Acta histochemica **109** (2007) 347—357

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acta histochemica
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Lectin histochemistry of gastrointestinal glycoconjugates in the greater horseshoe bat, *Rhinolophus ferrumequinum* (Schreber, 1774)

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Received 2 October 2006; received in revised form 21 February 2007; accepted 27 February 2007

KEYWORDS
Rhinolophus ferrumequinum;
Glycoconjugates;
Stomach;
Intestine;
Lectin histochemistry;
Immunohistochemistry

Summary

Mucins in the gastrointestinal tract of *Rhinolophus ferrumequinum* were investigated by histochemistry and lectin histochemistry to evaluate morphofunctional variations of different regions and their possible physiological and evolutionary implications. Histochemical methods included periodic acid-Schiff (PAS), Alcian blue (AB) at pH 2.5 and 1.0 and high-iron-diamine AB pH 2.5. Binding of lectins Con A, DBA, WGA, LTA, LFA, PNA and SBA; LFA, PNA and SBA with prior sialidase treatment; and paradoxical Con A were evaluated. The oesophagus lacked glands. The stomach was divided into a short cardias, a wide fundus and a brief pylorus. The surface muciparous cells secreted sulpho- and sialomucins with *N*-acetylgalactosamine (GalNAc) residues, *N*-acetyllactosamine and (β1,4 *N*-acetylglucosamine)$_n$ chains. Towards the pylorus, *N*-acetylgalactosamine residues disappeared and acidity decreased. Cardiac glands, neck cells in the fundic glands, pyloric and duodenal Brunner's glands all shared neutral, stable class-III mucins, mainly with *N*-acetylgalactosamine sequences. The intestine was divided into a duodenum, a jejuno-ileum and a short rectum. The goblet cells produced sulpho- and sialomucins with sialylated *N*-acetylgalactosamine sequences, (β1,4 *N*-acetylglucosamine)$_n$ and *N*-acetyllactosamine, whose sialylation increased towards the rectum. The main features of the mucins are probably associated with the requirements of fast absorption and food passage and in protection against mechanical and pathogenic injuries.

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doi:10.1016/j.acthis.2007.02.010

Introduction

It is well known that gastrointestinal secretions in vertebrates contain a number of mucosubstances that can differ according to cell type, anatomical region, functional status, pathological condition, age, sex and species (e.g., see Sheahan and Jervis, 1976; Filipe, 1979; Sato and Spicer, 1980; Allen, 1981; Suganuma et al., 1981; Pedini et al., 2001; Liquori et al., 2002; Choi et al., 2003; Schumacher et al., 2004). Thus, knowledge of variations in mucin composition along the gastrointestinal tract is of help in elucidating functional, pathologic and even taxonomic problems (e.g., Forman, 1972; Kamiya and Pirlot, 1975). Lectin histochemistry provides a tool for detecting differences between oligosaccharidic chains of glycoproteins (e.g., Brooks et al., 2002). It should be noted that while lectin-binding specificity is often quoted in terms of monosaccharide binding preference, for example a lectin may be said to be mannose-binding, lectins often recognize more complex structures in oligomers in which the specific monosaccharide is probably present in the sequence (Brooks et al., 2002). Thus, the monosaccharide binding specificity usually quoted generally refers to the monosaccharide that best inhibits lectin binding to complex (usually unidentified) glycoconjugates (e.g., Brooks and Carter, 2001), even if most workers refer to positive lectin labelling as indicative of presence in their sampled material of the monosaccharide or sequence that lectins best recognize. Nonetheless, lectin-binding studies are valuable for desease diagnostics and comparative purposes, in which they can detect variations between normal and pathologic conditions of given tissues, or between different regions in the same organ, or between homologous regions in specimens of different age, sex or species. Lectin histochemical studies have been widely reported in mammals, but not in Chiroptera. This order is characterised by several different dietary adaptations and, consequently, there is great morphofunctional variation between the gastrointestinal tracts of species (e.g., Grassé, 1955). Several studies on both gross and fine morphology (e.g., Rouk and Glass, 1970; Forman, 1972, 1973; Stutz and Ziswiler, 1984; Madkour et al., 1982; Tedman and Hall, 1985; Perrin and Hughes, 1992; Makanya and Maina, 1994; Makanya et al., 1997) have been carried out to relate variations in structure to different feeding habits. In contrast, few studies have investigated the glycoconjugates in the gastroenteric mucosa by histochemical methods (Rouk and Glass, 1970; Forman, 1971, 1972; Bhide, 1980, 1981; Perrin and Hughes, 1992) and there is only one published study using lectin histochemistry (Danguy et al., 1987). Additional data would be very useful in characterising the morphofunctional districts of the gastroenteric system and their variations among taxa, as well as their evolutionary and physiological implications.

In a previous study, we used histochemical, immunohistochemical and lectin histochemical methods to detect the cells secreting the gastric juice and mapped their variation along the gastric mucosa of the greater horseshoe bat, *Rhinolophus ferrumequinum* (Scillitani et al., 2005). In the present paper, we apply these methods to characterise the glycoconjugates in the gastrointestinal mucosa of the same species, in order to evaluate the variation in distribution of mucins and to provide clues as to their functional and evolutionary significance.

Material and methods

Two males and a female adult greater horseshoe bat, *R. ferrumequinum* (Schreber, 1774) were caught near Bari (Apulia) with the permission of the Italian Ministero dell'Ambiente. The specimens were sacrificed by ethyl ether overdose and their digestive tracts removed quickly. The samples were fixed in 10% formalin, dehydrated through graded alcohols and processed for embedding in paraffin wax by routine protocol (details given in Ferri et al., 1999). Serial sections, 4 μm thick, were cut by microtome.

Sections were dewaxed and rehydrated by routine protocol and were stained using the periodic acid-Schiff (PAS)–haemallum method (described by Mowry and Winkler, 1956), Alcian blue (AB) at pH 2.5 and pH 1.0, or with high-iron-diamine followed by Alcian blue (HID-AB) pH 2.5 (described by Spicer, 1965). Zymogen granules were identified with a modified Bowie's staining method according to Bonucci (1981).

