Histol Histopathol (2008) 23: 973-978

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Localization of influenza virus sialoreceptors in equine respiratory tract

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Summary. This study was performed to identify the equine respiratory tract areas which express the specific receptor for equine influenza virus; findings may be useful to provide new ways to treat the infectious disease. The present work aims to visualize *in situ* the presence of sialoderivatives in the horse respiratory tract in order to localize sialoderivatives acting as influenza virus receptors. To this purpose, nasal mucosae, trachea, bronchus and lung parenchyma were removed from 8 mature horses of both sexes. We performed sialic acid characterization by means of mild and strong periodate oxidation and saponification, combined with lectin histochemistry and sialidase digestion, in addition to the direct evidentiation of sialic acid residues. No differences were shown between sexes. Sialic acid residues are present in the nasal mucous cell secretion, where they are linked to galactose by means of α2-3 linkage and are mainly C_9 acetylated, and in the nasal and tracheal epithelial lining, where they are represented by periodate labile residues (α2-3)- and/or (α2-6)-linked to galactose. Specific receptors for equine influenza viruses are present at the nasal and tracheal epithelial lining cell coat levels, and in some trachea epithelial cells, but the horse possesses a preventive defence, which consists of the secretion of a mucous layer at nasal level, which could specifically inactivate the hemagglutinins of equine influenza virus; in addition, it expresses other sialoreceptors which can mask the influenza specific ones.

Key words: Horse, Respiratory tract, Lectin, Sialoderivatives, Influenza virus

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Introduction

Influenza virus particles infect host cells by means of hemagglutinin spikes, binding to sialoglycoconjugates acting as receptors on the host cell surface, and by means of neuraminidase spikes, cleaving terminal sialic acid from cell surface glycoconjugates, allowing the virion to escape from the host cell during the budding process (Werling, 2006). Influenza viruses are classified with the acronym $H_{1\text{-}16}N_{1\text{-}9}$ on the basis of their hemagglutinin (H) and neuraminidase (N) spikes (Suzuki, 2005). By means of mutations of H and N spikes, due to both shift and drift mechanisms, influenza viruses can adapt to the new host environments and enhance their transmission across different species (Rogers et al., 1983; Suzuki, 2005; Kogure et al., 2006; Shinya et al., 2006). Equine influenza viruses preferentially recognize the dimer sialic acid-α2-3-galactose (Kogure et al., 2006), which is the same receptor for avian influenza virus, while human influenza virus preferentially recognizes the dimer sialic acid-α2-6-galactose (Shinya et al., 2006).

This work aims to visualize *in situ* the presence of sialoderivatives in the horse respiratory tract, by means of mild and strong oxidation with 1mM and 44mM aqueous periodic acid solutions, and saponification with 0.5% KOH in 70% ethanol, combined with lectin histochemistry and sialidase digestion, in order to localize sialoderivatives acting as influenza virus receptors.

Materials and methods

Tissue preparation

Samples of nasal mucosae, trachea, bronchus and lung parenchyma were removed from 8 adult horses of both sexes, fixed at room temperature in Carnoy's fluid for 24 h and postfixed in 2% calcium acetate-4%

paraformaldehyde solution (1:1) for 3 h. Specimens were then dehydrated, embedded in paraffin wax and cut in serial sections 5 µm thick.

Histochemical procedures

Conventional histochemistry for the evidentiation of acidic carboxylated and sulphated groups was performed by means of the following reactions: Alcian Blue (AB) pH 2.5, Low Iron Diamine (LID), High Iron Diamine (HID) (Spicer et al., 1967; Pearse, 1968).

Mild and strong periodate oxidation with periodic acid (PO) were performed by immersing specimens in 1mM or 44mM periodic acid solution, respectively, for 15 min at room temperature, to reveal the presence of terminal sialic acid residues with O-acetyl substituents (Reid et al., 1978; Schauer, 1978).

Saponification was carried out by 0.5% potassium hydroxide in 70% ethanol for 30 min at room temperature (Reid et al., 1978).

