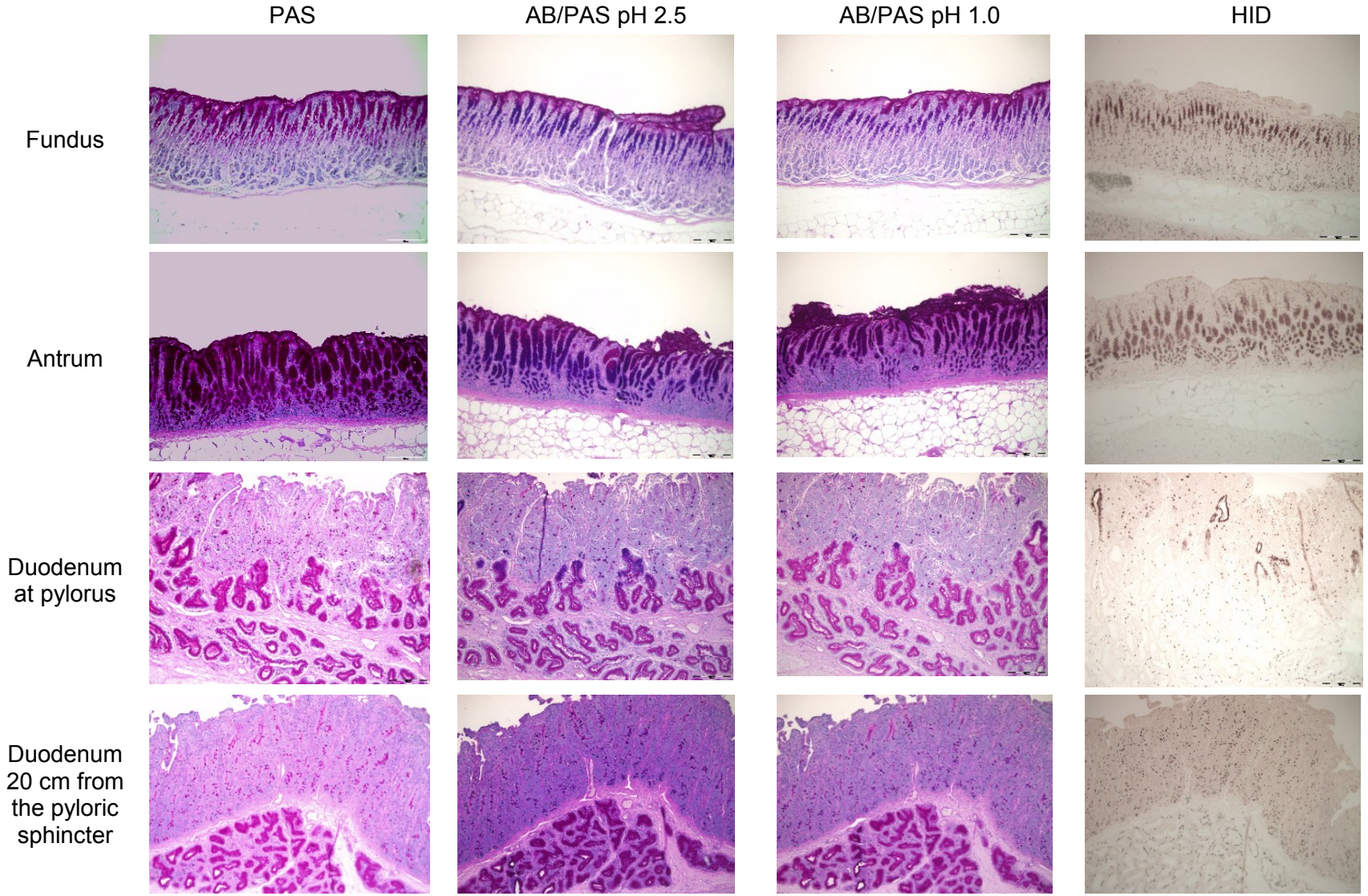

Figure 5.14. Histochemical staining of sections from a *T. circumcincta* infected, 9 months-old sheep, euthanased at day 28 p.i. Original magnification 100x.

mucin production by Brunner's glands increased with age, shown by the staining pattern of the central and peripheral area of the glands with PAS. In young sheep (6 months-old or less), only the peripheral area contained mucins while in the older animal (9 months-old), mucins were found also in the central area.

5.3.2.4. Neutral mucins

Neutral mucins were found most frequently in the SMCs of the abomasum and Brunner's glands in the duodenum. However, their presence in pit cells, MNCs and gland cells in the abomasum and goblet cells in the duodenum was dominated by acidic mucins.

5.3.3. Modification of mucins with parasitism

Infected tissues were thicker than non-infected tissues and the pit and MNC populations were expanded in the fundus and gland cells in the antrum. Comparisons of staining are based on intensity, not the number of cells stained.

5.3.3.1. *H. contortus* infection

At 4.5 months-old, the sulphated mucins produced by mucus-producing cells in both the fundus and antrum of the abomasum of the infected sheep were much less than in the non-infected sheep at the same age. In contrast, in the 6-9 months-old group, sulphation was increased compared with non-infected animals. The sulphation was noticeably less in fundic and antral mucins in the infected younger lambs (4.5 months-old) (Figure 5.8), however, in the older sheep (6-9 months-old) (Figures 5.9-5.10), there was greater sulphation of abomasal mucins. In non-infected sheep at 4 months-of-age, the mucins produced by pit cells in the fundus were strongly sulphated, whereas in the infected sheep, sulphation was mainly seen in the lower pit and MNCs. In contrast, in older infected sheep, pit cells and MNCs in the fundus and pit cells and gland cells in the antrum contained more sulphated mucins in infected sheep than in non-infected animals.

Older infected animals had less sialylated mucins in the fundic pit cells and MNCs than did younger infected animals (Figures 5.8-5.10). At 4.5 months old, the fundic pit cells and MNCs stained strongly for acidic mucins, while HID stained weakly, suggesting that sialylated mucins accounted for a large proportion of the

acidic mucins. In contrast, sialylation was comparatively less in older infected sheep (6-9 months-old). This trend was also seen in non-infected animals, making the effects of infection difficult to determine. In both infected and uninfected animals, the pit cells and MNCs showed strong staining for sialomucins. In the infected animals, these two cell populations in the fundus were greatly enlarged.

The total mucin stained with PAS in the goblet cells was small in both infected and non-infected animals compared with the large amount found in the Brunner's glands at all ages. There was no clear effect of infection on goblet cell mucins. Brunner's glands never showed staining for sulphated mucins, while goblet cells showed the presence of all three mucin types. In Brunner's glands, only the peripheral area contained mucins, except in the 9 months-old non-infected animal, in which mucins were present also in the central area of the gland.

5.3.3.2. *T. circumcincta* infection

As in sheep with *H. contortus* infection, the sulphation of abomasal mucins was reduced by infection in younger animals, however, in older animals (6-9 months-old), the sulphation of abomasal and duodenal mucins was greater in *T. circumcincta*-infected than uninfected animals. Reduced sulphation was clearly visible at 9 weeks- and 4.5 months-of-age in sheep infected with *T. circumcincta* (Figures 5.11 and 5.12). At 4.5 months old, there also was a remarkable decrease in mucin sulphation in the abomasum (Figure 5.12) compared with the non-infected sheep: pit cells in the fundus of the non-infected sheep contained a large proportion of sulphated mucins, while in the infected sheep, pit cells and MNCs stained weakly. There was a marked decrease in sulphation in the pit and gland cells of the antrum: non-infected sheep showed strong staining with HID, whereas there was weak staining in infected animals. Goblet cell sulphation decreased with *T. circumcincta* infection at 4.5 months old. In the 6 months-old sheep, sulphated mucins were mainly found in the pits of the antrum while in 9 months-old sheep, they were mostly contained in lower part of the pits and glands. Pit cells and MNCs in the fundus and goblet cells in the duodenum showed more sulphated mucins in infected sheep than in non-infected animals.

There were only small amounts of neutral mucins and total mucins in the SMCs in the fundus of *T. circumcincta* infected sheep at 9 weeks old (Figure 5.11)

and at 4.5 and 6 months old (Figures 5.12 and 5.13). That was less than in the uninfected animals at the same age. No effects on total mucins were seen in the antrum. Unlike *H. contortus* infected sheep, in the 9 months-old *T. circumcincta* infected sheep, the total mucins produced in the Brunner's gland were greater than in the uninfected animal. In the 4.5 and 6 months-old infected sheep, total mucins in Brunner's glands were reduced and unchanged respectively by infection.

In the 4.5 months-old sheep, the fundic pit cells and MNCs stained relatively strongly for acidic mucins, whereas the sialylation appeared to be comparatively less in older infected sheep (6-9 months-old). The distribution of cells staining for acidic mucins varied with individual sheep. At 9 weeks- and 9 months-of-age, the staining was in the lower pit and MNCs, but also spread through the pits at the other ages. Sialomucins were located within the cells of the pits, but there was very little seen in the lumen (Figure 5.15)

5.4. DISCUSSION

5.4.1. Total gastrointestinal mucins

The amount of mucin present in the cells of the abomasal tissue sections decreased with age. This differs from the chemical study of Ishihara et al. (1985) in which the total glycoprotein per gm of tissue were greater in sheep than in lambs. The total mucin in the milk-fed lambs was greater than in the weaned animals. *H. contortus* infection did not produce any alterations in the amount of total mucins in the abomasum and duodenum. After *T. circumcincta* infection, total mucins produced by Brunner's glands in infected sheep were less at 4.5 months old, unchanged at 6 months old and increased at 9 months old compared with non-infected animals at corresponding ages.

5.4.2. Sulphation of gastrointestinal mucins

Sulphated mucins were present in the fundic and antral SMCs in 3 days-old lambs, but not in the older animals (9 weeks-old to 9 months-old). In the duodenum, sulphated mucins were present in the goblet cells but not in the Brunner's glands, consistent with the study of Ohwada and Suzuki (1992) in adult sheep. In contrast, Pedini et al. (2001), reported that sulphomucins were present in

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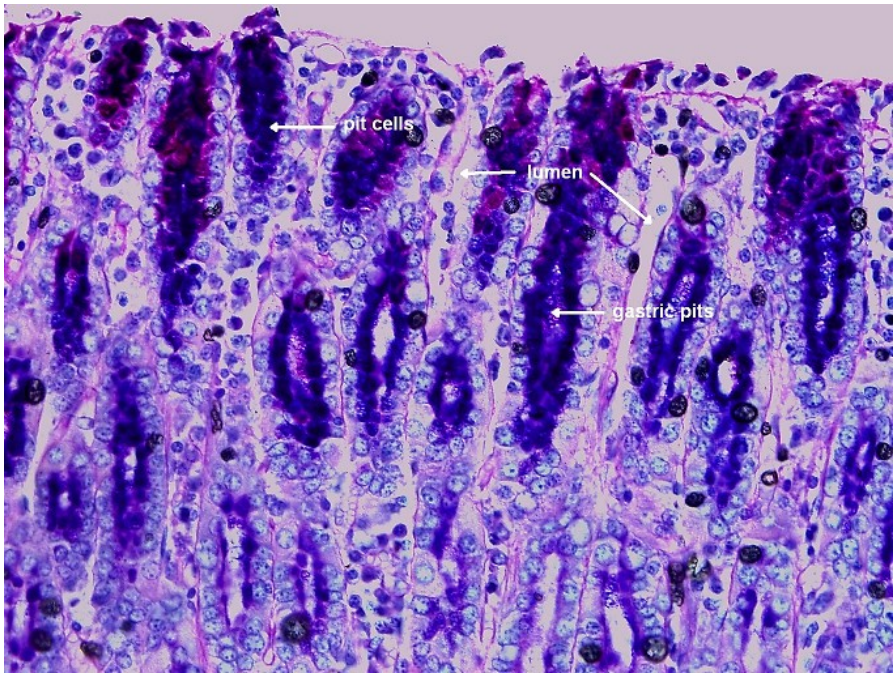

Figure 5.15. Sections of fundic tissue of a *T. circumcincta* infected, 4.5 months-old sheep stained with AB/PAS pH 2.5. Acidic mucins (blue) were present in the gastric pit and pit cells, but not in the lumen. Original magnification 400x.

both goblet cells and Brunner's glands in the duodenum, at least in some 2 months-old lambs (HID stained from 0-3), and that sulphation was therefore age-dependent.

Sulphated mucins were seen in the pit cells and MNCs in the fundus, pit cells and gland cells in the antrum and goblet cells in the duodenum. In gastric glands, the degree of mucin sulphation depends on their location: in the antrum, mucus cells lose their ability to add sulphate to mucins when they migrate from the gland to the surface of gastrointestinal tract (Kramer et al., 1978). The HID staining decreased with age in all cell types. Therefore, the effects of age on the degree of sulphation were mainly observed in MUC5AC producing pit cells, MUC6 producing MNCs and gland cells in the abomasum and MUC2 producing goblet cells in the duodenum. Although each species has its own distribution of mucosubstances throughout the gastrointestinal tract (Sheahan and Jervis, 1976), decreased sulphation in sheep was consistent with the trend of decreasing sulphate in other animals. This has been reported in the small intestine of rats (Shub et al., 1983), colon of mouse (Hill et al., 1990), the duodenum, ileum, caecum, colon and rectum of pigs (Brown et al., 1988; Turck et al., 1993) and duodenum of fallow deer (Scocco et al., 2001).

Weaning reduced the sulphation in fundic pit cells and MNCs, but there were no visible changes in sulphomucins in either the antrum or duodenum. The inability of Zeng et al. (2001) to infect milk-fed lambs with *T. circumcincta* may be related to the higher sulphation of abomasal mucins. This is supported by a number of studies. Sulphation increases the resistance of mucus to bacterial degradation (Rhodes et al., 1985; Amerongen et al., 1998). The sulphation of tracheobronchial mucin has been proposed to contribute to limit bacterial growth by reducing the availability of mucin as a source of nutrition, therefore, facilitating the resistance of mucin to infection by *P. aeruginosa* (Chance and Mawhinney, 2000). Sulphomucins are reported to prevent the colonisation of the gastric mucosa by *H. pylori* (Piotrowski et al., 1991). Syrian golden hamsters with highly sulphated mucin expelled worms faster than mucins of other breeds of hamsters with less-sulphated mucins (Shi et al., 1994). *S. venezuelensis* adult worms were not able to invade mucus where it was highly sulphated (Maruyama et al., 2002). Cebra (1999)

suggested that the high sulphation of mucins is a protective mechanism for the young animals, in which acquired immunity is not fully developed.

Infection with *H. contortus* or *T. circumcincta* reduced the sulphation of abomasal and duodenal mucins in young sheep (9 weeks- to 4.5 months-of-age), but increased sulphomucins in animals 6-9 months-of-age. In sheep 4.5 months-old, infection with *H. contortus* caused a marked decrease in sulphation in the pit cells and gland cells of the antrum, the pit and MNCs in the fundus and in the goblet cells of the duodenum (Figure 5.12). The increase in sulphation in older animals agrees with the observation of Newlands et al. (1990) that infection of immune 9-10 months-old sheep with *H. contortus* resulted in a significant increase in sulphomucins in the isthmus mucous cells, compared with unchallenged animals. This supports the earlier suggestion that the 8-9 months-old animals had natural immunity to *T. circumcincta* infection. Key modifications in the abomasum may be the increased sulphation, increased total mucin secretion and reduced sialylation (Table 3.5).

The host may increase sulphation as a protective mechanism to prevent pathogen establishment and degradation of mucins. Increased sulphation of sheep mucins would make the mucin molecules more resistant to attack by parasite enzymes (Ishihara et al., 1985; Rhodes et al., 1985; Amerongen et al., 1998). Alterations in the secretions of mucus-producing cells occur in animals infected with bacteria. The ability of microorganisms to degrade mucins may be partly responsible for alterations in the mucin structure. *H. pylori* caused increased mucin sulphation in the gastric mucosa (Liau et al., 1992). In contrast, *H. pylori* secreted a glycosulphatase capable of removing sulphate ester groups on C^6 of GlcNAc, Gal and Glc; this suggests that *H. pylori* is capable of overcoming interference with its colonisation of the gastric mucosa by host sulphomucins (Slomiany et al., 1992). Challenge with *S. typhimurium* led to increased sulphation of mucins in goblet cells in the villi, but not the crypts (Arnold et al., 1993). Cystic fibrosis (CF) mucins contained a higher proportion of sulphated O-glycans (Davril et al., 1999; Lamblin et al., 2001; Xia et al., 2005) than non-diseased (ND) mucins. Both CF and ND mucins contained GlcNAc-6-sulphate, Gal-6-sulphate and Gal-3-sulphate but CF mucins contained a higher amount (Xia et al., 2005).

5.4.3. Neutral and sialylated mucins

There is a great deal of species variation in the composition of mucins, but these features of sheep mucins are generally similar to those reported by Shehan and Jervis (1976). In the present study, acidic mucins were present mainly in the pit cells and MNCs in the fundus, pit cells and gland cells in the antrum and goblet cells in the duodenum of sheep. Neutral mucins were the predominant type in mature SMCs and Brunner's glands, but were also present in the other mucus-producing cells. Sialylated mucins were seen in Brunner's glands only in young lambs (3 days- to 9 weeks-of-age) and not in older animals (4-9 months-old). Goblet cells contained neutral and acidic mucins, as reported also by Ohwada and Suzuki (1992) who also noted that goblet cells in the crypts stained more strongly than those in the villi.

The distribution of cells staining for acidic mucins varied with individual sheep. Parasitised sheep have a greatly increased numbers of cells producing sialomucins in the pits and neck regions of the fundus, whereas staining for neutral mucins was reduced in SMCs, along with less total mucin. This was marked after *T. circumcincta* infection of sheep at 9 weeks old (Figure 5.11) and at 4.5 and 6 months old (Figures 5.12 and 5.13). Older infected animals had less sialylated mucins in the fundic pit cells and MNCs than did younger infected animals (Figures 5.8-5.10). At 9 weeks and 9 months old, the staining was in the lower pit and MNCs, but also spread through the pits at the other ages. No effects were apparent in the antrum.

In Chapter 3, chemical analysis showed there was a small proportion of sialic acid in the fundic and duodenal mucins. Histochemical study in this chapter showed that sialylated mucins were found within the pits and stored in pit cells and none was seen in SMCs. This may indicate that most of the mucins analysed chemically were from SMC secretions. It probably also reflects the rate of secretion to be higher in the SMCs compared with pit cells, contributing a greater percentage to the mucin content.

A limitation of histochemical staining to investigate sialylated mucins is the subjective assessment of staining intensity, changes in colour from magenta to purple and blue and area of secreting cells. Both sialomucins and sulphomucins are stained blue by AB/PAS pH 2.5, while co-expressed neutral and sialomucins are stained magenta by AB/PAS pH 1.0. Thus, mucins which stain blue with

AB/PAS pH 2.5 and magenta with AB/PAS pH 1.0 are assigned as sialylated mucins. This indirect assessment by comparing the intensity of blue staining at the two pH makes it difficult to determine quantitative changes in mucin sialylation with age and infection. Compared with identifying changes in sialylated mucins, the degree of sulphation was more easily determined.

In both the fundus and the antrum, pit cells and gland cells contained sialomucins and sulphomucins which were not seen in the SMCs, suggesting that gene expression changes as the cell matures and migrates from the pit to the surface. There was a difference in mucus glycoproteins between gastric mucosa of fundus and antrum, particularly the degree of sulphation. While the mucin sulphation in the fundus decreased with age, antral mucins remained strong staining, suggesting that antral mucins are highly sulphated. Ota et al. (1998) suggested that the mucous gel layer covering the gastric mucosa of corpus and antrum has different levels of protective ability or may have different functions. The higher sulphation in the antrum would support their suggestion and may explain why many infections with *H. contortus* and *T. circumcincta* are mainly in the fundus but not in the antrum.

The histochemical study on one or two animals from each group of sheep has demonstrated the decreasing sulphation of some mucins with age and the different effects of parasitism in younger and older sheep. This aspect of mucin composition could not be explained by the chemical methods used for monosaccharide analysis. Effects on sialylated mucins were not so clearcut using conventional AB/PAS histochemistry. Lectin histochemistry is another technique used to investigate the distribution and linkages of monosaccharides in glycoproteins. In Chapter 6, a preliminary study of lectin staining of the tissues from infected sheep is reported. In particular, the effects of infection and age on sialic acids are extended to include the changing pattern of sialic acid linkage during post-translational modification of the mucins.