Binding of seven horseradish peroxidase (HRP)-labelled lectins (all from Sigma, St. Louis, USA, except for LFA from EY Laboratories, San Mateo, California, USA) was assessed to determine the nature and the distribution of glycosidic residues in the oesophagogastric mucosa. The lectins employed, their concentrations and their sugar-binding preferences are summarized in Table 1. Lectin binding was performed as previously reported by Ferri and Liquori (1997) and Ferri et al. (2001). Briefly, rehydrated sections were exposed to 3% hydrogen peroxide for 10 min to inhibit endogenous peroxidase activity, and then incubated for 30 min

Table 1. Characteristics of the lectins utilized in this study

Lectin	Source and references	Major binding specificity	Lectin concentration (mg/ml)	Inhibitory sugar
Con A	*Canavalia ensiformis*[1]	D-mannose and/or D-Glucose	0.05	0.1 M MαM
WGA	*Triticum vulgaris* [2, 3, 4]	(GlcNAcβ1,4)*n*	0.02	0.01 M TACT
SBA	*Glycine max*[5, 6]	GalNAc	0.02	0.2 M GalNAc
PNA	*Arachis hypogaea* [7]	Galβ1, 3GalNAc	0.06	0.2 M Gal
LTA	*Tetragonolobus purpureus*[8, 9]	L-Fucα1, 6GlcNAc and L-Fucα1, 2Galβ1, 4[L-Fuc1,3] GlcNAcβ1,6R	0.10	0.2 M L-Fuc
DBA	*Dolichos biflorus*[10, 11]	GalNAcα1, 3GalNAc	0.02	0.2 M GalNAc
LFA	*Limax flavus*[12]	Sialic acid	0.02	0.2 M Sialic acid

Abbreviations: Fuc = fucose; Gal = galactose; GalNAc = *N*-acetylgalactosamine; GlcNAc = *N*-acetylglucosamine; MαM = methyl-α-mannopyranoside; TACT = *N*,*N*′,*N*″-triacetylchitotriose. References: [1]Finne and Krusius (1982), [2]Debray et al. (1983), [3]Gallagher et al. (1985), [4]Monsigny et al. (1980), [5]Pereira et al. (1974), [6]Bhattacharyya et al. (1988), [7]Lotan and Sharon (1978), [8]Pereira and Kabat (1974), [9]Debray et al. (1981), [10]Etzler and Kabat (1970), [11]Baker et al. (1983), [12]Miller et al. (1982).

at room temperature with peroxidase labelled lectin in 0.1 M phosphate-buffered saline (PBS). The activity of the HRP was then visualized histochemically with 0.005% 3–3′-diaminobenzidine (DAB: Sigma, St. Louis, USA) and 0.01% hydrogen peroxide in 0.05 M Tris–HCl buffer, pH 7.5 (Graham and Karnowsky, 1966), for 10 min. Finally, the sections were dehydrated through a graded ethanol series, cleared in xylene and mounted in DPX (Fluka BioChemika, Steinheim, Germany).

Controls: (1) substitution of the respective peroxidase-labelled lectin with PBS. (2) Incubation in the peroxidase-labelled lectin in the presence of the appropriate inhibitory sugar (concentrations are in Table 1), to confirm the specificity of lectin labelling. (3) Appropriate positive controls were included from different regions of the digestive system from two amphibians, *Bufo bufo* and *Triturus carnifex*, whose mucins were known to bind to the tested lectins as reported in previous studies (Liquori et al., 2002; Liquori et al., 2005).

Binding of LFA, PNA and SBA was also assessed after sialidase digestion. Reydrated sections were incubated in 1 U/10 ml sialidase (neuraminidase type V from *Clostridium perfrigens*, Sigma, St. Louis, USA) in 0.05 M/l acetate buffer, pH 5.5, containing 0.1% calcium chloride at 37 °C in a humid chamber for 30 min (Leathem and Atkins, 1983) before treatment with hydrogen peroxide and continuation of the method described above. LFA (a sialic acid binding lectin) labelling before and after sialidase digestion was included as a control to confirm that sialidase treatment was effective in removing sialic acid residues from tissues.

A variant of the Concanavalin A labelling method, paradoxical Concanavalin A labelling (PCS, periodate oxidation–borohydride reduction–Concanavalin A sequence), was also carried out to identify stable class III mucins, according to the method of Katsuyama and Spicer (1978). This method allows the grouping of complex carbohydrates in the mammalian digestive tract into three classes according to the modifications to Con A affinity induced by pre-treatments with periodate and sodium borohydrate. Class I glycoconjugates do not bind to Con A after periodate oxidation, whereas lectin affinities of class II and class III glyconjugates are not affected. Class II glycoconjugates lose Con A reactivity if the borohydrate reduction step follows after periodate oxidation, while the reactivity of class III glyconjugates is not affected, or even increases.

Combining different staining and labelling techniques, such as AB–PAS, PAS–Bowie, lectin–PAS, lectin–Bowie or PCS–Bowie, allowed the simultaneous visualization of different secreting cell types on the same section. Each experiment was repeated twice on specimens taken from the three different animals giving a total of six repetitions.

Staining/labelling in each experiment was assessed by at least two independent observers and scored as positive (+), moderately positive (−+), or negative (−) according to their intensity.

Results

The gastrointestinal tract of *R. ferrumequinum* was macroscopically short, scarcely convoluted without caeca. No gender differences or differences between animals were observed. Tables 2 and 3 summarize the histochemical staining and lectin labelling patterns observed.

Oesophagus

The mucosa was lined by a stratified squamous epithelium and was folded into several longitudinal plicae. No secreting cells were observed. The transition to the cardial area of the stomach was abrupt and marked by the change of the lining epithelium into a simple cuboidal one, illustrated in Fig. 1.

Stomach

The stomach was tubular in shape. The mucosa was folded into several plicae. Three regions were observed, a short cardias, a wide fundus and a small pylorus, characterised by multicellular glands with secretions specific to each region.

