INFECTION AND IMMUNITY, Oct. 1982, p. 179–182
0019-9567/82/100178-04$02.00/0

Vol. 38, No. 1

Effect of Decreased Salivation and pH on the Adherence of *Klebsiella* Species to Human Buccal Epithelial Cells

GARRISON H. AYARS, LEONARD C. ALTMAN,* AND MARSHA D. FRETWELL

Division of Allergy and Infectious Diseases, Department of Medicine, University of Washington, Seattle, Washington 98195

Received 19 January 1982/Accepted 8 June 1982

To assess the role of reduced salivary flow and intraoral pH on gram-negative bacterial colonization of the oropharynx, we studied in vitro *Klebsiella* adherence to normal human buccal epithelial cells at various pH values and to buccal cells from patients with pathological xerostomia (decreased saliva flow). Reduced pH significantly increased adherence of *Klebsiella pneumoniae* 84 to normal buccal epithelial cells ($P < 0.001$). In contrast, two clinical isolates of *K. oxytoca* showed no significant pH-dependent change in adherence. A corollary of this was that patients with pathological xerostomia had significantly increased adherence of *K. pneumoniae* 84 to their buccal epithelial cells as compared with normal controls ($P < 0.01$). These results suggest that reduced salivary flow and the concomitant reduction of intraoral pH may predispose patients to bacterial colonization with *K. pneumoniae*.

Aerobic gram-negative bacilli (GNB) rarely colonize the oropharynx of normal individuals. However, the incidence of oropharyngeal GNB colonization increases to 30 to 60% (1, 9, 10, 13) in certain pathological situations as compared with 3 to 6% (16) in control groups. Patients in intensive care units (10), those with pathological xerostomia (1), and elderly institutionalized patients (19) have an increased incidence of oropharyngeal colonization with GNB. Further, it has been reported that the frequency of respiratory infections with GNB is eight times greater in hospitalized patients who acquire GNB in their oropharynx than in comparably ill noncolonized patients (10). This association between oropharyngeal colonization and risk of pneumonia suggests that the ability of GNB to adhere to oral epithelial cells, and hence colonize this surface, may be an important bacterial virulence factor. Indeed, Higuchi et al., using an in vitro assay of GNB adherence, demonstrated that *Pseudomonas* organisms which bind poorly to normal human buccal epithelial cells (HBEC) adhere in increased numbers to buccal cells of hospitalized patients (8). This finding suggests that factors associated with hospitalization somehow alter oropharyngeal epithelial cells, making them more susceptible to GNB adherence.

To further examine the host factors that might predispose patients to GNB colonization, we elected to study the roles of saliva flow and pH in this interaction. Previous studies have shown that patients with reduced saliva flow secondary to Sjögren's syndrome or to radiation-induced sialadenitis have increased colonization with GNB (1, 13). Furthermore, it is known that pathologically reduced salivary flow is associated with a concomitant decrease in the pH of stimulated parotid saliva (6). We therefore studied the effect of altering pH on the in vitro adherence of three clinical isolates of *Klebsiella* species to normal HBEC. In a corollary study, we also examined *Klebsiella* adherence to buccal cells from patients with pathological xerostomia.

MATERIALS AND METHODS

Bacterial preparation. Three *Klebsiella* organisms obtained from human culture specimens were used; K84, a *K. pneumoniae* strain, and K90 and K181, both *K. oxytoca* strains. The bacteria were taken off fresh overnight trypticase soy plates with sterile cotton-tipped swabs and suspended at an optical density of 0.15 in 10 ml of M-9 minimal medium containing 0.2% glucose (15). The organisms were incubated for 1 h on a rotating rack at 37°C, and then 100 μCi of tritiated thymidine (New England Nuclear Corp., Boston, Mass.) and 1 ml of deoxyadenosine at 2.5 mg/ml (Calbiochem, La Jolla, Calif.) were added. The latter substance increased the thymidine dependence of the organisms, hence, enhancing labeling (14). The cultures were then incubated and tumbled for 3 additional hours and subsequently washed and centrifuged (12,000 × *g* for 10 min) three times with phosphate-buffered saline (PBS). After this, the bacteria were resuspended in pH-adjusted PBS (range 5.5 to 7.2) at a concentration of 10^9 organisms per ml based on optical density. Scanning electron micrographs were also per-

formed and showed that the three organisms were piliated.