Chapter 6

LECTIN HISTOCHEMISTRY OF THE ABOMASAL AND DUODENAL MUCINS

6.1. INTRODUCTION

Histochemical staining of glycoproteins in the sheep gastrointestinal tract with PAS, AB/PAS and HID reported in the last chapter provided information on the types of mucins present in the different mucus-producing cells. Changes with age and infection in the sulphation of mucins were clearly seen, as well as generally in the amounts of acidic and neutral mucins. This method, however, does not give detailed information on the nature and biochemical makeup of these glycoconjugates. Lectin histochemistry is reported to be a sensitive method for studying the carbohydrate composition of glycoconjugates (Spicer and Schulte, 1992; Danguy et al., 1998) and has been used to identify the elongation and termination of carbohydrate structures (Restrepo et al., 2000). The technique has been used to investigate mucus glycoproteins in the digestive tract of several vertebrate species (Madrid et al., 1997, 1998, 2000; Ferri et al., 2001).

Lectins are proteins which bind to specific sugar sequences in oligosaccharides or glycoproteins and are useful markers for the localisation and characterisation of glycoconjugates (Sharma and Schumacher, 1995a; Falk et al., 1998; Acosta-Serrano et al., 2001; Freitas et al., 2002). The mechanism of lectin binding to a sugar involves a combination of hydrogen bonding, hydrophobic interactions and metal ions coordination (reviewed by Lis and Sharon, 1998). Hydroxyl groups of carbohydrate interact with amine groups, hydroxyl and oxygen atoms of lectin protein molecules, forming hydrogen bonds. Hydroxyl groups of carbohydrates interact with two adjacent hydroxyl groups of the same amino acid on the protein core, resulting in the formation of bidentate hydrogen which is other common bond in carbohydrate-protein interactions. Co-operative bonding is the

interaction in which hydroxyl groups act simultaneously as donor and acceptor. Although very weak, Van der Waals forces in large numbers also contribute to binding. The stacking of monosaccharides on the side chains of the aromatic amino acids such as phenylalanine, tyrosine or tryptophan is other common type of interaction. Water bridges sometimes are also present in the interaction. The structure of the lectin protein molecule also plays an important role in determining the specificity of a lectin to a carbohydrate (reviewed by Rudiger and Gabius, 2001).

The selectivity of lectins for carbohydrates is not absolute and may also depend on the specific linkage between the terminal and penultimate sugar residues (Spicer and Schulte, 1992) rather than for a single peripheral sugar(s) e.g. *Limulus polyphemus* agglutinin has an affinity for sialic acid linked α-2,3 or α-2,6 to GalNAc, but not for sialic acid linked α-2,3 or α-2,6 to Gal (Slayter et al., 1984). Lectins may bind to sugars with similar molecular conformations e.g. soybean agglutinin (SBA) binds to both Gal and GalNAc and wheat germ agglutinin (WGA) binds to both GlcNAc and GalNAc (Wu et al., 1998). The structure of carbohydrates and their positions in an oligosaccharide also influence the attachment of a lectin (Goldstein and Hayes, 1978), so that monosaccharide residues linked in different ways to the side chain can be distinguished.

As binding of some lectins can be influenced by adjacent sugars, lectins with the same sugar specificity may produce variations in lectin staining patterns both between tissues and within tissues (Stanley and Phillips, 1999). Both BPL and PNA are specific for Galβ1,3GalNAc, however, their staining patterns are distinct. The binding of PNA to its receptor oligosaccharides can be inhibited by sialylation, even if sialic acid is not bound directly to the receptor sugars. Absence of lectin binding is therefore not the confirmation of the absence of a particular glycan structure, nor does positive staining confirm its presence, since there may be cross-reactions which interfere with the specificity. Treatment with neuraminidase (sialidase) is often used to remove sialic acids (Powell and Varki, 2001) which may be interfering with lectin binding. As well, after sialic acids are removed, the penultimate sugar residue is exposed and may be identified by binding to this sub-terminal carbohydrate.

Lectins bind to other glycoconjugates than mucins. O-linked oligosaccharides, the major glycans in mucins, can be removed by β-elimination by treatment with alkali (Ono et al., 1983). Provided there is complete removal of O-linked oligosaccharides, stronger staining of any sugars with lectins suggests that they are present on N-glycans. Conversely, loss of staining indicates that those sugars were located on O-linked oligosaccharides.

Altered lectin staining is usually a good indication of modification of the glycosylation, despite the limitations of the technique. Human gastric mucus has been probed with 27 different lectins to investigate glycan expression and the structure of the mucus layer (Jiang et al., 2004) and to demonstrate glycosylation changes in the pig intestine with age, weaning (Jaeger et al., 1989; Gelberg et al., 1992), different diets (More et al., 1987) and infection (Choi et al., 2003). Olson et al. (2002) found seven blood group A-like structures in infected rats that were not seen in control rats. They suggested that this change in glysosylation in mucins and other proteoglycans by transient induction of the enzyme blood group A glycosyltransferase may be a protective mechanism against microbial infection.

During infection with the fungal pathogen *C. albicans*, human small intestine exhibited degraded terminal mucin glycopeptides (de Repentigny et al., 2000). *P. aeruginosa* not only depleted high MW mucins, but some strains also possessed glycosidases, such as galactosidase, N-acetyl glucosaminidase and N-acetyl galactosaminidase, which would allow further degradation of the molecule (Aristoteli and Willcox, 2003). Bacteria, including the normal gut flora, can modulate some steps in the glycosylation process even without the presence of live bacteria, but only their soluble factors. The soluble product modulin, produced by *B. thetaiotaomicron*, interferes with fucosylation and galactosylation processes (Freitas et al., 2005). They also found the enzymes generating Fucα1,2Galβ1,4GalNAc and Galβ1,4GalNAc are switched-on and those producing NeuAcα2,6Gal are switched-off in goblet cells in the colon in infected mice. Changes in the carbohydrates of mucus glycoproteins could be a protective mechanism that the host uses to cope with pathogenic Helicobacter. Kawakubo et al. (2004) found when O-glycans terminating with α-1,4-linked GlcNAc were present, human gastric mucins were not altered by infection with *H. pylori*.

Changes in mucin glycosylation have been reported in rats parasitised by nematodes, such as *N. brasiliensis* (Nawa et al., 1994; Karlsson et al., 2000; Olson et al., 2002). During infection with *N. brasiliensis*, more sialic acids and GalNAc were present in the mucus granules of goblet cells in the small intestine of rats (Oinuma et al., 1995). In rats infected with *T. spiralis*, there was reduced sialylation and sulphation of secreted mucins during a primary infection and increased GalNAc and sialic acid during ejection (Gardiner, 1976). Karlsson et al. (2000) characterised four new sialylated oligosaccharides containing NANA/NGNA α2,3GalNAcβ1,4Galβ1, which were absent in non-infected animals.

Lectin staining has identified developmental changes in rats and in lambs. During postnatal development, sialylation changes to fucosylation in rat intestinal mucins, together with a change to more α-1,6 Fuc from more α-1,2 Fuc (Torres-Pinedo and Mahmood, 1984). The glycoprotein profiles in the intestine of 2 months-old lambs were studied by lectin histochemistry by Pedini et al., 2001. They identified that enterocytes and Brunner's gland cells contained a large amount of oligosaccharides terminating with sialic acid-Galβ1,3GalNAc while goblet cells contained sialic acids as termini, GalNAc as penultimate residues and also GlcNAc. A few goblet cells and Brunner's gland reacted with UEA, indicating a small amount of α-1,2 Fuc in epithelial cells and duodenal gland cells in sheep.

In the present study, a panel of 19 different lectins was used to stain gastrointestinal glycoconjugates in the fundic, antral and duodenal tissues collected from the sheep gastrointestinal tract. The lectins used in this experiment were chosen to be specific for the monosaccharides present in abomasal and duodenal mucins: Fuc, GlcN, GalN, Gal and sialic acids (Chapters 2-4) as well as for Man, a typical N-glycans sugar. In addition, sections were treated with neuraminidase and subjected to β-elimination. In this study, a preliminary observation on the alterations of carbohydrate residues on sheep gastrointestinal tissues after neuraminidase and β-elimination treatment was obtained from 3 lambs at 4.5 months old, each of them is non-infected, *H. contortus* infected or *T. circumcincta* infected.

6.2. MATERIALS AND METHODS

6.2.1. Animals and tissue samples

Fundic, antral and duodenal tissues were collected from (1) sheep 4-4.5 months-old, 6 months-old and 8-9 months-old (a) non-infected (b) *T. circumcincta*-infected and (c) *H. contortus*-infected and euthanased 28 or 21 days p.i (as described in Chapter 3) and (2) lambs 3 days-of-age, or 9 weeks-of-age (a) milk-fed (b) solid-fed and (c) solid-fed and infected with *T. circumcincta* (as described in Chapter 4). Tissues were collected, fixed and routinely processed for histology as described in Chapter 5, Sections 5.2.1-5.2.3.

One or two animals in each group were selected for staining with 19 lectins (those were chosen are marked with asterisk (*) in Appendix 5.3. The sugar specificities of the lectins are listed in Table 6.1. One section from all tissue blocks was stained with one of the 19 lectins. In 4.5 months-old animals, in which neuraminidase and β-elimination were carried out, 3 sections were used: one for lectin staining, one for neuraminidase treatment and subsequent lectin staining and one for β-elimination and subsequent lectin staining.

6.2.2. Lectin staining

Lectin histochemistry was performed according to the protocol (http://www.ihcworld.com/_protocols/general_IHC/immunoenzyme_pod.htm).

Sections were deparaffinised in xylene and hydrated in a graded ethanol series. They were then heated in a water bath at 95°C for 30 min in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) (Appendix 6.1.5) to retrieve the antigens. Sections were washed with Tris-buffered saline (TBS) washing buffer 1X (10X 0.5 M Tris-base, 9% NaCl, 0.5% Tween 20, pH 7.6) and then incubated in 200 ml TBS buffer containing 0.2 g trypsin (Appendix 6.1.6) at 37°C for 30 min. Sections were rinsed three times with washing buffer and incubated with biotinylated lectins (7.5 µg/ml in TBS) (Vector Labs, Appendix 6.1.10) at room temperature for 1 h. Finally, tissues were incubated with fluorescent-conjugated streptavidin (7.5 µg/ml in TBS buffer) (Alexa Fluor®546 and 488, Invitrogen, USA) (Appendix 6.1.11) at room temperature for 1 h in the dark. The sections then were washed and covered with coverslips.

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Table 6.1. Sugar specificity of 19 lectins used in this study

Lectins*	Abbre-viation	Major sugar specification	Inhibiting sugar
I. GalNAc group			
Bauhinia purpurea lectin	BPL	Gal(β-1,3) GalNAc	100 mM lactose
		Oligosaccharides ending with α-linked GalNAc	
Dolichos biflorus agglutinin	DBA	α-linked GalNAc	200 mM GalNAc
Griffonia simplicifolia lectin I	GSA-I	α-GalNAc	200 mM Gal/ 200 mM GalNAc
		α-Gal	
Maclura pomifera lectin	MPL	α-linked GalNAc	500 mM GalNAc
Soybean agglutinin	SBA	Oligosaccharides with terminal α/β-linked GalNAc or Gal	200 mM GalNAc
Psophocarpus tetragonolobus lectin	PTL	Galactoside (Gal/GalNAc)	100 mM GalNAc
II. GlcNAc group			
Griffonia simplicifolia lectin II	GSA-II	α/β-linked GlcNAc on non-reducing terminal	Chitin hydrolysate or 200 mM GlcNAc
Wheat germ agglutinin	WGA	GlcNAc and oligosaccharides with terminal GlcNAc or chitobiose	500 mM GlcNAc, chitin hydrolysate or 100 mM acetic acid
III. Gal group			
Peanut agglutinin	PNA	Gal(β-1,3)GalNAc	200 mM Gal
Ricinus communis agglutinin	RCA	Oligosaccharides ending in Gal& GalNAc	200 mM Gal or Lactose
IV. Fuc group			
Aleuria aurantia lectin	AAL	Fuc(α-1,6) GlcNAc	100 mM L-Fuc
		Fuc(α-1,3) LacNAc	
Lotus tetragonolobus lectin	LTA	(α-1,2)-linked Fuc	50-100 mM L-Fuc
		Fuc(α-1,3)GlcNAc	

Ulex europaeus agglutinin	UEA	(α-1,2)-linked Fuc	50-100 mM L-Fuc
V. Sialic acid group			
Maackia amurensis lectin	MAL	Sialic acid in α-2,3 linkage	Human glycophorin
Sambucus nigra agglutinin (Elderberry bark lectin)	SNA	Sialic acid attached terminal Gal in (α-2,3) & (α-2,6) linkage	500 mM lactose in buffered saline followed by 500 mM lactose in acetic acid
VI. Man group			
Concanavalin A	ConA	α-linked Man α-D-Glc	200 mM α-methyl mannoside/200 mM α-methyl glucoside mixture
Lens culinaris agglutinin	LCA	α-linked Man D-Glc	200 mM α-methyl mannoside/ 200 mM α-methyl glucoside mixture
Pisum sativum agglutinin	PSA	Man- in N-acetylchitobiose-linked α-Fuc in the receptor sequence	200 mM α-methyl mannoside/ 200 mM α-methyl glucoside mixture
VII. Oligosaccharide group			
Phaseolus vulgaris agglutinin	PHA	Oligosaccharides (also bi&triantennary branched Galβ1,4GlcNAc1,2Manα 1,6)	

* Lectins are divided into 7 groups depending on their specificity

Sections were viewed in a Nikon fluorescent microscope (Y-FL, Nikon, Japan) and photographed with an attached Canon digital camera (Power shot G1 PC 1004, Canon). The photographs were the permanent record as the fluorescence fades with time. The staining was orange if Alexa Fluor®546 was used and green with Alexa Fluor®488.

The negative control was substitution of TBS for the biotinylated lectin. The control for the specificity of staining was pre-incubation of biotinylated lectins with inhibitory sugars. Lectins (20 µg/ml) were incubated with the corresponding hapten sugar inhibitor at a concentration of 0.2 M (Table 6.1). Mixtures of the lectin and corresponding hapten sugar were incubated in a 37°C room on an orbital shaker for 1 h before they were used to stain sections.

6.2.3. Removal of O-linked oligosaccharides by β–elimination

To investigate whether lectins bound to specific sugars on O-linked oligosaccharides, these were removed by β–elimination prior to lectin staining. A preliminary investigation was carried out on three 4-4.5 months-old sheep, one from each of the non-infected, *T. circumcincta* infected and *H. contortus* infected groups. After the trypsin digestion step, sections were incubated in 0.17 M KOH containing 50% (v/v) dimethylsulphoxide and 10% (v/v) ethanol at 45°C for 1 h. Then sections were neutralised in 10 mM HCl and washed in 0.1 M sodium phosphate buffer (pH 7) before staining with lectins.

6.2.4. Neuraminidase treatment to remove terminal sialic acid residues

After the trypsin digestion step, sections were incubated with neuraminidase enzyme at 0.1 U/ml in 0.2 M sodium acetate buffer (pH 5.5) containing 1% (w//v) $CaCl_2$ at 37°C for 1 h before subsequent lectin staining.

6.3. RESULTS

6.3.1. β-elimination

The pattern and intensity of lectin staining after β-elimination in tissues from a non-infected, *H. contortus* infected and *T. circumcincta* infected 4-4.5 months-old sheep are reported in Table 6.2-6.4. Where there was only partial or no blocking by β-elimination of binding of lectins to their specific sugars or linkages, either these sugars were present on oligosaccharides other than O-linked oligosaccharides or that they were on O-glycans that are resistant to the β-elimination treatment.

The binding to fundic tissues of non-infected sheep by the nine lectins, AAL, SNA, GSA-II, GSA-I, SBA, DBA, BPL, PTL and RCA, was completely eliminated after O-linked oligosaccharides had been removed, indicating that they stained carbohydrates which were present on mucin-type oligosaccharides. After β-elimination, UEA, WGA, PHA and MPL has decreased staining. LTA, MAL and PNA did not stain either before or after treatment. After β-elimination, the staining intensity of the Man-specific group of lectins, ConA, LCA and PSA, variably showed no change, was slightly decreased or, in some cases, was stronger. In the antral tissue, UEA, AAL, DBA, MPL, BPL, PNA, PTL and RCA did not stain after O-glycans had been removed. The effects of β-elimination on the duodenal glycans were not as marked as on the abomasal glycans: AAL, MAL and DBA binding were completely eliminated and SBA staining intensity was reduced in both goblet cells and Brunner's glands.

β-elimination was not as effective on tissues from the sheep infected with *H. contortus* or *T. circumcincta* as it was on non-infected tissues. After O-linked oligosaccharide removal, the binding of fewer lectins was lost (reported in Tables 6.2-6.4).

6.3.2. Modifications of mucin sugars with age and infection

6.3.2.1. Sialic acids

There was more sialylated mucin in 3 days-old lambs than in older animals and the sialic acids in abomasal mucins had a different linkage pattern in these young animals. Differences with age on the sialylation of mucins were apparent only in very young sheep. Binding of MAL shows the presence of α-2,3-linked sialic acids, while SNA binds to both α-2,3- and α-2,6- linked sialic acids. Both SNA and

Table 6.2. Lectin staining of the fundus of 4.5 months-old sheep non-infected or infected with *H. contortus* or *T. circumcincta* and euthanased on day 21 or 28 p.i. respectively treated with neuraminidase and β-elimination. The changes in lectin staining after O-linked oligosaccharides removal and after sialic acid removal are marked by arrows.

	Non-infected			*T. circumcincta* infection			*H. contortus* infection		
Lectins	SMC	Pit cells	MNC	SMC	Pit cells	MNC	SMC	Pit cells	MNC
UEA	↓	↓	0	-	-	-	0	↓	-
AAL	↓↓	↓↓	↓↓	↓↓	↓↓	↓↓	↓↓	↓↓	↓↓
MAL	-	0	0	0	0	0	0	0	0
SNA	↓↓	↓↓	0	0	-	0	0	0	0
GSA-II	↓↓	↓↓	0	0	-	-	0↑	-↑	-
WGA	↓	↓	↓	-↑	-↑	-	-	-	-
GSA-I	↓↓	↓↓	0	-	-	0	-	-	0
SBA	↓↓	↓↓	↓↓	0	-	-	-	-	↓
DBA	↓↓	↓↓	↓↓	0	-	-	-	-	-
MPL	↓	0	0	-	-	0	-↑	0↑	0
BPL	↓↓	↓↓	0	↓	↓	0	-↑	-↑	0
PNA	0	0 ↑	0	0	↓↓	↓↓	0	0	0
PTL	↓↓	↓↓	0	-↑	-↑	-	↓↓	↓↓	↓
RCA	↓↓	↓↓	0	-	-	0	↓↓↑	↓↓↑	0
PHA	0	0	0	0	0	0	0	0	0

↓↓ lost staining, ↓ reduced staining, - no change with β-elimination

↑ increased staining with neuraminidase

Table 6.3. Lectin staining of the antrum of 4.5 months-old sheep non-infected or infected with *H. contortus* or *T. circumcincta* and euthanased on day 21 or 28 p.i. respectively treated with neuraminidase and β-elimination. The changes in lectin staining after O-linked oligosaccharides removal and after sialic acid removal are marked by arrows.

	Non-infected			*T. circumcincta* infection			*H. contortus* infection		
Lectins	SMC	Pit cells	Gland cells	SMC	Pit cells	Gland cells	SMC	Pit cells	Gland cells
UEA	↓↓	↓↓	↓↓	↓	↓	↓	↓	↓	↓
AAL	↓↓	↓↓	↓↓	↓↓	↓↓	↓↓	0	0	0
MAL	0	0	0	0	0	0	0	0	0
SNA	-	-	-	-	-	-	↓	↓	↓
GSA-II	-	-	↓↓	↓	↓	↓	↓	↓	↓
WGA	-↑	-↑	-↑	↓	↓	↓	↓	↓	↓
GSA-I	-	-	0↑	↓	↓	0	↓↓↑	↓↓↑	↓↓
SBA	-	-	↓↓	-	-	↓↓	-↑	-↑	↓↓↑
DBA	↓↓	↓↓	↓↓	↓	↓	-	-	-	↓↓
MPL	↓↓	↓↓	0↑	↓	↓	0	-	-	0
BPL	↓↓	↓↓	0	-	-	-	↓	↓	↓
PNA	↓↓	↓↓	0	↓↓	↓↓	0	↓↓	↓↓	↓↓
PTL	↓↓↑	↓↓↑	↓↓↑	↓	↓	↓	↓	↓	↓
RCA	0	↓↓	↓↓	0	↓	↓	0↑	-↑	-↑
PHA	-	-	0	-	-	-	0	0	0

↓↓ lost staining, ↓ reduced staining, - no change with β-elimination

↑ increased staining with neuraminidase

Table 6.4. Lectin staining of the duodenum of 4.5 months-old sheep non-infected or infected with *H. contortus* or *T. circumcincta* and euthanased on day 21 or 28 p.i. respectively treated with neuraminidase and β-elimination. The changes in lectin staining after O-linked oligosaccharides removal and after sialic acid removal are marked by arrows.

