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Lectin histochemistry for *in situ* profiling of rat colon sialoglycoconjugates

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Summary. The growing interest in glycoconjugates expressed and released by the epithelium of the intestinal mucosa is tightly related to the multiple functional roles attributed to sialic acid and its derivatives. In the present work, biotin and HRP conjugated lectins were used to detect the sialylation pattern and to identify specific structural features of sialoderivatives in the rat colon. In particular, the occurrence and distribution of sialic acids linked α2,6 to D-Gal/D-GalNAc and α2,3 to D-Gal were directly demonstrated with SNA and MAL II binding, respectively. In addition, in order to by-pass the specificity problems of SNA and MAL II as histochemical reagents, as well as to look for additional and complementary information about acetylation degree and sites, we combined sialidase digestion, potassium hydroxide deacetylation, and differential periodate oxidation with PNA and DBA binding. The data showed the distribution and structure of sialic acid-ß-D-Gal(1-3)-D-GalNAc and sialic acid-D-GalNac sequences, which proved to be widely distributed as cellular components or secretory products in surface goblet cells and crypt cells of the colonic epithelium. A high degree of *O*-acetylation, with acetyl groups mainly at 9 and 4 positions, was found, showing an increasing gradient from the proximal to distal portion of the colon. These results, which largely reproduce the sialylation pattern in other species, contribute new insights in defining the tissue specific expression of sialoderivatives in the colonic mucosa, and testify to their high heterogeneity which the wide range of sialic acid functional correlates in the intestinal tract depend on.

Key words: Sialoglycoconjugates, Sialic acid, Lectin histochemistry, Rat colon

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Introduction

It is well known that sialic acids, because of their size, negative charge and terminal location, present a high potential in modulating intermolecular and intercellular interactions in various pathophysiological events (Rutishauser and Landmesser, 1996; Kelm and Schauer, 1997; Varki, 1997; Bagriacik and Miller, 1999; Schauer, 2000, 2004). The variety of the sialic acid functional properties is greatly enhanced and modulated by their structural diversity, which is generated primarily by a combination of variations at C-5, with modifications (mostly the addition of *O*-acetyl groups) of any of the hydroxyl groups located at C-4, C-7, C-8 and C-9. An additional variety arises from their linkage to the underlying sugar chain, which can occur in a2-3 or α2-6 configurations, with Gal or GalNAc as the most common acceptor sugars, or in α2-8 configuration to form the polysialic acids (Schauer and Kamerling, 1997; Angata and Varki, 2002; Schauer, 2004). Such a structural heterogeneity can substantially modify the functional role of sialic acids in several biological processes (Kelm and Schauer, 1997; Angata and Varki, 2002).

In the gastrointestinal tract, the sialylation of the glycoconjugates, released by the epithelial cells and attached to the cell surface membranes, not only contributes further to the physicochemical properties of the intestinal mucins, but also increases the repertoire of the sialic acid tasks in various events, such as interactions with commensal and pathogenic micro-organisms, inflammation, neoplastic transformation, and cancer metastasis (Bresalier et al., 1996; Wargovich et al., 2004; Bodger et al., 2006; Chandrasekaran et al., 2006). Indeed, altered glycosylation is a universal feature of cancer cells (Fukuda, 1996; Hakamori, 1996, 2002; Konno et al. 2002). In particular, the expression of certain sialylated sugar chains, as well as changes of sialic acid *O*-acetylation, have been associated with colorectal adenomas and carcinomas (Iwakawa et al., 1996; Grabowski et al., 2000; Brockhausen et al., 2001; de Albuquerque Garcia Redondo et al., 2004; Shen et al.,

2004; Seales et al., 2005; Chiricolo et al., 2006). Since colorectal cancer is the third most common malignancy in the world, and it represents the main cause for cancer deaths in Europe and the USA (Xing et al., 2006), growing research interest has been focused on elucidating the sialoglycoconjugates of the epithelial cells of the mammalian colon. An additional current problem posed by the intestinal sialylation pattern is the potential that sialic acids, as terminal sugars in the glycan chains, have as specific binding sites for microorganisms, thereby regulating intestinal flora and pathogenity (Reddy et al., 1996; Angata and Varki, 2002; Freitas et al., 2002; Pastoriza Gallego and Hulen, 2006).

The development of physical methods has allowed us to analyze *extra situm* the occurrence of a large variety of sialic acids in the mucins isolated from different parts of the human intestinal tract (Robbe et al., 2003, 2004) and, more recently, in the mouse gut (Rinninger et al., 2006), also providing information on their detailed structure. In the present work, we investigated the expression of sialoglycoconjugates in the rat colon by applying lectin histochemistry. The use of lectins, using two distinct methodological approaches, was aimed at identifying and characterizing in situ specific structural features of sialic acids. In particular, direct lectin binding to sialic acid residues was addressed to both localize terminal sialic acids and specify the type of their linkage to particular underlying sugar residues. Chemical pretreatments and sialidase predigestion, combined with appropriate lectins, were employed to visualize sialylated glycan sequences, as well as to clarify the degree and position of acetylated substituents in sialic acid residues. The double approach resulted in a comprehensive investigation of the tissue-specific sialoglycosylation pattern and it contributed new insights into the in situ characterization of sialoderivatives of the rat colon as a basis for a better understanding of the dynamic relationships between endogenous and exogenous carbohydrates in the intestinal environment.

Materials and methods

Animals

Eight 11-12 week-old female Sprague-Dawley rats (Morini, Reggio Emilia, Italy) were housed in plastic cages with wire tops and bottoms and maintained at constant environmental temperature (20-22°C) on a 12 h light-dark cycle, according to internationally accepted guide lines. All rats were maintained on a standard diet (AIN-76; American Institute of Nutrition, 1977) whose components were purchased from Piccioni Laboratories (Gessate, Milano, Italy). Food and water were provided *ad libitum*.

Tissue processing

The anaesthetized animals were sacrificed by cervical dislocation under the supervision of authorized investigators. The entire colon was removed immediately and washed thoroughly in 0.1 M phosphate buffered saline solution (PBS) to remove luminal contents. Two cm segments from each colon were taken, 1 cm distal to the cecum in the proximal colon and 2 cm from the anus in the distal colon. The segments were placed for 24 h at room temperature in Carnoy's fluid and postfixed in a 2% calcium acetate - 4% paraformaldehyde solution (1:1) for 3 h (Menghi et al., 1985). After dehydration through a graded series of ethanols, specimens were embedded in paraffin wax. Sections were serially cut at 5 μm.

Lectin histochemistry

Sambucus nigra (SNA), specific for sialic acid α2,6-linked to D-Gal/D-GalNAc residues, and *Maackia amurensis* (MAL II), which specifically binds sialic acid α2,3-linked to D-Gal residues, both conjugated with biotin, were purchased from Vector Laboratories (Burlingame, CA, USA). Horseradish peroxidase (HRP)-conjugated lectins, *Arachis hypogaea* (PNA) and *Dolichos biflorus* (DBA) were used for detection of ß-D-Gal(1,3)-D-GalNAc and D-GalNAc, respectively (Sigma Chemicals, St. Louis, MO, USA).