Cardias: The luminar epithelium of the mucosa was of simple cuboidal type, with cells having their apical cytoplasm filled with mucous granules positive for PAS (Fig. 1A), AB pH 1.0 and 2.5, and stained brown with the HID-AB reaction. Furthermore, they labelled strongly for binding of lectin DBA and LFA, and weakly for WGA (Fig. 1B), SBA, and PNA. Sialidase pre-treatment abolished labelling with LFA, and did not affect labelling with PNA or SBA. The cardial glands were muciparous, short, and of simple tubular shape. Their secretory product was weakly positive for PAS (Fig. 1A), AB pH 2.5 and 1.0, and stained weakly with HID-AB. Furthermore, the glandular cells labelled for binding of DBA, PNA, WGA (Fig. 1B), LFA and Con A after the periodate oxidation–borohydrate reduction. Binding of LFA was abolished by sialidase pre-treatment, whereas binding of PNA was not affected. The transition to the fundus was marked by the appearence in the glands of the first parietal and chief cells.

Fundus: The luminal epithelial cells of the fundus showed the same basic histochemical patterns of the cardial surface epithelial cells (Figs. 1C, D). The fundic glands were mostly simple and tubular shaped, with an isthmus, a neck and a basal region (Figs. 1C, D). Three main glandular cell types were observed: the neck, the parietal, and the chief cells. Neck cells were found not only in the neck region of the glands but also in the basal region, interspersed among the other cells (Fig. 1D). The mucins in the neck cells mainly differed from those in the surface cells in being negative for AB pH 2.5, AB pH 1.0 and LFA, and positive for C binding after periodate oxidation–borohydrate reduction (Fig. 1D). Although they did not produce mucins, the cytoplasm of the parietal cells were positive for DBA binding (Fig. 1C) and labelled weakly with WGA, Con A, PNA and SBA. The chief cells presented secretory granules that only stained with the Bowie reaction (Fig. 1C). In the fundic glands of the aboral region, the chief cells disappeared and only neck and parietal cells were found (Fig. 1D).

Pylorus: The luminal epithelial cells showed the same histochemical features as in the previous

Table 2. Histochemical staining of gastrointestinal mucous cells in *R. ferrumequinum*

Cell type	Staining			
	PAS	AB pH 2.5	AB pH 1.0	HID AB 2.5
Cardias				
Surface muciparous cell	+	+	+	Brown
Glandular muciparous cell	−+	−+	−+	Brown
Fundus				
Surface muciparous cell	+	+	+	Brown
Neck cell	−+	−	−	−
Pylorus				
Surface muciparous cell	+	−+	−+	Weak brown
Glandular muciparous cell	−+	−+	−+	Weak brown
Duodenum				
Goblet cell	+	+	+	Brown
Brünner's glandular cell	+	−	−	−
Jejuno-ileum				
Goblet cell	+	+	+	Brown
Rectum				
Goblet cell	+	+	+	Brown

Table 3. Lectin-binding pattern of gastrointestinal mucous cells of *R. ferrumequinum*

Cell type	Labelling									
	Con A	Paradoxical Con A	DBA	PNA	Sialidase PNA	SBA	Sialidase SBA	WGA	LTA	LFA
Cardias										
Surface muciparous cell	−	−	+	−+	−+	−+	−+	−+	−	+
Glandular muciparous cell	−	+	+	+	+	−	−	+	−	−+
Fundus										
Surface muciparous cell	−	−	+	+	+	+	+	−+	−	+
Neck cell	−+	+	+	+	+	−+	−+	+	−	−
Pylorus										
Surface muciparous cell	−	−	−	+	+	−	−+	−	−	−+
Glandular muciparous cell	−	+	−+	+	+	+	+	−	−	−+
Duodenum										
Goblet cell	−	−	+	+	+	−	+	+	−	+
Brünner's glandular cell	−	+	−	+	+	+	+	−	−	−
Jejuno-ileum										
Goblet cell	−	−*	−	+†	+‡	−	+	+	−	+
Rectum										
Goblet cell	−	−	+	−	−+	−	+	+	−	+

*Some goblet cells were weakly positive for paradoxical Con A.
†Positivity decreased towards the rectum.
‡Positivity increased towards the rectum.

regions, but differed in being only weakly positive for AB pH 2.5 and AB pH 1.0. They were positive for PNA binding (Fig. 1E) and weakly positive for LFA, but did not bind DBA, SBA and WGA. Sialidase pre-treatment did not change the PNA binding pattern, but resulted in a weak binding of SBA (Fig. 1F) and abolition of LFA labelling. The pyloric glands consisted of muciparous cells of one type. Parietal, chief and neck cells were not observed. Mucins in the glandular cells stained weakly with PAS, AB pH 2.5, and AB pH 1.0. Furthermore, they were positive for paradoxical Con A labelling, and bound PNA (Fig. 1E), SBA (Fig. 1F) and, weakly, DBA, PNA and LFA. Sialidase pre-treatment resulted in abolition of LFA labelling, whereas SBA labelling was not affected.

Intestine

The intestine was divided into a small and a large intestine. The small intestine was in turn divided into two main regions, a duodenum and a jejuno-ileum. The large intestine was very short, slightly larger in diameter than the small intestine, and was represented only by the rectum.

Duodenum: The duodenal mucosa had long, convoluted villi that were finger- or tongue-shaped in section. The luminal epithelium of the villi contained two main cell types: enterocytes and goblet cells. The goblet cells produced mucins positive to PAS, AB pH 2.5 and AB pH 1.0, and stained with HID-AB. Positive binding of lectins DBA, PNA, WGA and LFA were seen. Sialidase pre-treatment eliminated positivity to LFA and did not affect the binding of PNA. SBA binding was enhanced by sialidase pre-treatment (Figs. 2A, B). Tubular Lieberkühn crypts were noted at the base of villi, and multicellular Brunner's glands opened into them. Brunner's glandular cells contained PAS positive and AB-negative mucins. They labelled with the paradoxical Con A method, and for binding of PNA, SBA (Fig. 2A), DBA and WGA.