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Lectins	Non-infected		*T. circumcincta* infection		*H. contortus* infection	
	Goblet cells	Brunner's glands	Goblet cells	Brunner's glands	Goblet cells	Brunner's glands
UEA	-	-	-	-	↓↓	↓
AAL	↓↓	↓↓	↓↓	↓↓	↓↓	↓↓
MAL	↓↓	0	↓↓	0	↓↓	0
SNA	-	-	-	-	↓↓	↓↓
GSA-II	-	↓	↓	-	-	↓
WGA	-	-	-	-	↓	↓
GSA-I	0	0	0	0	0	0
SBA	↓	↓	↓	↓	↓	↓
DBA	↓↓	↓↓	0	0	↓	↓
MPL	0	-	-	-	0	-
BPL	0	-	0	-	0	0
PNA	-	-	↓↓	↓↓	↓↓	↓↓
PTL	-↑	↓	-	-	↓	-
RCA	0	↓	0	↓	0	↓
PHA	0	0	0	0	0	0

↓↓ lost staining, ↓ reduced staining, - no change with β-elimination

↑ increased staining with neuraminidase

MAL bound to duodenal mucins in all animals, whereas only SNA stained abomasal mucins, except in 3 days-old lambs. This indicates that abomasal mucins in 3 days-old lambs contained both α-2,6- and α-2,3-linked sialic acids, whereas the α-2,3 linkage was not present in older lambs from 9 weeks- to 9 months-of-age (Figure 6.1). Goblet cells in the duodenum stained more strongly with SNA in 3 days-old lambs than in older sheep (Figure 6.2). Duodenal mucins contained both α-2,3- and α-2,6-linked sialic acids at all ages.

Sialylation was less in the fundus and duodenum of sheep infected with either *H. contortus* or *T. circumcincta* compared with that in non-infected tissues at 4.5 months-of-age (Figure 6.3). Nine weeks-old lambs infected with *T. circumcincta* had less sialic acids in duodenal mucins than did weaned uninfected lambs (Figure 6.4).

6.3.2.2. Fucose

The principal linkage of peripheral Fuc was α-1,2-linked Fuc. Three lectins were used with specificities for Fuc in different linkages: LTA for α-1,2-linked Fuc or Fucα1,3GlcNAc; UEA for α-1,2-linked Fuc and AAL for α-1,6- or α-1,3-linked Fuc. In non-infected tissues, there was weak staining for α-1,6- or α-1,3-linked Fuc (AAL), whereas no mucus-producing cells contained α-1,2-linked Fuc or Fucα1,3GlcNAc, as detected by LTA. UEA binding was stronger than for the other two lectins, showing the presence of α-1,2-linked Fuc. LTA binding may be sensitive to the presence of other sugars. If adjacent sialic acid caused the difference in LTA and UEA binding, it could not be confirmed by removal of terminal sialic acids by neuraminidase, as this did not change the binding pattern of any of the Fuc-specific lectins.

UEA staining revealed a trend of increasing fucosylation in abomasal and duodenal mucins with age. In 3 days-old lambs, both fundic and duodenal tissues showed a small amount of mucins terminating with α-1,2-linked Fuc residues, compared with 9 weeks-old lambs and 4-9 months-old sheep. There was a marked increase in α-1,2-linked Fuc in SMCs in the fundus at 4-9 months-of-age, compared with younger animals (Figure 6.5). In the antrum, SMCs and pit cells did not stain for α-1,2-linked Fuc at 3 days-old, but stained very strongly from 9 weeks-old to 9 months-old. In addition, gland cells produced more α-1,2-linked Fuc in

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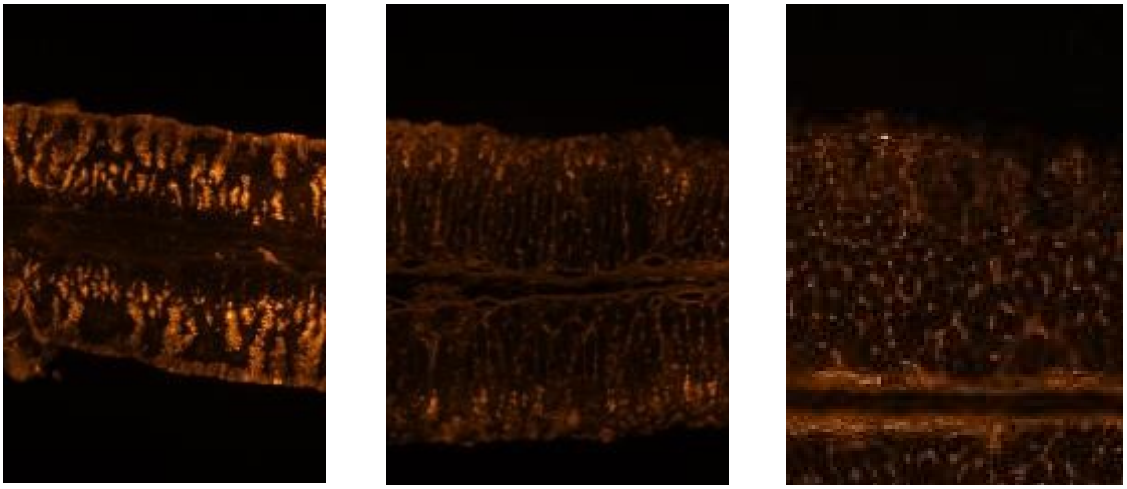
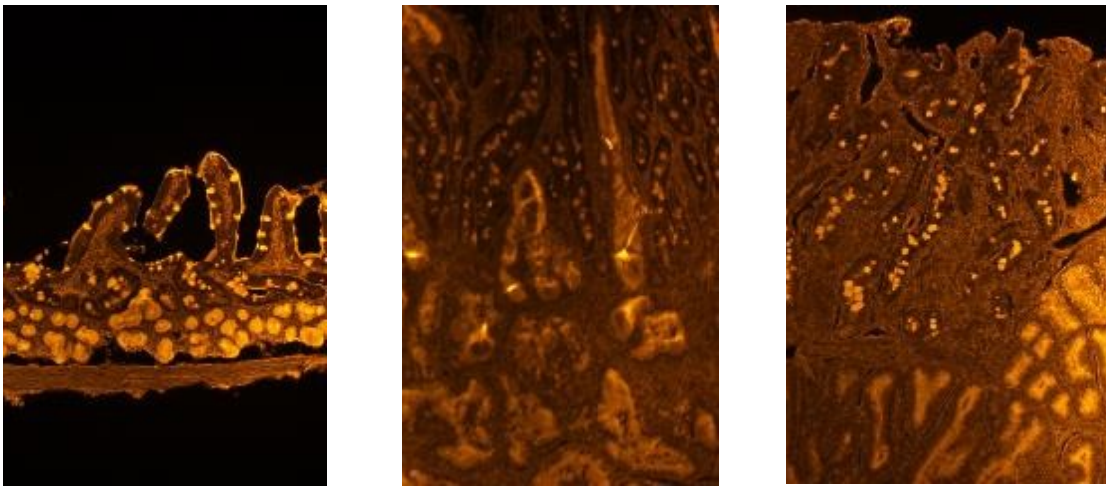


A B C

Figure 6.1. Lectin staining with MAL (α-2,3-linked sialic acids) of the fundus of non-infected, 3 day-old (A), 9 week-old, solid-fed (B) and 4.5 month-old lamb. Original magnification 100x.



A B C

FIgure 6.2. Lectin staining with SNA (α-2,3- and α-2,6-linked sialic acids) of the duodenum of non-infected, 3 day-old (A), 4.5 month-old (B) and 9 month-old lamb (C). Original magnification 100x.

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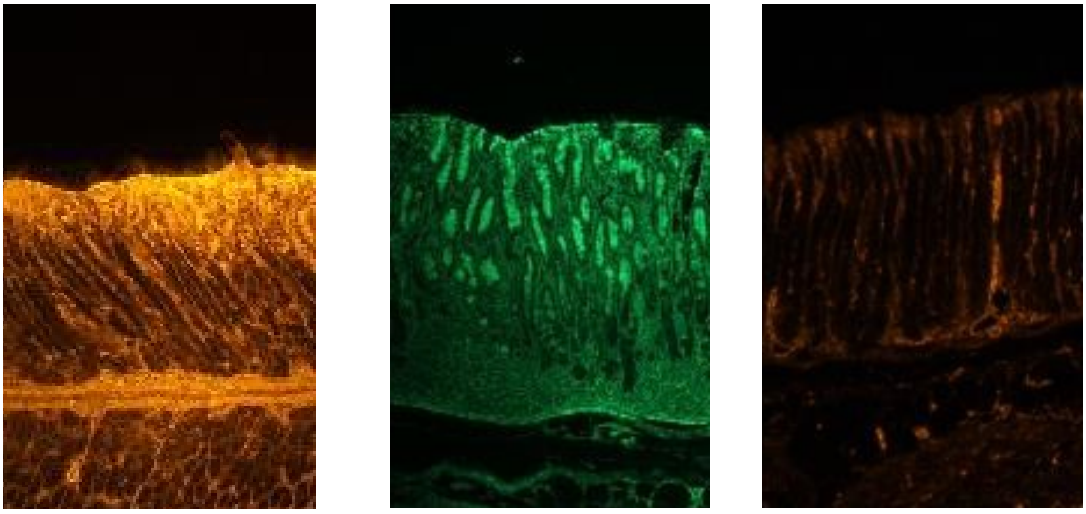

Figure 6.3. Lectin staining with SNA (α-2,3- and α-2,6-linked sialic acids) of the fundus of non-infected (A), *H. contortus*-infected (B) and *T. circumcincta*-infected sheep at 4.5 months old. Original magnification 100x.

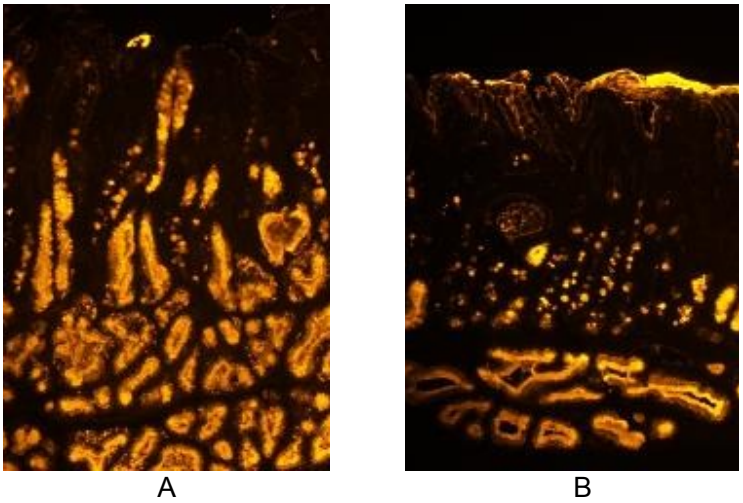

FIgure 6.4. Lectin staining with SNA (α-2,3- and α-2,6-linked sialic acids) of the duodenum of 9 weeks old, solid-fed, non-infected (A) and solid-fed, *T. circumcincta*-infected lamb (B). Original magnification 100x.

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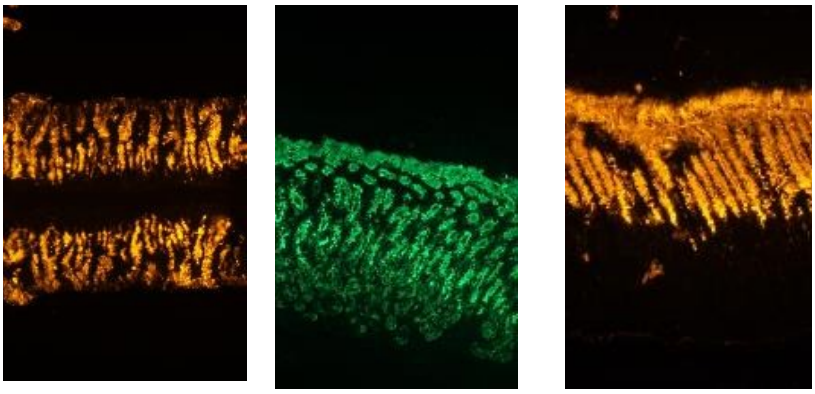

A B C

Figure 6.5. Lectin staining with UEA (α-1,2-linked Fuc) of the fundus of non-infected sheep at 3 days old (A), 6 months old (B) and 9 months old (C). Original magnification 100x.

sheep older than 3 days-of-age (Figure 6.6). Similarly, in the duodenum, both goblet cells and Brunner's glands produced more Fuc with age, shown by the increasing intensity of UEA binding from 3 days-old to 9 months-old (Figure 6.7). In contrast to UEA binding, there was no LTA (α-1,2-linked Fuc and Fucα1,3GlcNAc) binding to mucus-producing cells and AAL (α-1,6- and α-1,3-linked Fuc) staining was weak in the fundus at all ages, but increased in the antrum from 3 days-of-age to 6 months-of-age (Figure 6.8) as well as in the duodenum (Figure 6.9).

Infection with either *H. contortus* or *T. circumcincta* reduced α-1,2-linked Fuc binding (UEA) in antral (Figure 6.10) and duodenal tissues (Figure 6.11) at 9 months-of-age. Whereas there was no LTA binding (α-1,2-linked Fuc and Fucα1,3GlcNAc) in any tissues in non-infected sheep at all ages, it was weak in the pits in the fundus of the 6 months-old *T. circumcincta* infected sheep and moderate in the 9 months-old animal (Figure 6.12), but did not bind to either the antrum or the duodenum. There was no binding of LTA to tissues from the *H. contortus* infected sheep. The effects of infection on AAL staining were variable in different animals, but consistently less in both goblet cells and Brunner's glands in the duodenum of all infected sheep from 9 weeks- to 9 months-old, compared with non-infected animals at the same age (Figure 6.13).

After β-elimination, AAL staining was lost completely in all tissues of both non-infected and infected sheep, while UEA staining was lost only in the antrum of non-infected animals and in goblet cells of *H. contortus* infected sheep. Removal of terminal sialic acids by neuraminidase did not change the binding pattern of any of the Fuc-specific lectins.

6.3.2.3. N-acetyl galactosamine

A total of 7 lectins that bind specifically to GalNAc in different linkages were used: GSA-I, SBA, DBA, MPL, BPL, PNA and PTL. The binding intensities and patterns depend both on the position of the GalNAc residues in the oligosaccharides, as well as on the specificity for the linkages. The same binding patterns were not shown by three pairs of lectins chosen to be specific for the same linkage: GSA-I and SBA (specific for α/β-GalNAc), DBA and MPL (α-GalNAc) and BPL and PNA (Galβ1,3GalNAc). As there was no consistent staining intensity of any of the GalNAc-specific lectins to the mucus-producing cells in the fundus, antrum and duodenum, the effects of age or infection could not be determined.

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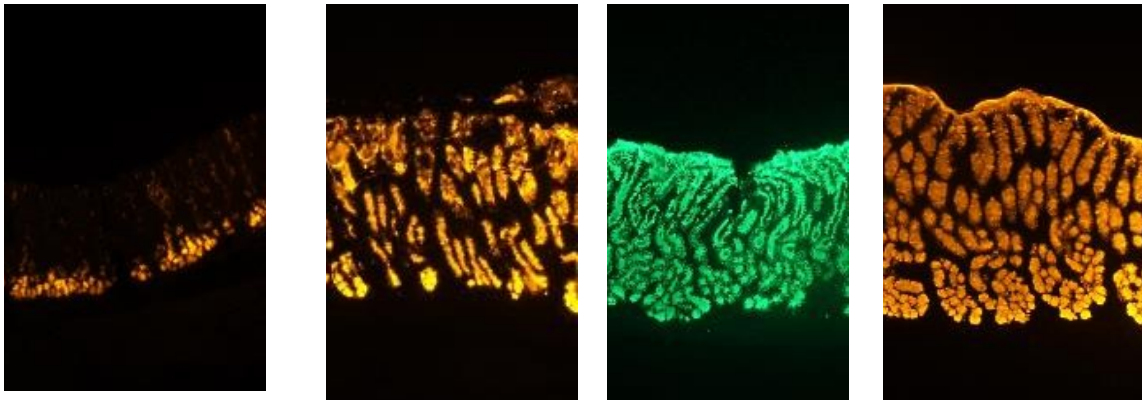
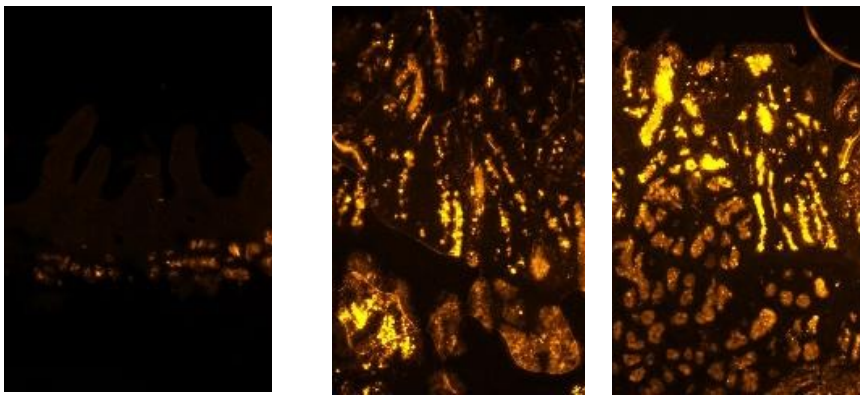


A B C D

Figure 6.6. Lectin staining with UEA (α-1,2-linked Fuc) of the antrum of non-infected sheep at 3 days old (A), 4.5 months old (B), 6 months old (C) and 9 months old (D). Original magnification 100x.



A B C

Figure 6.7. Lectin staining with UEA (α-1,2-linked Fuc) of the duodenum of non-infected sheep at 3 days old (A), 4.5 months old (B) and 9 months old (C). Original magnification 100x.

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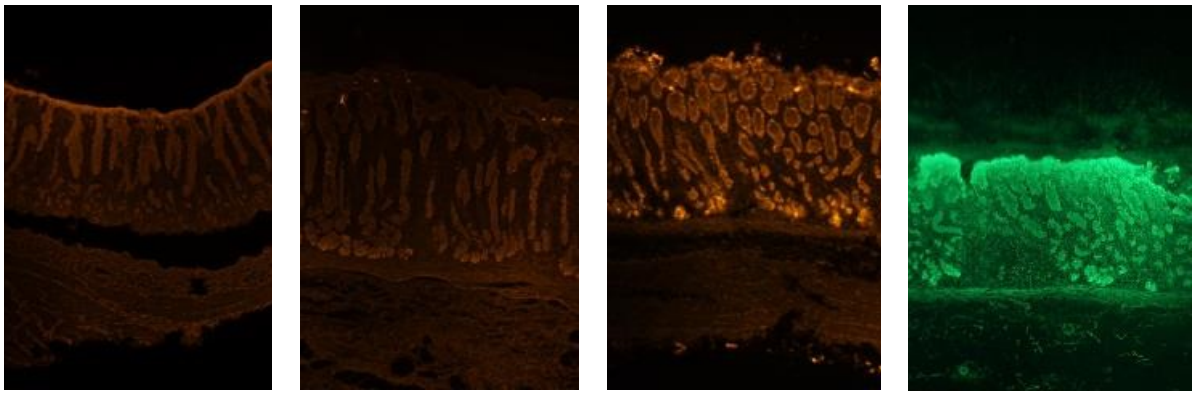
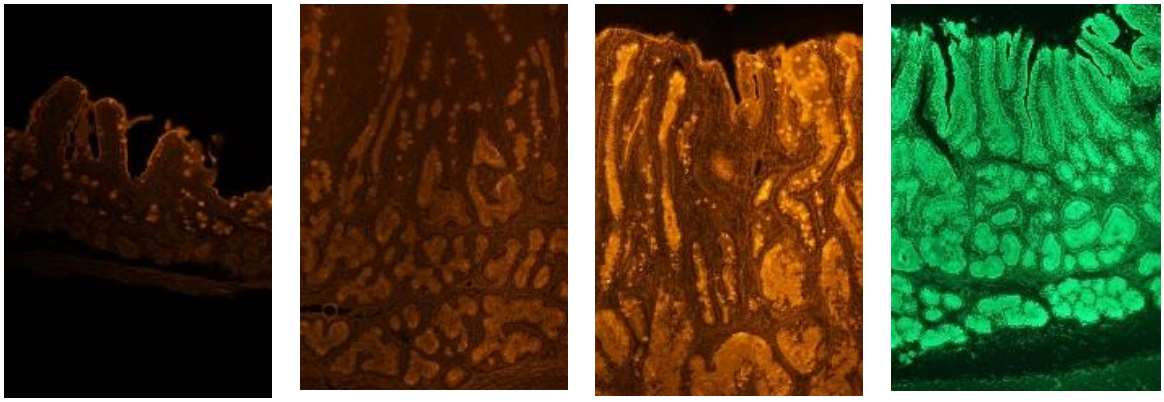


A B C D

Figure 6.8. Lectin staining with AAL (α-1,6- and α-1,3-linked Fuc) of the antrum of non-infected sheep at 3 days old (A), 9 weeks old, solid-fed (B), 4.5 months old (C) and 6 months old (D). Original magnification 100x.