Biotinylated lectin histochemistry was performed according to Hsu et al. (1981). After inactivation of the endogenous peroxidase activity with 0.3% H_2O_2 in methanol for 30 min, sections were treated with avidin and then with biotin (AB blocking Kit, Vector). To minimize non specific binding, specimens were covered with 1% bovine serum albumin (BSA, Sigma) in PBS and washed three times in PBS. Subsequently, SNA and MAL II lectins were applied in a solution of PBS (100 μg/ml) containing 1% BSA for 1 h at room temperature in a humidified chamber. The samples were then rinsed three times in PBS and incubated for 45 min with an avidin-biotin-peroxidase reagent (ABC Kit, Vector) and again washed in PBS. The peroxidase binding sites were visualized with a solution of 3,3'-diaminobenzidine (DAB, Vector).

Histochemical staining using HRP-conjugated lectins was performed as previously detailed (Accili et al., 1999); briefly, endogenous peroxidase was blocked as above and sections were incubated for 30 min at room temperature with PNA and DBA lectins at a concentration of 60 μg/ml and 100 μg/ml, respectively, in PBS. Peroxidase was developed as above.

Chemical and enzymatic treatments

Adjacent sections, from serially cut samples, were subjected to PNA and DBA binding with and without the following pretreatments:

1) digestion with 0.5 U/ml sialidase from Clostridium perfringens (Type V, Sigma) for 16 h at 37°C in 0.1 M acetate buffer, pH 5.5, containing 10 mM $CaCl_2$

2) mild periodate oxidation (PO) with a 1 mM aqueous

solution of periodic acid, for 15 min at room temperature. Mild oxidation abolishes the staining with sialidase/PNA-DBA or KOH/sialidase/PNA-DBA when sialic acids do not contain acetyl groups in the side chain (Roberts, 1977)
3) strong periodate oxidation (PO) with a 44 mM aqueous solution of periodic acid, for 15 min at room temperature. This treatment selectively oxides sialic acid side chains lacking in C9 acetyl substituents as well as penultimate ß-Gal linked via an α2-6 bond. Thus, by comparing the results from mild and strong oxidation, the occurrence of C9-acetyl groups can be detected. In addition, the type of linkage between sialic acid and penultimate ß-Gal can be elucidated (Roberts, 1977)
4) deacetylation with 0.5% potassium hydroxide in 70% ethanol solution for 30 min at room temperature. This treatment, by detaching acetyl substituents, renders sialic acids, which contain acetylated groups in C4, susceptible to sialidase digestion (Moschera and Pigman, 1975)
The designs of the employed procedures were carried out as follows:
a. SNA, MAL II PNA, DBA
b. 1 mM PO/ PNA, 1 mM PO /DBA
c. 44 mM PO/ PNA, 44 mM PO / DBA
d. Sialidase/ PNA, Sialidase /DBA
e. KOH/ sialidase/ PNA, KOH/ sialidase/ DBA
f. 1 mM PO/sialidase/ PNA, 1 mM PO/sialidase/ DBA:
g. 44 mM PO/ sialidase/ PNA, 44 mM PO/ sialidase/ DBA
h. 1 mM PO/ KOH/ sialidase/ PNA, 1 mM PO/ KOH/ sialidase/ DBA
i. 44 mM PO/ KOH/ sialidase/ PNA, 44 mM PO/ KOH/ sialidase/ DBA

The histochemical experiments specific for demonstration of sialic acid derivatives were formulated according to previous reports (Schulte and Spicer, 1985; Schulte et al., 1985; Menghi et al., 1992; Accili et al., 1994; Gabrielli et al., 2004).

Controls

Controls to verify the lectin specificity included substitution of the conjugated lectins for the respective unconjugated lectins, as well as pre-incubation of lectins with the corresponding hapten sugars at a concentration of 0.1-0.4 M (Accili et al., 1994).

Controls for sialidase digestion aimed to determine the influence of the enzyme-free buffer (Plendl et al., 1989) and to investigate the efficacy and the specificity of treatments.

The effects of the oxidation pretreatments on the lectin binding were also checked.

Results

Proximal colon

Tables 1 and 2 illustrate the detailed distribution of carbohydrate moieties visualized by the different staining procedures.

Both SNA and MAL II exhibited moderate reactivity on colonocytes of the lining epithelium (Fig. 1A,B). Some goblet cells strongly reacted with MAL II within the surface epithelium and at crypt upper portions (Fig. 1B).

PNA affinity, restricted to colonocytes (inset in Fig. 2A), extended to surface goblet cells after sialidase digestion which also induced a moderate reactivity at the luminal border of some crypt cells (Fig. 2A). All these binding sites were almost unchanged by differential oxidation pretreatments (Fig. 2B). The deacetylation, aimed at removing sialidase resistant sialoderivatives, promoted a strong PNA positivity in the crypt cells (Fig. 2C), which was unmodified by periodate oxidation (Fig. 2D). In deacetylated and predigested samples, a moderate increase of PNA reactivity, resistant to periodate oxidation, was observed also in colonocytes which showed staining at their luminal cell borders (Fig. 2C,D).

The DBA binding, mostly occurring at the crypt upper portions (Fig. 3A), was enhanced after sialidase digestion at surface goblet cells and crypt cells, where sialic acids, based on the effect of periodate oxidation, proved to be heterogeneously provided of acetyl substituents at their lateral chain (Fig. 3B,C). The occurrence of sialic acid-D-GalNAc sequences was found also at the luminal surface of the colonocytes (Fig. 3B). Deacetylation prior to sialidase digestion induced an increase in DBA reactivity, resistant to periodate oxidation, only at crypt fundus cells (Fig. 3D).

Distal colon

The patterns of lectin binding in the epithelium of the colon distal segment are reported in Tables 1 and 3.

SNA stained very weakly at the surface epithelium (Fig. 4A), while MAL II revealed sialic acid (α2,3)-D-Gal terminal sequences differently distributed at all epithelial structures. The crypt fundus cells, in particular, exhibited a marked staining (Fig. 4B).

New PNA binding sites, after sialidase digestion,

Table 1. SNA and MAL II binding pattern in the rat proximal and distal colon.

	Proximal Colon		Distal Colon	
LECTINS	SNA	MAL II	SNA	MAL II
Colonocytes	0-1	1	0-1	1-2
Surface goblet cells	0	2	0	1
Crypt upper portion	0	2-3	0	2
Crypt fundus	0	0	0	3-4

Results are expressed by a subjective scale ranging from 0 to 4, with 0 being unreactive and 4 being strongly reactive.