Lectin staining was performed as previously described by Menghi (1984). The following lectins were used: PNA from *Arachis hypogaea* [terminal ß-D-galactose(1-3)-N-acetylgalactosamine], RCA I from *Ricinus communis* [terminal ß-D-galactose(1-4)-N-acetylglucosamine], SNA from *Sambucus nigra* [N-acetylneuraminic acid(α2-6)-galactose/N-acetyl-galactosamine], MAA from *Maackia amurensis* [α-sialyl(2-3)lactose]. After inhibition of endogenous peroxidase activity by 0.3% H_2O_2/methanol for 30 min, tissue sections were immersed in horseradish peroxidase conjugated lectins (PNA; RCA I) (0.02-0.2 mg ml^{-1}) in 0.05M phosphate-buffered saline (PBS), pH 7.2 for 30 min at room temperature. Biotynilated lectins (SNA; MAA), dissolved in the same buffer, were used at a concentration of 20mg ml^{-1}, and, after rinsing with PBS, the sections were incubated in streptavidin/peroxidase complex. After rinsing in PBS and 0.5M Tris/HCl buffer, pH 7.4, sections were treated with diamino-benzidine-H_2O_2 medium (Graham and Karnovsky, 1966) for 10 min at room temperature before dehydration and mounting in Permount.

Before lectin histochemical staining, some sections were incubated, at 37°C for 16 hours, in 0.86U/mg Prot of sialidase (Type V, from *Clostridium perfringens*) dissolved in 0.1M sodium acetate buffer, pH 5.5, containing 10mM $CaCl_2$ (Scocco et al., 2002).

PNA, RCA I and Sialidase were purchased from Sigma Chemical Co.; SNA and MAA were obtained from VECTOR Laboratoires Inc.

On the basis of sialic acid acceptor sugar forming the dimer receptor for influenza viruses, the following histochemical procedures were performed as sequential treatments for discriminating sialoderivatives:

a) PNA - RCA I
b) 1mM - 44mM PO/PNA - RCA I
c) Sialidase/PNA - RCA I
d) KOH/Sialidase/PNA - RCA I
e) 1mM - 44mM PO/Sialidase/PNA - RCA I
f) 1mM - 44mM PO/KOH/Sialidase/PNA - RCA I

Table 1. Response of samples to lectins and sequential treatments.

Histochemical treatments	Nasal mucosae			Trachea		Bronchus		Bronchiole	Lung	Sugar moieties visualized
	Epithelial cells	Goblet cells	Nasal glands	Epithelial cells	Tracheal glands	Epithelial cells	Goblet cells	Epithelial cells	Alveolar cells	
PNA	–/±[a]	±/+	–/±	–	–	–	±	–/±	–	ß-Gal residues in terminal non-reducing position linked (1-3)GalNAc
Sial/PNA	±[a]	+/++	±	±/+[a,b]	–/+	–	±	–/±	–	Terminal disaccharide sialic acid-ß-Gal with sialic acid not C_4 acetylated linked (1-3)GalNAc
KOH/Sial/PNA	±[a]	+/++	±	±/+[a,b]	–/+	–	±	–/±	–	Sialic acid with and without C_4 acetyl groups linked to ß-Gal(1-3)GalNAc
1PO/Sial/PNA	–/±[a]	+	–/±	–	–/±	–	±	–/±	–	Presence of sialic acid substituted on the polyhydroxyl side chain with acetyl groups
44PO/Sial/PNA	–	±/+	–	–	–	–	–	–	–	Sialic acid C_9 and/or $C_{7,9}$ and/or $C_{8,9}$ and/or $C_{7,8,9}$ O-acetylated linked (α2-3)Gal(1-3)GalNAc
RCA I	±	+/++	±/+	–	–	–	+	–	–	ß-Gal residues in terminal non-reducing position linked (1-4)GlcNAc
Sial/RCA I	±	+/++	±/+	–	–	–	+	–	–	Terminal disaccharide sialic acid-ß-Gal with sialic residues not acetylated at C_4 linked (1-4)GlcNAc
KOH/Sial/RCA I	±	+/++	±/+	–	–	–	+	–	–	Sialic acid with and without C_4 acetyl groups linked to ß-Gal(1-4)GlcNAc
MAA	±[a]	–	±	±/+[a,b]	+	–/±	–	–	–	Detection of sialic acid linked (α2-3)Gal
SNA	±/+[a]	–	–/±	±/+[a,b]	+	–/±	–	–	–	Detection of sialic acid linked (α2-6)Gal