A B C D

Figure 6.9. Lectin staining with AAL (α-1,6- and α-1,3-linked Fuc) of the duodenum of non-infected sheep at 3 days old (A), 9 weeks old (B), 4.5 months old (C) and 6 months old (D). Original magnification 100x.

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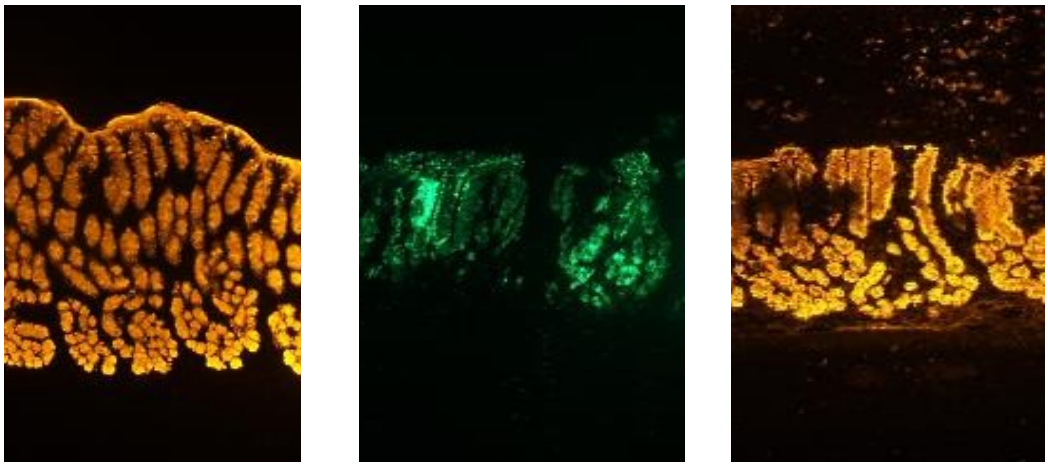
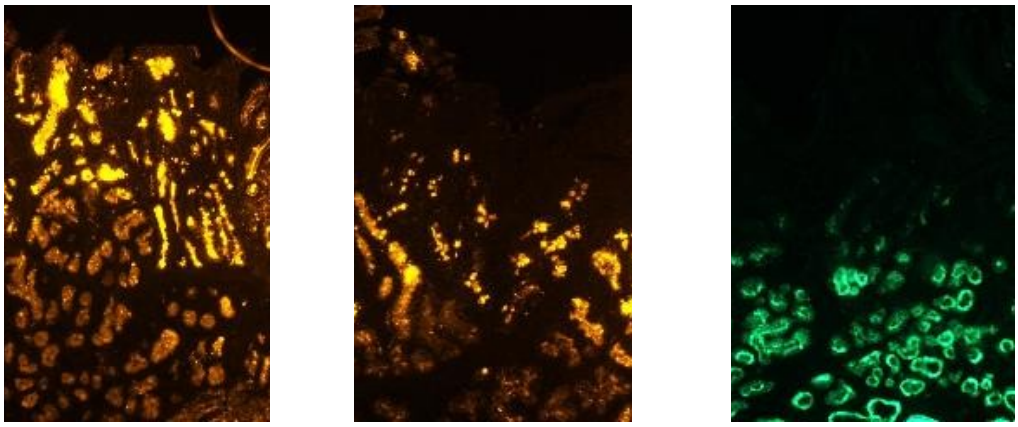


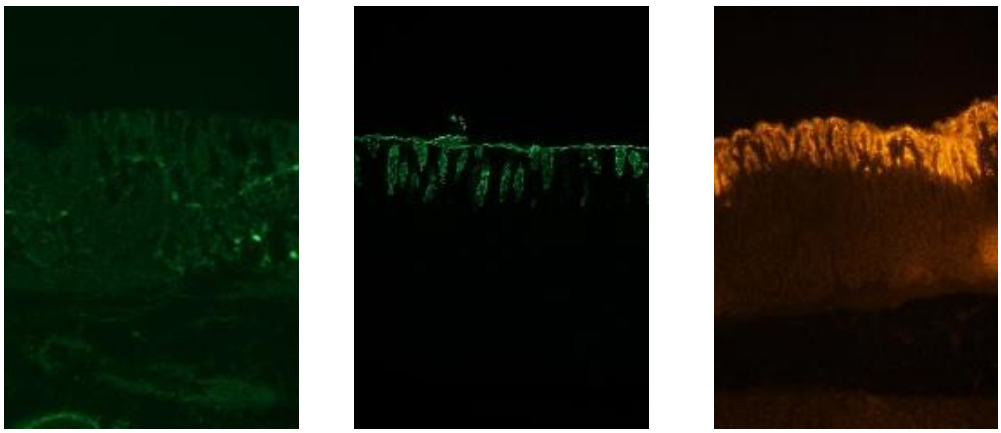
A B C

Figure 6.10. Lectin staining with UEA (α-1,2-linked Fuc) of the antrum of non-infected (A), *H. contortus*-infected (B) and *T. circumcincta*-infected sheep at 9 months old. Original magnification 100x.



A B C

Figure 6.11. Lectin staining with UEA (α-1,2-linked Fuc) of the duodenum of non-infected (A), *H. contortus*-infected (B) and *T. circumcincta*-infected sheep at 9 months old. Original magnification 100x.



A B C

Figure 6.12. Lectin staining with LTA (α-1,2-linked Fuc and Fucα1,3GlcNAc) of the fundus of *T. circumcincta*-infected sheep at 4.5 months old (A), 6 months old (B) and 9 months old (C). Original magnification 100x.

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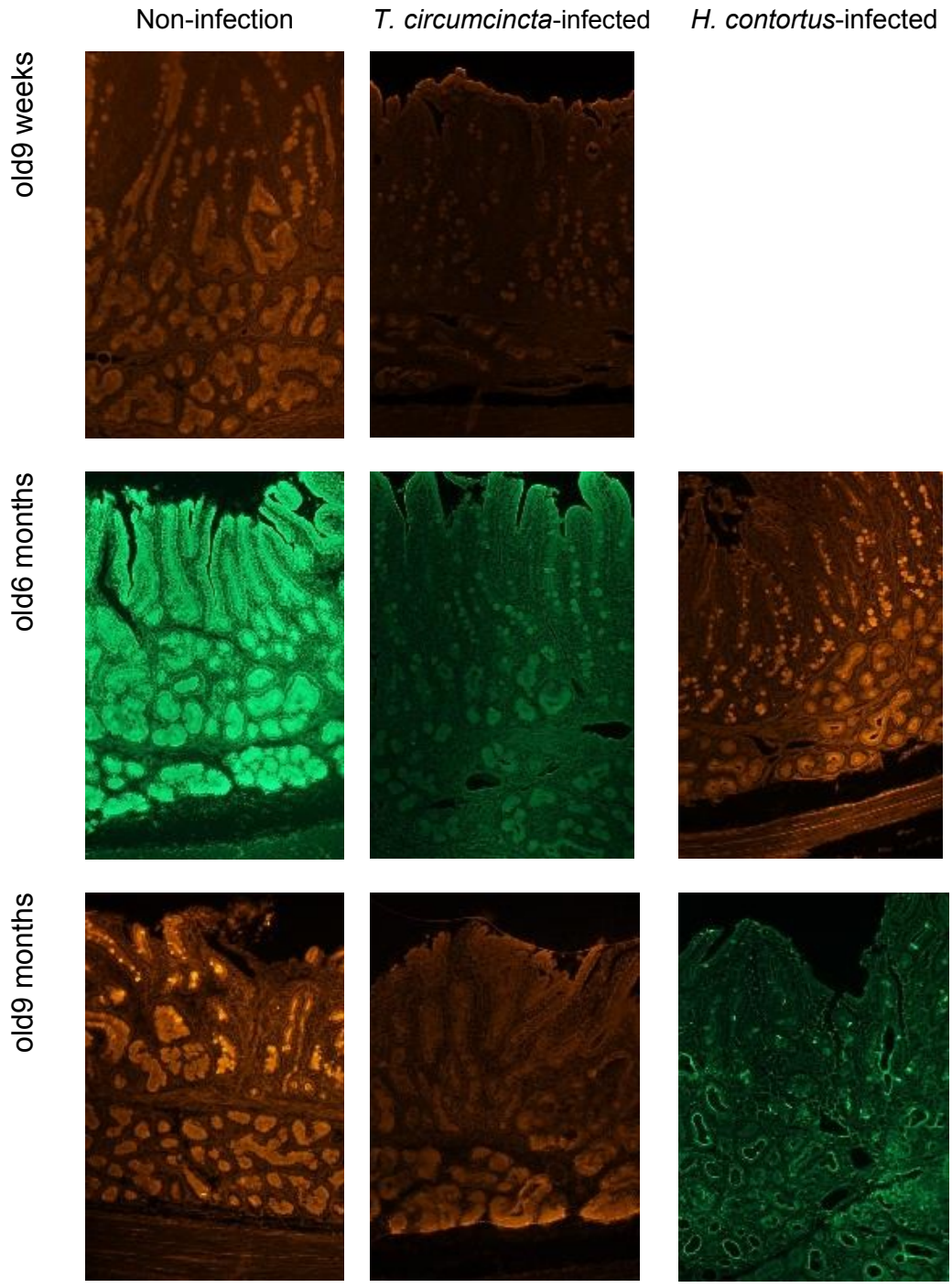

Figure 6.13. Lectin staining with AAL (α-1,6- and α-1,3-linked Fuc) of the duodenum of 9 week-old, 6 month-old and 9 month-old sheep, non-infected or infected with *H. contortus* or *T. circumcincta*. Original magnification 100x.

Neuraminidase treatment did not reduce the variation in lectin staining intensity, but did provide additional information about the presence of specific GalNAc linkages. Pit cell mucins in uninfected sheep contained the Galβ1,3GalNAc linkage, as shown by the lectins BPL and PNA. Whereas there was no PNA staining in untreated fundic tissues of the 4.5 months-old uninfected sheep, PNA bound to pit cells after removal of terminal sialic acids by neuraminidase. In contrast, BPL stained pit cells without treatment and staining was unchanged after terminal sialic acids had been removed (Figure 6.14).

Pit cells in the antrum of solid-fed lambs stained very strongly with PNA while those in milk-fed lambs did not stain (Figure 6.15). In the duodenum, DBA stain with goblet cells and Brunner's glands in weaned but not in unweaned lambs (Figure 6.16), however, duodenal tissues in milk-fed lambs stained with PTL more strongly than in solid-fed lambs (Figure 6.17).

PTL binding to GalNAc or Gal in fundic SMCs and pit cells in *T. circumcincta* infected sheep and to the antrum and duodenum of non-infected sheep was increased by neuraminidase treatment (Figure 6.18). This indicated either the presence of galactosides at the subterminal position on the oligosaccharides or interference with PTL binding by the presence of sialic acids. Similarly, neuraminidase treatment increased MPL (α-GalNAc) and BPL (Galβ1,3GalNAc) staining of fundic tissues and GSA-I and SBA (α/β-GalNAc) binding in the antrum of the *H. contortus* infected 4.5 months-old sheep.

6.3.2.4. Galactose

Gal residues were detected by RCA binding. In the fundus, the amount of Gal was lower at 3 days-old than in older animals (9 weeks- to 9 months-of-age), however, it was not noticeably different in any mucus-producing cells from 4-9 months-of-age (Figure 6.19). RCA staining of antral mucins varied from weak to moderate in SMCs, pit cells and gland cells and showed no age effects. However, in the duodenum, a large amount of Gal was present in both goblet cells and Brunner's glands in 3 days-old lambs and generally less in older sheep up to 6 months-of-age (Figure 6.20). There was a small increase in the amount of Gal produced by goblet cells and Brunner's glands in 9 months-old sheep compared

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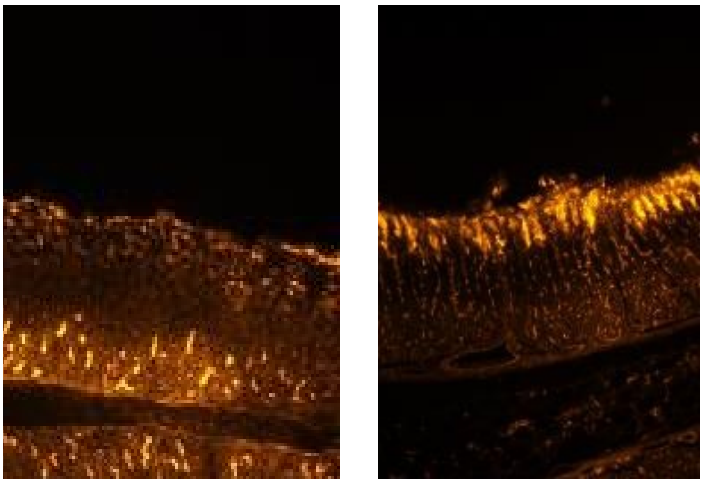
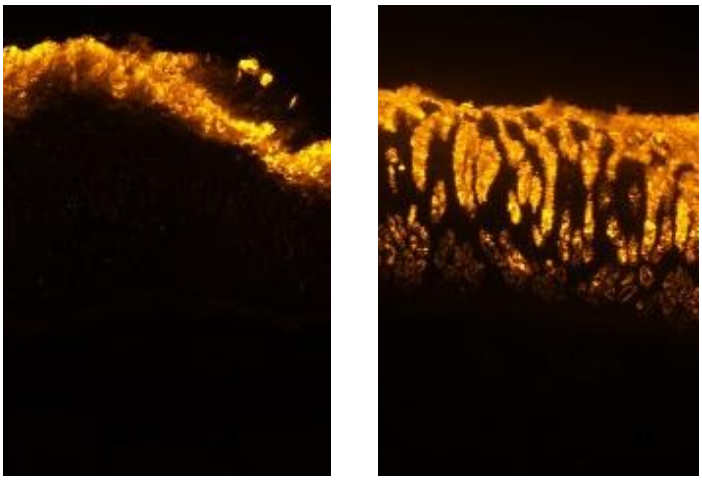


A B

Figure 6.14. Lectin staining with PNA (Galβ1,3GalNAc) of the fundus of a non-infected sheep at 4.5 months old before (A) and after (B) neuraminidase treatment. Original magnification 100x.



A B

Figure 6.15. Lectin staining with PNA (Galβ1,3GalNAc) of the antrum of 9 weeks-old, milk-fed (A) and solid-fed (B) lamb. Original magnification 100x.

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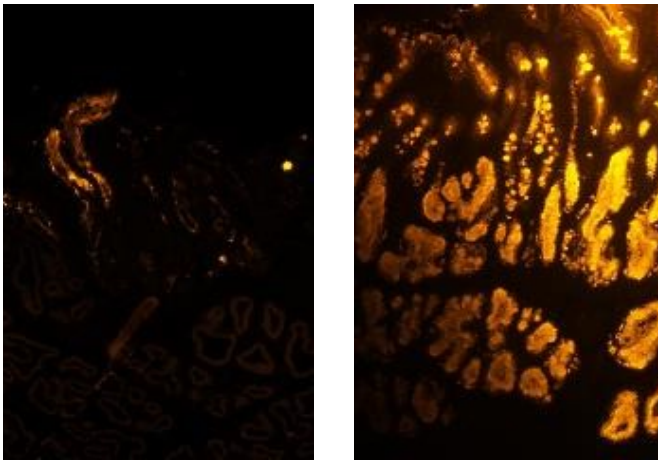
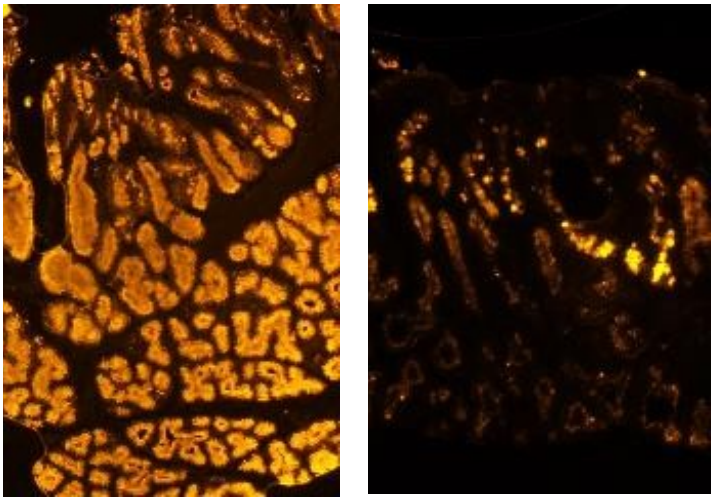


A B

Figure 6.16. Lectin staining with DBA (α-GalNAc) of the duodenum of 9 weeks-old, milk-fed (A) and solid-fed (B) lambs. Original magnification 100x.



A B

Figure 6.17. Lectin staining with PTL (galactoside) of the duodenum of 9 weeks-old, milk-fed (A) and solid-fed (B) lambs. Original magnification 100x.

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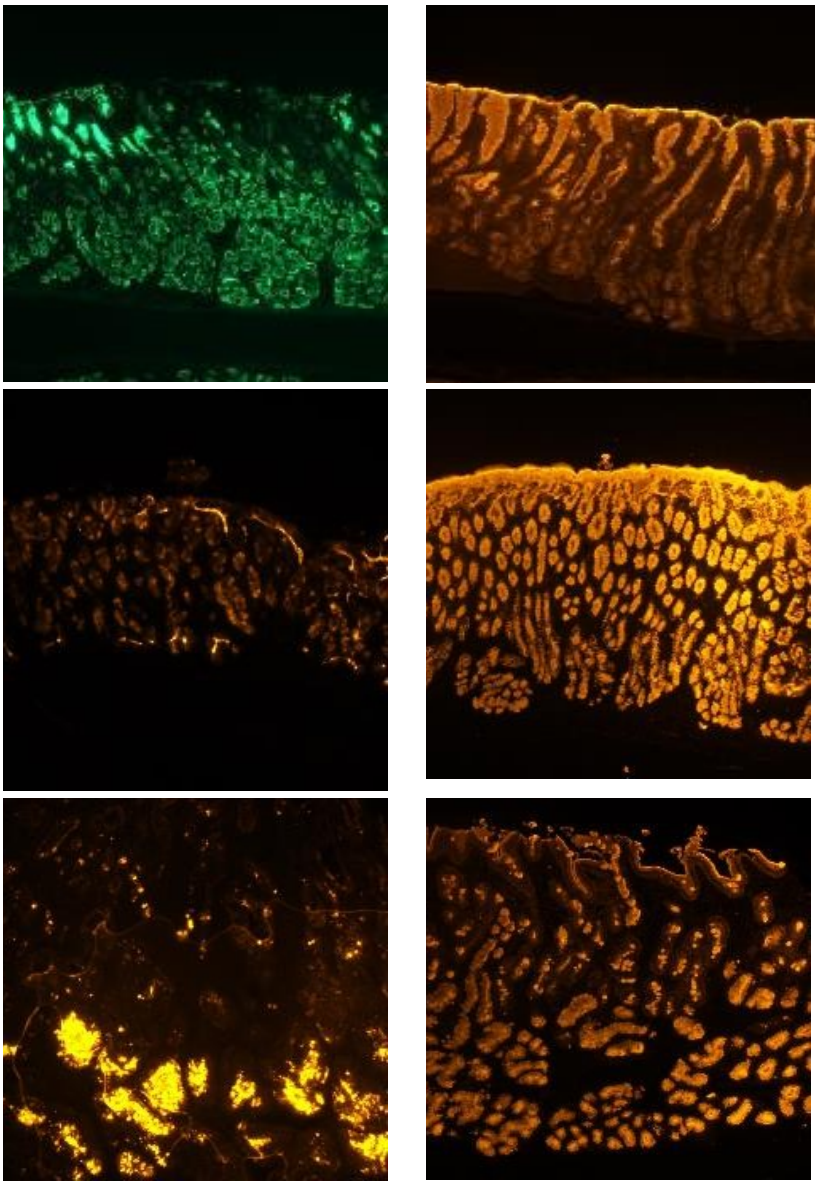

Figure 6.18. Lectin staining with PTL (galactoside) of the fundus of *T. circumcincta* infected sheep (top), the antrum of non-infected sheep (middle) and the duodenum of non-infected sheep (bottom) at 4.5 months old after neuraminidase treatment. Before treatment (left), after treatment (right). Original magnification 100x.

