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Effect of Neuraminidase on Adherence of *Pseudomonas aeruginosa* to Human Buccal Epithelial Cells. Inhibition of Adhesion by Monosaccharides

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A b s t r a c t

The aim of this study was to evaluate the action of *Clostridium perfringens* neuraminidase on the adherence of 28 strains of *Pseudomonas aeruginosa* which were isolated from humans, different animals and environment to human buccal epithelial cells (BECs). Two reference strains – NCTC 6749 and ATCC 27853 were also examined. Incubation of cells with the enzyme significantly increased bacterial adherence (a mean number of bacteria adhering to cells amounted 19.62 ± 9.20, for controls – 7.54 ± 5.86). The reference strains of *Pseudomonas aeruginosa* showed the following adherence: NCTC 6749 – 43.04 (control 20.83) and ATCC 27853 – 22.21 (control 5.51). This study demonstrates that asialogangliosides function as receptors on buccal epithelial cells for *P. aeruginosa* strains. Monosaccharides inhibition studies showed an inhibition of adhesion of *P. aeruginosa* (two reference strains – NCTC 6749 and ATCC 27853, two hospital strains – 80/85 and 351) to normal BECs in the presence of N-acetylneuraminic acid and N-acetylgalactosamine. D-galactose is the best inhibitor of bacterial adhesion to neuraminized BECs. All monosaccharides used had a significant effect on *P. aeruginosa* adherence to trypsinized BECs. These data suggest a difference in the receptors on the three types of BECs.

K e y w o r d s: neuraminidase, monosaccharides, adherence, BECs, *Pseudomonas aeruginosa*

Introduction

The adhesion of a pathogen to an epithelial surface is the initial step in an infection. The ability of *Pseudomonas aeruginosa* to colonize the upper respiratory tract has been correlated with its ability to adhere to human buccal epithelial cells (BECs) (Johanson *et al*., 1980). *P. aeruginosa* adheres poorly to functional and intact BECs (Woods *et al*., 1980b). Bacterial binding can be substantially increased by modification of the epithelial surface by the *P. aeruginosa* exoproducts, particularly a neuraminidase (Cacalano *et al*., 1992; Saiman *et al*., 1992). Pilin, the major adhesin of *P. aeruginosa*, adheres to cell surfaces *via* the Gal Nac β1-4 Gal moiety of certain asialylated glycolipids including asialo GM1 (Saiman and Prince, 1993). The *P. aeruginosa* exoproduct, neuraminidase, may increase the availability of such receptors by cleaving terminal sialic acid residues from cell surface gangliosides (Cacalano *et al*., 1992).

The role of neuraminidase in the pathogenesis of infection has not been clearly defined. This enzyme has long been postulated to facilitate interactions between prokaryotes and mammalian hosts (Drzeniek, 1972). Bacterial neuraminidase was first discovered in culture filtrates of *Vibrio cholerae* and *Clostridium perfringens* (quote from Leprat and Michel-Briand, 1980). *P. aeruginosa* was first reported to produce neuraminidase by Shilo (1957). Leprat and Michel-Briand (1980) further characterized the neuraminidase produced by a clinical strain of *P. aeruginosa* isolated from a child with *cystic fibrosis* (CF) and suggested a role for enzyme in the pathogenesis of infection. Cacalano *et al*., (1992) examined the properties of PAO1 neuraminidase to determine its potential role in facilitating *Pseudomonas* sp. colonization of the respiratory epithelium. They demonstrated that the *P. aeruginosa* neuraminidase was 1000-fold more active than the *C. perfringens* enzyme in releasing sialic acid from respiratory epithelial cells. This effect correlated with the increased adherence of PAO1 to epithelial cells after exposure to PAO1 neuraminidase.