Table 2. PNA and DBA binding patterns, with and without chemical and enzymatic pretreatments, in the rat proximal colon. The comparative evaluation of the results of the different treatments provides data on the acetylation degree and position of sialic acids linked to D-Gal(b1,3)D-GalNAc sequences (PNA), and sialic acid linked to preterminal D-GalNAC residues (DBA).

	PNA	Sial/PNA	1 PO/ Sial/PNA	44PO/Sial/ PNA	KOH/Sial/ PNA	1PO/KOH/ Sial/PNA	44PO/KOH/ Sial/PNA	Visualized sialic acids
Colonocytes	1	1	1	0	2*	2*	2*	C_4- and C_9- acetylated Sia linked α2-3 to Gal. Acetylation in C_7 and /or C_8 is also possible
Surface goblet cells	0	3	3	3	3	3	3	C_4-non acetylated Sia with acetyl groups in C_9, linked α2-3 to Gal. Acetylation in C_7 and /or C_8 is also possible
Crypt upper portion	0	0-1*	0-1*	0-1*	1-2	1-2	1-2	C_4-non acetylated and C_4-acetylated Sia, both with acetyl groups in C_9, linked α2-3 to Gal. Acetylation in C_7 and /or C_8 is also possible
Crypt fundus	0	0	0	0	3	3	3	C_4- acetylated Sia with acetyl groups in C_9, linked α2-3 to Gal. Acetylation in C_7 and /or C_8 is also possible
	DBA	Sial/DBA	1PO/Sial/ DBA	44 PO/ Sial/DBA	KOH/ Sial/DBA	1PO/KOH/ Sial/DBA	44PO/KOH/ Sial/DBA	Visualized sialic acids
Colonocytes	1	1-2*	1	1	1-2*	1	1	Non-acetylated Sia
Surface goblet cells	1-2	3	2	2	3	2	2	C_4-non acetylated Sia partly containing acetyl groups in C_9 or $C_{7,9}$ or $C_{8,9}$ or $C_{7,8,9}$
Crypt upper portion	2	3-4	2-3	2-3	3-4	2-3	2-3	C_4-non acetylated Sia partly containing acetyl groups in C_9 or $C_{7,9}$ or $C_{8,9}$ or $C_{7,8,9}$
Crypt fundus	1-2	2-4	2	2	4	3	3	C_4-non acetylated and C_4-acetylated Sia, partly containing acetyl groups in C_9 or $C_{7,9}$ or $C_{8,9}$ or $C_{7,8,9}$

Results are expressed by a subjective scale ranging from 0 to 4, with 0 being unreactive and 4 being strongly reactive. Sia: sialic acids. *: Staining at the cell luminal border.

Table 3. PNA and DBA binding patterns, with and without chemical and enzymatic pretreatments, in the rat distal colon. The comparative evaluation of the results of the different treatments provides data on the acetylation degree and position of sialic acids linked to D-Gal(ß1,3)D-GalNAc sequences (PNA), and sialic acids linked to preterminal D-GalNAC residues (DBA).

	PNA	Sial/PNA	1 PO/ Sial/PNA	44PO/ Sial/PNA	KOH/ Sial/PNA	1PO/KOH/ Sial/PNA	44PO/KOH/ Sial/PNA	Visualized sialic acids
Colonocytes	1-2	1-2	1-2	0-1	2-3*	2-3*	2-3*	C_4 acetylated Sia with acetyl groups in C_7 and /or C_8 and C_9, linked α2-3 to Gal
Surface goblet cells	0	3	3	0-2	3	3	0-2	C_4-non acetylated Sia with acetyl groups in the lateral chain, partly in C_9 in sugar units α2-3-linked to Gal
Crypt upper portion	0	1-2	1-2	0-1	3-4	3-4	1-3*	C_4-non acetylated and C_4- acetylated Sia with acetyl groups in the lateral chain, partly in C_9 in sugar units α2-3-linked to Gal
Crypt fundus	0-1	1**	1**	0	3*	3*	2-3*	C_4-non acetylated and C_4-acetylated Sia with acetyl groups in C_7 and/or C_8 and, partly, in C_9, linked α2-3 to Gal
	DBA	Sial/DBA	1PO/ Sial/DBA	44 PO/ Sial/DBA	KOH/ Sial/DBA	1PO/KOH/ Sial/DBA	44PO/KOH/ Sial/DBA	Visualized sialic acids
Colonocytes	1	1	1	0-1	1	1	0-1	No Sia linked to D-GalNAC
Surface goblet cells	0	1-2	1-2	0-1	2	2	0-1	C_4-non acetylated and C_4- acetylated Sia with acetyl groups in C_7 and /or C_8 and, partly in C_9
Crypt upper portion	2**	3	3	0-1	3-4	3-4	0-1	C_4-non acetylated and C_4- acetylated Sia with acetyl groups in the lateral chain
Crypt fundus	0	0	0	0	0	0	0	No Sia linked to D-GalNAC

Results are expressed by a subjective scale ranging from 0 to 4, with 0 being unreactive and 4 being strongly reactive. Sia: sialic acids. *: Staining at the cell luminal border; **: staining in few cells.

were mainly found at some surface goblet cells and in the crypt upper portions, while occasional binding was detected at the crypt fundus (Fig. 5A). The sialidase induced reactivity was largely reduced only after strong oxidation (Fig. 5B). Deacetylation, prior to sialidase degradation, produced new PNA affinity sites at the apical surface of the crypt fundus cells, as well as at the luminal border of the colonocytes. PNA binding strongly increased also in the cells of the crypt upper portions (Fig. 5C). All of these induced reactivity sites turned out to be unmodified after pretreatment with 1 mM PO and partially resistant to 44 mM PO (Fig. 5D).

DBA affinity was induced by sialidase digestion in the surface goblet cells and in the crypt upper portions (Fig. 6A). The resistance to mild oxidation and susceptibility to strong oxidation indicated sialic acids heterogeneously acetylated at their side-chain (not shown). The presence of acetyl substituents at the sialic acid pyranose ring could be deduced by the efficacy of alcoholic deacetylation to modify the sial/DBA reactivity in surface goblet cells and upper crypt cells (Fig. 6B), which resisted mild oxidation, not strong oxidation (not shown).

Controls

No staining was evidenced in sections exposed to unconjugated lectins or to conjugated lectins preincubated with their appropriate inhibitory sugars. Controls to check the efficacy and specificity of the enzymatic digestions were as expected. The enzyme-free buffer solution, as well as the chemical treatments, produced a light aspecific decrement of reactivity in several sites. Controls to test the effect of periodate oxidation on lectin binding showed that 1 mM PO did not modify PNA and DBA reactivity, whereas pretreatment with 44 mM PO abolished PNA staining and greatly decreased DBA labelling (not shown).