Jejuno-ileum: This was the longest area of the intestine and is referred to as the jejuno-ileum because a clear-cut distinction between a jejunum and an ileum was not possible. The villi appeared as transverse folds packed in a zig-zag pattern (Fig. 2C). The luminal epithelium of this region appeared similar in structure to that of the duodenum, with enterocytes and goblet cells with the same staining and labelling properties. However, one difference was that goblet cells here did not label with the lectin DBA. Some goblet cells showed a weak positivity to paradoxical Con A. PNA labelling decreased in intensity towards the rectum (Fig. 2D). Sialidase pre-treatment resulted in abolition of LFA binding and did not affect binding of PNA in the anterior tract, but an increase in labelling in the posterior tract was seen. Furthermore, the goblet cells were negative for SBA labelling, but became positive after sialidase

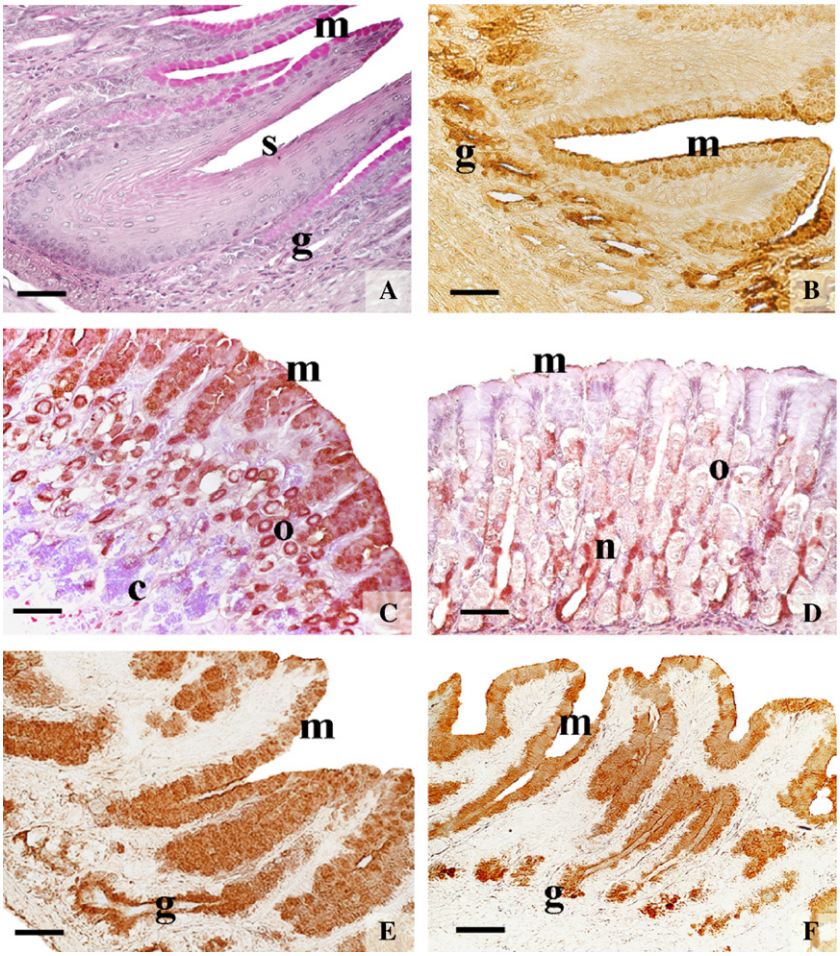

Figure 1. Gastroesophageal mucosa of *Rhinolophus ferrumequinum*. (A) Oesophagogastric transition, PAS–haemallum. The transition from the oesophagus to the cardias is marked by abrupt changes in the mucosa. The lining epithelium of the oesophagus (centre) is a stratified squamous epithelium (s) and no secreting cells are seen. The mucosa of the cardias (right and left) presents a simple cuboidal epithelium (m) and simple tubular glands (g), both with cells producing a PAS-positive mucus. (B) Cardias. WGA labelling. The intensity of labelling of the mucins in the secreting cells increases from the surface (m) to the fundus of the glands (g). (C) Fundus, oral mucosa. DBA–Bowie. The simple cuboidal cells of the luminal epithelium (m) secrete mucins that appear brown with DBA-labelling. The cytoplasm of the parietal cells in the glands (o) also label brown because of inner membrane-linked glycoproteins. The chief cells (c) have pepsinogen granules stained blue by the Bowie reaction. (D) Fundus, aboral mucosa. Paradoxical Con A–Bowie. Chief cells are lacking. Paradoxical Con A labelling is seen in the neck cells (n) interspersed in the glands. Mucous surface cells (m) and parietal cells (o) are negative. (E) Pylorus. PNA. The pyloric glands consist of muciparous cells and no neck, chief or parietal cells are seen. Mucins in both the surface epithelial (m) and glandular (g) cells are positive. (F) Pylorus. SBA labelling. Mucins in the secreting cells have an increasing lectin-binding positivity from the lumen towards the glandular fundus. All bars = 50 μm.

treatment. Lieberkühn crypts were seen at the base of villi.

Rectum: Here, villi disappeared, substituted instead by low ridges in which enterocyte and goblet cells were arranged into several, short tubular crypts (Figs. 3A–D). The mucins in the goblet cells showed staining and labelling properties similar to those of the jejuno-ileum, except they were positive for DBA labelling (Fig. 3A) and negative for PNA labelling. Sialidase pre-treatment resulted in an increase in labelling positivity with PNA and a strong increase in SBA labelling intensity (Fig 3B), whereas LFA labelling was abolished (Fig. 3D).