Bacterial adherence to tissues is a result of interactions between surface molecules on the bacteria and plasma membrane receptors on host cells. Blocking of the binding sites on the bacterial surfaces with competitive specific carbohydrates completely prevented the bacterial adhesion process *in vitro* (Prince, 1992; Sheth *et al*., 1994). Blocking monosaccharides could afford to characterize the receptors on epithelial cells.

The present study was designed to investigate adherence of 28 *P. aeruginosa* strains to neuraminidase-treated human buccal epithelial cells. A partial characterization of the receptors for these bacteria on either intact or trypsinized and neuraminized buccal epithelial surfaces also was performed.

Experimental

Material and Methods

Strains. *P. aeruginosa* strains were isolated from humans (8 strains – 76/68, 80/85, PA2, 351, 516, 571, I, 5s), flowers – *Zantedeschia aethiopica* (1 strain – XVII), community sewage (2 strains – 1, 2) and different animals: deer (1 – J33), chicken (1 – XVI), dog (2 – II, III), fox (1–38A), minks (2–193, 227), cattle (2–39, 43), swine (3–8, 6, 32B), chinchillas (1–16), fish (3–19c, 28, 35), cat (1 – IV). All strains were confirmed as species belonging to *P. aeruginosa* with NEFERM test 24 (Lachema). Strains were stocked at 4°C in a semi-solid maintenance medium (triptic soy; 0,7% agar). Two reference strains: ATCC27853 and NCTC 6749 were also examined.

Culture conditions. Bacteria were grown at 37°C in triptic soy broth overnight, pelletted by centrifugation, twice washed in phosphate buffer saline (PBS), pH 7.2 and resuspended to a cell concentration of 10^8 CFU/ml.

Buccal epithelial cells. Human buccal epithelial cells (BECs) were collected from healthy people, nonsmoking volunteers by scraping with wooden applicator sticks. Buccal cells were removed from the applicator sticks by agitation in 10 ml PBS. Buccal cells were then washed three times (centrifugation 10 min with PBS at 200 × g) to remove any unattached bacteria. A total cell count was then determined employing a hemocytometer. The concentration of BECs was adjusted to 2.0×10^5 cells per ml.

Effect of neuraminidase on human epithelial cells. Sialic acid from human buccal epithelial cells was released by adding 2.5 U (2.27 mg) of the *Clostridium perfringens* neuraminidase (Sigma) in the PBS, pH 6.5. The cells with enzyme were incubated for 60 min at 37°C. Buccal cells were then washed by centrifugation (200 × g for 10 min) three times with PBS and adjusted to a concentration of 2.0×10^5 cells per ml (Cacalano *et al*., 1992).

Trypsinization of buccal cells. Buccal cells were incubated with 2.5 μg/ml trypsin in phosphatic buffer, pH 8.2 (Sigma) for 30 min at 37°C. The trypsinized cells were washed three times with PBS and the cell concentration was adjusted to 2.0×10^5 cells per ml (Woods *et al*., 1980a).

Adhesion assay. Adherence of *P. aeruginosa* to buccal epithelial cells was assayed by method of Woods *et al*. (1980a, b). The buccal epithelial cells suspension (0.2 ml) and bacterial suspension (0.2 ml) were mixed and incubated at 37°C for 2 h in a shaking water bath. After incubation, the bacterium – buccal cell mixture was again washed three times by centrifugation (10 min at 200 × g) to remove any unattached bacteria. Smears were made, air dried, fixed in methanol, and stained with Giemsa staining solution. The number of bacteria adhering to buccal epithelial cells was counted under a light microscope. In each experiment, the first 30 well-defined epithelial cells were observed. Three independed trials were used to obtain the mean number of bacteria adhering to cells in each experiment. The results were statistically worked out by means of 1-factor variance analysis counted with the method of least squares.

