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Research paper

Histochemical study of the effects on abomasal mucins of *Haemonchus contortus* or *Teladorsagia circumcincta* infection in lambs

H.V. Simpson[a,*], S. Umair[b], V.C. Hoang[a], M.S. Savoian[c]

[a] Institute of Veterinary, Animal and Biomedical Sciences, Massey University, Private Bag 11-222, Palmerston North, New Zealand
[b] The Hopkirk Research Institute, AgResearch Ltd., Private Bag 11-008, Palmerston North, New Zealand
[c] Institute of Fundamental Sciences, Massey University, Private Bag 11-222, Palmerston North, New Zealand

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ABSTRACT

Previously, chemical analysis of gastric fundic mucin showed that infection of sheep with *Haemonchus contortus* or *Teladorsagia circumcincta* changed the proportions of monosaccharides and decreased terminal mucin fucosylation and sialylation. To identify the effects of these parasites on the two mucin-secreting cell lineages, fundic and antral tissues were collected for histochemistry from 69 lambs aged from 3–4 to 9–10 months-of-age which had received a single infection of either *H. contortus* or *T. circumcincta* and euthanased at Day 21 or 28 post- infection respectively. All fundic tissues were stained separately with: (1) with Periodic Acid Schiff (PAS) for all mucins; (2) Alcian Blue (AB) pH 2.5 for acidic mucins (sialylated and sulphated); (3) AB pH 1 for sulphated mucins and (4) High Iron Diamine (HID) for sulphated mucins. Antral and fundic tissues from 24 lambs were also stained for acidic and neutral mucins or with specific lectins for α-1-linked fucose and for α-2,3- and α-2,6-linked sialic acids. Only mucin sulphation appeared to differ visually in uninfected lambs over this age range: there was weak staining with HID in tissues from lambs 3–6 months-of-age, but was generally more intense in those over 7 months-of-age. Sulphomucins were not apparent in surface mucous cells (SMC) or generally in the upper pits. Sialylomucins were located predominantly in the pits and glands, with small amounts of sialylated mucins in SMC and on the luminal surface, mainly in younger animals up to 6 months-of-age and less in the older animals. Parasitism markedly reduced the predominantly neutral surface mucin5AC of the SMC and pit cells, despite pit elongation in both antrum and fundus, whereas the acidic Muc6 secreted by mucus neck cells (MNC) increased along with MNC hyperplasia. Sulphated mucins were present mainly from the mid-pits downward and heavy staining was more common in older animals. In these sheep, the markedly reduced neutral mucin in the SMC and pit cells in both antrum and fundus contrasts with reported hypersecretion of mucus in the intestine, which is believed to aid in parasite expulsion. It has been proposed that intestinal goblet cell hypersecretion occurs only in resistant animals, therefore reduced mucins in the abomasum may be indicative of susceptibility to abomasal parasites.

1. Introduction

Gastrointestinal and other epithelial surfaces are overlaid with a protective mucus gel containing mainly secreted high M.W. mucins, as well as ions, antibodies and other molecules which reduce physical and chemical damage to the epithelium (Allen, 1981; Powell, 1981; Lichtenberger, 1995). Mucins consist of a polypeptide core to which are attached mainly O-linked oligosaccharides between GalNAc and hydroxyl groups of serine or threonine (Kobata, 1992), as well as by N-linkages, in which GlcNAc is linked to an amine group of asparagine (Kobata, 1992). Different epithelial tissues express genes for large secreted gel-forming mucins and smaller membrane-bound mucins. In the stomach, the secreted mucins are Muc5AC and Muc6 (Ho et al., 1995; Byrd et al., 1997), in contrast to Muc2 in the intestine and also Muc6 in the duodenum (de Bolos et al., 2001). The lower M.W. membrane-bound mucins are mainly Muc1 in the stomach and Muc2 and 3 in the intestine (Andrianifahanana et al., 2006).