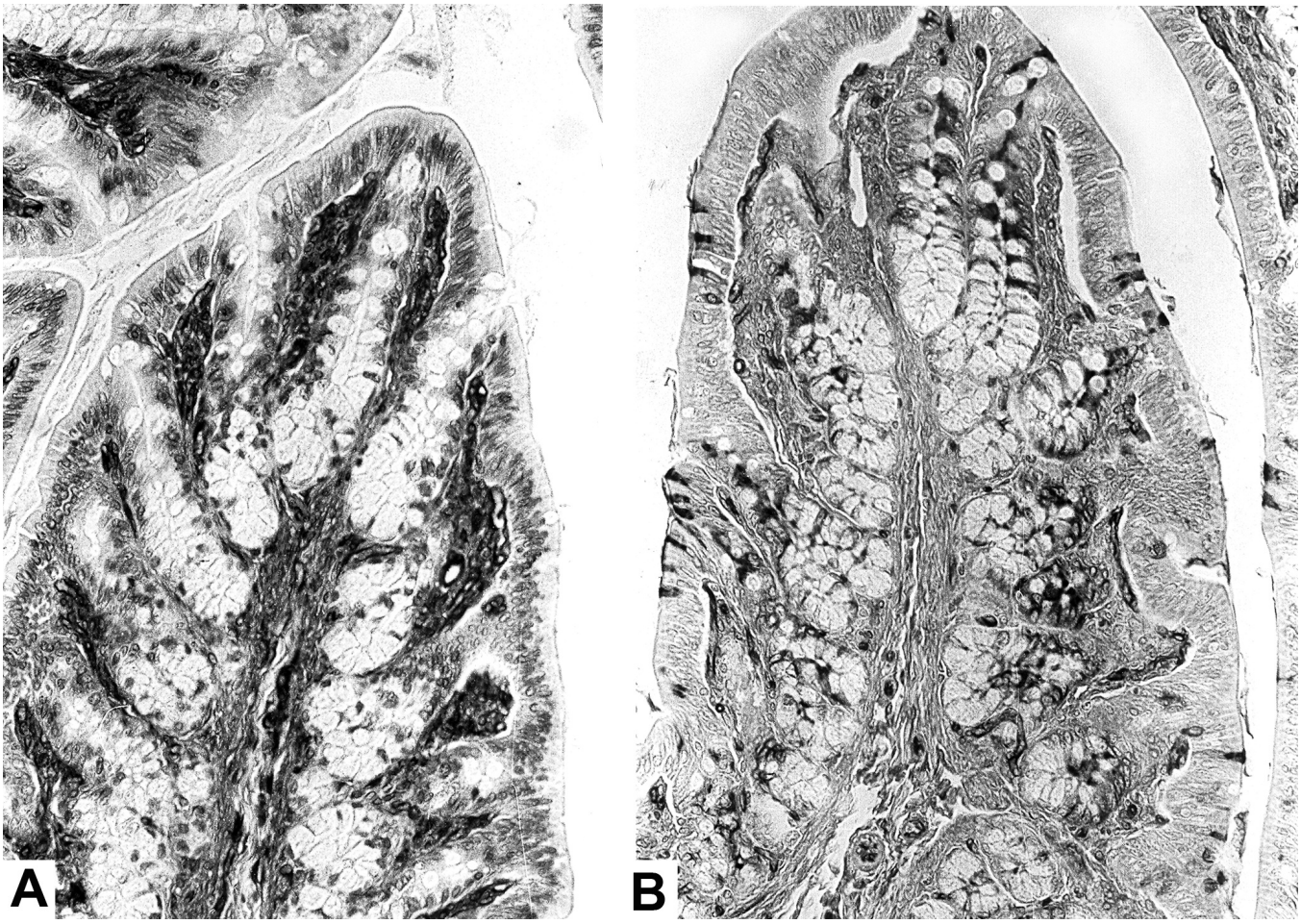

Fig. 1. Rat proximal colon. SNA (**A**) and MAL II (**B**) lectin patterns. The most marked affinity sites are produced by MAL II at surface goblet cells and crypt upper portions. x 260