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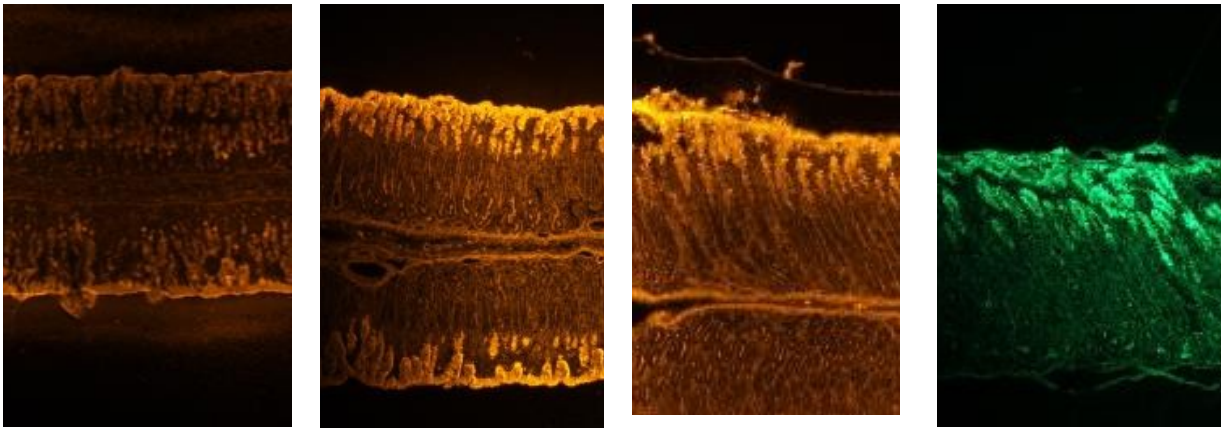
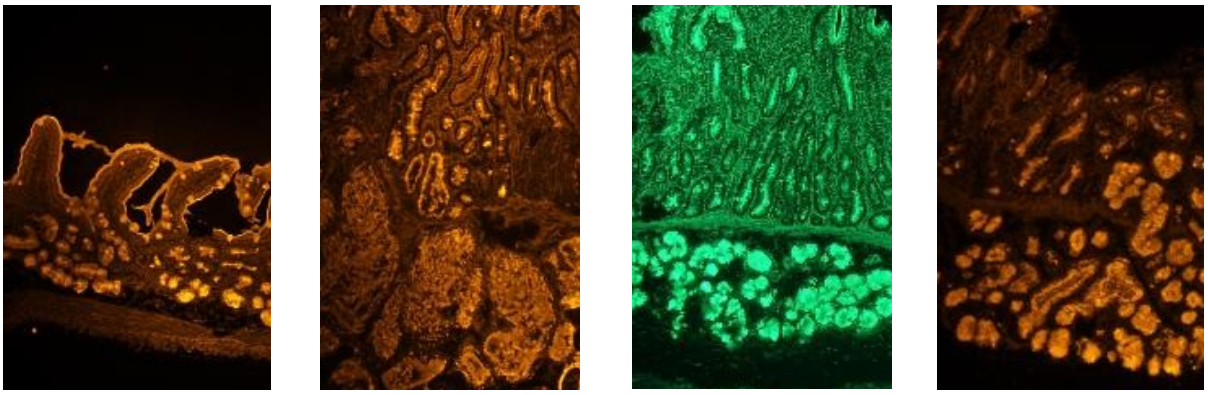


A B C D

Figure 6.19. Lectin staining with RCA (Gal) of the fundus of non-infected sheep at 3 days old (A), 9 weeks old, solid-fed (B), 4.5 months old (C) and 6 months old (D). Original magnification 100x.



A B C

Figure 6.20. Lectin staining with RCA (Gal) of the duodenum of non-infected sheep at 3 days old (A), 4.5 months old (B), 6 months old (C) and 9 months old (D). Original magnification 100x.

with 4-6 months-old animals. Fundic tissues from milk-fed lambs did not stain with RCA whereas SMCs and pit cells in solid-fed lambs stained strongly (Figure 6.21).

Neither *H. contortus* nor *T. circumcincta* infection caused marked changes in the staining for Gal residues in the fundus, antrum and duodenum. After neuraminidase treatment to remove terminal sialic acid residues, the staining of RCA increased in the antrum of the 4.5 months-old *H. contortus* infected sheep (Figure 6.22), but not in the fundus and duodenum. No effects of treatment were seen in tissues from the non-infected and *T. circumcincta* infected sheep.

6.3.2.5. N-acetyl glucosamine

The presence of terminal GlcNAc was detected by both WGA and GSA-II, although the intensity of WGA binding was greater than GSA-II staining. WGA can also bind GalNAc. Both showed that GlcNAc decreased with age. GSA-II staining showed decreased GlcNAc in fundic mucins in 4-9 months-old animals, compared with younger animals. Similarly, WGA stained strongly to fundic mucus-producing cells of sheep 3 days- to 9 weeks-of-age, but stained moderately at 4.5-6 months old and weakly at 9 months old (Figure 6.23). There was a large amount of GlcNAc in the antrum of non-infected sheep, shown by staining with WGA, which increased in intensity after sialic acid removal (Figure 6.24). In the duodenum, Brunner's glands produced a larger amount of GlcNAc than goblet cells in all animals.

The effects of parasitism on GlcNAc residues varied with age. In *H. contortus* infection, there was a decrease in GlcNAc in the fundus at 4-6 months old, but an increase at 8-9 months old compared with non-infected sheep (GSA-II binding). WGA also showed stronger staining to fundic mucus-producing cells in sheep at 9 months-old compared with the non-infected sheep. This trend was also found in *T. circumcincta* infection. In contrast to the effects of infection on the fundic tissues, there were no marked changes in either GSA-II or WGA staining in antral and duodenal mucins in both infections.

6.3.2.6. Mannose

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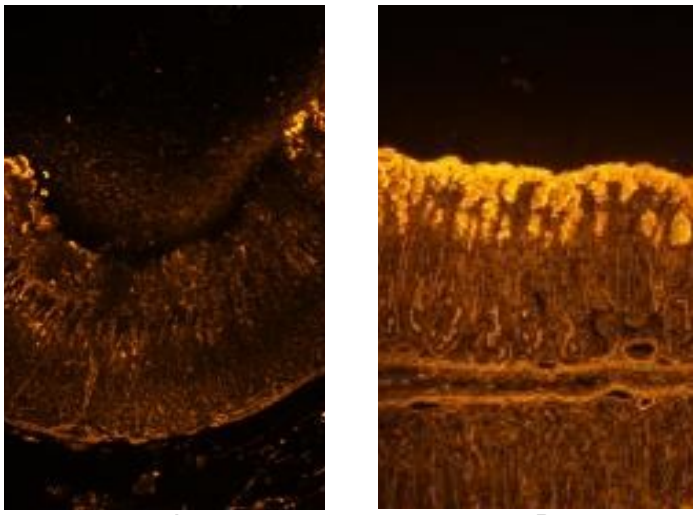

A B

Figure 6.21. Lectin staining with RCA (Gal) of the fundus of milk-fed (A) and solid-fed (B) lambs. Original magnification 100x.

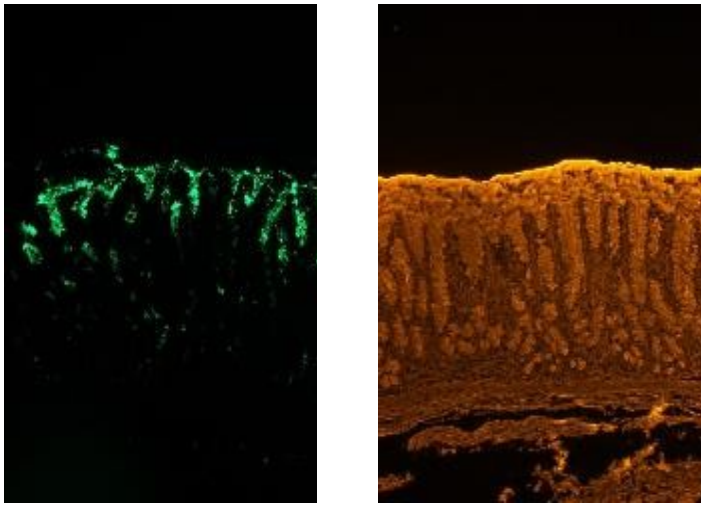

Figure 6.22. Lectin staining with RCA (Gal) of the antrum of *H. contortus* infected sheep, 4.5 months old after neuraminidase treatment. Before treatment (left), after treatment (right). Original magnification 100x.

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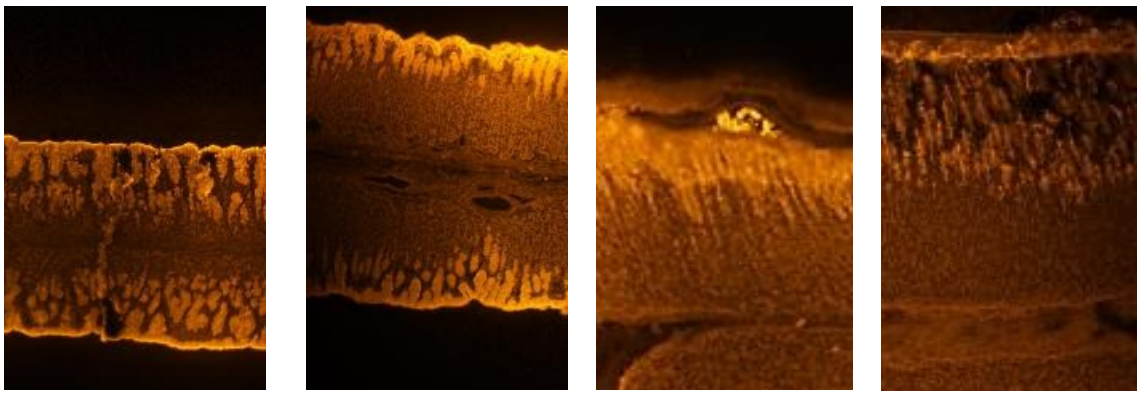

A B C D

Figure 6.23. Lectin staining with WGA (GlcNAc) of the fundus of non-infected sheep at 3 days old (A), 9 weeks old (B), 4.5 months old (C) and 9 months old (D). Original magnification 100x.

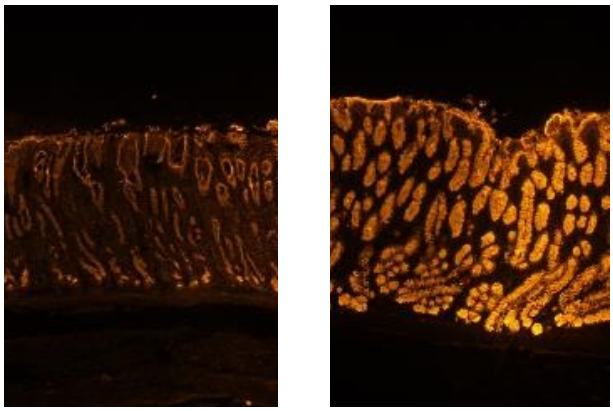

Figure 6.24. Lectin staining with WGA (GlcNAc) of the antrum of non-infected sheep after neuraminidase treatment. Before treatment (left), after treatment (right). Original magnification 100x.

ConA, PSA and LCA, which all bind specifically to Man and Glc-linked oligosaccharides, did not stain mucus-producing cells but stained the background weakly (Figure 6.25). This suggests the absence or a very small amount of mucins containing N-glycans in abomasal and duodenal tissues.

6.3.2.7. Oligosaccharides

In non-infected sheep at 4-9 months old, PHA stained cell membranes, but did not stain mucus-producing cells. Staining was seen in the fundus and antrum of 3 days-old lambs and milk-fed and solid-fed animals at 9 weeks-of-age, in the fundus of *T. circumcincta* and *H. contortus* infected sheep at 9 months-of-age and in the antrum of *H. contortus* infected sheep at 4.5-6 months old. Duodenal mucins did not show any binding to PHA.

6.4. DISCUSSION

This was a pilot study to determine whether lectin staining would be a useful technique for investigating changes in mucin carbohydrates in parasitised tissues. As only one sheep in each group was chosen for study and individual animal differences were significant for some lectins, more reliable identification of the effects of age and infection will need processing of all tissues for a selected panel of lectins. A total of 19 lectins were used to identify those which would be most useful in a larger study. Such a large number is not manageable if staining intensity is a key assessment factor and not only presence or absence of lectin binding to particular cells. To compare intensities, all sections need to be processed in a single batch for each lectin, together with positive and negative controls. Sections subjected to β-elimination to identify lectin binding to O-linked glycans should be processed in the same batch with the non-treated sections. Similarly, treatment with enzymes, such as neuraminidase, to remove terminal sugars should also be carried out in the same batch with the non-treated sections.

Fluorescent labeling of the lectins was used to evaluate the feasibility of using image analysis to identify quantitative changes in staining. A disadvantage was that labeled cells were very brightly fluorescent, making differentiation of cell types difficult and particularly to separate the staining of the surface mucus layer from staining of the SMCs. As fluorescence is not stable over time, sections must

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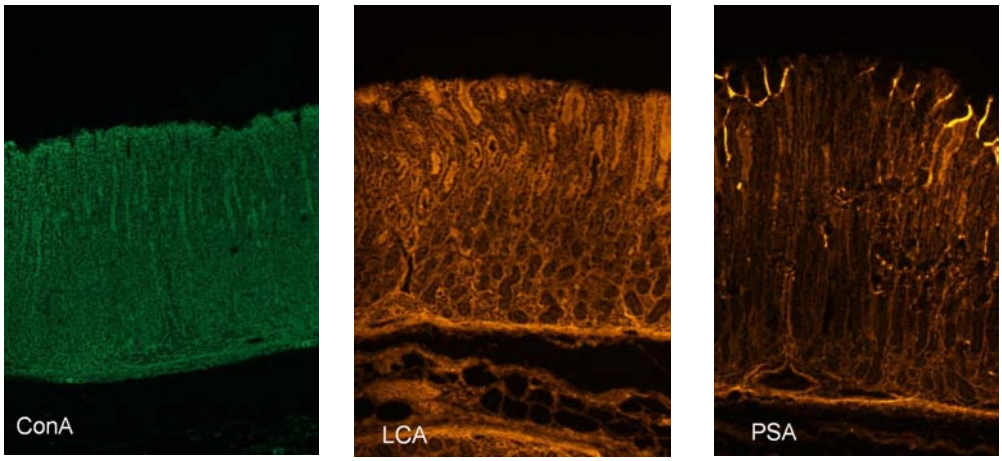

Figure 6.25. Lectin staining of the fundus tissue with ConA, LCA and PSA (Man/Glc). Original magnification 100x.

be photographed for a permanent record. For this study, the intensity of staining was subjectively assessed.

As mucin glycans are predominantly O-linked, lectin binding to mucins and not other glycans can be verified by their removal by β-elimination and assessing any residual staining. In this study, β-elimination prevented binding of many lectins to uninfected tissues, but fewer lectins failed to stain after treatment of infected tissues. This could be caused by larger amounts of N-linked glycans in infected tissues or, more likely, by the O-glycans being more resistant to alkali treatment in infected sheep. Two types of O-linked oligosaccharides have been recognised: one type is labile to treatment and removed after short term treatment, whereas resistant O-linked oligosaccharides need longer treatment (Gomez-Santos et al., 2007). In this study, β-elimination was carried out for one hour, resulting in some lectins still binding to tissues (Tables 6.2 to 6.4), suggesting the presence of resistant O-glycans, particularly after infection. In future studies, longer incubation time would be needed to completely remove the O-glycans. The more resistant O-glycans in parasitised tissues may be significant and contribute to the changes in the properties of the mucin glycoproteins.

Differences in binding of lectins with specificity for the same linkage, e.g. LTA and UEA-I, may result from preferences for location of the sugar in different positions on the oligosaccharide. It is known that LTA and UEA-I preferentially label Fuc residues located in the outer regions of the oligosaccharide chains, whereas AAL can also bind to those Fucα-1,6-linked to the innermost GlcNAc residues (Pereira and Kabat, 1974; Sugii and Kabat, 1982; Osawa and Tsuji, 1987). UEA has been reported to have binding only to O-linked oligosaccharides (Madrid et al., 1998; Sakamoto et al., 2000), while LTA is restricted to N-linked glycoproteins (Schulte and Spicer, 1983). Another reason for strong UEA binding (α-1,2 linkage), but not LTA (both α-1,2 and α-1,3 linkages), may be that LTA has an unreported preference for the latter linkage.

The Man-specific lectins were used to assess binding to N-glycans, which form only a small proportion of mucin oligosaccharides, but are more common in other glycans. As expected, binding of these lectins was not reduced after β-elimination and, in some cases, the staining intensity was stronger. This indicates that there were N-linked oligosaccharides which were masked by bulky O-glycans

and after these had been removed, N-linked oligosaccharides were exposed to lectins and were stained more strongly.

Lectin binding provided general information about the monosaccharides in the different mucin-producing cells in the gastrointestinal tract, most reliably about the terminal sugars. In the fundus of uninfected sheep, SMCs contained Fuc predominantly in the α-1,2 linkage and sialic acids in the α-2,6- but not α-2,3-linkage, except at 3 days-of-age; Gal, GlcNAc and α/β-linked GalNAc were also demonstrated. Pit cells contained large amounts of Fuc, GlcNac, GalNAc and moderate amounts of Gal and α-2,6-sialic acids. MNCs stained for most sugars. Although there are well recognised species differences in mucin glycans (Sheehan and Jervis, 1976), these patterns are consistent with reports for guinea-pigs (Lueth et al., 2005), rat gastric mucosa (Suganuma et al., 1985) and the bovine abomasum (Sommer et al., 2001). Positive SNA staining for sialic acid has been proposed as a marker for SMCs in the bovine abomasum (Sommer et al., 2001). Goblet cells showed the presence of Fuc, GlcNAc, GalNAc, Gal and sialic acids in both α-2,3 and α-2,6 linkages.

6.4.1. Effects of age and weaning on lectin binding

The most marked effects of age were increasing fucosylation and decreased sialylation. Other changes were increased staining for Gal and decreased GlcNAc in the fundus and reduced Gal in the duodenum. Terminal GlcNAc was abundant in both the fundus and antrum (WGA and GSA-II binding) and was reduced in animals over 4 months-old in the fundus (Figure 6.23), but not noticeably in the antrum. A large amount of Gal (RCA) was present in both goblet cells and Brunner's glands in 3 days-old lambs and generally less in sheep up to 6 months-of-age, but a small increase in the 9 months-old sheep (Figure 6.20). The staining for Gal was lower in the fundus at 3 days-old than in animals over 9 weeks-of-age (Figure 6.19). This differed from the results of chemical analysis which showed an increase in the percentage of Gal in fundic mucins in 3 days-old lambs was a little higher (47%) than in sheep 4-9 months old (40%) although lower in both milk-fed (38%) and weaned (24%) 9 weeks-old lambs. As Gal is a monosaccharide internal in the oligosaccharides, lectin binding may reflect changes in the position of Gal in the oligosaccharide chain rather than total Gal in the mucin.