Monosaccharide inhibition studies. The effect od D-fucose, D-galactose, D-arabinose, N-acetylneuraminic acid, N-acetylglucosamine and N-acetylgalactosamine (Sigma) on the adhesion of *P. aeruginosa* (two reference strains – NCTC 6749 and ATCC 27853, two hospital strains – 80/85 and 351) to trypsinized, neuraminized and normal BECs was examined. In the experiment the bacteria were preincubated with the appropriate sugar for 30 min at 37°C before use in the adhesion assay (bacteria with buccal cells were incubated for 45 min) (McEachran and Irvin, 1985).

Results

Modification of epithelial cells by the *Clostridium perfringens* neuraminidase. The effects of enzyme on human buccal epithelial cells were tested in the adherence of *P. aeruginosa* strains to BECs after exposure to the *C. perfringens* neuraminidase. If asialogangliosides function as receptors in this model system, we would expect that neuraminidase-treated cells should bind the increased number of *P. aeruginosa*. As shown in Table I, the *P. aeruginosa* strains adherence to buccal epithelial cells was increased (a mean number of bacteria adhering to cells amounted 19.62 ± 9.20) after the BECs were incubated with *C. perfringens* neuraminidase as compared to the PBS-treated control (7.54 ± 5.86). The *P. aeruginosa* strains showed differentiated adherence to neuraminidase-treated cells; the numbers of adhering bacteria ranged from 5.36 to 53.29 (controls – 0.63–16.27). A very high adherence was observed for strain isolated from fish (35) – 23.61 (control 0.63) and a low adherence was observed for strain isolated from cat (IV) – 18.97 (control – 13.55). The reference strains of *P. aeruginosa* showed the following adherence: NCTC 6749–43.04 (control 20.83) and ATCC 27853–22.21 (control 5.51). The neuraminidase of *C. perfringens* did not increase the adherence of two strains isolated from minks (193) and bovine (39) to BECs.

Table I
Effect of the *C. perfringens* neuraminidase on the adhesion of *P. aeruginosa* strains to human buccal epithelial cells

P. aeruginosa strains	The mean numbers of bacteria adhering to 1 buccal cell	
	Examined samples	Controled samples
76/68	21.60*	9.35**
80/85	10.15	3.16
PA2	22.88	5.52
351	22.57	6.44
516	19.30	9.85
571	24.93	10.33
I	21.51	4.35
5s	29.44	9.84
II	9.71	2.64
III	16.73	7.00
IV	18.97	13.55
39	24.68	32.0
43	20.72	5.15
8	25.56	9.03
6	15.41	4.64
32B	30.65	16.27

P. aeruginosa strains	The mean numbers of bacteria adhering to 1 buccal cell	
	Examined samples	Controled samples
XVI	11.55	4.21
193	5.36	6.40
227	21.55	4.60
16	15.09	6.68
38A	20.69	5.09
J33	15.09	8.35
19c	9.13	4.05
28	53.29	7.17
35	23.61	0.63
1	12.01	4.4
2	17.61	7.33
XVII	9.69	3.07
Total (28strains)	19.62 ± 9.20	7.54 ± 5.86
ATCC 27853	22.21	5.51
NCTC 6749	43.04	20.83

* – sample with neuraminidase; ** – sample with PBS

Table II
Effect of various monosaccharides on the adhesion of *P. aeruginosa* to human buccal epithelial cells

Monosaccharide	Buccal cell type	% of control *P. aeruginosa* strains			
		ATCC 27853	NCTC 6749	351	80/85
None	Normal	100.0	100.0	100.0	100.0
	Neuraminized	100.0	100.0	100.0	100.0
	Trypsinized	100.0	100.0	100.0	100.0
N-Acetylglucosamine (10 mg/ml)	Normal	71.7	90.8	50.35	78.8
	Neuraminized	28.6	35.1	24.9	40.0
	Trypsinized	18.5	12.3	8.2	2.95
N-Acetylgalactosamine (10 mg/ml)	Normal	22.0	33.95	35.0	49.5
	Neuraminized	28.0	66.0	47.3	60.4
	Trypsinized	24.0	13.7	9.4	2.6
N-Acetylneuraminic acid (2 mg/ml)	Normal	32.0	48.7	17.55	17.9
	Neuraminized	26.7	57.0	26.0	20.3
	Trypsinized	21.5	15.0	15.2	1.09
D-Arabinose (0.2 M)	Normal	82.9	55.0	56.6	46.4
	Neuraminized	24.9	41.5	42.2	31.3
	Trypsinized	19.2	12.3	8.5	6.5
D-Fucose (0.2 M)	Normal	51.7	90.0	12.6	123.0
	Neuraminized	29.0	85.0	24.2	14.75
	Trypsinized	28.0	7.65	6.4	2.4
D-Galactose (0.2 M)	Normal	47.65	43.9	25.6	54.25
	Neuraminized	23.5	37.2	17.7	35.4

