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Evidence for Sialyl Glycoconjugates as Receptors for *Bordetella bronchiseptica* on Swine Nasal Mucosa

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The nature of the receptors for *Bordetella bronchiseptica* was investigated by using the in vitro adherence assay system. The results indicated that sialyl glycoconjugates acted as receptors on swine nasal mucosa. These results were obtained by two independent approaches: (i) inhibition of epithelial cell adherence with sialic acid-containing compounds but not with compounds lacking sialic acid residues and (ii) loss of adherence after treatment of epithelial cells with periodate or neuraminidase. *B. bronchiseptica* seems to have strong affinity for mucin. This may help the bacterium to colonize the mucosal surfaces of the swine nasal cavity.

The capacity of a certain microorganism to colonize epithelial surfaces may be related to its ability to bind to the relevant surfaces (1, 10, 18). Since the adherence of bacteria to mucosal surfaces may constitute the initial, critical step in colonization and infection, analysis of the adherence mechanisms would be important in understanding the early events in host-parasite interactions. Previous studies have demonstrated that microbes adhere to epithelial surfaces via certain receptors that can be blocked by the addition of substances which mimic the receptors concerned. Examples of substances which can inhibit bacterium-receptor interactions are D-mannose or its derivatives for *Escherichia coli* (19–21) and L-fucose for *Vibrio cholerae* (9) and *Campylobacter jejuni* (3, 17).

Although *Bordetella bronchiseptica*, the pathogen of atrophic rhinitis in swine, has been found to be capable of attaching to a variety of cell types (2, 8, 15, 16, 23, 26, 29, 31), little is known regarding the nature of the receptors on the natural target cells. The purpose of the present study was to characterize the receptors for *B. bronchiseptica* on swine nasal mucosa by using the in vitro adherence assay system as a tool to gain insight into the nature of the chemical compounds involved.

MATERIALS AND METHODS

Bacterial strains. The *B. bronchiseptica* strains used in the present study were phase I A19, S1, and H16, which were the primary isolates from pigs affected with atrophic rhinitis. Cultures were maintained on Bordet-Gengou agar (Difco Laboratories, Detroit, Mich.) containing 7% defibrinated sheep blood and were stored at 4°C. Inocula for the adherence assay were collected from 18-h cultures on Bordet-Gengou agar, washed twice with phosphate-buffered saline (PBS; pH 7.0), and suspended in Hanks balanced salt solution (Nissui Seiyaku, Tokyo, Japan) containing 0.03% sodium bicarbonate (HBSS). The final suspensions were adjusted to 4×10^8 CFU/ml.

Nasal epithelial cells. Epithelial cells were obtained by gently scraping the ventral turbinate mucosae of pigs (slaughtered at an abattoir) with a sterile soft brush, which was immediately immersed in PBS. The cells were then harvested by centrifugation, suspended in erythrocyte lysis buffer (0.14 M NH_4Cl, 0.017 M Tris [pH 7.2]), and allowed to stand for 5 min at ambient temperature. After sedimentation for 5 min at 200 × *g*, the cells were washed three times and suspended in HBSS to a density of 2×10^6 cells per ml with the use of a hemacytometer.

Mucin. Crude nasal mucin was obtained from the nasal PBS washings of five piglets. The slightly turbid mucin suspensions were collected, pooled, and centrifuged for 10 min at 1,700 × *g* to remove cells and debris. The presence of mucin was ascertained by observing the characteristic fern-ing appearance of mucin when placed on a glass microscope slide. This crude mucin preparation was quantitated by determining the protein concentration by the Lowry method (14) and was adjusted to a concentration of 300 μg of protein per ml.

Adherence assay. The assay for determining the amount of bacteria attached to epithelial cells was as follows. To 6×10^5 epithelial cells were added 6×10^7 bacteria and HBSS up to 0.6 ml. A control without the addition of bacteria was also prepared. The mixtures were incubated for 30 min in a shaking water bath at 37°C before the interaction was terminated by the addition of 5 ml of ice-cold PBS. Unattached bacteria were eliminated by repeated washings with 15 ml of PBS through a polycarbonate membrane filter (12-μm-pore size; Nuclepore Corp., Pleasanton, Calif.). The filter was then removed, and the cells were transferred onto a glass microscope slide by slight touching. After being air dryed, the slide was fixed and stained with May-Grünwald and Giemsa solutions. Bacteria adhering to each of the first 50 epithelial cells were counted by direct-light microscopy at a 1,000× magnification. The results were expressed as the mean number of bacteria attached per epithelial cell. Three replicate assays were performed on different days.

