Role of Salivary Protease Activity in Adherence of Gram-negative Bacilli to Mammalian Buccal Epithelial Cells In Vivo

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ABSTRACT Serious illness is accompanied by markedly increased susceptibility to colonization of the respiratory tract by gram-negative bacilli and an increase in the number of such organisms which adhere to regional epithelial cells during incubation in vitro. Trypsinization of cells from normal subjects causes a similar increase in bacillary adherence. We studied bacillary adherence to buccal cells in vitro, protease activity of upper respiratory secretions with a fibrin plate technique, and the amount of fibronectin on the surface of buccal cells with a direct radioimmunobinding assay. Among 10 patients seriously ill with acute respiratory failure bacillary adherence to buccal cells and protease activity in secretions were increased compared with controls and cell-surface fibronectin was decreased; all patients were colonized in vivo with gram-negative bacilli. These changes were persistent and 80% of the patients died. Serial determinations were made in eight patients undergoing coronary artery bypass surgery. Following surgery, protease activity and bacillary adherence increased and cell-surface fibronectin decreased; 38% of coronary artery bypass patients became colonized. In these uncomplicated patients the changes observed were transient, largely returning to normal by the third postoperative day. Increased protease activity of secretions and alterations in epithelial cell surfaces as reflected by loss of buccal cell-surface fibronectin occur swiftly after major illness and appear to underlie enhanced cell adherence of bacilli and colonization of the upper respiratory tract. These findings suggest new approaches to the prevention of nosocomial pneumonia.

INTRODUCTION

Recent observations suggest that adherence of potentially pathogenic bacteria to epithelial cells of mucosal surfaces is the initial event in colonization and subsequent infection by these microorganisms (1–4). Bacterial adherence to mammalian epithelial cells is dependent upon specific recognition systems between bacteria and tissue cells. The ability of bacteria to adhere to epithelial cells is, in the majority of cases, associated with surface structures known as pili in gram-negative bacteria (5) and fibrillae in gram-positive bacteria (6). Mammalian hosts possess several defense mechanisms that function at mucosal surfaces to prevent bacterial colonization. These include secretory fluid flow, epithelial cell desquamation, secretory antibodies, and secretory glycoproteins that inhibit bacterial adherence by competing with epithelial cell surfaces for bacterial binding sites (7).

Serious illness is associated with a markedly increased susceptibility of the upper respiratory tract to colonization by gram-negative bacilli (8). This susceptibility is paralleled by increased adherence of gram-negative bacilli to buccal cells in vitro (8), a finding that suggests that altered cellular adherence may account for bacillary colonization of ill patients. Bacillary adherence to normal buccal epithelial cells in vitro can be increased by brief exposure of the cells to trypsin (9). Fibronectin, a large molecular weight protein, is known to be highly sensitive to trypsin (10) and is present on the surface of normal oropharyngeal epithelial cells (11). The present study was designed

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to investigate the roles of proteases present in oropharyngeal secretions and cell-surface proteins in colonization of the upper respiratory tract by gram-negative bacilli.

METHODS

Two groups of patients were studied. One group consisted of 10 patients with acute respiratory failure (ARF)[1] due to noncardiogenic pulmonary edema of various etiologies. ARF was defined as a clinical syndrome characterized by diffuse infiltration of the lungs radiographically, severe arterial hypoxemia due in large part to intrapulmonary shunting, reduced lung compliance, and reduced lung volumes (12). The mean age of the ARF group was 58.6 yr, and 8 of 10 patients died of respiratory failure. Controls were normal individuals who were matched by age with study patients. The second group of study patients consisted of eight individuals undergoing elective coronary artery bypass (CAB) surgery. The mean age of this group was 53.4 yr, and controls were age-matched normal individuals.

Adherence of gram-negative bacilli to buccal epithelial cells was assayed by previously published techniques (13). Briefly, buccal cells were obtained by vigorous scraping of the mucosa with a moistened cotton-tipped swab. The cells were suspended in phosphate-buffered saline (PBS), pH 7.4, and washed three times over 10-μm filters (Gelman Sciences, Inc. Ann Arbor, Mich.) to rid the cells of nonadherent bacteria. Washed cells were adjusted to a concentration of 10^4/ml and incubated in PBS for 1 h with *Pseudomonas aeruginosa* that had been radiolabeled during growth with [^{14}C]lysine; the ratio of bacteria to buccal cells was 100:1. After incubation with *P. aeruginosa* buccal cells were again washed three times over 10-μm filters. The filters, containing 10^4 buccal cells, were solubilized and cell-associated radioactivity was counted on a Searle Radiographics, Inc. (Chicago, Ill.) Mark III scintillation counter equipped with computer conversion of counts per minute to disintegrations per minute. For each assay, the specific activity of the *P. aeruginosa* preparation was determined and buccal cell-associated radioactivity was expressed as bacteria per cell that had adhered during incubation in vitro.