Results are given in arbitrary units as follows: - negative; ± weak; + moderate; ++ strong staining. [a]: cell coat, [b]: some cells.

g) KOH/1mM - 44mM PO/Sialidase/PNA - RCA I

Controls

The respective peroxidase-conjugated lectins were omitted and/or their hapten sugars were added in control sections. As controls for enzyme digestion, sections were incubated with the respective enzyme-free buffers under the same experimental conditions. Additional controls for SNA and MAA were performed by means of sialidase treatment preceded or not by saponification before incubating sections with lectin solutions.

Results

No differences were observed between sexes.

AB and LID reaction showed overlapping binding sites; in particular, goblet cells of nasal mucosae and glandular cells of both nasal and tracheal lamina propria glands were strongly positive. The cell coats of nasal mucosae, trachea and bronchiole were moderately positive to AB and LID. HID showed a strong reactivity in the goblet cells of nasal mucosae and bronchi.

The responses of samples to lectin incubation and sequential treatments are reported in Table 1, also showing the sugar moieties visualized by the respective treatment.

No staining occurred in sections treated with unconjugated lectins or after incubation with hapten sugar in lectin-HRP or biotynilated solution. The immersion of sections in enzyme-free buffer solution resulted in unmodified binding.

The most significant features are visualised in Figs. 1-3, and explained in the relative captions.

Discussion

Conventional histochemistry showed the presence of carboxylated carbohydrates in the nasal and tracheal glands, and in the goblet cells of nasal and bronchi mucosae, which also express highly sulphated glucidic material. Carboxylated material is also present at the epithelial cell coat level of trachea, bronchi and bronchioles.

Direct visualization of sialic acid residues, performed by means of MAA and SNA lectins, having nominal specificity towards two different dimers of sialic acid and ß-galactose, distinguishable on the basis of α2-3 and α2-6 linkage, showed the presence of both the dimers, mainly localized in some cells and in the cell coat of epithelial lining of the trachea, and in the cell coat of nasal mucosae. Lesser amounts of such dimers are present in the bronchus epithelia cell coat. In nasal glands, the dimer sialic acid-α2-3-galactose prevails over the sialic acid-α2-6-galactose one, while tracheal glands display a reverse pattern.

Indirect detection of sialic acid residues performed using periodate oxidation, saponification, sialidase digestion and lectin incubation showed that both the