Controls

No labelling was observed in control sections exposed to DAB-H_2O_2 after substitution of PBS for

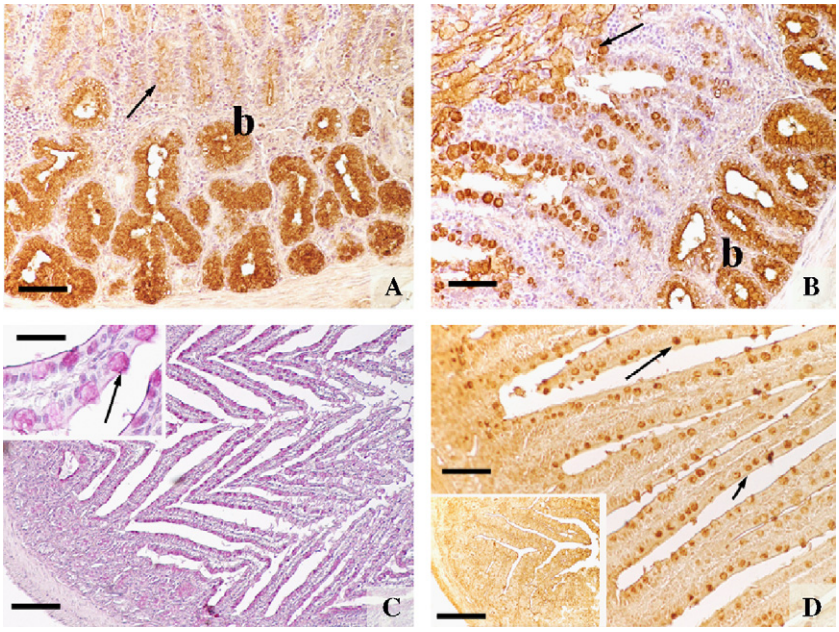

Figure 2. Intestinal mucosa of *R. ferrumequinum*. (A) Duodenum. PNA labelling. Well-developed Brunner's glands are seen in the submucosa. The cells in the Brunner's glands (b) are positive, whereas the goblet cells (arrow) are negative. Bar = 100 μm. (B) Duodenum. Sialidase digestion then PNA labelling. The sialidase pre-treatment renders the goblet cells (arrow) positive for PNA binding, whereas the labelling of the Brunner's gland cells (b) is not affected. Bar = 100 μm. (C) Jejuno-ileum. Periodic acid-Schiff (PAS)–haemallum. The villi in this region are arranged in a zig-zag fashion. Goblet cells (insert) are PAS-positive. Main bar = 200 μm, insert bar = 50 μm. (D) Jejuno-ileum. PNA labelling. Goblet cells (arrow) in the oral mucosa of jejuno-ileum are positive for PNA binding, whereas they are negative in the aboral mucosa (insert). Main bar = 100 μm, insert bar = 260 μm.

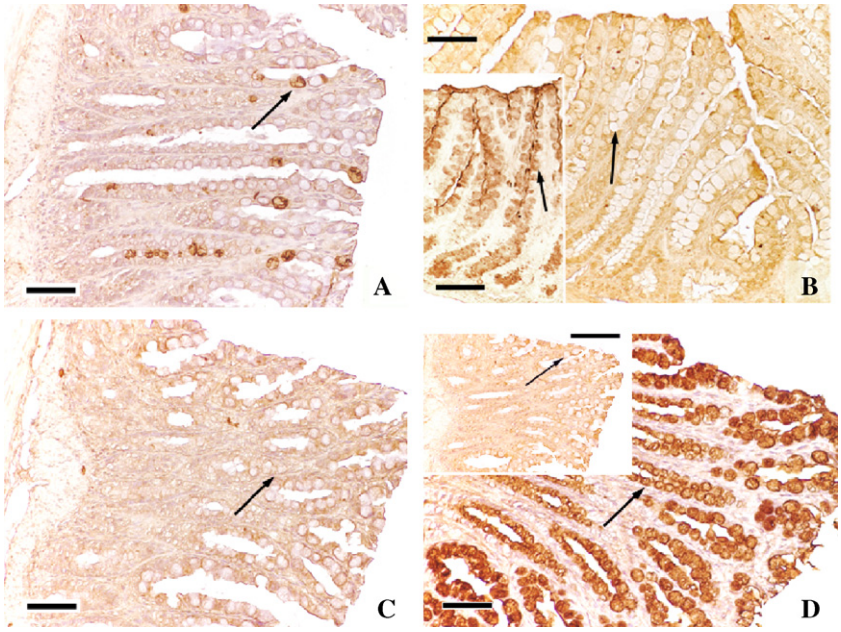

Figure 3. Rectal mucosa of *R. ferrumequinum*. The mucosa presents low ridges with goblet cells arranged into crypts. (A) DBA labelling. Some goblet cells are positive for DBA binding (arrow). Main bar = 100 μm. (B) SBA labelling before and (insert) after sialidase treatment. Goblet cells (arrow) do not label for SBA binding but after removal of sialic acid (insert) they are positive. Main bar = 100 μm, insert bar = 120 μm. (C). LFA with sialic acid. Goblet cells (arrow) do not label for LFA because incubation in the presence of sialic acid inhibits binding. Main bar = 100 μm. (D) LFA labelling before and (insert) after sialidase treatment. Goblet cells (arrow) label for LFA binding but after removal of sialic acid (insert) they are negative. Main bar = 100 μm, insert bar = 200 μm.

lectin–HRP conjugates. Hapten sugars significantly inhibited the corresponding lectin labelling in all cases (e.g., Fig. 3C). LFA labelling was always abolished by sialidase pre-treatment (Fig. 3D), thus confirming that sialidase digestion was effective in cleaving sialic acid residues.

Discussion

The main regions of the gastrointestinal system of *R. ferrumequinum* differ in the type and distribution of mucus that is produced. Mucus is produced by a number of cell types and differs both in quantity of acidic and neutral glycoconjugates, and in the lectin-binding pattern.

The oesophagus has no secreting activity, since no glands were observed. Oesophageal glands are lacking also in two Egyptian species, *Rousettus aegyptiacus* and *Taphozous nudiventris* (Madkour et al., 1982).