The terminal sialic acids were mainly attached to the glycans in an α-2,6 linkage in sheep gastrointestinal mucins, as in ovine submaxillary mucins, which expressed 86% of the sialic acids in an α-2,6 linkage (Hill et al., 1977a). Similarly, in the human intestine NeuAcα2-6Gal-specific SNA stained strongly than NeuAcα2-3Gal-specific MAL (Huet et al., 1998; Freitas and Cayuela, 2000; Freitas et al., 2001). Sialylation was higher in 3 days-old lambs than in any other sheep. There was a change in the linkage of sialic acids to abomasal mucin glycans during the postnatal period. In 3 days-old lambs, both α-2,3- and α-2,6-linked sialic acids were present, while there were only α-2,6-linked sialic acids in animals over 9 weeks-of-age. Switching off the production of α-2,3-sialic acids in abomasal mucins may be a large contributor to the decrease in sialylation with age. In mice, colonisation of the colon by the normal microflora reduced NeuAcα2-6Gal expression in colonocytes and NeuAcα2-3Gal in cells in the bottom of the crypt (Freitas et al., 2002). Stem cells in the base of antral glands of lambs contained α-2,3-linked sialic acids, but not gland cells, pit cells and SMCs, suggesting changes in gene expression modulate the linkage of sialic acids as these cells mature. In contrast, both sialic acid linkages were present in the goblet cells of older sheep, but not in Brunner's glands, as MAL (α-2,3 linkage) bound weakly to goblet cells, but did not stain Brunner's glands in the sheep duodenum. Goblet cells contained small amounts of α-2,3-linked sialic acids and larger amounts of α-2,6-linked sialic acids.

Both fundic and duodenal tissues of 3 days-old lambs contained a smaller proportion of mucins terminating with Fuc residues than did 9 weeks-old lambs and 4-9 months-old sheep. Both UEA and AAL staining increased in fundic, antral and duodenal tissues. Pedini et al. (2001) reported that very few goblet cells and gland cells in the duodenum of lambs reacted with UEA, indicating only small amounts of Fuc, whereas Ohwada and Suzuki (1992) described moderate amounts of Fuc in epithelial cells and duodenal glands of adult sheep. Increased fucosylation (UEA staining) appears to be a feature of maturation in sheep gastrointestinal tissues as in pigs (Gelberg et al., 1992).

The most apparent differences between milk-fed and weaned lambs were in the binding of Gal and GalNAc specific lectins to the antrum and duodenum. Pit cells in the antrum of solid-fed lambs stained very strongly with PNA (Galβ1,3GalNAc), while those in milk-fed lambs did not stain (Figure 6.15). In the

duodenum, DBA (α-GalNAc) stained goblet cells and Brunner's glands in weaned but not in unweaned lambs (Figure 6.16), however, duodenal tissues in milk-fed lambs stained with PTL (GalNAc or Gal) more strongly than in solid-fed lambs (Figure 6.17). Fundic tissues from solid-fed lambs stained more strongly with RCA (Gal) than those in milk-fed lambs (Figure 6.21), the opposite of the results from chemical analysis (Chapter 4). As discussed above, the total Gal in mucins may not reflect lectin binding, which may be influenced by the position of Gal within the core, backbone and peripheral structures in the oligosaccharide.

6.4.2. Effects of infection

Parasitism had visible effects on the intensity of lectin binding to abomasal and duodenal mucins, although these were not always the same in sheep at different ages or during infection with the two species of nematode. Chemical changes in mucins resulted in β-elimination not being as effective in tissues from sheep infected with *H. contortus* or *T. circumcincta*: binding of 9 lectins was removed in fundic tissues of non-infected sheep after O-linked oligosaccharides had been removed, whereas, there appeared to be more O-glycans resistant to alkali treatment in infected tissues. Further evidence of modifications of mucin oligosaccharides comes from the different patterns of PHA staining. Although duodenal mucins did not show any binding to PHA, it did occur in the fundus of *T. circumcincta* and *H. contortus* infected sheep at 9 months-of-age and in the antrum of *H. contortus* infected sheep at 4.5-6 months old.

Reduced fucosylation of duodenal mucins, particularly in older animals, was a prominent effect of parasitism by either *H. contortus* or *T. circumcincta*, as was previously demonstrated by chemical analysis. Binding of UEA to α-1,2-linked Fuc in duodenal mucins was reduced by either parasite only in the older sheep (9 months-of-age) (Figure 6.11), whereas AAL binding to α-1,6- and α-1,3-linked Fuc was consistently less in both goblet cells and Brunner's glands in the duodenum of all infected sheep from 9 weeks to 9 months old (Figure 6.13). LTA did not bind to α-1,2-linked Fuc or Fucα1,3GlcNAc in either the antrum or duodenum of infected or uninfected sheep. The α-1,6 linkage is present on mucin-type oligosaccharides while the α-1,2 linkage is present in other glycoconjugates rather than mucins.

H. contortus infection was shown by chemical analysis (Chapter 3) to reduce the fucosylation of fundic mucins at all ages, whereas older *T. circumcincta* infected sheep showed minimal effects. Whereas previously it appeared that abomasal mucins were unaffected, lectin staining has shown the involvement of the antrum in parasitism of the 9 months-old sheep by both parasites (Figure 6.10), shown by reduced α-1,2 linked Fuc binding (UEA). Parasitism had minimal effects on fundic mucin fucosylation, but there were differences caused by the two species. Whereas LTA did not bind to uninfected tissues or those from the *H. contortus* infected sheep, in *T. circumcincta* infected sheep there was evidence of α-1,2-linked Fuc or Fucα1,3GlcNAc in pit cells, from weak and moderate staining in the 6 and 9 months-old animals respectively (Figure 6.12). A further difference between tissues from sheep infected with the two abomasal parasites was that after β-elimination, UEA staining was lost in goblet cells only in *H. contortus* infected sheep.

Overall, infection reduced mucin sialylation, particularly in the younger animals, as also shown by chemical analysis (Chapter 3). Reduced lectin binding was apparent only in duodenal mucins in 9 weeks-old lambs infected with *T. circumcincta* (Figure 6.4), in the fundus and duodenum of sheep infected with either parasite at 4.5 months-of-age (Figure 6.3), but not in older sheep. As discussed previously (Section 3.4.3), the decreased sialylation in the abomasum may be a component of the host response or the parasites may be responsible though secretion of glycosidases.

The binding of lectins to other monosaccharides was also altered in parasitised animals, particularly with *H. contortus* infection in younger sheep. Most of the differences were apparent only after the tissues had been treated with neuraminidase to remove sialic acids. Whereas RCA binding to Gal residues in all tissues of sheep infected with *H. contortus* or *T. circumcincta*, only in the antrum of the 4.5 months-old *H. contortus* infected sheep (Figure 6.18) was staining of RCA increased by neuraminidase treatment. Similar effects were seen in staining for the different GalNAc linkage. Neuraminidase treatment increased MPL (α-GalNAc) and BPL (Galβ1,3GalNAc) binding to fundic tissues and GSA-I and SBA (α/β-GalNAc) binding in the antrum of the *H. contortus* infected 4.5 months-old sheep. In contrast, PTL binding to GalNAc or Gal in fundic SMCs and pit cells in *T.*

circumcincta infected sheep was increased by neuraminidase treatment (Figure 6.15) as was also in the antrum and duodenum of non-infected sheep, but not in those infected with *H. contortus*.

In contrast, both parasite infections had similar effects on GlcNAc residues in mucins. There were no marked changes in antral and duodenal mucins (GSA-II and WGA), however in the fundus, there was a decrease in GlcNAc at 4-6 months old, but an increase at 8-9 months old compared with non-infected sheep. Fundic mucins of 8-9 months-old sheep infected with *H. contortus* also previously showed an increase in the proportion of GlcNAc (Table 3.3).

This lectin study has shown that there are subtly different effects of parasitism by *H. contortus* and *T. circumcincta* on gastrointestinal mucins at different ages. Altered oligosaccharide structure was apparent from the binding of PHA only to fundic tissues of parasitised older sheep and to antral tissues of 4-6 months-old *H. contortus* infected sheep, but not to any tissues from uninfected sheep. As well, parasitised abomasal and duodenal tissues were resistant to the binding of many lectins after β-elimination, although this varied between the two infections (Tables 6.3 and 6.4). There were also numerous examples of different binding of individual lectins with and without β-elimination and neuraminidase treatment to tissues after the two infections. Despite only a representative animal being studied from each group, significant effects of parasitism have emerged warranting more extensive studies on these and other tissues from infected animals.

Chapter 7

GENERAL DISCUSSION

Mucus, the protective layer lining the gastrointestinal tract, plays an important role in lubricating and protecting the epithelium against degradation by acid and pepsin. As it is also the location where pathogens first make physical contact with the host, they may modulate this layer to favour their colonisation and establishment in the host. There have been many studies about the changes in mucins, the main component of mucus, during infections with a variety of pathogens in rats, pigs, cattle and humans (Chapter 1, Sections 1.5.3-1.5.7) and a few studies in rats during nematode parasitism, however, almost nothing is known about mucin composition in sheep parasitised by nematodes.

Sheep develop immunity to nematodes over several months of their life and generally do not become resistant until 9-10 months-of-age (Smith et al., 1985). As it is possible that the structure of mucin carbohydrates plays a role in susceptibility to parasitism, the sheep included in this study were infected at different ages from the youngest, which were trickle infected from 5-9 weeks-of-age, to the oldest at 9 months-of-age. The parasites used for infection were *H. contortus* or *T. circumcincta*, the two most common abomasal parasites of sheep in New Zealand. The youngest infected lambs were parasite-naive before infection with *T. circumcincta* over a 5 week period. The older animals had previously experienced field infections and were infected at least 4 weeks after parasite removal with anthelmintic drenches. Infection was with a single dose of either *H. contortus* or *T. circumcincta* and, at necropsy, the animals covered three age ranges from 4 to 9 months.

Mucus was collected from both the fundus, where the worms were present, and also from the duodenum, as effects of abomasal nematodes on duodenal mucins might suggest more widespread involvement of the immune system. For each age group, mucin profiles in infected sheep were compared with those in non-infected animals. Little is known about the changes in mucins with age, which may be important in susceptibility to parasites. The presence of specific epitopes on mucin carbohydrates is known to be a determinant of susceptibility to pathogens (Mouricout, 1997; Kawakubo et al., 2004). The design of the experiment involving the youngest lambs followed the protocol used by Zeng et al. (2001), which showed that early weaned lambs were much more susceptible than milk-fed animals to infection with *T. circumcincta*. Three groups were studied, milk-fed, early weaned and weaned lambs infected from weeks 5-9 with a twice weekly infection with *T. circumcincta* larvae, to investigate whether there might be differences in mucin composition between milk- and solid-fed lambs which could contribute to lower susceptibility in unweaned lambs.

As the mucin profile is likely to change during the course of the infection (Manjili et al., 1998; Rowe et al., 2009), a single time point was selected for mucin collection for each parasite. The times chosen were 21 days p.i. for *H. contortus* infection, 28 days p.i. after the single infection and 35 days after the first dose of the trickle infection with *T. circumcincta*. These times were selected to coincide with the presence of egg laying adult worms, as the luminal stages of abomasal parasites are responsible for causing the pathophysiology associated with these species (Lawton et al., 1996; Simpson et al., 1997; Scott et al., 2000). However, this was only one time point during the infection and the progressive effects occurring during the whole infection may be very informative. As well, the timing of changes in mucin profiles may vary in individual sheep, depending on the level of immunity, maturity of the animal and host effects on parasite development.

Mucins collected from uninfected sheep from 3 days-of-age to 9 months-old showed significant changes, particularly in the early postnatal period, in the proportions of the monsaccharides Fuc, GalNAc, GlcNAc, Gal and sialic acids. The most marked differences were in sulphation and in the terminal sugars Fuc and sialic acids between 3 days- and 9 weeks-of-age, whereas further increase in fucosylation

and decreases in sialylation and sulphation occurred more slowly. Sulphated mucins were not found in SMCs and Brunner's glands, except in 3 days-old and 9 weeks-old lambs respectively. Neutral mucins were present in SMCs, pit cells and MNCs in the fundus, SMCs, pit cells and gland cells in the antrum and goblet cells and Brunner's glands in the duodenum. Sialylated mucins were not present in the SMCs of sheep older than 3 days-old. Changes in the linkages involved in sialylation were also apparent in other cells. Fundic mucins of 3 days-old lambs contained both α-2,3 and α-2,6-linked sialic acids, while by 9 weeks-of-age, only α-2,6-linked sialic acids were present. Maturation of antral gland cells was also observed: α-2,3-linked sialic acids were present in stem cells in the base of antral glands, but not in gland cells, pit cells and SMCs, suggesting changes in gene expression modulate the linkage of sialic acids as these cells matured.

The most noticeable effects of milk feeding were the larger amounts of mucus in all mucus-producing cells in the fundus and more sulphomucins in the pit cells and MNCs. These may contribute to the resistance of unweaned lambs to parasites, however, milk products themselves seem to play an anti-nematode role. A variety of milk and milk components inhibit the motility of *T. circumcincta* larvae in vitro (Zeng et al., 2003). Milk and its glyconconjugates similarly contribute to resistance to infection to a variety of microorganisms (Coppa et al., 1990; Kunz and Rudloff, 1993; Newburg, 1996).

Infection with either species of parasite changed the monosaccharide composition of fundic and duodenal mucins, particularly the terminal residues of the side chains, decreasing both fucosylation and sialylation. This was also seen in rats infected with intestinal parasites, suggesting that the terminal sugar residues may be involved in the immune response and either facilitate or inhibit the establishment of pathogens (Ishikawa, 1994; Holmen et al., 2002; Yamauchi et al., 2006). Differences in the glycoconjugates of goblet cell mucins influence selective colonisation by different nematode species (Ishikawa et al., 1994; Yamauchi et al., 2006). The relative importance of the host and parasite in altering the terminal sugars is unknown, however, the reduced fucose and sialic acids caused in the duodenum by abomasal parasites suggests the host response is important.

Alternatively, changes in terminal sugars may be caused by parasite glycosidases, either to use mucins as a source of nutrients or to modify them to facilitate their survival in the host. Pathogens can modulate host glycans either by degrading host glycans or exposing underlying saccharides with which their lectins can interact (Dwarakanath et al., 1995b; Vimr et al., 2004). Secretion of glycosidases is common in pathogens of epithelial surfaces (Slomiany et al., 1992a, b; Wadstrom et al., 1996; Sonnenburg et al., 2005), including *F. hepatica* (Irwin et al., 2004). Sialic acids are important attachment sites and promote survival of a range of pathogens (Varki, 1997; Ramphal and Arora, 2001; Nasir-ud-Din et al., 2003), but together with sulphation reduce the susceptibility of mucins to degradation (Rhodes et al., 1985; Amerongen et al., 1998; Chance and Mawhinney, 2000; Becker and Lowe, 2003). Not only do glycosidases modify the sugar composition, but they also change the physical properties of mucins, such as viscosity, gel forming ability and solubility which can also influence pathogen colonisation and development (reviewed by Bansil et al., 1995; Forstner et al., 1995).

Reduced fucosylation and sialylation of duodenal and abomasal mucins was common to infection with either *H. contortus* or *T. circumcincta*. Other changes in the monosaccharide profiles were similar in the duodenal, but not fundic mucins. There were no major differences between the monosaccharide profiles of either duodenal or abomasal mucins after a trickle infection with *T. circumcincta* in 9 weeks-old lambs or a single infection in 4-4.5 months-old animals, whereas older lambs showed much lesser effects in the abomasum. This difference with age was much less marked in 8-9 months-old sheep infected with *H. contortus*, suggesting that older sheep were fully susceptible to that species, but less susceptible to *T. circumcincta*. By 8-9 months-of-age, sheep are more likely to have been exposed to *T. circumcincta* than to *H. contortus* in the field before they were brought indoors for experiments, as this is the predominant abomasal species in cooler areas. At this age, they would be expected to have developed protective immunity to *T. circumcincta*, so that the *H. contortus* infection may have been a primary infection and the *T. circumcincta* a challenge infection.

A typical effect of nematode infection is hyperplasia of mucus-producing cells (Newlands et al., 1990) and increased mucus secretion. This does vary with time after infection, as MUC 5AC expression decreased by day 22 after *H. contortus* infection, more so in resistant sheep (Ingham et al., 2008; Rowe et al., 2009). Newland et al. (1990) investigated the mucin profiles of sheep 6 and 12 weeks after immunisation and subsequent challenge. They reported that after 6 weeks, the mucins in challenged sheep resembled those in immune sheep, but by 12 weeks, were similar to those in naïve sheep, indicating that the sheep had again become susceptible to parasites. The expression of glycosylation-related genes changed during the course of infection of rats infected with *N. brasiliensis*; the activities of sialylatransferase, fucosyltransferase and sulphotransferase were upregulated, but varied during the infection (Yamauchi et al., 2006). Increased mucus secretion may be induced by nematode chemicals or the host immune response (Miller, 1987; Nawa et al., 1994).

Changes in mucin glycosylation during infection with gastrointestinal nematodes have been suggested to result from Th2 cytokines which are involved in protective immunity (Lawrence, 2003; Yamauchi et al., 2006; Li et al., 2009). The typical Th2 response involves the cytokines IL-4, IL-5, IL-9, IL-10 and IL-13 (Gasbarre, 1997; Balic et al., 2000; Madden et al., 2004). Whereas a primary infection of sheep with *H. contortus* did not induce IL-4, IL-5 and IL-13, there was a significant induction of these cytokines in immune sheep after challenge infection (Meeusen et al., 2005): IL-5 and IL-13 increased 3 days after challenge and IL-4 levels peaked on day 5. They suggested that IL-4 is involved in rapid worm expulsion, while IL-5 and IL-13 are critical for delayed rejection. Specific components of the protective response differ with the host-parasite system (Lawrence, 2003), but similar effects have been reported in rodents. Mice resistant to *T. muris* produce high levels of Th2-type cytokines, while susceptible animals produced low levels (Else et al., 1992). IL-4 regulated expulsion of worms in mice infected with *Heligmosomoides polygyrus* (Urban et al., 1991), as blocking IL-4 resulted in preventing worm expulsion in a challenge infection. A similar observation was found in IL-13 in mice infected with *T. muris* (Bancroft et al., 1998) and IL-9 with *T. spiralis* infection (Faulkner et al., 1997).

There is scanty information on the direct effects of cytokines on mucins, most coming from studies of cells derived from the respiratory tract, where an allergic Th2 type response has many features in common with the Th2 response associated with parasitism of the gastrointestinal tract by nematodes. In asthma, increased mucus secretion is associated with the Th2 response (reviewed by Andrianifahanana et al., 2006), IL-4 (Dabbagh et al., 1999), IL-9 (Louahed et al., 2000) and IL-13 (Whittaker et al., 2002), all stimulate mucus production and increased glycoconjugates in mucus-producing cells. The common pathway appears to be through IL-13, as neither IL-4, IL-9 nor any Th1 factors induced mucus secretion in the absence of IL-13, which probably acts directly on the epithelial cells (Whittaker et al., 2002). The IL-4R, common for IL-13 and IL-4 is essential (Whittaker et al., 2002; Kuperman et al., 2005). Several other cytokines either increase or decrease mucus secretion or mucin gene expression (Andrianifahanana et al., 2002), such as TNF-α, which is suggested to mediate the reduction in numbers of goblet cells and increased sulphation of mucins in goblet cells in the villi during Salmonellosis in mice (Arnold et al., 1993). IL-4 induced N-acetyl galactosaminyltransferase expression and increased the density of mucin O-glycans and mucins producing more of the short chains T- (GalNac-) and Tn- (Galβ1,3GalNAc-) in human colonic carcinoma cells in vitro (Bennett et al., 1998; Kanoh et al., 2008). Mucin biosynthesis and expression of the rate limiting enzyme GCNT3 have recently been shown to be upregulated in goblet cells after infection of cattle with *Cooperia oncophera* (Li et al., 2009).

An interesting observation in the present experiments is that the difference in mucin profiles in the two infections was found mainly in the abomasum, not in the duodenum. This suggests that the changes in mucin profile in the duodenum reflect the effects of host immunity being expressed beyond the tissue where the parasites are present. There could also be caused by parasite ES released into the abomasal lumen provoking changes in mucins in more distal parts of the gastrointestinal tract. In this case, the ES of the two species might be expected to have a greater effect at the site of release and also to produce similar effects where the parasites were located, not a more distant site. Reports of the rat liver parasite *T. taeniaformis* causing gastric and intestinal pathology may also indicate that host responses to parasites can be expressed in distant tissues (Cook and Williams, 1981; Rikihisa et al., 1986; Abella et

al., 1997; Konno et al., 1999). Mucosal hyperplasia occurred in both the stomach and small intestine, the size and weight of both organs increased (Cook and Williams, 1981) and the hexosamine concentration in the hyperplastic gastric mucosa doubled and there were many fewer AB-positive cells than PAS-positive cells (Rikihisa and Lin, 1984). While parasite products could not be ruled out, it is likely that products of the host inflammatory response are causing this effect in these other organs, as a parabiotic experiment showed effects in both the donor and recipient (Cook and Williams, 1981). Another indication of widespread effects of immune responses is the secretion of antibodies to gastrointestinal nematode carbohydrates, such as carbohydrate larval antigens (CARLA) (Harrison et al., 2003b; 2008) in saliva.