Inhibition experiments. As possible inhibitors of bacterial adherence, the following saccharides and glycoconjugates (obtained from Sigma Chemical Co., St. Louis, Mo.) were used: L-fucose, D-glucose, D-galactose, D-mannose, D-glucosamine, D-galactosamine, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, lactose, *N*-acetylneuraminic acid, *N*-acetylneuramin-lactose, human α_1-acid glycoprotein, bovine submaxillary mucin (type I), mixed disialogangliosides (type II), and mixed monosialogangliosides (type III). An inhibition text mixture consisted of 0.3 ml of epithelial cells, 0.15 ml of bacteria, and 0.15 ml of an inhibitor in HBSS. The bacteria were preincubated for 30 min at 37°C with the inhibitor. The adherence was compared with that in the control mixture lacking added inhibitors. The control mix-

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TABLE 1. Inhibition of *B. bronchiseptica* A19 adherence to swine nasal epithelial cells by different compounds

Compound (20 mg/ml)	Adherence[a]	% Inhibition	Concn (mg/ml [mM]) required for 50% inhibition
None	14.6 ± 0.8		
L-Fucose	15.5 ± 0.9	0	>20 (>122)
D-Glucose	15.0 ± 1.3	0	>20 (>111)
D-Galactose	15.6 ± 0.2	0	>20 (>111)
D-Mannose	15.5 ± 0.3	0	>20 (>111)
D-Glucosamine	14.7 ± 0.8	0	>20 (>92.8)
D-Galactosamine	15.6 ± 0.9	0	>20 (>92.8)
N-Acetyl-D-glucosamine	15.4 ± 1.5	0	>20 (>90.4)
N-Acetyl-D-galactosamine	14.8 ± 0.8	0	>20 (>90.4)
Lactose	14.7 ± 0.8	0	>20 (>58.4)
N-Acetylneuraminic acid	6.6 ± 0.8	55	10
N-Acetylneuramin-lactose	5.0 ± 0.1	66	10
α_1-Acid glycoprotein	6.2 ± 0.7	58	10
Bovine submaxillary mucin	2.1 ± 0.3	86	0.078
Porcine nasal mucin, crude	ND[b]	ND	0.075
Disialogangliosides, mixed	1.8 ± 0.2	88	0.078
Monosialogangliosides, mixed	0.5 ± 0.1	97	0.078

[a] Mean number of bacteria attached per epithelial cell ± standard error of the mean, determined from triplicate assays.
[b] ND, Not determined.

ture was always prepared and incubated simultaneously with the mixtures containing added inhibitors. The concentrations of inhibitors given in Results are the final ones in the test mixtures.

Periodate and neuraminidase treatments. To characterize the attachment sites on cell surfaces, we treated swine nasal epithelial cells and bovine erythrocytes, which are known to be agglutinated by *B. bronchiseptica* (26, 27), either for 5 min at 4°C with 10 mM sodium metaperiodate (Nakarai Chemicals, Kyoto, Japan) or for 30 min at 37°C with 0.1 U of *Arthrobacter ureafaciens* neuraminidase (Nakarai) per ml, both of which were dissolved in 10 mM sodium acetate buffer (pH 5.5) containing 0.85% NaCl. The control cell suspension without periodate or neuraminidase was incubated simultaneously under identical conditions. Three washes with HBSS for epithelial cells or with PBS for erythrocytes were done before the cells were used for the adherence assay and the hemagglutination test.

Hemagglutination test. The number of bacterial cells to be added to the test system was initially determined by titrating the bacteria for bovine erythrocyte-agglutinating activity. The penultimate lowest concentration of bacteria that produced complete agglutination was used for the test. Periodate- or neuraminidase-treated bovine erythrocytes (0.5%) in PBS were combined with an equal volume (25 μl) of bacteria in a microtiter plate. The mixtures were incubated for 2 h at 37°C before they were examined for agglutination.