Swabs of the posterior pharynx, buccal mucosa, and specimens of either expectorated sputum or aspirated tracheal secretions were cultured on sheep's blood agar and MacConkey's agar. Colonies of gram-negative bacilli were identified by standard techniques (14). "Colonization" was defined as the presence of any colonies of *Enterobacteriaceae* or *Pseudomonadaceae* in upper respiratory tract specimens.

Measurement of protease activity in secretions was performed using the ^{125}I-labeled fibrin plate method of Unkeles et al. (15). Fibrin plates were prepared by distributing 0.1 ml of radioactive fibrinogen solution (100 μg/ml) (Amersham Corp., Arlington Heights, Ill.) over the surface of a 35-mm plastic petri dish with a spreader. The plates were dried a minimum of 1 d at 45°C and stored at this temperature until use. Fibrinogen was converted to fibrin by overlayering the plates with 2 ml of RPMI 1640 (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.) containing 10% fetal bovine serum and incubating at 37°C for 18 h. The medium was then removed and the plates were washed three times with 2.0 ml of incubation buffer to remove all traces of serum. Nonstimulated saliva was obtained from patients and controls and sterilized by filtration through 0.45-μm filters. 1-ml aliquots were adjusted to 1 mg/ml protein in 0.1 Tris HCl, pH 8.1, containing 10 μg plasminogen, and deposited onto separate ^{125}I-fibrin plates. All determinations were performed in triplicate. After incubation at 37°C for 20 h, the entire reaction mixture from each plate was withdrawn and counted directly in a gamma counter. Results were expressed as counts per minute ^{125}I released per milliliter of secretions.

Plasma fibronectin was purified by affinity chromatography on gelatin:Sepharose (Pharmacia Fine Chemicals, Piscataway, N. J.) as described by Vuento and Vaheri (16) using 2 ml plasma/ml beads. Bound fibronectin was eluted with a liner gradient of L-arginine (0–2 M, gradient volume 200 ml) in 0.05 M Tris/HCl, pH 7.5. Fractions eluted between 50 and 75% of the gradient volume were pooled, dialyzed against 0.1 M ammonium bicarbonate, and lyophilized. This material yielded a single band on electrophoresis in 5% polyacrylamide gels in the presence of sodium dodecyl sulphate. Antifibronectin antisera were prepared by immunizing rabbits with 1 mg of purified fibronectin in complete Freund's adjuvant. Three injections were administered at biweekly intervals and the animals were bled 1 wk after the last injection. The rabbit sera were initially absorbed by passage through a gelatin-Sepharose column and a Sepharose column to which fibronectin-depleted plasma had been coupled. Antisera were then immunochemically purified with fibronectin bound to Sepharose beads. After washing, antibody was eluted with 3.0 M potassium iodide in PBS. The resultant preparation had a molecular weight of ~160,000 on nonreduced polyacrylamide gel electrophoresis in sodium dodecyl sulphate. Double immunodiffusion assays with purified antifibronectin and with normal human plasma yielded a single precipitin band.

For quantitative determination of buccal cell surface fibronectin, a direct radioimmune binding assay was developed. Antibodies to fibronectin were radiolabeled with ^{125}I according to the procedure of Fraker and Speck (17). A solution of 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (Pierce Chemical Co., Rockford, Ill.) in chloroform was prepared at a concentration of 1 mg/ml. A 2-μl aliquot of this solution was transferred to the reaction vessel containing a small magnetic stirring bar and 100 μl of reaction mixture. The iodination was started when all the chloroform had evaporated from the reaction vessel. The sample to be labeled (100 μg), potassium ^{125}iodide (15 μCi) nonradioactive potassium iodide (11 μl of a 1-mg/100-ml solution), and pH 7.4 borate buffer, to make the total volume of 100 μl, were added to the reaction vessel and the reaction was allowed to proceed in an ice bath, with stirring, for 5 min. The reaction was terminated by decanting the mixture from the residual glycoluril. 1 μg of labeled antibody (sp act 10^4 cpm/μg) was then incubated with 10^4 epithelial cells in PBS at 37°C for 1 h. The cells were then washed thoroughly with PBS, placed in a scintillation vial, the cell-associated counts per minute counted on a gamma counter, and the results expressed as counts per minute ^{125}I-antifibronectin bound per 10^4 epithelial cells.