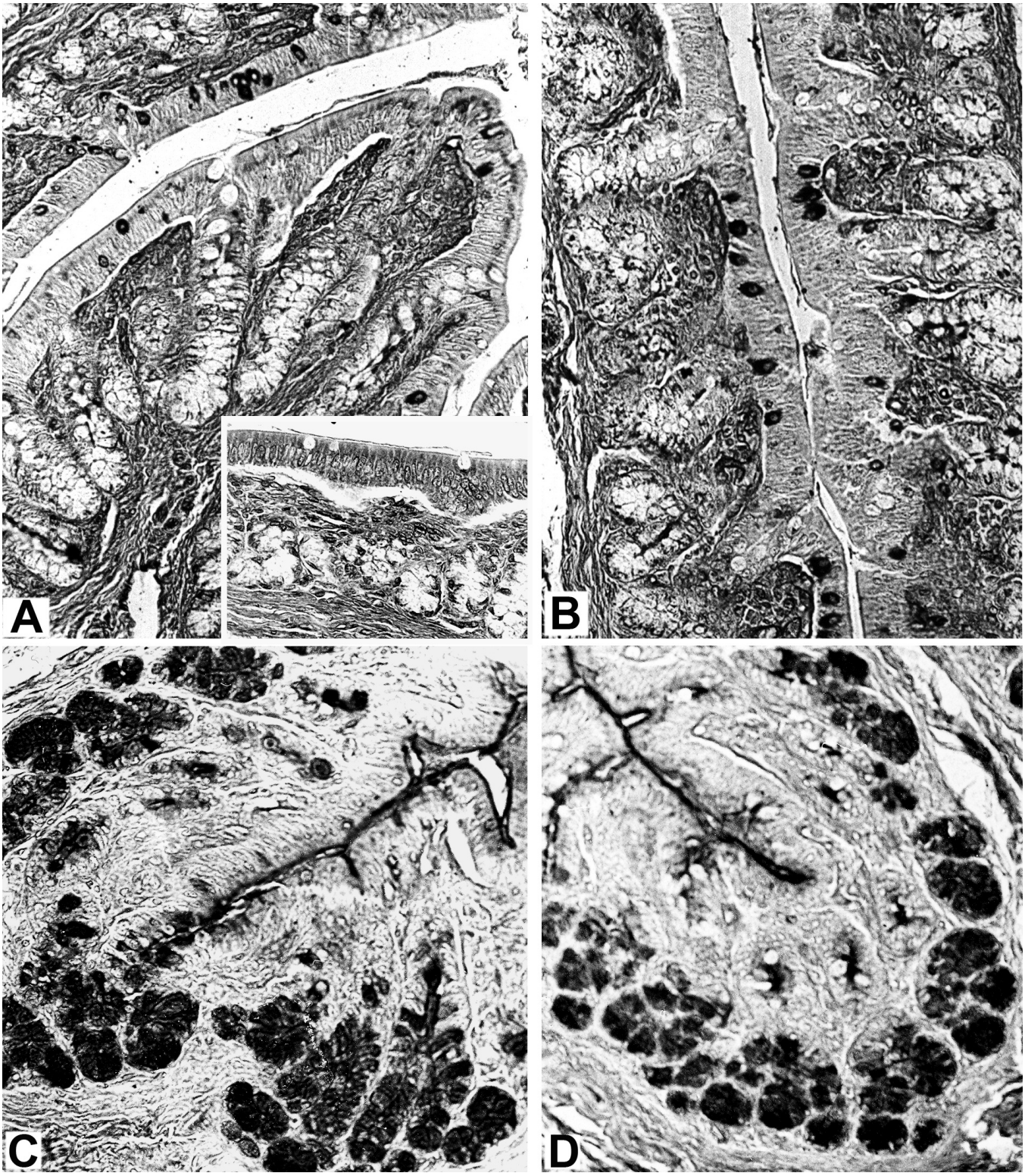

Fig. 2. Rat proximal colon. PNA binding pattern (inset) shows a weak staining in the surface colonocytes and connective tissue. **A.** Sialidase predigestion induces new PNA reactivity at surface goblet cells. Some crypt cells exhibit staining at the luminal border. All these affinity sites maintain after mild oxidation **(B)**. In the tissues subjected to KOH/sialidase pretreatment, the PNA reactivity strongly increases in the crypts, mainly at the fundus, and it appears at the epithelial luminal border **(C)**. A similar PNA binding pattern is produced after 44 mM PO/KOH/sialidase treatment **(D)**. x 250

Discussion

In the present study, the detection of the sialylation pattern of glycoconjugates expressed along the rat colon, as well as the identification of distinct sialoderivatives, were achieved by using a panel of lectins combined with

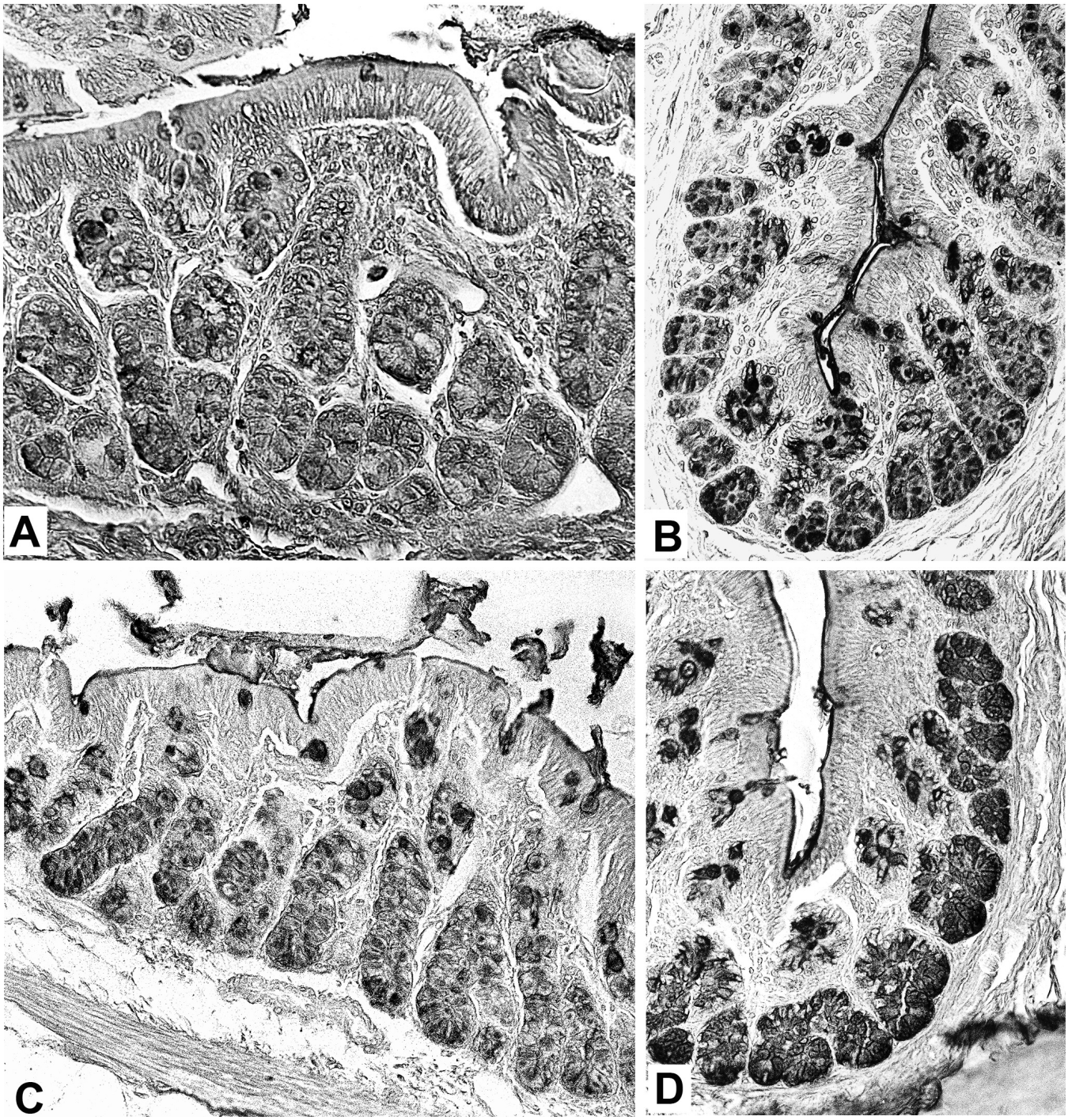

Fig. 3. Rat proximal colon. The DBA reactivity, mainly located in the crypt cells **(A)**, greatly increases after sialidase digestion and extends to the colonocyte luminal surface **(B)**. The sialidase/DBA staining is partly prevented by mild oxidation pretreatment, mainly in the crypts **(C)**. Deacetylation results in a stronger sialidase/DBA reactivity at the fundus of the crypts **(D)**. x 250

enzymatic and chemical treatments. In particular, the direct visualization of sialoglycoconjugates containing Sia(α2,3)-D-Gal and Sia(α2,6)-D-Gal/GalNAc sequences, as recognized by the highly specific and sensitive MAL II and SNA lectins, respectively, was supplemented with the indirect demonstration of Sia-D-Gal(ß1,3)-D-GalNAc and Sia-D-GalNAc sequences, revealed by sialidase induced PNA and DBA reactivity. Further data on structural features of sialic acids originated from the comparative evaluation of the effects that deacetylation and differential periodate oxidation produced on sialidase/PNA and sialidase/DBA binding patterns. On the whole, a high expression of sialoglycoconjugates was evidenced in the rat colonic mucosa, differently located mainly in the surface goblet cells, which are interspersed among colonocytes, as well as in the crypt cells. At this latter site, differential sialylation patterns were visualized in the crypt upper portion, near the surface epithelium, and in the crypt fundus, consistently with the occurrence of distinct cell populations along the crypt.