The stomach of *R. ferrumequinum* has no caeca or particular specializations, similar to other insect-feeding Chiroptera (e.g., Rouk and Glass, 1970; Forman, 1971; Perrin and Hughes, 1992), thus it can regarded as primitive and near to the probable ancestral condition of the order. Similar to the majority of Chiroptera (e.g., Forman, 1971), *R. ferrumequinum* presents surface epithelial gastric cells with acid mucins, that tend to become neutral towards the pylorus. In the cardias, the surface epithelium produces sulphated mucins whose lectin-binding patterns suggest the presence of *N*-acetylgalactosamine (GalNAc) sequences, *N*-acetyllactosamine and (β1,4 *N*-acetylglucosamine)$_n$. The muciparous cells in the cardiac glands differ from the surface ones in producing stable class-III mucins (Katsuyama and Spicer, 1978). The surface muciparous cells in the fundus are similar to those of the cardias area. The mucins in the fundic surface epithelial cells of three African bats (*Hypsignatus monstruosus*, *Hipposideros caffer* and *Miniopterus minor*) are similar to those of *R. ferrumequinum* in labelling for binding of DBA and WGA, but they differ in being negative for PNA binding (Danguy et al., 1987). In the fundic glands, the only muciparous cells are the neck ones, the mucins of which differ mainly from those in the surface cells in being neutral and stable class III glycoconjugates with D-mannose or D-glucose residues. Positive labelling of parietal cells by some lectins is not due to the presence of mucus, but to glycoproteins linked to the membranes of the tubulo-vesicular system (see Scillitani et al., 2005). The pyloric surface epithelial cells produce mostly neutral mucins with monosaccharide residues similar to those of the previous regions, but without *N*-acetylgalactosamine or β1,4 *N*-acetylglucosamine sequences. In the pyloric glands, mostly neutral, stable class-III mucins were observed, with *N*-acetyllactosamine and *N*-acetylgalactosamine. Data about the lectin-binding of mucins in the pylorus of Chiroptera are lacking in the literature, but some data from other mammals can be found in Schumacher et al. (2004). Similar to most of the species sampled by Schumacher et al. (2004), both the surface pyloric cells and the glandular ones of *R. ferrumequinum* are positive for PNA binding, but differ in being negative for WGA and Con A binding.

Our analyses allowed us to divide the intestine of *R. ferrumequinum* into three main areas: a duodenum, a jejuno-ileum and a rectum. The intestine of the co-generic *R. hildebrandti* from Africa is similar to that of *R. ferrumequinum* in having a long small intestine and a short rectum, but differs in that it lacks a distinct duodenum with long villi and Brunner's glands, these being replaced by a region with villi arranged in a honeycombed fashion (Makanya and Maina, 1994). Villi longer than in the rest of the intestine are also found in the duodenum of the Australian bats *Pteropus poliocephalus* and *P. alecto* (Tedman and Hall, 1985) as well as in *R. ferrumequinum*. The 'zig-zag' arrangement of the villi of the small intestine of *R. ferrumequinum* is found in other species of Chiroptera as well as in Insectivora and marsupials (e.g., Lennep, 1962; Stutz and Ziswiler, 1984; Zhukova, 1989). Thus, Zhukova (1989) regards this as a primitive feature of mammals.

The goblet cells in the duodenum of *R. ferrumequinum* present sulpho- and sialomucins with *N*-acetyllactosamine, (β1,4 *N*-acetylglucosamine)$_n$ and sialylated *N*-acetylgalactosamine sequences. Brunner's gland cells in the duodenum present neutral, stable class-III mucins with *N*-acetylgalactosamine residues. The cytoplasm of Brunner's gland cells contain glycoconjugates with residues of D-mannose and/or D-glucose and β1,4 *N*-acetylglucosammine sequences. The jejuno-ileum contains goblet cells with sulpho- and sialomucins, some of which are of the stable class-III type, with *N*-acetyllactosamine, (β1,4 *N*-acetylglucosamine)$_n$ and sialylated *N*-acetylgalactosamine. Towards the rectum, the *N*-acetyllactosamine sequences tend to be sialylated. The goblet cells in the rectum contain sulpho- and sialomucins with *N*-acetylgalactosamine and *N*-acetyllactosamine chains, both with terminal sialic acid. Among the three aforementioned African bats studied by Danguy et al. (1987), *Hypsignatus monstruosus* has the same

main labelling pattern in the gastrointestinal tract for DBA, PNA and WGA binding as that of *R. ferrumequinum*, reported here, but some differences are seen in the other two species. Schumacher et al. (2004) report the goblet cells in the duodenum to be generally negative for binding of the lectins tested in the study reported here, except for WGA positivity in three species, and PNA in one. We report that *R. ferrumequinum* duodenal goblet cells are positive for both WGA and PNA binding. Furthermore, Brunner's glands in general are found to be positive for PNA, WGA and Con A binding, whereas in the study reported here, *R. ferrumequinum* mucins were positive for PNA binding only. On the basis of the shared affinities in lectin-binding of muciparous cells in pyloric and Brunner's glands, Schumacher et al. (2004) conclude that these cells developed from a common staminal precursor. In *R. ferrumequinum*, the muciparous cells of pyloric and Brunner's gland share the same kind of labelling pattern with five out of six lectins tested, and are both positive for paradoxical Con A binding, and binding of PNA and SBA after sialidase treatment, thus confirming the hypothesis of a common precursor. Paradoxical Con A labelling indicates that stable class-III mucins are found in the fundic neck, pyloric and Brunner's muciparous cells in *R. ferrumequinum*; a similar pattern was reported in the rat by Katsuyama and Spicer (1978). These authors conclude that this finding is indicative of a common developmental pathway for the cited cells.