Epithelial cells. HBEC were obtained from the oral mucosae of patients and controls by vigorous scraping with cotton-tipped swabs. The swabs were then swirled in 5 ml of PBS to dislodge the cells, which were then freed from nonadherent endogenous bacteria by three serial centrifugations at 400 × *g* for 10 min. The HBEC were then resuspended at a concentration of 2×10^5 cells per ml in pH-adjusted PBS.

In vitro adherence assay. The adherence assay was done by combining 200 μl of the radiolabeled bacteria to 200 μl of the HBEC (GNB-to-HBEC ratio, 5,000:1). Both solutions were pH-adjusted, and the mixture was incubated in a shaking water bath at 37°C for 1 h. The nonadherent bacteria were removed by pouring the mixture over 10-μm polycarbonate filters (Nuclepore Corp., Pleasanton, Calif.) on a vacuum manifold (Millipore Corp., Bedford, Mass.) and washing extensively with pH-adjusted PBS. The filters were dried in scintillation vials, scintillation cocktail was added, and the filters were then counted for 1 min in a Packard liquid scintillation spectrometer (Packard model no. 3002, La Grange, Ill.). A known number of organisms was also counted in a similar fashion to determine the specific activity (counts per organism) for each bacterial strain. Specific activities for the three organisms ranged from 0.001 to 0.003 and were determined in each experiment. To express our results, we used these values to convert the absolute number of counts into the number of GNB per HBEC. In preliminary studies, visual counting was compared with counting by the radioisotope method, and a correlation coefficient of 0.973 was found. The pH range examined was from 5.5 to 7.2. The pH of normal stimulated saliva is between 7.2 and 7.6 (11). In our statistical analysis we used 7.2 as our reference value, with statistical significance ($P < 0.05$) determined by the Student *t* test.

Salivary flow, pH, and strain K84 adherence to HBEC of xerostomia patients and controls. For our studies with HBEC from xerostomia patients we measured strain K84 adherence at pH 7.2, using the same radioisotope adherence techniques described above. Of the 14 patients with xerostomia, 9 had Sjögren's syndrome and 5 had radiation-induced sialadenitis secondary to head and neck cancer therapy. Of these 14 patients, 8 had saliva studies (3 Sjögren's and 5 radiation sialadenitis) to determine representative salivary flow rates and pH. Six normal control patients had similar studies. Unilateral, lemon drop-stimulated parotid saliva was collected for 10 min, using a modified Carlson-Crittenden saliva cup (18). The saliva was collected under oil in 15-ml conical centrifuge tubes (Falcon Plastics, Oxnard, Calif.), a method which we had previously determined would maintain pH stability for up to 6 h. Saliva volume and pH were measured immediately after collection.

RESULTS

Effect of pH on *Klebsiella* adherence to normal HBEC. Figure 1 summarizes the results of studies that examined the effect of pH on the adherence of three *Klebsiella* strains to normal HBEC. K84, a strain of *K. pneumoniae*, exhibited low adherence of 10.8 ± 2.2 (mean ± standard error of the mean) organisms per HBEC at a pH of 7.2, which increased to a maximum of 74.2 ± 14.9 GNB per HBEC at a pH of 6.5 (panel A). This difference is significant at $P < 0.001$. In contrast, adherence of K90 (panel B) and K181 (panel C), two *K. oxytoca* strains, was low at a pH of 7.2 and was not significantly affected by reducing pH in the range studied.

