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NOTES

In Vivo Identification of Sialic Acid as the Ocular Receptor for *Pseudomonas aeruginosa*

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In vivo bacterial adherence of *Pseudomonas aeruginosa* to the immature ocular epithelium was mediated by a sialic (*N*-acetylneuraminic) acid (NANA) receptor. Saturation of binding sites on the bacterial surface by NANA prevented attachment of the organism to the epithelial cell membrane receptor. Additionally, a significant number of animals receiving NANA-treated organisms were protected from septicemia and death. In vivo protection studies showed excellent correlation with scanning electron microscopy, in that the number of adherent organisms at the corneal surface decreased dramatically in the presence of NANA. These studies exhibit a strong correlation with clinical cases of human infant ocular infection.

The importance of bacterial adherence as a prerequisite for colonization and infection at mucosal surfaces is well established for numerous tissues, including the eye (3, 7, 10, 12, 13). Numerous in vitro (11, 14) but few in vivo studies (1) have addressed this problem. For example, in vitro studies using the monosaccharide mannose have shown that this sugar mediates the binding of *Escherichia coli* to the surface of various eucaryotic cells (10, 11). Additional in vitro studies have suggested the importance of mucin and a sialic acid receptor in facilitating *Pseudomonas aeruginosa* binding to the lower respiratory tract (12) and to tracheobronchial mucin (14). Nothing is known, however, about the putative site required for *P. aeruginosa* attachment to the mucosal cell membrane in vivo. Our data here show that attachment of *P. aeruginosa* to the eye is mediated by a sialic acid receptor at the ocular surface of mouse pups and that by blocking this receptor with the sugar inhibitor *N*-acetylneuraminic acid (NANA), bacterial adherence is prevented, thus protecting a significant number of animals from septicemia and death.

In vivo bacterial adherence was examined by scanning electron microscopy procedures as described before (7, 8). In brief, 5-day-old mouse pups were injected beneath the fused eyelid with 2.5 μl of a final concentration of 10^7 CFU of *P. aeruginosa* ATCC 19660 (5–7). The bacterial inoculum was mixed immediately before injection with either phosphate-buffered saline (PBS) or one of several monosaccharides (Sigma Chemical Co.) at a final concentration of 25 mg/ml (Table 1). The selection of sugars was based on biochemical research establishing their presence in human ocular mucus (9), and on our own cytochemical studies showing that the murine ocular surface is rich in sialic acid residues (15). *N*-Acetylmannosamine (manNac) and pyruvate were used in combination, as they are the C_6 and C_3 subunits of NANA.

Before the sugar was combined with *P. aeruginosa*, each was adjusted to a neutral pH (7.0 to 7.5). Eyes were enucleated at 5 to 10 min after bacterial inoculation and fixed for 3 h at 4° C in a mixed fixative containing 2.0% OsO_4, 2.5% glutaraldehyde, and 0.2 M PO_4 buffer (pH 7.4). Specimens were examined in an ETEC autoscan scanning electron microscope operating at 10 kV. Survival of mouse pups was also observed and statistically analyzed by using χ^2 analysis to determine if any of the sugars significantly enhanced survival over that with PBS.

Scanning electron microscopy showed that compared with the other sugars or the PBS control (Fig. 1A), NANA dramatically decreased the number of adherent bacteria observed at the surface of the eye (Fig. 1B). In the NANA-treated group, a few random organisms (0.17/μm^2 of corneal surface) were seen adhering to surface cells. In contrast, in PBS controls, the number of organisms (12.5/μm^2 of corneal surface) was significantly increased. Our observations suggest that NANA saturation of binding sites on the bacterial surface prevents attachment of the organism to the epithelial cell membrane receptor which contains sialic acid or a sialic acid analog. In addition, mice given a NANA-treated inoculum showed increased survival rates ($P \leq 0.05$) compared with those with any of the other sugars or PBS (Table 1). Although manNac appeared to afford protection compared with that afforded by the other sugars, these data were not significant when compared with PBS control data. The data from a combination of manNac and pyruvate corroborate that conclusion. Similar protection was observed whether the NANA was from *E. coli* or sheep submaxillary glands or was synthetic (Sigma).

To establish the optimum amount of NANA which afforded protection, various concentrations of the sugar were examined. A low concentration (1%) of NANA had no protective effect, while concentrations of 3% and greater provided significant protection.