As far as the functional implications of our findings are concerned, variation in morphology and mucin composition certainly reflects differences in functions of the gastrointestinal districts of *R. ferrumequinum*. In bats, the capacity to store food in the gastrointestinal tract is limited because of flight constraints that do not allow an excessive increase in body weight (see McNab, 1973). Thus, the passage of food in the gastrointestinal tract is fast and rates of absorption are high (e.g., Keegan, 1977; Tedman and Hall, 1985). This probably explains the shortening of the gastrointestinal system and the lack of glands in some regions. The oesophagus is short and without glands to allow the food to enter the stomach rapidly. Cardiac glands lubricate the area and the food reaches the fundus where the gastric juice is produced. Scillitani et al. (2005) found that the composition of the gastric juice changes along the fundus because of the progressive disappearance of the chief cells. In the aboral fundus, the acidic environment could also act as a barrier against pathogens. The pylorus and the Brunner's gland in the duodenum secrete stable class-III neutral mucins, with the function of lubricating the area and neutralizing the gastric juice. The goblet cells can also contribute to this function (see Schumacher et al., 2004). In the intestine, mucins have a number of important functions: among these, the regulation of the exchanges between the lumen and the enterocytes and the secretion of molecules with antimicrobial properties. The villi folded in a zig-zag fashion increase the absorbing efficiency of the intestine and reduce the time of passage through the intestine. The changes in composition of the mucus towards the rectum suggest a change in its functions: probably in the aboral area the lubrication becomes predominant, while it has been hypothesized that the shortening of the colon has the role of reducing the absorption of water that in other mammals takes place there (Okon, 1977).

The low specialization of the gastrointestinal tract of *R. ferrumequinum* among Chiroptera, as well as some features shared with members of the the Insectivora, suggests that the organization of the digestive tract of this species could be primitive for the order and, possibly, for the whole eutherian clade. Further studies will support these working hypotheses and will clarify whether the cited shared features are due to common ancestry or to convergence.

References

Allen A. Structure and function of gastrointestinal mucus. In: Johnson LR, editor. Physiology of the gastrointestinal tract. New York: Raven Press; 1981. p. 617–39.

Baker DA, Sugii S, Kabat EA, Ratcliffe RM, Hermentin P, Lemieux RU. Immunochemical studies on the combining sites of Forssman hapten reactive hemagglutinins from *Dolichos biflorus*, *Helix pomatia* and *Wistaria floribunda*. Biochemistry 1983;22:2741–50.

Bhattacharyya L, Haraldsson M, Brewer CF. Precipitation of galactose-specific lectins by complex-type oligosaccharides and glycopeptides: studies with lectins from *Ricinus communis* (Agglutinin I), *Erythrina indica*, *Erythrina arborescens*, *Abrus precatorius* (Agglutinin), and *Glycine max* (Soybean). Biochemistry 1988;27:1034–41.

Bhide SA. Observations on the stomach of the Indian fruit bat *Rousettus leschenhaulti* (Desmarest). Mammalia 1980;4:571–9.

Bhide SA. Structure and some aspects of histochemistry of the stomach of two Indian bats. Curr Sci 1981.

Bonucci E. Manuale di Istochimica. Rome: Lombardo; 1981.

Brooks SA, Carter TM. *N*-acetylgalactosamine, *N*-acetylglucosamine and sialic acid expression in primary breast cancer. Acta Histochem 2001;103:37–51.

Brooks SA, Dwek MV, Schumacher U. Functional and molecular glycobiology. Oxford: BIOS Scientific Publishers Limited; 2002.

Choi BY, Sohn YS, Choi C, Chae C. Lectin histochemistry for glycoconjugates in the small intestines of piglets naturally infected with *Isospora suis*. J Vet Med Sci 2003;65(3):389–92.

Danguy A, Lenglet G, Kiss Jr R, De Launoit Y, Na Kongo ML, Kiss Sr R. Distribution of fluorochrome-coupled lectins in the gastrointestinal tract of African bats. Arch Biol (Bruxelles) 1987.

Debray H, Decout D, Strecker G, Spik G, Montreuil J. Specificity of twelve lectins towards oligosaccharides and glycopeptides related to *N*-glycosylproteins. Eur J Biochem 1981;17:41–55.

Debray H, Pierce-Crétel A, Spik G, Montreuil J. Affinity of ten insolubilized lectins towards various glycopeptides with the *N*-glycosylamine linkage and related oligosaccharides. In: Bog-Hansen TC, Spengler GA, editors. Lectins, vol. 3. Berlin: Walter de Gruyter & Co; 1983. p. 337–50.

Etzler ME, Kabat EA. Purification and characterization of a lectin (plant hemagglutinin) with blood group A specificity from *Dolichos biflorus*. Biochemistry 1970; 9:869–77.

Ferri D, Liquori GE. Glycohistochemistry of the external nasal gland of two lizards (*Podarcis sicula campestris* and *Chalcides chalcides*). Acta Histochem Cytochem 1997;30:1–6.

Ferri D, Liquori GE, Natale L, Santarelli G, Scillitani G. Mucin histochemistry of the digestive tract of the red-legged frog *Rana aurora aurora*. Acta Histochem 2001;103:225–37.

Ferri D, Liquori GE, Scillitani G. Morphological and histochemical variations of mucous and oxynticopeptic cells in the stomach of the seps, *Chalcides chalcides* (Linnaeus, 1758). Journal of Anatomy 2001;194:71–7.

Filipe MI. Mucins in the human gastrointestinal epithelium: a review. Invest Cell Pathol 1979;2(3):195–216.

Finne J, Krusius T. Preparation and fractionation of glycopeptides. Methods Enzymol 1982;83:269–77.

Forman GL. Histochemical differences in gastric mucus of bats. J Mammal 1971;52:191–3.

Forman GL. Comparative morphological and histochemical studies of stomachs of selected North American bats. Univ Kansas Sci Bull 1972;26:591–729.

Forman GL. Studies of gastric morphology in North American Chiroptera (Emballonuridae, Noctilionidae and Phyllostomatidae). J Mammal 1973;54:909–23.

Gallagher JT, Morris A, Dexter TM. Identification of two binding sites for wheat-germ agglutinin on polylactosamine-type oligosaccharides. Biochem J 1985;231: 115–22.

Graham RC, Karnowsky MJ. The early stages of absorption of injected peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. Adv Carbohyd Chem Biochem 1966;35: 127–31.

Grassé PP. Ordres des Chiroptères (Chiroptera Blumenbach, 1774). Anatomie. In: Grassé PP, editor. Traité de Zoologie. Anatomie, Systematique, Biologie. Tome 17. Fascicule 2. Mammifères. Les ordres: Anatomie, Èthologie, Systematique. Paris: Masson et cie; 1955. p. 1729–80.