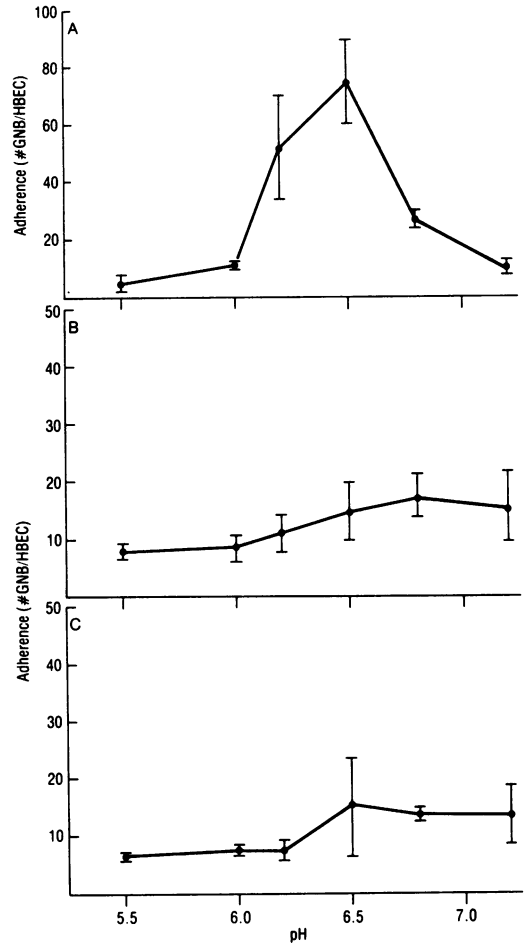

FIG. 1. Influence of pH on the adherence of (A) *K. pneumoniae* 84, (B) *K. oxytoca* 90, and (C) *K. oxytoca* 181 to HBEC. Adherence is expressed as the number of GNB per HBEC. The pH values studied were 5.5, 6.0, 6.2, 6.5, 6.8, and 7.2.

Salivary flow, pH, and adherence in xerostomia patients versus controls. In the eight xerostomia patients studied, the mean stimulated salivary flow was 0.30 ± 0.06 ml per min (mean ± standard error of the mean) compared with 0.76 ± 0.08 ml per min in six controls ($P < 0.001$). This confirms that stimulated salivary flow measurements are discriminating in separating xerostomia patients from normal individuals (3, 20). The mean pH of the stimulated saliva in the

TABLE 1. Adherence of *K. pneumoniae* 84 to buccal epithelial cells of xerostomia patients and controls

Patient population	No.	Adherence[a]	P
All xerostomia	14	41.6 ± 9.0	<0.01
Sjögren's	9	34.0 ± 7.8	<0.05
Radiation sialadenitis	5	61.4 ± 25.0	<0.005
Controls	22	18.3 ± 4.9	

[a] Expressed as number of strain K84 (mean number of bacilli) per HBEC ± standard error of the mean. The pH used in these experiments was 7.2.

xerostomia patients was 6.39 ± 0.41 versus 7.45 ± 0.05 in the controls ($P < 0.025$), thus confirming that the pH of saliva from patients with xerostomia is significantly reduced.

Next we compared strain K84 adherence to HBEC from xerostomia patients and controls (Table 1). In the 14 xerostomia patients (9 Sjögren's and 5 radiation sialadenitis), the mean number of bacilli per HBEC was 41.6 ± 9.0 compared with 18.3 ± 4.9 in 22 controls ($P < 0.01$). Similarly, strain K84 adherence to buccal cells of the Sjögren patients and the radiation-induced sialadenitis patients, when analyzed separately, was statistically different from that of controls ($P < 0.05$ and $P < 0.005$, respectively).