These experiments have raised the possibility that mucin profiles are a useful marker for the host immune response to parasites. More specific changes in the mucin glycans during infection, such as linkages, the length and density of oligosaccharide side chains and specific sequences may need to be identified. These will require more sophisticated techniques such as mass spectrometry, nuclear magnetic resonance and light scattering to study the alterations in glycosylation during parasitism. The mucin profiles in genetically different animals may be able to be used to identify resistant, resilient and susceptible individuals. If changes in mucin profiles are also seen in other organs in the body, gastrointestinal mucins may not be required, but instead mucins could be obtained from accessible sources such as the salivary gland or nasal cavity to facilitate the diagnosis of susceptibility or resistance of sheep to parasites.

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Appendix 1

SHEEP

1.1. Animals

Between 2 and 4 month of age male sheep were brought inside a shed and kept in pens of up to 6 animals, fed chaff and water ad libitum and cleaned once daily. A parasite-free stage (before experimental single infections) was reached by a double dose (1ml/5kg, calculated with the weight of the heaviest animal in the group) of Matrix (Ancare, New Zealand) followed by a single dose (1ml/10kg) of Matrix 2 days later. The parasite-free stage was controlled via FEC.

1.2. Faecal floats (to determine the presence of nematode eggs in faeces)

Faeces were collected per rectum. Two grams of feaces were macerated and mixed with saturated NaCl solution (approximately 30 mls). The mixture was poured into a vial so as to form a meniscus at its surface, over which a coverslip was placed. After 15 minutes, the coverslip was carefully unlifted (with a deft upward motion) so as to take with it the surface layer of mixture, placed on a microscope slide and examined under 10x objective. The absence of any nematode eggs after the examination of 2 faecal floats on two successive days was considered to indicate the absence of nematode parasites and a negative result.

1.3. Faecal Egg Count (FEC)

A modification of the McMaster technique (modified by Stafford, West and Pomroy, 1994) was used. The collected faecal sample from the sheep (2g for quantification) was placed in a small sieve in a scale pan. 30ml saturated sodium chloride (universal glass filled) was poured over the faecal sample which was mashed, submerged in the salt solution, with a pestle. The sieve with the large detritus left behind was discarded then. For qualification the float method was used: The salt solution/faecal mix was poured back into the universal glass and a coverslip placed

on top on which any eggs present in the faecal sample will stick to after floatation. After 10 min the coverslip was placed onto a slide and the sample examined for any eggs. For quantification, both sides of the McMaster slide were filled with the salt solution/faecal mix and the number of eggs within the grids was counted. The total number was multiplied by 50 to get the eggs per gram of faeces (e.p.g.).

1.4. Infections

Once the sheep were parasite-free and free of drench residues (5 weeks after drench with Matrix for *H. contortus* infection and 4 weeks for *T. circumcincta*) they were infected. After counting (100µl in 0.5ml iodine), the desired number was taken out from the *H. contortus* (approximately 10,000 L_3) or *T. circumcincta* (approximately 50,000 L_3) larval culture stock and sheep were infected orally via syringe.

1.5. Larval Culture Stock

For the maintenance of larval culture stocks, sheep were regularly infected with either *H. contortus* or *T. circumcincta*. Their faeces were collected each into a bag that was attached to a harness. The presence of nematode eggs in the faeces was monitored from day 18 p.i for both *H. contortus* and *T. circumcincta* infections by FEC (qualification/float method). Once present, FECs (quanification/McMaster slide) were carried out and faeces collected after there were more than 200 eggs per gram. Faeces were placed in trays, moistened and mixed with vermiculite. The trays were covered with glass plates and incubated in a warm room (27 to 28°C) for 10 to 14 days for both *H. contortus* and *T. circumcincta*. The mixture was kept moist and turned over twice during the incubation. Larvae were harvested using a modified Baermann technique (Baermann, 1917): The faeces mixture was placed into a sieve lined with tissue and the sieve was put into a tray filled with RO water leading to L_3 entering the RO water. After 24h, the RO water containing the larvae was placed into another sieve lined with tissue on top of a funnel attached to a short tube with a closable end where the larvae were collected after another 24h. *H. contortus* larvae were stored at 10°C, and *T. circumcincta* at 4°C, in RO water in culture flasks.

Appendix 2

LAMB EXPERIMENT

2.1. Lamb-feeding plan

200 g milk was dissolved in 1 litre of water at 37º- 40ºC

	Feeding time	Volume of milk per feed per lamb
Day 1- day 7	7.30 12.30 5.30 9.00	150- 250 ml
Day 8- day 28	7.30 1.30 8.30	250- 350 ml
Day 29- day 63	9.30 5.30	400- 750 ml

2.2. Milk bottle washing protocol

Rinse first with cold water and then warm water and dish-washing liquid. Bottles are left in bleach solution. When in use, rinse the bottle thoroughly with water.

2.3. Milk replacer components

From a 10 kg bag (21MJ/kg metabolisable energy) of Anlamb to be made up at 200 g/L for lambs:

28% (max) fat

26% (min) protein

37% lactose

6% minerals

Nil fibre

1% (max) salt

3% moisture

supplementary vitamins A, B1, C, D3, E and K3.

Appendix 3

GEL ELECTROPHORESIS

3.1. Solutions

All solutions were kept at 4°C unless stated otherwise.

Acrylogel 30%

40% acrylogel was diluted to 30% by diluting 15ml of 40% solution with 5ml MilliQ water.

Tris-HCl 1.5M, pH 8.8

100ml were made up of 18.165g Tris-HCl in MilliQ water and the pH was adjusted to 8.8 then; filterd through Whatman No.1 filter.

SDS 10% (w/v)

3.5g SDS were diluted to a final volume of 35ml MilliQ water. The solution was filtered through a 0.2µm filter and stored at room temperature.

Tris 1M, pH 6.8

10ml were made up of 1.211g Tris in MilliQ water and the pH was adjusted to 6.8; filtered through Whatman No.1 filter.

Ammoniumpersulfate (AMPS) 10% (w/v)

0.1g was dissolved in 1ml MilliQ water (prior to use), kept for a maximum time of 5 days.

SDS Gel Loading Buffer (4x)

200mM Tris-HCl	6.055g
8% SDS	2g
24% Glycerol	6ml
4% β-Mercaptoethanol	1ml
0.016% Bromphenolblue	0.004g

The solution was made up to 25ml of MilliQ water, filtered through a 0.2µm filter and the pH adjusted to 6.8. The solution was stored at room temperature.

Tris-Glycine Electrophoresis Buffer (5x)

125mM Tris	7.5686g
1250mM Glycine	46.9375g
0.5% SDS (SDS 10% solution)	25ml

Make up to a final volume of 500ml. The pH was adjusted to 8.3; filtered through Whatman No.1 filter.

3.2. Preparation of SDS-PAGE gradient 4-15%

	15% (5 ml/gel)	4% (5 ml/gel)
H_2O	1.1	3.6485
30% acrylamide	2.5	0.65
1.5 M Tris pH 8.8.	1.3	0.65
1.5 M Tris pH 6.8		
10% SDS	0.05	0.025
10% AMPS	0.05	0.025
TEMED	0.002	0.0015

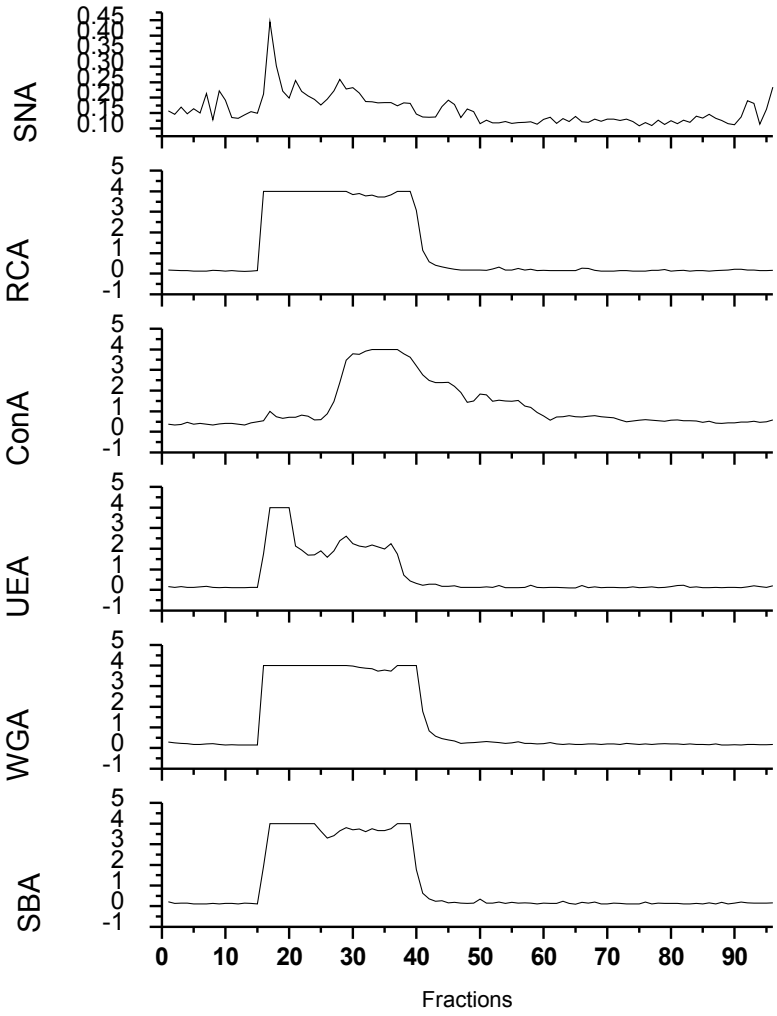
3% stacking gel was prepared as follows:

	3% stacking gel (2 ml/gel)
H_2O	1.625
30% acrylamide	0.198
1.5 M Tris pH 8.8.	
1.5 M Tris pH 6.8	0.15
10% SDS	0.012
10% AMPS	0.012
TEMED	0.0024

Appendix 4

REACTIVITY OF MUCIN FRACTIONS TO DIFFERENT LECTINS

Fractions collected from reduced abomasal mucins were used for investigating the sensitivity of lectins in carbohydrate detection. 100 µl of each fraction was coated onto the plate. After being washed and incubated with blocking solution, biotinylated lectins (2 µg/ml) was added and incubated in 1 hour. Unabsorbed lectins were washed, plate was incubated with phosphatase- conjugated streptavidin. Finally, p-nitrophenylphosphate was added to detect the reaction.

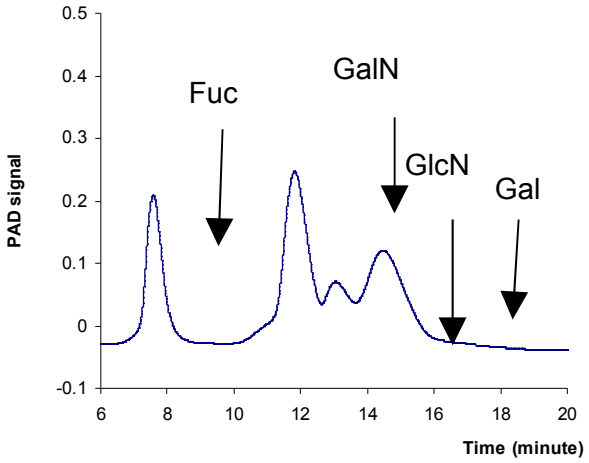


Appendix 5

HIGH PERFORMANCE ANION EXCHANGE CHROMATOGRAPHY

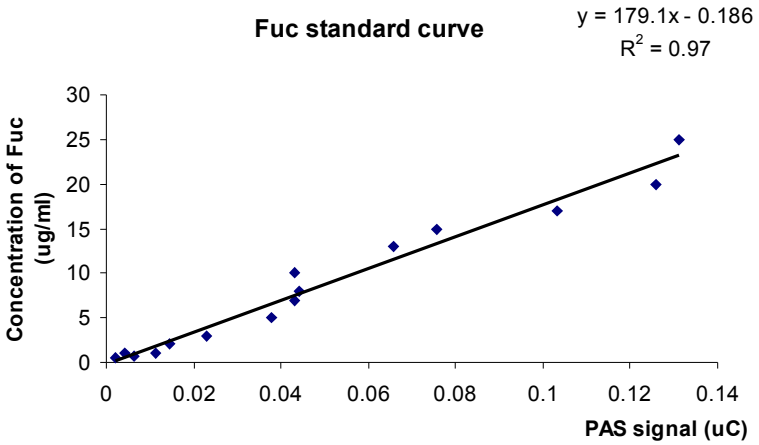
5.1. Monosaccharide separation sequence on HPAEC

The mixtures of monosaccharide in hydrolysed abomasal and duodenal mucins were injected into HPAEC. They are separated in less than 20 minutes, no interfering peaks were seen.



5.2. Standard curve of monosaccharides

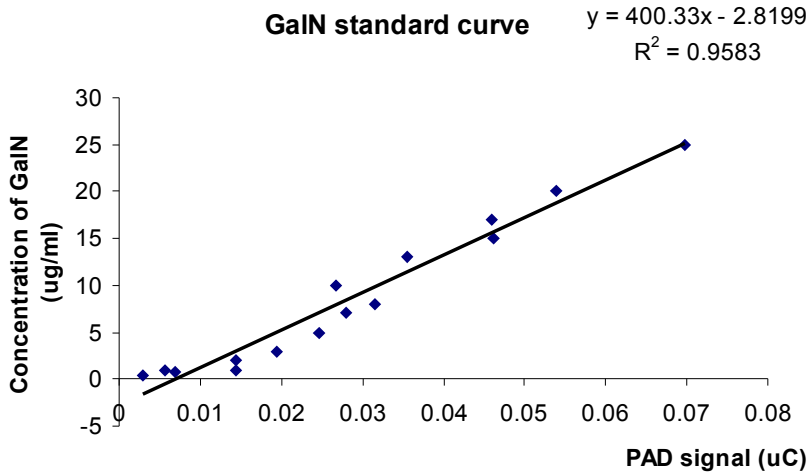
5.2.1. Standard curve of Fucose



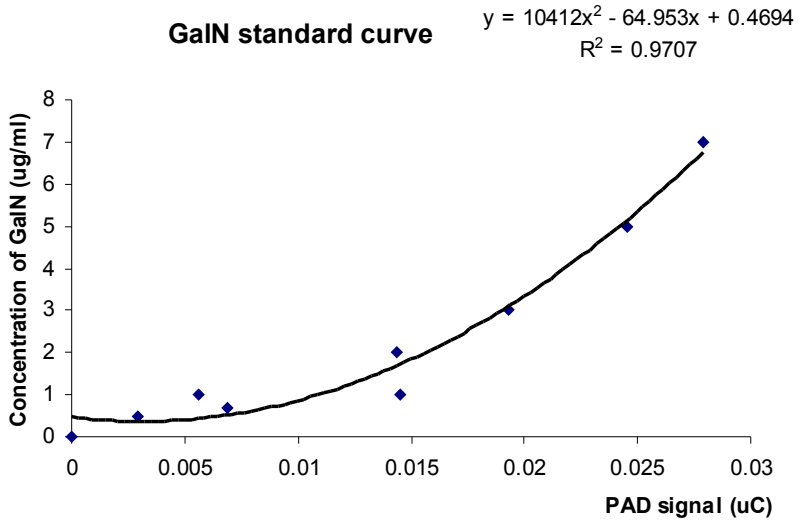
PAD signal (uC)

5.2.2. Standard curve of Galactosamine

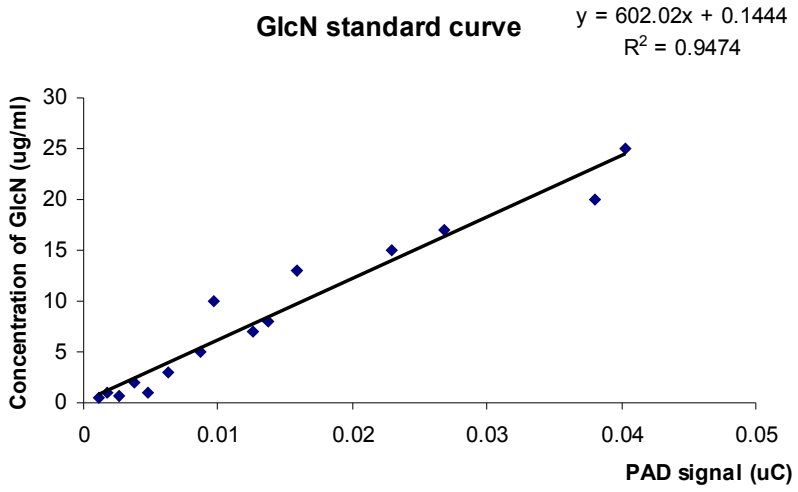
5.2.2.1. Standard curve of GalN with the concentration ranged from 2- 25 µg/ml



5.2.2.2. Standard curve of GalN with the concentration ranged from 0- 2 µg/ml

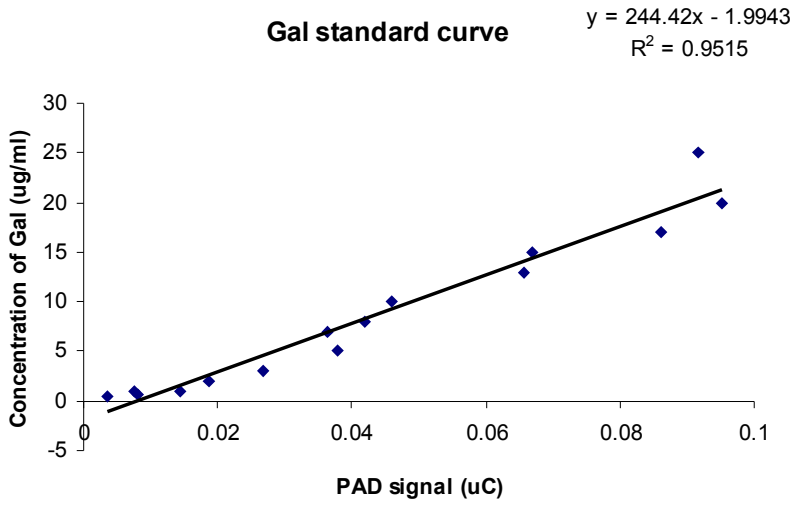


5.2.3. Standard curve of Glucosamine

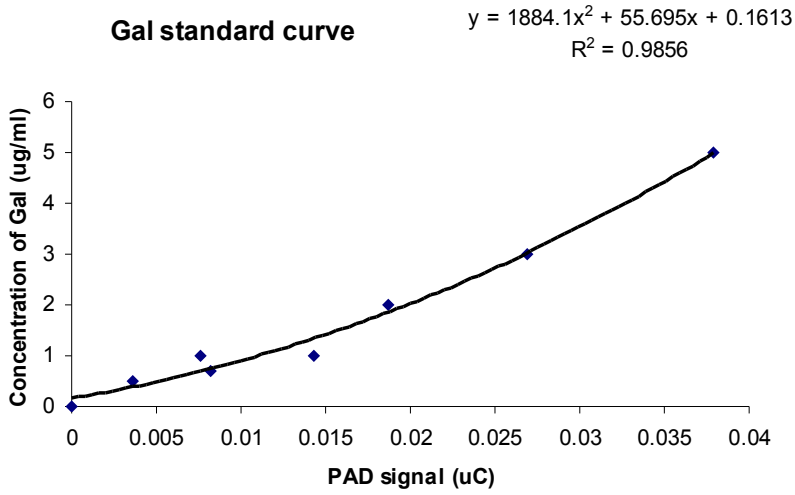


5.2.4. Standard curve of Galactose

5.2.4.1. Standard curve of Gal with the concentration ranged from 2- 25 µg/ml



5.2.4.2. Standard curve of Gal with the concentration ranged from 0- 2 µg/ml



5.2.5. The peak area and peak height of each monosaccharide corresponding to its concentration in the mixtures

Concentration (µg/ml)	Fucose			Galactosamine			Glucosamine			Galactose		
	Retention time	Peak area	Peak height	Retention time	Peak area	Peak height	Retention time	Peak area	Peak height	Retention time	Peak area	Peak height
0.5	8.79	0.00203	0.00469	13.89	0.00291	0.0053	15.15	0.00119	0.00218	16.86	0.00362	0.00491
0.7	8.71	0.00633	0.0133	14.23	0.00692	0.012	15.39	0.00264	0.00502	17.37	0.00818	0.00977
1	8.95	0.00407	0.00943	14.51	0.00559	0.00982	15.62	0.00172	0.00342	17.67	0.00759	0.00894
2	8.72	0.0145	0.03044	14.09	0.01435	0.02377	15.24	0.00374	0.00746	17.25	0.01873	0.01973
3	8.76	0.02289	0.04756	14.24	0.01933	0.03034	15.45	0.00634	0.01204	17.49	0.02689	0.02743
5	8.71	0.03788	0.07547	14.12	0.02456	0.03493	15.33	0.00873	0.01682	17.39	0.03793	0.03729
7	8.67	0.04322	0.08929	13.75	0.02789	0.03981	15.07	0.01259	0.02322	16.86	0.03644	0.03928
8	8.17	0.04418	0.09075	13.18	0.03158	0.04291	14.47	0.01372	0.02568	16.21	0.04181	0.04341
10	8.95	0.0432	0.08688	14.47	0.02678	0.03788	15.62	0.00975	0.0199	17.82	0.04599	0.04384
13	8.57	0.06563	0.13566	13.81	0.03555	0.05091	14.92	0.01583	0.0325	16.99	0.0657	0.06426
15	8.57	0.07563	0.15621	13.48	0.0461	0.06251	14.75	0.02296	0.04529	16.54	0.06681	0.07186
17	8.55	0.1034	0.19891	13.82	0.04604	0.07139	14.92	0.02687	0.0532	17.00	0.08612	0.0825
20	8.40	0.12597	0.23853	13.31	0.05396	0.09553	14.45	0.03808	0.07344	16.33	0.09522	0.10026
25	8.35	0.13122	0.25745	12.83	0.06984	0.11434	14.12	0.04036	0.08444	15.65	0.09155	0.11029