Monosaccharide inhibition. A great variability in the inhibiting of the adhesion of bacteria to normal, neuraminized and trypsinized BECs was observed among the monosaccharides tested (Table II). N-acetylglucosamine enhanced the binding of *P. aeruginosa* to normal BECs and inhibited the binding to trypsinized and less frequently to neuraminized buccal epithelial cells. N-acetylgalactosamine demonstrated a significant effect on inhibition of *P. aeruginosa* adherence to trypsinized BECs, lesser to normal

and only slight to neuraminized BECs. N-acetylneuraminic acid inhibited the binding of *P. aeruginosa* to three types of BECs. This monosaccharides demonstrated the lowest effect on adhesion of *P. aeruginosa* NCTC 6749 to normal and neuraminized BECs. D-arabinose slightly inhibited the binding of *P. aeruginosa* to normal BECs, better to neuraminized and significantly to trypsinized. D-fucose demonstrated different effects on adhesion. This sugar inhibited adhesion of 351 strain to three types of BECs: 1) it enhanced adhesion of 80/85 strain to normal BECs and inhibited to neuraminized and trypsinized BECs, 2) it had no effect on adhesion of NCTC 6749 to normal and neuraminized BECs and inhibited to trypsinized, 3) it had a quite significant effect on inhibition of binding of ATCC 27853 to three types of BECs. D-galactose inhibited binding to all BEC types but with better inhibition for trypsinized and neuraminized BECs.

Discussion

It is known that *P. aeruginosa* adherence *in vivo* and *in vitro* to respiratory tract cells occurs only after varioux kinds of injury, including pretreatment of epithelial cells with enzymes (Woods *et al.*, 1980a). One of these enzymes seems to be neuraminidase (Cacalano *et al*., 1992; Saiman *et al*., 1992). In the respiratory tract there are several potential substrates for the action of neuraminidase. The glycoproteins which comprise respiratory mucins are highly sialylated and epithelial membranes have abundant sialylated ganglioside and other glycoconjugate components (Cacalano *et al*.,1992). This enzyme is active against a range of substrates expected to be present in the respiratory tract, including α 2,3-linked sialic acids found in sialyllactose as well as the sialic acid residues present on epithelial cell surfaces (Cacalano *et al*., 1992). Asialogangliosides that contain Gal Nac β1–4 Gal sequence have been shown to act as receptors for at least two discrite *Pseudomonas* adhesions, pilin (Paranchych *et al*., 1991), and exoenzyme S (Baker *et al*., 1991). These asialoganglioside receptors appear to be of major importance in mediating *Pseudomonas* attachment, in modifying membrane glycolipids and exposing these potential receptors what is consistent with the observation that neuraminidase treatment of the epithelial monolayers increased attachment, and correlated with the release of sialic acid from the monolayers (Cacalano *et al*., 1992). Because the *C. perfringens* neuraminidase used in our study increased bacterial adherence asialogangliosides could function as receptors on buccal epithelial cells for *P. aeruginosa* strains. These receptors have been detected in the increased amounts on the surface of cystic fibrosis respiratory cells (de Bentzman *et al*., 1996). The milieu of the CF (especially hyperosmolarity) may specifically activate the expression of several genes, including the neuraminidase gene *nanA*, which initiate colonization and facilitate longterm infection (Cacalano *et al*., 1992, Lanotte *et al*., 2003). The increased number of asialoGM1 receptors in CF patients who had been infected by *P. aeruginosa* might be related to the fact that their cells had been previously exposed *in vivo* to *P. aeruginosa* exoproducts, particularly neuraminidase (Saiman and Prince, 1993). Imundo *et al*. (1995) have identified asialo GM1 as a receptor for *P. aeruginosa* adherence on CF bronchial cell lines.