Both Sia-D-Gal(ß1,3)-D-GalNAc and Sia-D-GalNAc sequences were identified, showing some differences in their distribution between the colonic proximal and distal portions. Thus, sialic acids linked to D-Gal(ß1,3)-D-GalNAc, besides being homogeneously located in the surface goblet cells, were mostly detected at the crypt fundus in the proximal colon, while they were more highly expressed at the crypt upper portion in the distal colon. Sialic acids linked to D-GalNAc, which proved to be generally less abundant than sialic acids linked to D-Gal(ß1,3)-D-GalNAc, were mainly located at the crypt fundus in the proximal colon and in the surface goblet cells in the distal one.

The analysis of the acetylated sialoderivatives allowed the tissue-specific distribution of sialoglycoconjugates along the rat colon to be further detailed. It resulted in an increasing gradient of *O*-acetylated sialic acids from proximal to distal colon, closely resembling previous findings from human intestinal mucins (Robbe et al., 2003). The most common acetyl substitutions were identified at the glycerol-like side chain, with or without the presence of additional acetyl esters at the pyranose ring. In particular, a large part of the *O*-acetylated sialic acids showed *O*-acetylation at 9 position. They were widely distributed, with a high concentration in the surface goblet cells of the proximal colon. Here, the resistance of sial/PNA reactivity to strong periodate oxidation accounted for a tissue-specific expression of C_9 acetylated sialic acids linked α2,3 to D-Gal(ß1,3)-D-GalNAc preterminal sequences. The binding pattern of MAL II lectin supported this rule.

Although sialic acids are known to show usually only one *O*-acetyl group (Schauer, 2004), di- and tri-*O*-acetylated sialic acids are reported as a common feature in the human colon (Corfield and Schauer, 1982; Robbe et al., 2003). In contrast, a recent analysis, performed by gas chromatography coupled to mass spectrometry, was

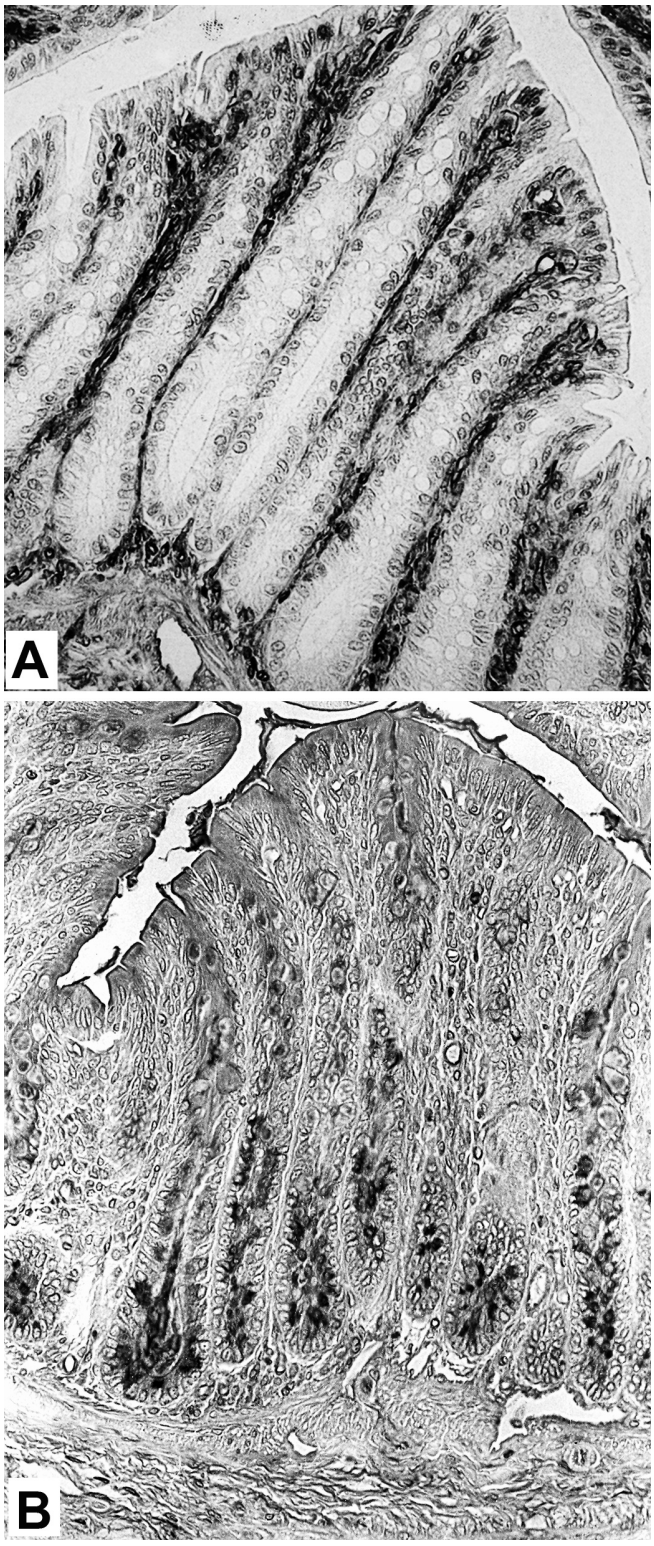

Fig. 4. Rat distal colon. SNA lectin weakly stains the surface epithelium **(A)**. MAL II binds to surface epithelium and crypt cells, with the strongest reactivity at the crypt fundus **(B)**. x 250

not able to detect a significant amount of oligo *O*-acetylated sialic acids in the mouse gut (Rinninger et al., 2006). On the other hand, species-specific differences in tissue sialylation patterns among closely related taxa, such as humans, chimpanzees, mice and rats, were recently documented (Althelde et al. 2006). In the rat colon, our results pointed out a large occurrence of sialoderivatives with acetyl groups at both the pyranose ring and the side chain. Indeed, *O*-4-acetylation was found to be largely associated with lateral acetylation, mainly with 9-*O*-acetylation, in sialoderivatives located in surface colonocytes and crypt cells. In the proximal colon, the effect of oxidation treatment on PNA and DBA affinity, consequent to deacetylation and sialidase digestion, suggested a high presence of C7-8-9 and C4 acetylated sialic acids in D-Gal(ß1,3)-D-GalNAc and D-GalNAc sequences in the crypt fundus cells which exhibited a marked and diffuse cytoplasmic staining. Conversely, in the distal colon, the corresponding binding patterns indicated a large occurrence of such