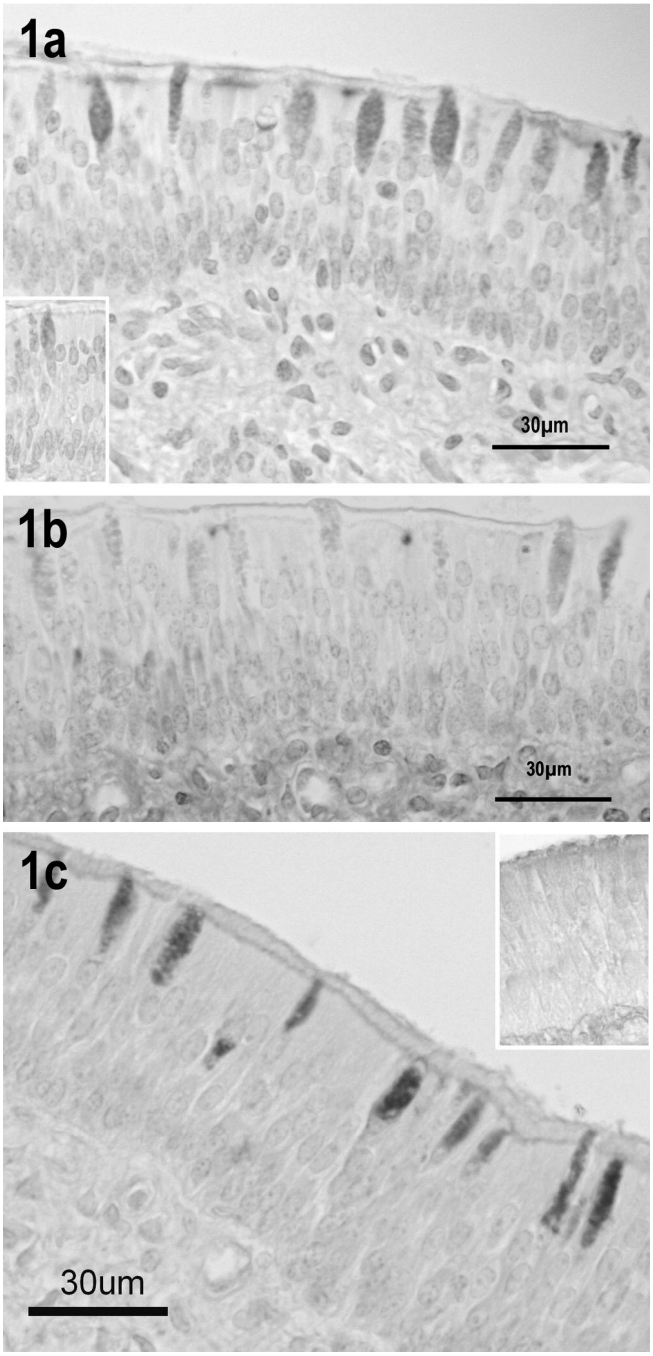

Fig. 1. Horse nasal mucosae. Sialidase/PNA **(a)** enhances goblet cell reactivity towards PNA (inset) and promotes cell coat positivity. 44 mmol/l PO/Sialidase/PNA treatment **(b)** strongly decreases the Sialidase/PNA positivity of goblet cells, indicating the presence of sialic acid (α2-3/6)- linked to galactose; residual positivity indicates the presence of sialic acidα–2-3-galactose dimer. HID **(c)** shows a strong reactivity of goblet cells which fail to react with MAA (inset).

dimers directly evidenced are (1-3)- linked to N-acetyl-galactosamine, since only PNA reactivity was affected by enzymatic digestion, with or without prior saponification, while RCA I reactivity was unmodified by the same treatments. Saponification with KOH did not affect sialidase/PNA reactivity of samples, showing the lack of sialic acid residues acetylated at C_4.

Mild oxidation destroys the unsubstituted sialic acid residues, which are indicated as periodate labile (Schauer, 1978; Menghi and Materazzi, 1994). Strong oxidation affects the sialic acid residues substituted at the C_7 and/or C_8 and/or $C_{7,8}$ level, in addition to C_9 acetylated sialic acid (α2-6)- linked to galactose, while it doesn't oxidize the C_9 and/or $C_{7,9}$ and/or $C_{8,9}$ and/or $C_{7,8,9}$ acetylated sialic acid (α2-3)- linked to galactose (Reid et al., 1978).

Sequential treatments demonstrate the presence, in some cells and in the cell coat of trachea epithelial lining, of periodate labile sialic acid (α2-3)- and (α2-6)-linked to galactose(1-3)-N-acetyl-galactosamine. The presence of sialic acid(α2-6)-galactose dimer is in contrast with data previously reported by Suzuki and co-workers (2000), and this fact could be ascribed to different fixatives and staining methods used. The present data, obtained by means of sequential treatments, is further confirmed by direct methods (see Fig. 2b).