DISCUSSION

In the past, emphasis on the study of nosocomial pneumonias has focused on the characteristics of the microorganisms involved. More recently, investigators have examined host factors that may predispose patients to infection (8). We propose that salivary flow and pH are important host factors in preventing GNB adherence and colonization of oropharyngeal epithelial cells and subsequent nosocomial respiratory infections. This hypothesis is based on our finding that a strain of *K. pneumoniae* showed increased adherence to buccal cells of normal individuals at reduced pH, suggesting that low oral pH may predispose to abnormal adherence and colonization with this pathogen. In contrast, adherence of two strains of *K. oxytoca*, a less common respiratory pathogen, was not enhanced at reduced pH. As a corollary, the adherence of *K. pneumoniae* 84 to buccal cells of xerostomia patients was increased. This suggests that reduced salivary flow induced some alteration of the buccal epithelial surface that permitted enhanced bacterial adherence. This alteration may be related to the decreased intraoral pH seen in xerostomia patients but could be due to other factors. An interesting possibility is that decreased salivary flow could result in increased saliva proteolytic activity, a condition which Woods et al. have shown is related to increased GNB adherence (23). The mechanism they propose is that proteolytic activity damages cell surface fibronectin, uncovering bacterial binding sites present on the cell surface. Although it had been previously reported that patients with Sjögren's syndrome and radiation-induced sialadenitis have increased colonization with GNB (1, 13), our data are the first to demonstrate that bacterial adherence, the in vitro correlate of colonization, is also increased in these patients with a strain of *K. pneumoniae*.

Decreased salivary flow and lowered oral pH may be important in many patient populations at risk for nosocomial pneumonia. Indeed, alterations in saliva production may be a "common denominator" that underlies increased oropharyngeal colonization by GNB in such diverse groups as patients in intensive care units (10), those undergoing elective surgery (8), elderly institutionalized individuals (19), as well as those with pathological xerostomia (1, 13). Factors common to these individuals and to other groups of hospitalized patients that may disrupt normal saliva production include decreased oral intake, normally a major stimulus to salivation, drugs that interfere with saliva production (e.g., anticholinergics and phenothiazines), and decreased mentation, which interferes with perception of stimuli to salivation. The net result is decreased saliva flow and reduced oral pH, conditions that we have found to result in increased adherence of *K. pneumoniae* 84 to buccal epithelial cells.

Salivation may prevent GNB adherence by many mechanisms other than regulation of oral pH. Stimulated saliva has a cleansing action that retards bacterial attachment (12). Salivary glycoproteins can competitively inhibit the adherence of certain bacterial species (17, 22). Saliva also contains immunoglobulin A antibodies that can bind specifically to bacteria (21) and agglutinins that promote bacterial clumping and facilitate clearance (7). Also, there are components of saliva that have direct antibacterial action such as lactoferrin (2) and lysozyme (5). As such, it may be that Upton Sinclair's adage that " nature castigates those that don't masticate" (4) should have been directed to physicians in the management of hospitalized patients.

ACKNOWLEDGMENTS

The work was supported by Public Health Service grants DE-02600 and DE-07063 from the National Institutes of Health.

We thank Rosemary Bacina and Coralie Baker for expert technical assistance and Jane Fowler and Alexis Raphael for typing this manuscript.