These results suggest that a sialic acid-specific adhesin on the surface of *P. aeruginosa* mediates the binding to and subsequent colonization of the ocular epithelium. The free NANA inhibition of binding to the epithelial surface may be partial or transitory. Direct inspection of inoculated eyes at day 15 (eyelids open) showed that the incidence of infection was lower in the animals which received an inoculum

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TABLE 1. Effects of PBS and various monosaccharides on mouse pup survival

Inoculum containing *P. aeruginosa*[a]	No. surviving/no. infected for day:			
	1	2	3	10[b]
PBS	7/29	5/29	3/29	3/29 (10)
NANA (synthetic)	7/10	6/10	6/10	6/10 (60)
Glucose	5/17	3/17	3/17	1/17 (6)
Glucosamine	6/17	2/17	2/17	1/17 (6)
Galactose	3/20	2/20	2/20	0/20 (0)
Galactosamine	4/22	3/22	3/22	3/22 (14)
Mannose	4/18	1/18	1/18	0/18 (0)
manNac	14/33	11/33	10/33	9/33 (27)
Fucose	5/18	4/18	4/18	2/18 (11)
manNac + pyruvate	3/11	0/11	0/11	0/11 (0)

[a] Five-day-old mouse pups were challenged with 10^7 *P. aeruginosa* beneath the fused eyelid. The PBS or sugars (25 mg/ml) were combined with bacterial suspensions at room temperature.

[b] Percentage of mice surviving at the end of the experiment when eyes are open (approximately 15 days of age) is shown within parentheses.

TABLE 2. Effects of PBS and various NANA concentrations on mouse pup survival

Inoculum containing *P. aeruginosa*[a]	No. surviving/no. infected for day:			
	1	2	3	10[b]
PBS	10/17	5/17	5/17	3/17 (18 and 18)
NANA (%)				
1	13/18	8/18	4/18	3/18 (17 and 5)
2	12/18	7/18	4/18	4/18 (22 and 16)
3	17/19	12/19	12/19	11/19 (58 and 36)
4	16/18	11/18	11/18	8/18 (44 and 27)

[a] Five-day-old mouse pups were challenged with 10^7 *P. aeruginosa* beneath the fused eyelid.

[b] Percentage of mice surviving at 15 days of age (when eyes are open) and percentage of survivors exhibiting corneal opacity, respectively, are shown within parentheses.

containing either 1 or 2% NANA versus PBS-treated organisms (Table 2). Animals with corneal opacity were observed with more frequency in the 3 and 4% NANA groups versus the PBS-treated group. This may be due, in part, to the increased number of survivors that could be examined for corneal opacity after administration of the higher concentrations of the sugar. Several additional studies were also performed to assess the role of NANA in *P. aeruginosa* adherence. One such study involved the removal of sialic acid residues in an attempt to block bacterial adherence. When ≤2 U of neuraminidase per ml (Sigma type V) were combined with a suspension of *P. aeruginosa* immediately before inoculation beneath the fused eyelid of 5-day-old pups, survival was increased significantly ($P \leq 0.02$) (Table 3). The enzyme was found to be capable of releasing sialic acid under the conditions used (0.01 M PBS, pH 7.35) and did not adversely affect bacterial viability (CFU) under these conditions. Another study used NANA (25 to 40 mg/ml) in an attempt to prevent bacterial adherence in an adult mouse model of ocular bacterial infection (4). NANA- or PBS-treated organisms were topically applied to the scarified corneas of the 37-day-old adult mice. At 18 to 24 h, animals were examined directly, and no difference in the degree of corneal opacity was observed. This indicates that a different mechanism is operative in mediating bacterial adherence to the scarified ocular surface of the adult animal (Hazlett, unpublished data). The age of the animal or scarification