The same sialoderivatives are present in the cell coat and in the goblet cells of nasal mucosae; in addition, nasal goblet cells express a great amount of C_9 and/or $C_{7,9}$ and/or $C_{8,9}$ and/or $C_{7,8,9}$ acetylated sialic acid (α2-3)- linked to galactose.

Findings obtained with direct and indirect methods can be overlapped, with the exception of the detection of sialoderivatives in the nasal goblet cells, in particular of the C_9 acetylated sialic acid residues (α2-3)- linked to galactose, evidenced by sequential treatments, which failed to react to MAA. This fact could be ascribed to the strong presence of sulphated groups, which could represent a steric hindrance for the internalization of MAA and/or SNA lectins, or contribute to the formation

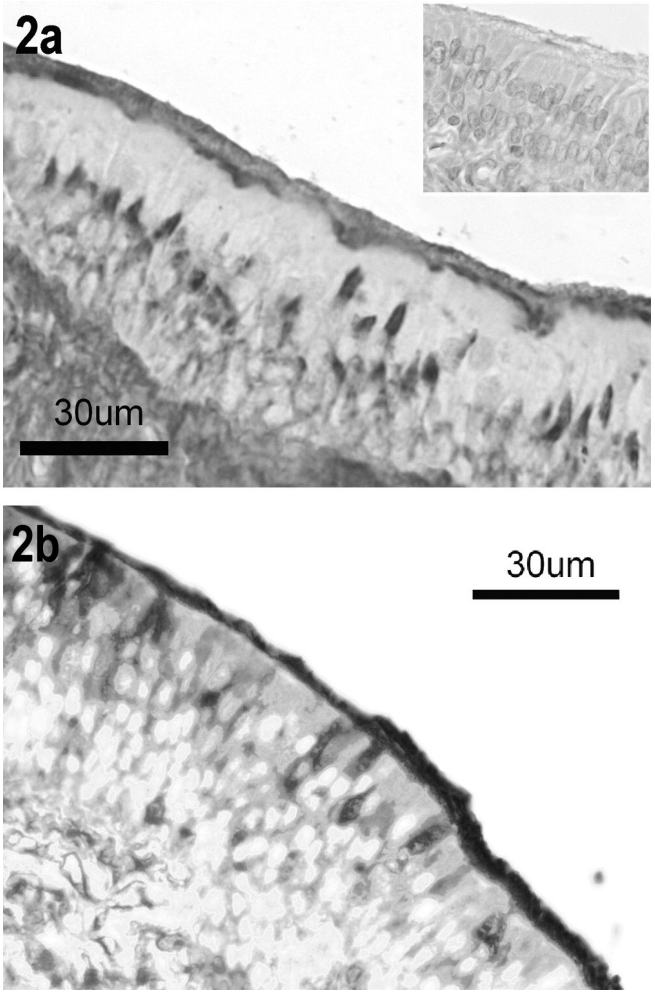

Fig. 2. Horse trachea. Sialidase/PNA promotes the appearance of positive binding sites at cell coat level and in some epithelial cells **(a)** which are negative to PNA (inset). SNA shows binding sites in some cells and at cell coat level demonstrating the presence of sialic acid-α2-6-galactose dimer **(b)**.