Kamiya T, Pirlot P. Comparative gastric morphology of Old World bats. I. In light microscopy. J Mammal Soc Japan 1975;6:145–54.

Katsuyama T, Spicer SS. Histochemical differentiation of complex carbohydrates with variants of the concanavalin A–horseradish peroxidase method. J Histochem Cytochem 1978;26(4):233–50.

Keegan DJ. Aspects of the assimilation of sugars by *Rousettus aegypticus*. Comparative biochemistry and physiology. Part A. Physiology 1977;58:349–52.

Leathem AJC, Atkins NJ. Lectin binding to paraffin section techniques. In: Bullock GK, Petrusz P, editors. Techniques in immunocytochemistry, vol. 2. London, New York: Academic Press; 1983. p. 39–60.

van Lennep EW. The histology of the mucosa of the small intestine of the long-nosed bandicoot (Marsupialia: *Perameles nasuta* Geoffroy) with special reference to intestinal secretion. Acta Anat 1962;50: 73–89.

Liquori GE, Scillitani G, Mastrodonato M, Ferri D. Histochemical investigations on the secretory cells in the oesophagogastric tract of the Eurasian green toad, *Bufo viridis*. Histochem J 2002;34:517–24.

Liquori GE, Zizza S, Mastrodonato M, Scillitani G, Calamita G, Ferri D. Pepsinogen and H,K-ATPase mediate acid secretion in gastric glands of *Triturus carnifex* (Amphibia, Caudata). Acta Histochem 2005; 107(2):133–41.

Lotan R, Sharon N. Peanut (*Arachis hypogaea*) agglutinin. Methods Enzymol 1978;50:361–7.

Madkour GA, Hammouda EM, Ibrahim IG. Histology of the alimentary tract of two common Egyptian bats. Ann Zool 1982;19:53–74.

Makanya AN, Maina JN. The morphology of the intestine of the insectivorous horseshoe bat (*Rhinolophus hildebrandti*, Peters): a scanning electron and light microscopic study. Afr J Ecol 1994;32(2):158–68.

Makanya AN, Maina JN, Mayhew TM, Tschanz SA, Burri PH. A stereological comparison of villous and microvillous surfaces in small intestines of frugivorous and entomophagous bats: species, inter-individual and craniocaudal differences. J Exp Biol 1997;200(18): 2415–23.

McNab BK. Energetics and distribution of vampires. J Mammal 1973;54:131–44.

Miller RL, Collawn Jr. JF, Fish WW. Purification and macromolecular properties of sialic acid specific lectin from the slug *Limax flavus*. J Biol Chem 1982;257(13): 7574–80.

Monsigny M, Roch AC, Serre C, Maget-Dana R, Delmotte F. Sugar–lectin interactions: how does wheat-germ agglutinin bind sialoglycoconjugates? Eur J Biochem 1980;104:147–53.

Mowry RW, Winkler CH. The coloration of acidic carbohydrates of bacteria and fungi in tissue sections with special reference to capsule of *Cryptococcus*

neoformans, pneumococci, and staphylococci. Am J Pathol 1956;32:628–9.

Okon E. Functional anatomy of the alimentary canal in the fruit bat, *Eidolon helvum*, and the insect bat, *Tadarida nigeriae*. Acta Zool 1977;58:83–93.

Pedini V, Scocco P, Gargiulo AM, Ceccarelli P. Carbohydrate histochemistry of lamb duodenum. Acta Histochem 2001;103(3):315–23.

Pereira MEA, Kabat EA. Specificity of purified hemagglutinin (lectin) from *Lotus tetragonolobus*. Biochemistry 1974;13:3184–92.

Pereira MEA, Kabat EA, Sharon N. Immunochemical studies on the specificity of Soybean agglutinin. Carbohyd Res 1974;37:89–102.

Perrin MR, Hughes JJ. Preliminary observations on the comparative gastric morphology of selected Old World and New World bats. Z Saugetierkunde 1992;57: 257–68.

Rouk CS, Glass BP. Comparative gastric histology of five North and Central American bats. J Mammal 1970.

Sato A, Spicer SS. Ultrastructural cytochemistry of complex carbohydrates of gastric epithelium in the guinea pig. Am J Anat 1980;159(3):307–29.

Schumacher U, Duku M, Katoh M, Jorns J, Krause WJ. Histochemical similarities of mucins produced by Brünner's glands and pyloric glands: a comparative study. Anat Rec Pt A, Discov Mol, Cell, Evol Biol 2004;278(2):540–50.

Scillitani G, Zizza S, Liquori GE, Ferri D. Histochemical and immunohisto-chemical evidence for a gradient in gastric juice production in the greater horseshoe bat, *Rhinolophus ferrumequinum* (Schreber, 1774). Acta Chiropterol 2005;7(2):301–8.

Sheahan DG, Jervis HR. Comparative histochemistry of gastrointestinal mucosubstances. Am J Anat 1976; 146(2):103–31.

Spicer SS. Diamine methods for differentiating mucosubstances histochemically. J Histochem Cytochem 1965;13:211–34.

Stutz H, Ziswiler V. Morphological and histological investigations of the digestive tract of Middle European bats (Mammalia: Chiroptera). Myotis 1984; 21–22:41–6.

Suganuma T, Katsuyama T, Tsukahara M, Tatematsu M, Sakakura Y, Murata F. Comparative histochemical study of alimentary tracts with special reference to the mucous neck cells of the stomach. Am J Anat 1981;61(2):219–38.

Tedman RA, Hall LS. The morphology of the gastrointestinal tract and food transit time in the fruit bats *Pteropus alecto* and *P. poliocephalus* (Megachiroptera). Aust J Zool 1985.

Zhukova NF. Peculiarities of intestinal morphology with respect to chiropteran phylogeny. In: Hanák V, Horácek I, Gaisler J, editors. European bat research. Praha: Charles University Press; 1989. p. 25–31.