RESULTS

The effects of different saccharides and glycoconjugates on the adherence of *B. bronchiseptica* A19 to swine nasal epithelial cells are shown in Table 1. All of the sialic acid-containing compounds prevented adherence. The greatest inhibition was achieved with mucins and gangliosides, which were effective even at a concentration of 78 μg/ml. Three other compounds containing sialic acids, i.e., *N*-acetylneuraminic acid, *N*-acetylneuramin-lactose, and α_1-acid glycoprotein, were more than 100-fold less effective. None of the compounds lacking sialic acid residues showed the slightest inhibition. These effects were also found with two other *B. bronchiseptica* strains (S1 and H16). The results suggest that the added substances inhibited adherence by competing with epithelial cell receptors for the specific binding sites on the bacterial surface, indicating a possible recognition by the bacterium of sialic acid-containing structures.

To confirm the involvement of sialic acid residues in adherence, we treated two cell types, i.e., swine nasal epithelial cells and bovine erythrocytes, with either periodate or neuraminidase. Neuraminidase impaired most of the receptivity of both types of cells, as did sodium metaperiodate, a selective oxidizing agent for α-glycols (Table 2).

DISCUSSION

Our results show that the epithelial cell adherence of *B. bronchiseptica* strains may be explained by the recognition of sialic acid-containing structures on the host cell surface. The binding specificity of *B. bronchiseptica* for sialic acid residues was shown by two lines of evidence. (i) Only sialic acid-containing compounds and not compounds lacking sialic acid residues inhibited epithelial cell adherence. (ii) The loss of adherence was seen after treatment of epithelial cells with either periodate or neuraminidase. There may be differences in the adhesive specificity of the organisms for intact and injured cells. Judging from the results of trypan blue exclusion tests, the isolated nasal epithelial cells used in the present study seemed not to have any viability despite being fresh. However, the cell surface sialic acid-dependent bacterial adherence was also observed with intact bovine erythrocytes.

B. bronchiseptica appeared to have a strong affinity for mucins and gangliosides. The minimum concentration of these compounds required for 50% inhibition of bacterial adherence was 78 μg/ml. Among the monosaccharides, only *N*-acetylneuraminic acid had inhibitory activity. This inhibition was, however, less effective, indicating that the binding sites on the microorganism recognized bound sialic acids more strongly than free ones.

There are several reports suggesting that sialic acids may serve as receptors for certain organisms. *Mycoplasma pneumoniae* and *M. gallisepticum* have been known to bind sialyl glycoconjugates (4–7, 24, 28). In 1982, Lindahl et al. (12) reported that the colonization factor antigen I fimbria on enterotoxigenic *E. coli* was a sialic acid-specific lectin. Lindahl and Wadström also described the recognition of sialic acid residues by the K99 hemagglutinin of enterotoxigenic *E. coli* (13). More recently, a novel fimbrial type (S

TABLE 2. Effect of pretreatment of swine nasal epithelial cells and bovine erythrocytes with periodate or neuraminidase on *B. bronchiseptica* A19 adherence and hemagglutination

Treatment[a]	Adherence[b] (% reduction)	Hemagglutination[c]
None	15.0 ± 0.8	+
Periodate	3.8 ± 0.4 (75)	−
Neuraminidase	1.4 ± 0.1 (91)	−

[a] See the text for procedures.
[b] Mean number of bacteria attached per epithelial cell ± standard error of the mean, determined from triplicate assays.
[c] +, Positive hemagglutination; −, negative hemagglutination.

fimbria) on *E. coli* characterized by its specific binding to sialyl galactoside was found by Korhonen and co-workers (11, 22). *Pseudomonas aeruginosa* was also shown to adhere to mucin strands (30), and only mucin and *N*-acetylneuraminic acid were found to inhibit the epithelial cell adherence (25).

It is of great interest to speculate on the significance of bacterial adherence to mucin in the pathogenesis of respiratory tract infections. The secretion of mucin, which prevents adherence to host cell surfaces by blocking the binding sites on certain bacteria, may facilitate elimination of the organisms by ciliary movement. However, it can also be envisaged that if the mucociliary clearance system is impaired and the mucus stagnates, mucus gel may form a matrix to which pathogens bind easily. It is, therefore, considered that mucin is one of the receptors for *B. bronchiseptica*, and the affinity for respiratory mucin may provide an explanation for why this organism is a frequent colonizing agent in swine nasal mucosa.

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