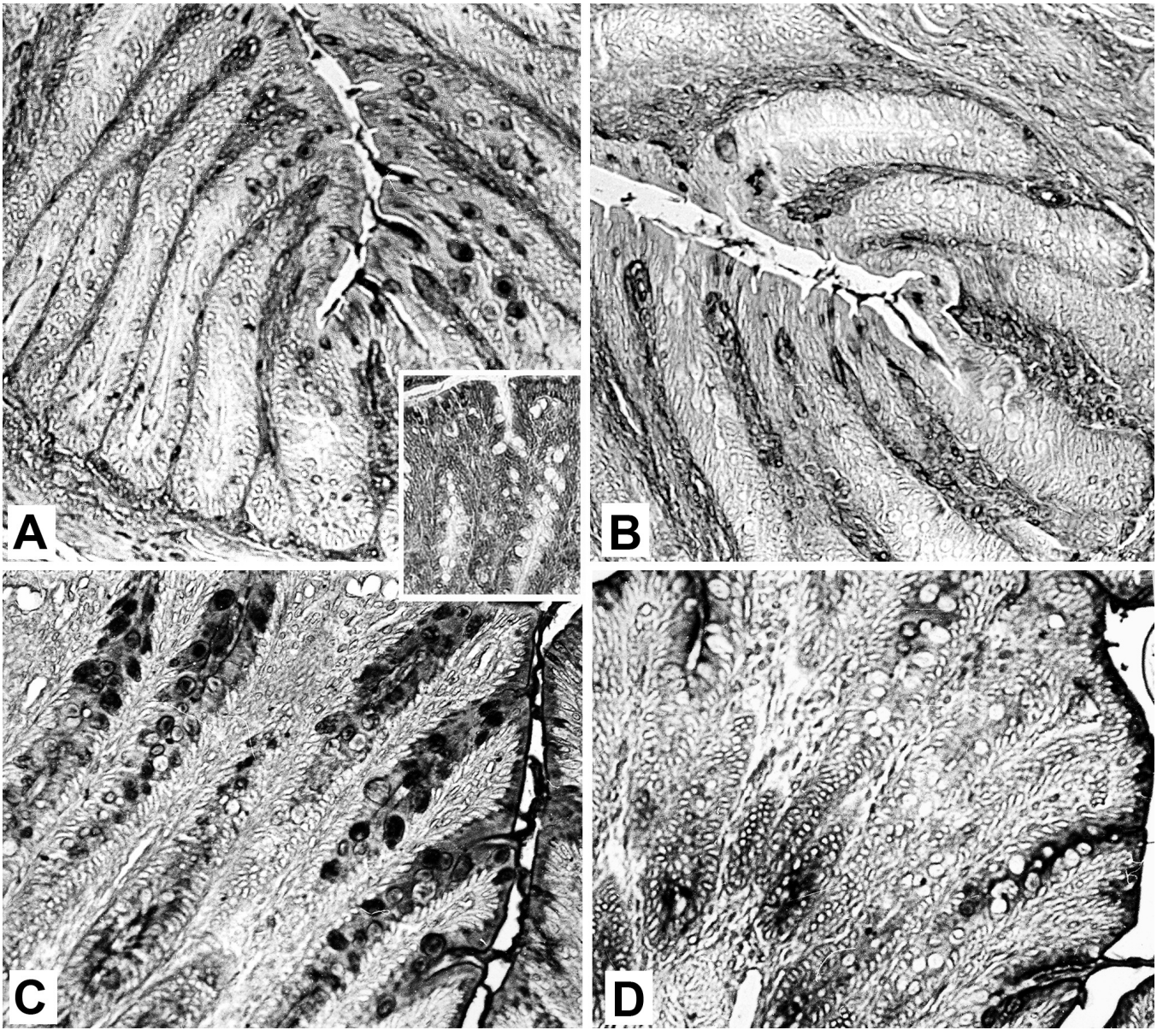

Fig. 5. Rat distal colon. PNA reactivity is induced by sialidase pre-digestion **(A)**, when compared with native PNA binding pattern (inset), at surface goblet cells and in a few crypt cells at both the upper portion and fundus. The lectin binding is prevented by strong oxidation followed by sialidase/PNA **(B)**. KOH/sialidase/PNA produces a strong staining at crypt cells and at the surface luminal border **(C)**. Strong oxidation pretreatment reduces reactivity at the crypt upper portion **(D)**. x 250 (inset, x 180).

sialoderivatives in the cells of the crypt upper portion, but a lower expression in the crypt fundus, restricted to the luminal cell surface. The different distribution patterns along the colon were only partially reproduced by the results of the sialic acid direct demonstration. Thus, MAL II, recognizing sialic acid linked α2,3 to D-Gal, fairly confirmed and integrated the PNA binding patterns, induced by the chemical and enzymatic pretreatments, at any reactive site in the proximal colon, except the crypt fundus where no MAL II staining could be detected. As already suggested for other tissues (Schulte and Spicer, 1984; Accili et al., 1994, 2001), the large occurrence of cytoplasmic 4-O- and 9-O-acetylated sialic acids, as here evidenced by the indirect lectin binding, as well the eventual presence of other sialoderivatives, such as 8-*O*-methylated sialic acids identified in both glycoproteins and glycolipids of the mouse gut (Rinninger et al., 2006), might prevent, by steric hindrance, the accessibility of MAL II lectin to this site. On the other hand, the high expression of sialoglycoconjugates in crypt fundus cells is confirmed by their strong stainability when the conventional histochemical methods for demonstration of carboxylated glycocomponents are applied (unpublished data). At the fundus of the distal colon crypts, instead, a marked staining was produced by MAL II, consistent with the indirect demonstration of Sia(α2,3)-D-Gal(ß1,3)-D-GalNAc sequences, located at the luminal cell surface where, probably, MAL II binding to its affinity sites is not negatively influenced.

In its turn, SNA lectin, which specifically binds Sia(α2,6)-D-Gal/GalNAc, failed to stain the colonic epithelium of either the proximal or distal portions, although it markedly reacted with connective tissue and endothelia. Similar patterns were produced by SNA in the normal human colon (Sata et al., 1991; Dall'Olio and Trere, 1993; Murayama et al., 1997) as well as in a normal intestinal epithelium cell line (de Albuquerque Garcia Redondo et al., 2004). In addition, in genetically altered mice with deficiency in the sialyltransferase ST6Gal-I, it was suggested that Sia(α2,6)-D-GalNAcα1-O-Ser/Thr (sialyl-Tn epitope), which can also be recognized by SNA, is very rare in normal mouse tissues (Martin et al. 2002). However, in spite of the negative binding patterns produced by SNA, the occurrence of O-acetylated sialic acid, linked to D-GalNAc preterminal sugar, is here clearly indicated by the DBA reactivity, induced by chemical and enzymatic treatments at different sites along the rat colon. Actually, SNA needs an intact glycerol side chain for recognition (Shibuya et