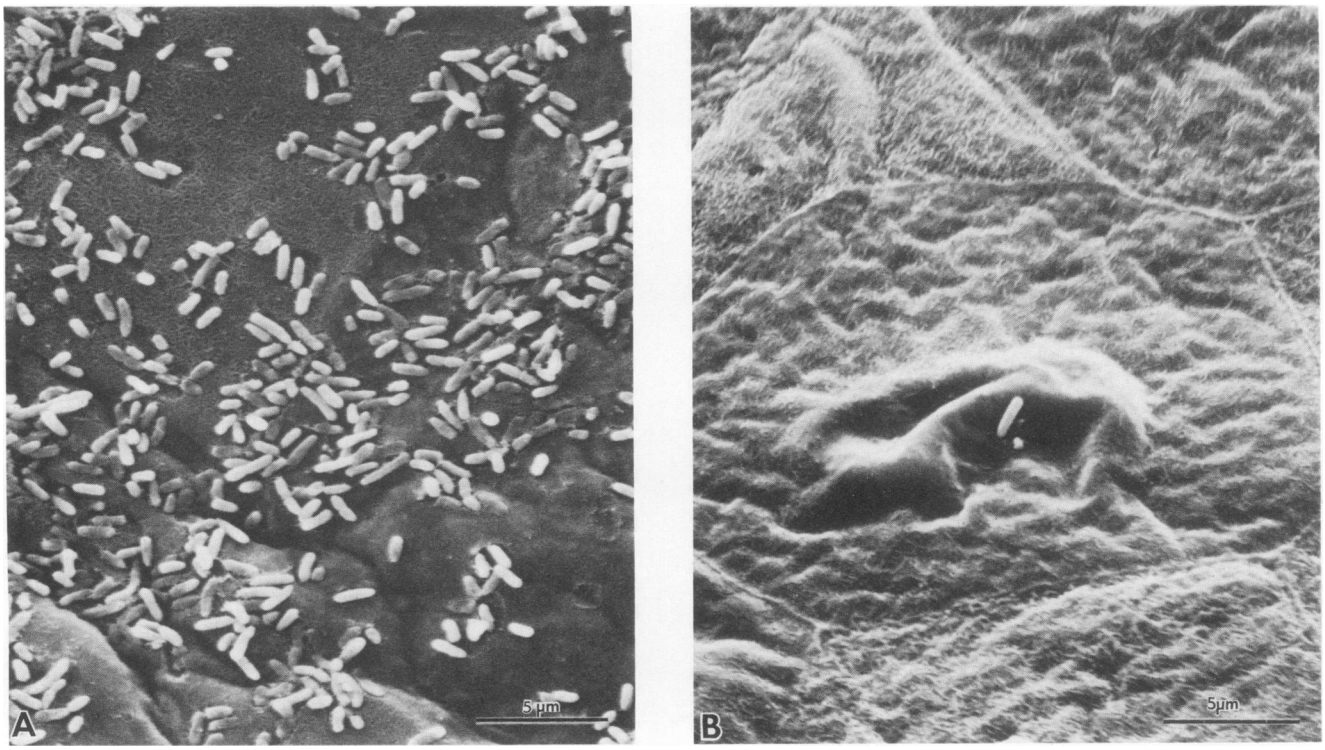

FIG. 1. Binding of *P. aeruginosa* to peripheral corneal epithelium 5 to 10 min after bacterial inoculation: (A) after PBS; (B) after 40 mg of NANA per ml. Magnification, ×3,600.

TABLE 3. Effects of PBS and neuraminidase concentrations on mouse pup survival

Inoculum containing *P. aeruginosa*[a]	No. surviving/no. infected for day:				
	1	2	3	10[b]	
PBS	3/9	1/9	1/9	1/9	(11 and 11)
Neuraminidase concn (mg/ml)					
1	3/10	0/10	—[c]	—	—
2	7/9	6/9	4/9	4/9	(44 and 33)

[a] PBS or neuraminidase was combined with bacterial suspensions of 10^7 *P. aeruginosa* at room temperature and injected beneath the fused eyelid of 5-day-old mouse pups.

[b] Percentage of mice surviving at 15 days of age (when eyes are open) and percentage of survivors exhibiting corneal opacity, respectively, are shown within parentheses.

[c] —, No mice survived past day 2.

itself may result in expression of receptors different from those at the unwounded ocular surface of the infant mouse. In this regard, it is known that in the adult eye, organisms bind preferentially to wounded areas and do not adhere to or infect an unscarified cornea (8).

This report provides the first in vivo evidence for identification of a new receptor-adhesin system which mediates *P. aeruginosa* binding and colonization of the untraumatized corneal epithelial surface. This animal model is important to develop further, as it shows excellent correlation with clinical reports of similar *P. aeruginosa* infections in newborn human infants (2). In these cases, no trauma to the ocular surface is required to initiate bacterial infection and, despite vigorous antibiotic treatment, subsequent death (2). Utilization of this animal model in further studies may provide valuable information regarding treatment of this "opportunistic" mucosal pathogen.

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