The action of neuraminidase is observed not only on the substrates of respiratory tract. The effect of enzyme on adherence of *P. aeruginosa* to unwounded cornea was also studied (Shingh *et al*., 1991). Incubation of the postnatal day immature mouse cornea with neuraminidase to remove sialylated residues significantly enhanced binding. Treatment of the fibronectin (the most important ECM protein) by neuraminidase showed an important increase of *P. aeruginosa* adhesion (Rebiere-Huet *et al*., 2004). Interestingly, neuraminidase-treated erythrocytes were more easily agglutinated by *P. aeruginosa* (Gilboa-Garber, 1982).

Monosaccharides inhibition experiments showed a participation of different surface in the receptors involved in the attachment of *P. aeruginosa*. Both N-acetylneuraminic acid and N-acetylgalactosamine inhibited the adhesion to normal BECs. D-galactose, inhibited bacterial adhesion to neuraminized BECs most efficiently. All monosaccharides used had a significant effect on *P. aeruginosa* adherence to trypsinized BECs. These results are in agreement with the observation made by other authors on the adhesive properties of *P. aeruginosa* to different types of epithelial cells of respiratory tract (Ramphal and Pyle, 1983; McEachran and Irvin, 1985; Vishwanath and Ramphal, 1985). Studying nature of receptors for *P. aeruginosa* in the respiratory tract tissue, McEachran and Irvin (1985) demonstrated the presence of two classes of receptor sites in the adherence of *P. aeruginosa* to human buccal epithelial cells; the high affinity-low copy number site was found on trypsinized BECs and a low affinity-high copy number class of binding sites was found to be trypsin sensitive. N-acetylneuraminic acid, N-acetylglucosamine (Ramphal and Pyle, 1983, Kuroki *et al*., 1989, Yamaguchi and Yamada, 1991), D-arabinose and D-fucose (McEachran and Irvin, 1985) have been found to inhibit the adherence of mucoid and non mucoid strains of *P. aeruginosa*

to injured tracheal cells, to buccal cells and to tracheobronchial mucus (Vishwanath and Ramphal, 1985) and may be served as receptors for this organism in the respiratory tract. Ko *et al*. (1987) observed that blocking of the binding sites on *P. aeruginosa* ATCC 27853 surfaces with N-acetylneuraminic acid (NANA) completely prevented the bacterial adhesion process *in vitro*. In our study this carbohydrates also demonstrated a significant effect on inhibition of binding of *P. aeruginosa* ATCC 27853 to the three types of BECs. Gilboa-Garber (1972) has described two lectins in *P. aeruginosa* one of which, termed Ps-GAL, binds specifically to D-galactose and its derivatives. McEachran and Irvin (1985) observed an enhanced adhesion of a mucoid and non mucoid strain of *P. aeruginosa* to human BECs in the presence of D-galactose. This result disagree with our work. Our sugar inhibition date for D-galactose indicates that the Ps-GAL lectin is involved in the adhesion of *P. aeruginosa* to human buccal epithelial cells. Because common oligosaccharides and polymeric saccharides (dextran) (Wolska *et al*., 2005) are the inhibitors of the adherence of *P. aeruginosa* to buccal epithelial cells, the application of carbohydrates might be an alternative to antibiotic in cases of resistance to conventional treatment.

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