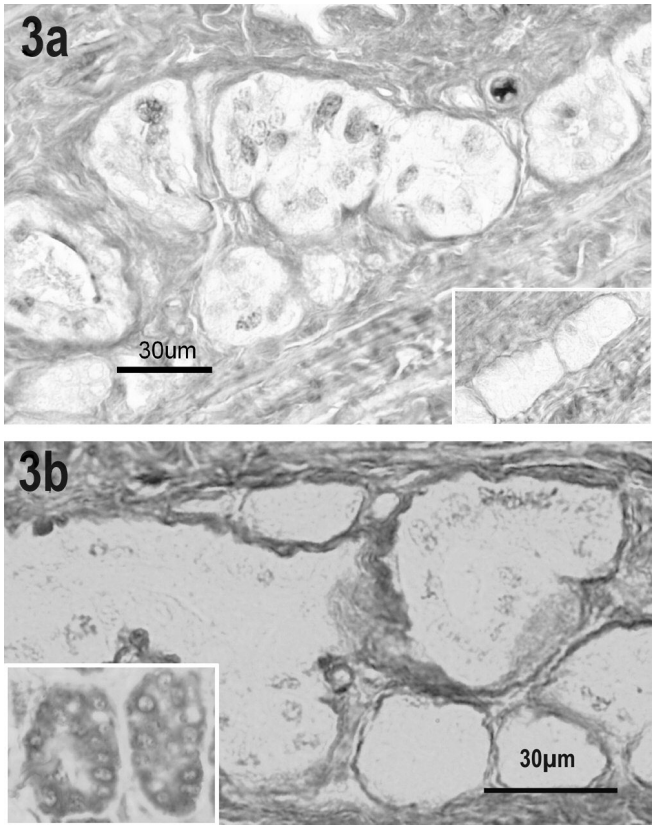

Fig. 3. Horse trachea. Tracheal glands show some positive cells after Sialidase/PNA treatment **(a)** with respect to negative PNA (inset). 1 mmol/l PO/Sialidase/PNA strongly decreases glandular cell positivity **(b)** demonstrating that sialic acid residues are prevalently periodate labile; inset: reactivity of tracheal glands to MAA.

of a highly negative charge, which can negatively affect the lectin binding (Spicer and Schulte, 1992).

In this specific situation, the availability of the sequential treatments, which evidenced the sialoderivatives acting as receptors specifically recognized by the equine influenza virus (Rogers et al., 1983; Kogure et al., 2006; Shinya et al., 2006), is very important. In addition, the sequential treatment method allows an even more precise characterization of sialoderivatives on the basis of their acetylation degree (Scocco et al., 2002; Scocco and Pedini, 2006).

On the basis of obtained data, we can affirm that equine influenza virus specific sialoreceptor is present on the cell coat and in some cells of the trachea epithelial lining; at these levels the dimer sialic acid-α2-3-galactose shows periodate labile sialic acid residues. The same receptor is also present at nasal cell coat level, but in a lesser quantity.

Sialoglycoconjugates showing the dimer sialic acid-α2-3-galactose, having the sialic acid residues C_9 and/or $C_{7,9}$ and/or $C_{8,9}$ and/or $C_{7,8,9}$ acetylated, are present in the nasal goblet cell secretions; we can hypothesize that these sialoderivatives, forming the mucous layer on the nasal mucosae, could have a specific role in the defence against the equine influenza virus, performed by agglutination of virus.

In addition, sialoderivatives of both surface and secretion types, characterized by the dimer sialic acid-α2-6-galactose, specifically recognized by human influenza virus, are expressed by the same tissue structures; it is likely that these sialoglycoconjugates perform an unspecific defence, masking the specific receptors (Reutter et al., 1982; Hanaoka et al., 1989; Zimmer et al., 1992; Nieuw-Amerongen et al., 1995).

Finally, the presence of sialoderivatives characterized by the sialic acid (α2-6)- linked to galactose and C_9 acetylated sialic acid residues (α2-3)-linked to galactose in the nasal mucous secretions, could contribute to the defence of the early respiratory passageways against human A- and C-like influenza viruses (Rogers et al., 1983; Zimmer et al., 1992; Kogure et al., 2006; Shinya et al., 2006).

It may be useful to have a complete picture of sialoderivatives expressed by different tracts of the equine respiratory tract, to provide new ways to treat infectious diseases (Totani et al., 2003; Suzuki, 2005) and to evaluate possible new recognition sites in case of virus mutation and/or transmission among different species (Suzuki, 2005; Shinya et al., 2006).

Acknowledgements. The authors wish to express a specific gratitude to Prof D. Werling for his suggestion to perform this work. We are grateful to Sara Tulczyjew for linguistic revision.

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Accepted February 18, 2008