LITERATURE CITED

1. **Brown, L. R., S. Dreizen, S. Handler, and D. A. Johnston.** 1975. Effect of radiation induced xerostomia on human oral microflora. J. Dent. Res. **54**:740–750.
2. **Bullen, J. J., C. G. Ward, and S. N. Walles.** 1974. Virulence and the role of iron in *Pseudomonas aeruginosa* infection. Infect. Immun. **10**:443–450.
3. **Chisholm, D. M., and D. K. Mason.** 1973. Salivary gland function in Sjögren's syndrome. A review. Br. Dent. J. **135**:393–399.
4. **Deutsch, R. M.** 1977. The new nuts among the berries. Bull Publishing, Palo Alto, Calif.
5. **Donaldson, D. M., R. R. Roberts, H. S. Larsen, and J. G. Tew.** 1974. Interrelationship between serum beta-lysin, lysozyme and the antibody complement system in killing of *Escherichia coli*. Infect. Immun. **10**:657–666.
6. **Englander, H. R., I. L. Shklair, and L. S. Fosdick.** 1959. The effects of saliva on the pH and lactate concentrations in dental plaques. J. Dent. Res. **38**:848–853.
7. **Gibbons, R. J., and J. Van Houte.** 1980. Bacterial adherence and the formation of dental plaque, p. 61–104. *In* E. H. Beachey (ed.), Bacterial adherence. Chapman and Hall, New York, N.Y.
8. **Johanson, W. G., Jr., J. H. Higuchi, T. R. Chaudhuri, and D. E. Woods.** 1980. Bacterial adherence to epithelial cells in bacillary colonization of the respiratory tract. Am. Rev. Respir. Dis. **121**:55–63.
9. **Johanson, W. G., Jr., A. K. Pierce, and J. P. Sanford.** 1969. Changing oropharyngeal bacterial flora of hospitalized patients. Emergence of gram-negative bacilli. N. Engl. J. Med. **21**:1137–1140.
10. **Johanson, W. G., Jr., A. K. Pierce, J. P. Sanford, and G. D. Thomas.** 1972. Nosocomial respiratory infections with gram-negative bacilli. Ann. Intern. Med. **77**:701–706.
11. **Karshan, M., F. Krasnow, and L. E. Krejal.** 1931. A study of blood and saliva in relation to immunity and susceptibility to dental caries. J. Dent. Res. **11**:573–589.
12. **Laforce, F. M., J. Hopkins, R. Trow, and W. L. Want.** 1976. Human oral defenses against gram-negative rods. Am. Rev. Respir. Dis. **114**:929–935.
13. **MacFarlane, T. W., and D. K. Mason.** 1974. Changes in the oral flora in Sjögren's syndrome. J. Clin. Pathol. **27**:416–419.
14. **Metzler, D. E.** 1977. Biochemistry: the chemical reactions of living cells, p. 875. Academic Press, Inc., New York.
15. **Roberts, R. B., P. H. Abelson, D. B. Cowie, E. T. Bolton, and R. J. Britten.** 1963. Studies of biosynthesis in Escherichia coli. Carnegie Institute of Washington publication no. 607, Washington, D.C.
16. **Rosenthal, S., and I. B. Tager.** 1975. Prevalence of gram-negative rods in normal pharyngeal flora. Ann. Intern. Med. **83**:355–357.
17. **Slots, J., and R. J. Gibbons.** 1978. Attachment of *Bacteroides melaninogenicus* subsp. *asaccharolyticus* to oral surfaces and its possible role in colonization of the mouth and periodontal pockets. Infect. Immun. **19**:254–264.
18. **Sproles, A. C., and L. D. Schaeffer.** 1974. An advanced design of the Carlson-Crittenden cup for the collection of parotid saliva. Biomater. Med. Devices Artif. Organs **2**:95–101.
19. **Valenti, W. M., R. G. Trudell, and D. W. Bentley.** 1978. Factors predisposing to oropharyngeal colonization with gram-negative bacilli in the aged. N. Engl. J. Med. **298**:1108–1111.
20. **Whaley, F., J. Williamson, D. M. Chisholm, et al.** 1973. Sjögren's syndrome: sicca components. Quart. J. Med. **166**:279–304.
21. **Williams, R. C., and R. J. Gibbons.** 1972. Inhibition of bacterial adherence by secretory immunoglobulin A: a mechanism of antigen disposal. Science **177**:697–699.
22. **Williams, R. C., and R. J. Gibbons.** 1975. Inhibition of streptococcal attachment to receptors on human buccal epithelial cells by antigenically similar salivary glycoproteins. Infect. Immun. **11**:711–718.
23. **Woods, D. E., D. C. Straus, W. J. Johanson, Jr., and J. A. Bass.** 1981. Role of salivary protease activity in adherence of gram-negative bacilli to mammalian buccal epithelial cells in vivo. J. Clin. Invest. **68**:1435–1440.