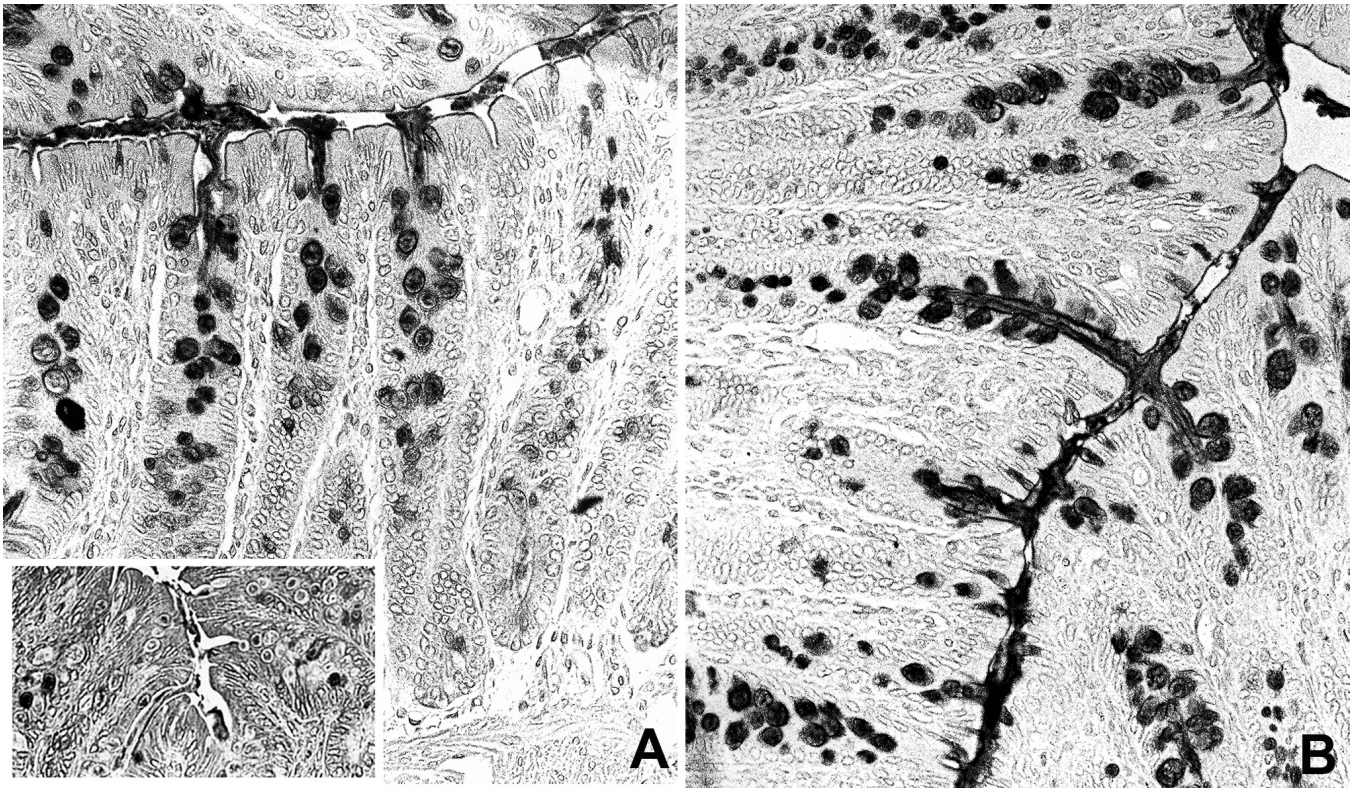

Fig. 6. Rat distal colon. DBA binding pattern shows reactivity mainly at some cells in the crypt upper portion (inset in **A**). After sialidase digestion, new DBA reactive sites can be observed in the surface goblet cells and at the crypt upper portion **(A)**. The staining is increased by deacetylation pretreatment **(B)**. x 250 (inset, x 180).

al., 1987), which for many lectins correlates with the inability to bind 9-O-acetylated sialic acid (Angata and Varki, 2002). Nevertheless, studies on SNA have shown that this lectin can bind sialic acids with or without 9-O-acetyl groups (Brinkman-Van der Linden et al., 2002). The failure of SNA to recognize affinity sites in the rat colonic epithelium is unclear, at present. The high density of negative charges, due to sialylated glycocomponents and sulphated groups (personal communication), which results in a hydration coat, might influence the SNA ability to visualize sialic acids linked α2,6 to D-Gal/D-GalNAc in the rat colon.

Despite previous data reporting high levels of Neu5Ac in the human and mouse gut, and relatively abundant content of Neu4,5Ac_2 in the mouse colon (Morimoto et al., 2001; Robbe et al., 2003; Rinninger et al., 2006), we detected a poor expression of sialic acids lacking in acetyl groups either at C4 or at the side-chain. These were specifically located as Sia-D-GalNAc sequences in the rat proximal colon. The present findings do not rule out that a higher content of unsubstituted sialic acids can exist in the rat colon, linked to acceptor sugars other than those here investigated, as well as being present as short lateral chains, thus undetectable by indirect lectin binding.

In summary, the wide and heterogeneous distribution of sialic acids linked to D-Gal preterminal sugar, and to a lesser extent to D-GalNAc, as well as the diversity of their structural features pointed out in the rat colon, are in good agreement with data already reported for other species (Karlsson et al., 1997; Rinninger et al., 2006). As previously proposed (Robbe et al., 2003), the functional meaning of such a concentration of sialylated components, which cause a high negative charge density, may be generally related to the characteristic viscoelastic properties of intestinal mucins, greatly contributing to water and electrolyte transport in the distal portion of the gut. The increasing gradient of O-acetylated sialic acids from proximal to distal colon, as here documented, might reflect a corresponding functional gradient in the requirement of a protective and lubricant action played by these molecules.

As far as the role attributed to the sialic acid in pathological events is concerned, the present results, which combine the visualization of distributional sites with the structural characterization of sialoderivatives in the normal colonic mucosa, may provide a base to better evaluate in the rat the relationships between sialylation pattern and malignant transformation and metastatic potential of cancer cells.

Finally, on the basis of studies which have reported interactions of a number of intestinal bacterial strains to sialic acids (Corfield et al., 1992; Pastoriza Gallego and Hulen, 2006), an additional, essential aspect of the sialic acid biology concerns host-pathogenic and host-symbiotic interactions in the intestinal tract. In this context, the variety of sialic acid structures, expressed at different sites of both the proximal and distal colon, might greatly contribute to increase the repertoire of potential binding sites for microorganisms, which has been proposed to explain the regio-specific colonization of bacteria in the intestine (Robbe et al., 2003). An important role, in particular, may be attributed to the high level of *O*-acetylation, mainly at 9 position, which the sialylated components show in the rat colonic mucosa. Indeed, since the acetylation of sialic acids is known to inhibit enteric bacterial sialidases (Corfield et al., 1992; Robbe et al., 2003), the cell type-specific expression of *O*-acetylated sialoderivatives, showing an increasing gradient from the proximal to distal colon, besides playing a general defensive role, can act in selecting specific strains which, by the production of sialate O-acetylesterases, can be more competitive than others in developing colonies in this area.

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