The characteristics of mucins influence the susceptibility or resistance of a host to pathogens which attach to specific cell surface carbohydrate structures, often the oligosaccharides of mucins (Hakansson et al., 1996; Wadstrom et al., 1996; Freitas et al., 2002; Magalhães et al., 2009). Altered mucin glycosylation can therefore

* Corresponding author at: Institute of Veterinary, Animal and Biomedical Sciences, Massey University, Private Bag 11-222, Palmerston North 4442, New Zealand.
E-mail address: H.V.Simpson@massey.ac.nz (H.V. Simpson).

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compromise the protection provided by gastrointestinal mucins, which depends largely on their oligosaccharide chains (Mall et al., 1997). The proportions of neutral, sialo- and sulphomucins are altered by bacteria (Ota et al., 1998; Kobayashi et al., 2009), including the normal gut flora (Enss et al., 1992), protozoa (Choi et al., 2003) and by diseases such as cancer, ulcerative colitis and cystic fibrosis (Ehsanullah et al., 1982; Raouf et al., 1992; Xia et al., 2005). Mucins also play a role in the immunity to intestinal nematode parasites (reviewed by Hasnain et al., 2013) through goblet cell hyperplasia and increased secretion of mucus (Muc2) and associated protective proteins, increased mucin sulphation and ectopic expression of gastric type Muc5AC.

The changes in abomasal mucins seen with age and in the presence of nematodes may be tissue-specific, and even parasite-specific, and differ from those in the intestine. The mucin secreting cells in the stomach are the upward migrating surface mucous cell (SMC)/pit cell lineage and the downward migrating zymogenic mucous neck cells (MNC), the immature chief cells. In the antrum, Muc5AC is produced by SMC and pit cells and Muc6 by basal gland cells. In the gastric fundus, Muc5AC is secreted by SMC and pit cells and Muc6 by MNC, to form a luminal mucus gel of alternating layers of the two mucins (Ota and Katsuyama, 1992). Chemical analysis of abomasal mucins showed altered glycosylation of fundic mucin during infection of sheep with *Haemonchus contortus* or *Teladorsagia circumcincta*: both parasites decreased mucin fucosylation, sialylation and sulphation (Hoang et al., 2010a,b), but changes in the proportions of other monosaccharides differed during infection with the two parasites (Hoang et al., 2010a). Miller et al. (1983) and Newlands et al. (1990) observed histologically that in 9–10 months-old control animals there were neutral mucins in the SMCs, both neutral and acidic mucins in the pits and mainly acid mucins in the isthmus and neck zones, but in infected animals, there was less neutral mucin in the surface zone and neutral and acidic mucins in the neck, but significantly increased sulphomucins in the isthmus mucous cells. This is consistent with the reduced expression of Muc5AC in sheep infected with *H. contortus* (Rowe et al., 2009).

Newborn lambs are relatively resistant to nematode parasites until weaning, become susceptible and develop resistance over several months (Manton et al., 1962; Urquhart et al., 1966; Dineen et al., 1978; Smith et al., 1985; Stear et al., 1996) and generally become resistant by 9–10 months-of-age (Smith et al., 1985). Successive changes in mucin secretion and composition over the same time period may be a factor determining susceptibility, as mucin composition influences infection of humans and animals with bacteria (Ota et al., 1998; Kobayashi et al., 2009) and protozoa (Choi et al., 2003). With increasing age in mammals, gastrointestinal sulphation decreases (Shub et al., 1983; Turck et al., 1993; Scocco et al., 2001) and there is increasing fucosylation and decreased sialylation, caused by changing enzyme activities during development (Shub et al., 1983; Torres-Pinedo and Mahmood, 1984). In very young and unweaned lambs, the thick layer of gastric mucins, which are more highly sulphated and sialylated than in older animals (Hoang et al., 2010a,b), may contribute to resistance to parasitism until after weaning (Zeng et al., 2001). The changes in monosaccharide composition during the first weeks of life, particularly reduced sialylation and sulphation and increased fucosylation, continue over the next 9–10 months (Ishihara et al., 1985; Ohwada and Suzuki, 1992; Hoang et al., 2010a,b).