Appendix 5.3. Monosaccharide composition of abomasal and duodenal mucins in sheep

Infection	Age	Day of infection	Sample No	Abomasum				Duodenum			
				Fuc (%)	GalN (%)	GlcN (%)	Gal (%)	Fuc (%)	GalN (%)	GlcN (%)	Gal (%)
H. contortus infected sheep	4.5	21	1, 2	10.61	21.75	24.41	43.24	13.51	29.46	24.08	32.95
	4.5	21	3*, 4*	6.09	18.03	41.11	34.77	13.71	26.02	15.59	44.67
	4.5	21	5, 6	2.22	16.59	38.56	42.64	15.16	25.2	34.5	25.13
				6.3	**18.8**	**34.7**	**40.2**	**14.1**	**26.9**	**24.7**	**34.3**
				4.2	*2.7*	*9.0*	*4.7*	*0.9*	*2.3*	*9.5*	*9.8*
	6	21	7, 8	3.59	26.12	22.37	47.93	7.34	16.77	19.93	55.96
	6	21	9*, 10*	10.58	27.69	14.63	47.09	16.12	23.23	28.48	32.17
	6	21	11, 12	16.27	36.1	12.95	34.69	12.17	21.28	23.57	42.97
				10.1	**30.0**	**16.7**	**43.2**	**11.9**	**20.4**	**24.0**	**43.7**
				6.4	*5.4*	*5.0*	*7.4*	*4.4*	*3.3*	*4.3*	*11.9*
	8	21	13*, 14*	8.13	14.73	18.46	58.67	4.55	8.56	26.39	60.5
	8.5	21	15, 16	1.62	17.68	34.59	46.12	0.19	5.89	28.22	65.7
	9	21	17*, 18*	5.35	26.71	23.13	44.82	9.93	2.53	22.21	65.32
				5.0	**19.7**	**25.4**	**49.9**	**4.9**	**5.7**	**25.6**	**63.8**
				3.3	*6.2*	*8.3*	*7.6*	*4.8*	*3.0*	*3.1*	*2.9*
T. circumcincta infected sheep	4.5	21	19*, 20*	8.82	7.08	16.5	67.6	10.27	29.8	8.77	51.15
	4.5	21	21*, 22*	9.86	8.28	27.96	53.89	14.59	17.22	25.5	42.68
	4.5	21	23, 24	6.22	12.72	13.74	67.32				
				8.3	**9.4**	**19.4**	**62.9**	**12.4**	**23.5**	**17.1**	**46.9**
				1.9	*3.0*	*7.5*	*7.8*	*3.1*	*8.9*	*11.8*	*6.0*

T. circumcincta infected sheep (continued)	6 6 6	21 21 21	25, 26 27*, 28* 29, 30	1.36 6.17 7.98 **5.2** *3.4*	35.55 29.99 24.45 **30.0** *5.6*	8.68 6.82 15.68 **10.4** *4.7*	54.4 57.02 51.9 **54.4** *2.6*	13.87 18.97 30.01 **21.0** *8.3*	6.16 6.93 7.5 **6.9** *0.7*	71.63 38.66 40.7 **50.3** *18.5*	8.34 35.44 21.79 **21.9** *13.6*
	9 9	21 21	31*, 32* 33, 34 35, 36	23.76 23.11 **23.4** *0.5*	19.95 20.09 **20.0** *0.1*	11.91 13.81 **12.9** *1.3*	44.38 42.98 **43.7** *1.0*	5.82 7.81 **6.8** *1.4*	30.46 24.64 **27.6** *4.1*	6.55 15.21 **10.9** *6.1*	57.18 52.34 **54.8** *3.4*
Non-infected sheep	4 4.5 4.5		37*, 38* 39, 40 41, 42	11.73 12 13.99 **12.6** *1.2*	14.76 25.34 33.62 **24.6** *9.5*	31.26 19.27 15.13 **21.9** *8.4*	42.25 43.4 37.26 **41.0** *3.3*	14.3 9.05 **11.7** *3.7*	50.07 36.64 **43.4** *9.5*	25.35 30.15 **27.8** *3.4*	10.28 24.15 **17.2** *9.8*
	6 6 6		43*, 44* 45, 46 47, 48	14.70 6.89 27.36 **16.3** *10.3*	29.19 23.83 19.96 **24.3** *4.6*	17.77 24.00 16.11 **19.3** *4.2*	38.35 45.27 36.56 **40.1** *4.6*	22.45 31.36 **26.9** *6.3*	44.09 18.38 **31.2** *18.2*	7.19 15.14 **11.2** *5.6*	26.27 34.86 **30.6** *6.1*
	9 9 9		49*, 50* 51, 52 53, 54	24.41 29.06 14.73 **22.7** *7.3*	34.69 21.45 27.55 **27.9** *6.6*	5.23 9.24 12.53 **9.0** *3.7*	35.68 40.25 45.18 **40.4** *4.8*	38.16 31.31 22.53 **30.7** *7.8*	25.47 15.91 23.34 **21.6** *5.0*	9.80 24.72 22.76 **19.1** *8.1*	26.57 28.05 31.37 **28.7** *2.5*
La	3 day-old		55*, 56*								

mbs			57*, 58* 59, 60	**2.5**	**24.9**	**25.4**	**47.2**	**1.9**	**26.8**	**32.7**	**38.6**
Lambs (continued)	9 week-old, milk-fed		61*, 62*	13.40	45.00	9.91	31.69	13.21	50.36	5.85	30.59
			63*, 64*	18.93	33.48	9.40	38.18	30.97	53.17	9.99	5.87
			65, 66	8.36	36.74	9.46	45.44				
				13.6	**38.4**	**9.6**	**38.4**	**22.1**	**51.8**	**7.9**	**18.2**
				5.3	*5.9*	*0.3*	*6.9*	*12.6*	*2.0*	*2.9*	*17.5*
	9 week-old, solid-fed		67*, 68*	22.36	36.64	16.19	24.82	18.67	36.08	15.82	29.43
			69*, 70*	20.83	43.78	13.68	21.72	24.12	31.75	18.02	26.12
			71, 72	16.25	40.29	19.21	24.25	19.51	36.06	20.16	24.28
				19.8	**40.2**	**16.4**	**23.6**	**20.8**	**34.6**	**18.0**	**26.6**
				3.2	*3.6*	*2.8*	*1.7*	*2.9*	*2.5*	*2.2*	*2.6*
	9 week-old, solid-fed+ *T. circumcincta* infection	35	73*, 74*	11.64	30.39	16.71	41.25	12.60	41.01	5.87	40.52
			75*, 76*	15.99	34.88	6.08	43.05	25.22	39.38	5.30	30.10
			77, 78	3.07	41.03	18.50	37.40	20.25	46.85	19.37	13.53
				10.2	**35.4**	**13.8**	**40.6**	**19.4**	**42.4**	**10.2**	**28.1**
				6.6	*5.3*	*6.7*	*2.9*	*6.4*	*3.9*	*8.0*	*13.6*

* Tissues collected from those abomasum and duodenum were used for histochemistry and lectin histochemistry.

+ Figure in bold represents the mean value

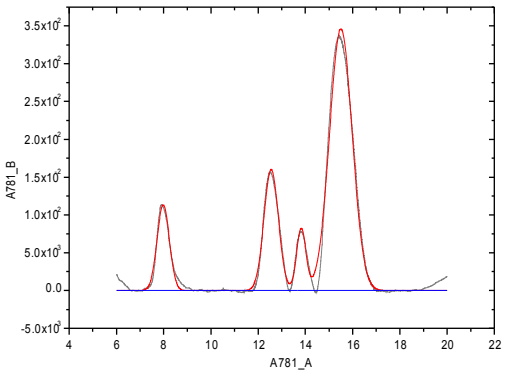
Figure in italics represents the standard deviations

§ Odd figure represents abomasal mucin whereas even figure represents duodenal mucin of the same sheep

5.4. Peak of monosaccharides fitted with Gaussian distribution

Peak Analysis Title

Source File: A781	Data Set: A781_B	Date:8/2/2008
Chi^2=7.403737194E-7	COD=0.98893	# of Data Points=4201
SS=0.003101425510	Corr Coef=0.99445	Degree of Freedom=4189



Fitting Results

Peak #	Peak Type	AreaFitT	FWHM	MaxHeight	CenterGrvty	AreaFitTP
1	Gaussian	0.0073	0.60538	0.01133	7.98201	11.29103
2	Gaussian	0.01212	0.71002	0.01604	12.55089	18.73969
3	Gaussian	0.00399	0.45942	0.00817	13.82345	6.17372
4	Gaussian	0.04127	1.11981	0.03462	15.50135	63.79556
		0.06469				

BaseLine: CONSTANT

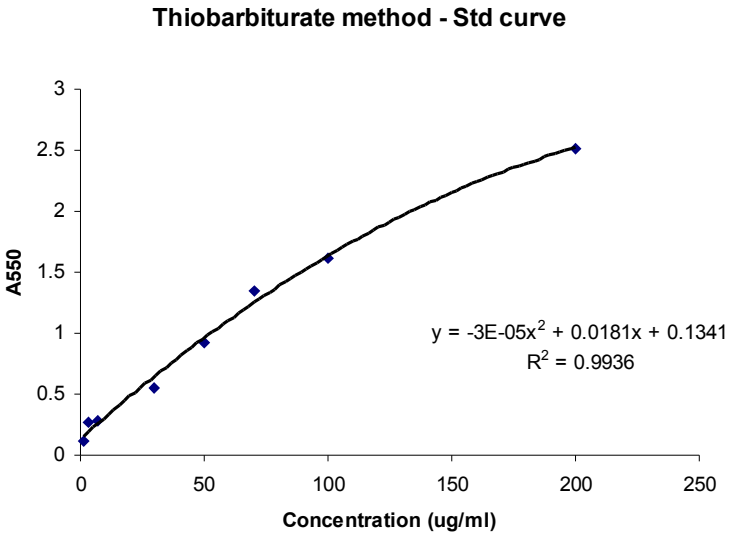
The following fields can be moved into the plot to show various peak characteristics. First, move proper fields into the plot, save this template, then assign these fields with proper options in Peak Characterization Report Plot dialog box, or directly in the initialization file.

Field 8 Field 9 Field 10 Field 11

Field 12 Field 13 Field 14 Field 15

Field 16 Field 17 Field 18 Field 19 Field 20

5.5. The correlation between concentration of N-acetyl neuraminic acid (NANA) and A_{550}.


Thiobarbiturate method - Std curve
A550
Concentration (ug/ml)
y = -3E-05x2 + 0.0181x + 0.1341
R2 = 0.9936
0
0.5
1
1.5
2
2.5
3
0
50
100
150
200
250

Appendix 6

TISSUE PREPARATION AND STAINING

6.1. Solution

6.1.1. Carnoyd's fluid

The solution was made up with ethanol: chloroform: glacial acetic acid at the ratio of 6:3:1, stored at room temperature in the fume hood (because of its toxicity).

6.1.2. Preparation of 4% paraformaldehyde

Weight out enough paraformaldehyde powder (Sigma) for the volume of 4% solution. For 100 ml use 4 g and add 80 ml MQ water.

Heat carefully at ~60^{o}C (solution is cloudy white) for about 60 min with stirrer on a hot plate with beaker covered with foil in a fume hood.

Make sure the solution temperature does not exceed 65^{o}C. If it does, start again. Solution will be cloudy, add 10 µl aliquots of 1M NaOH (BDH chemicals) until the solution becomes clear.

The solution is allowed to cool down to room temperature and then filter through filter 0.22 µm into 100 ml volumetric flask (Schott) and make up to 100 ml with MQ water (MilliPore system). Then it is divided into falcon tubes (20 ml each aliquot), then can be used for tissue fixing.

They are frozen at -20^{o}C and thawed once used. Solution can be thawed at room temperature or at 37^{o}C in emergency. After being thawed, solution needs to be mixed well by shaking several times. Then it is ready to use.

6.1.3. 2% calcium acetate

2 g of calcium acetate is dissolved in 100 ml MQ water and stored at 4^{o}C

6.1.4. Ethanol 90%, 70% and 40%

These solutions were prepared from 95% ethanol diluted with MQ water, store at room temperature in capped bottle to prevent evaporation.

6.1.5. Sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0)

Tri-sodium citrate (dehydrate)	2.94 g
Distilled water	1000 ml

Mix to dissole. Adjust pH to 6.0 with 1 N HCl and then add 0.5 ml of Tween 20 and mix well.

Store this solution at room temperature for 3 months or at 4^{o}C for longer storage.

Washing buffer: 10X TBS- Tween 20 (0.5 M Tris base, 9% NaCl, 0.5% Tween 20, pH 7.6)

Trizma base	61 g
NaCl	90 g
Distilled water	1000 ml

Mix to dissolve and adjust to pH 7.6 using concentrated HCl and then add 5 ml Tween 20

Store this solution at room temperature. Dilute 1:10 with distilled water before use and adjust if necessary.

6.1.6. Enzyme digestion buffer

0.2 g trypsin was dissolved in 200 ml above TBS- Tween 20 washing buffer 1X, incubate at 37^{o}C for 30-60 min before incubate with sections.

6.1.7. Preparation of Schiff's reagent

200 ml of distilled water was warmed to 37^{o}C then add 1 g of pararasaniline. The mixture was shaken until dissolved.

3.8 g sodium bisulphite and 1 ml concentrated HCl was added. The whole mixture was shaken well. Keep shaking and cool with water. Put away in dark overnight.

Add 1 g of activated charcoal. Shake well. Filter. Store in refrigerator.

6.1.8. Preparation of Alcian blue solution

pH 1.0: 1 g Alcian blue in 100 ml 0.1 M HCl

pH 2.5: 1 g Alcian blue in 100 ml acetic acid 3%

6.1.9. Preparation of diamine solution

Dissolve 120 mg or N.N- dimethyl-m-phenylenediamine dihydrochloride and 20 mg N,N- dimethyl-p- phenylenediamine dihydrochloride in 50 ml distilled water.

Then add 1.4 ml 40% ferric chloride

Then solution pH should be between 1.5- 1.6.

6.1.10. Biotinylated lectins

Lectins, except *Maackia amurensis* agglutinin already pre- diluted, were diluted in PBS buffer.

PBS buffer was made up by dissolving 4 g NaCl; 0.2 g KCl; 0.575 g Na_2HPO_4; 0.1 g KH_2PO_4 in 500 ml MQ water, pH 7.2. Buffer was sterilized by autoclaving and keep at 4°C.

Biotinyalted lectins	Source	Abbre.	Concentration (mg/ml)	Volume per 200 µl buffer (µl)
Concanavalin A	*Canavalia ensiformis* (Jack bean) seeds	ConA	1	1.5
Soybean agglutinin	*Glycine max*	SBA	1	1.5
Wheat germ agglutinin	*Triticum vulgaris* (wheat germ)	WGA	1	1.5
Dolichos biflorus	*Dolichus biflorus* (horse gram) seeds	DBA	1	1.5

agglutinin				
Ulex europaeus agglutinin I	*Ulex europaeus* (furze gorse) seeds	UEA I	1	1.5
Ricinus communis agglutinin I	*Ricinus communis* (castor bean) seeds	RCA I	1	1.5
Peanut agglutinin	*Arachis hypogaea* (peanuts)	PNA	1	1.5
Griffonia (Bandeiraea) simplicifolia lectin II	*Griffonia (Bandeiraea) simplicifolia* seeds	GSA II	1	1.5
Griffonia (Bandeiraea) simplicifolia lectin I	*Griffonia (Bandeiraea) simplicifolia* seeds	GSA I	1	1.5
Lotus tetragonolobus lectin	*Lotus tetragonolobus* seeds	LTA	1	1.5
Maackia amurensis lectin II		MAA	1	1.5
Psophocarpus tetragonolobus lectin II	Winged bean	PTL	2	0.75
Maclura pomifera lectin	*Maclura pomifera* (osage orange tree) seeds	MPL	2	0.75
Sambucus nigra lectin	*Sambucus nigra* (elderberry) bark	SNA	2	0.75
Pisum sativum agglutinin	*Pisum sativum* (garden pea) seeds	PSA	5	0.3
Lens culinaris agglutinin	*Lens culinaris* (lentil) seeds	LCA	5	0.3
Bauhinia purpurea lectin	*Bauhinia purpurea* alba seeds	BPL	2	0.75
Aleuria	*Aleuria aurantia*	AAL	2	0.75

aurantia lectin	mushrooms			
Phaseolus vulgaris agglutinin	*Phaseolus vulgaris*	PHA	5	0.3

6.1.11. Flourescent- conjugated streptavidin

1 mg streptavidin Alexa Fluor® 546 and 488 was dissolved in 1ml autoclaved PBS buffer, stored at 4°C, protected from light (covered with foil) and ready to dilute for staining.

* Note for tissue fixation use glass Petri dishes to fix tissues (plastic Petri dishes are not suitable, damaged when chloroform is in use), tissue sections lie in the lid of the Petri dish, covered with Carnoyd's fluid, lay the bottom of the dish on top the tissues so that it is held flat during the fixation. Paraformaldehyde needs to be shaken thoroughly, filtered first before use. After being trimmed and cut, tissues were kept in plastic vials sealed with paraffin, stored in ethanol 70%.

6.2. Staining

6.2.1. Periodic acid Schiff – PAS staining

Sections were dewaxed and hydrated through grades of ethanol. Then, they were oxidized in 0.5% periodic acid for 5 min. Sections were washed in running tap water and incubated with Schiff's reagent for 10 min. They were washed in running tap water and incubated with Mayer's haematoxylin (Sigma) for 30 seconds. Sections were washed in water, dehydrated through grades of ethanol, cleared and mounted by entellan solution (Merck, Germany). Sections were covered by coverslip and looked under light microscopy.

6.2.2. Alcian blue pH 2.5 and pH 1.0

Sections were de-paraffinized and hydrated. Then they were stained with Alcian blue with pH 2.5 and pH 1.0 for 10 min. Sections were washed with running tap water and continue from step 2 as above PAS staining.

6.2.3. High iron diamine – HID staining (Spicer's technique)

Sections after being de-paraffinzed and hydrated were stained with freshly prepared diamine solution for 24 hours.

Rinse sections with distilled water. Dehyrate section, clear and mount with ettelan solution.