In the present experiments, fundic tissues were collected for histochemistry from 69 lambs aged from 3–4 to 9–10 months-of-age, which had received a single infection of either *H. contortus* or *T. circumcincta* and euthanased at Day 21 or 28 p.i. respectively. Egg- laying adult worms are present at this time and, together with other luminal stages of these parasites, cause the pathophysiology associated with abomasal nematodes (Lawton et al., 1996; Simpson et al., 1997; Scott et al., 2000). The tissues were stained according to Spicer (1965) with Periodic Acid Schiff (PAS) for all mucins, Alcian Blue (AB)/PAS pH 2.5 for both sialylated and suphated mucin and High Iron Diamine (HID) and AB/PAS pH 1 for sulphated mucin. Inclusion of AB/PAS pH 1 in the protocol allows distinction of sialomucins and sulphomucins. Antral tissue was also examined from 27 of these animals from which mucins had been chemically analysed (Hoang et al., 2010a). Lectin histochemistry was performed with UEA-1 (*Ulex europaeus* agglutinin-1) for α-1,2-linked Fuc; MAL (*Maackia amurensis* lectin) for α-2,3-linked sialic acids and SNA (*Sambucus nigra* agglutinin) for α-2,3- and α-2,6-linked sialic acids on a smaller number of tissues to detect fucosylation and sialylation of mucins.

2. Materials and methods

2.1. Sheep and parasite infection

Tissues were collected from a total of 69 male sheep, mainly Romney or Romney cross, aged between 3 and 10 months, including 27 sheep used for chemical analyses of mucins (Hoang et al., 2010a). Animals were obtained at 2–3 months-of-age from the field, where they had been exposed to field infections. When brought indoors, they were immediately drenched with 2 mL/5 kg Matrix (Ancare, New Zealand), followed 2 days later with a second dose (1 mL/5 kg). Absence of eggs in faeces confirmed the removal of nematodes. The animals were fed *ad libitum* with lucerne chaff and had free access to water.

Larvae for infection were cultured from faeces of sheep infected with pure strains of *H. contortus* or *T. circumcincta* and stored at 10 °C or 4 °C respectively prior to use. A total of 14 lambs remained uninfected controls, 31 received 10,000 L3 *H. contortus* and 24 were given 50,000 L3 *T. circumcincta*. The lambs were grouped by age at tissue collection of 3–4, 5–6, 7–8 or 9–10 months-of age consisting of uninfected ($N = 5, 4, 2$ and 3); *H. contortus*-infected ($N = 6, 8, 12$ and 5) and *T. circumcincta*-infected ($N = 7, 9, 5$ and 3) animals respectively. Animals were euthanased by captive bolt and exsanguination on Day 21 post-infection (p.i.) for *H. contortus* or Day 28 p.i. for *T. circumcincta* infection. All infected sheep had worms in the abomasum at necropsy and no worms were seen in the control animals.

2.2. Tissue collection

After euthanasia, the abomasum was removed, opened along the greater curvature and gently washed with saline 4 times to remove worms and adherent debris. A 2 × 2 cm piece of tissue was collected from a fundic fold, and in 27 lambs, also from the centre of the antrum. Tissues were fixed overnight in Carnoy's fluid, then for 3 h in a solution of 4% paraformaldehyde and 2% calcium acetate. They were kept in 70% ethanol until routinely processed and embedded in paraffin wax. Sections were cut 5 μm thick.

2.3. Histochemistry

Reagents were obtained from Sigma except Alcian blue, *N,N*-dimethyl-m-phenylendiamine dihydrochloride and *N,N*-dimethyl-*p*-phenylendiamine monohydrochloride (Fluka) and sodium metabisulfite (Riedel-de Haen). All fundic tissues were stained separately with: (1)PAS for all carbohydrate residues; (2) AB pH 2.5 for acidic mucins (sialylated and sulphated); (3) AB pH 1 for sulphated mucins and (4) HID for sulphated mucins (Spicer, 1965). Antral and fundic tissues from 27 lambs were also stained with AB pH 2.5 or pH 1.0, followed by counterstaining with PAS. Sections were examined with bright field illumination using an Olympus BX51 microscope outfitted with a 10X (N.A. 0.3) lens. Images were acquired with a MicroPublisher 5.0 RTV camera