To analyze the effects of proteases in secretions on buccal cell membranes in vivo, buccal cells were obtained and washed as before. Cell membranes were isolated and labeled with ^{125}I as described (18). Aliquots of the labeled membrane preparation containing ~20,000 cpm were dissolved in buffer and placed on 5% acrylamide-0.17% bisacrylamide sodium dodecyl sulphate slab gels (19). The gels were dried onto filter paper by heating under reduced pressure for 3 h in a Bio-Rad drying apparatus (Bio-Rad Laboratories, Richmond, Calif.). ^{125}I was visualized by autoradiography for 12 h on Kodak RP/R2 x-ray film (Eastman Kodak Co., Rochester, N. Y.).

[1] *Abbreviations used in this paper*: ARF, acute respiratory failure; CAB, coronary artery bypass; PBS, phosphate-buffered saline.

Effects of salivary proteases on buccal cells in vitro were studied also. Nonstimulated saliva was obtained from patients and controls and sterilized by filtration through 0.45-μm filters. Aliquots of these secretions were incubated with buccal cells obtained from normal controls for 1 h at 37°C and the cells were subsequently assayed for *P. aeruginosa* adherence and surface fibronectin as described before.

RESULTS

The respiratory tracts of each of the ARF patients were colonized with gram-negative bacilli. Adherence of *P. aeruginosa* to the cells of these patients in vitro was significantly greater than to cells from age-matched controls ($P < 0.01$, Table I). Cell surface fibronectin was reduced in the ARF group ($P < 0.01$) and salivary protease activity was increased compared to controls ($P < 0.01$). Repeated measurements in several of these patients showed no significant changes and it was not possible to determine the sequence of the alterations, since all of the patients remained seriously ill for prolonged periods and 80% died.

The CAB group was chosen because the onset of major stress could be anticipated. Results of this group are shown in Table II. 1 d before surgery (day 0), all values were similar to control subjects; none of the CAB patients was colonized with gram-negative bacilli preoperatively. 24 h postoperatively (day 1), in vitro adherence of *P. aeruginosa* and salivary protease activity were significantly increased and cell surface fibronectin was significantly decreased ($P < 0.01$) compared to controls. These alterations persisted on day 2 but by day 3 each parameter had returned towards control values. Three of the eight CAB patients (38%) were colonized with gram-negative bacilli by day 2. None of the patients developed clinically evident infection nor experienced any postoperative complications.

TABLE I
P. Aeruginosa Adherence, Buccal Cell Surface Fibronectin, and Protease Activity in Secretions in ARF Patients and Controls

	P. aeruginosa adherence*	Cell surface fibronectin‡	Protease activity§
ARF (10)	12.3±2.2‖	1.28±0.04‖	5.67±0.14‖
Control (10)	2.4±0.9	3.19±0.04	2.11±0.10

* Measured by radiolabel adherence assay. Data are given as mean±SEM number of bacteria attached per epithelial cell.
‡ Measured by radioimmunoassay; values represent mean ±SEM counts per minute ^{125}I-antifibronectin bound to 10^4 buccal cells times 10^{-3}.
§ Values represent mean±SEM counts per minute ^{125}I released from insoluble ^{125}I-fibrin matrix exposed to 1.0-ml secretions for 20 h at 37°C times 10^{-4}.
‖ Significantly different from the value for the controls ($P < 0.01$) by Student's *t* test.

Autoradiograms of ^{125}I-labeled membrane preparations from one of the CAB patients' buccal cells on each of the 4 d are shown in Fig. 1. A decrease in a large molecular weight protein on days 1 and 2 is evident, a finding that paralleled the decrease in cell-surface fibronectin as assayed immunologically.

Treatment of buccal cells from normal subjects with sterilized secretions from ill patients resulted in increased bacillary adherence and decreased cell surface fibronectin compared to treatment with saliva from normal controls. Representative data are shown in Table III. Attempts to restore cell surface fibronectin by in vitro incubation of deficient cells with purified plasma fibronectin were unsuccessful, presumably due to the inability of plasma-derived fibronectin to bind to these cells.

DISCUSSION

The previous observation that upper respiratory tract epithelial cells recovered from seriously ill patients bound increased numbers of gram-negative bacilli in vitro (8) suggested that altered adherence might explain the remarkable susceptibility of such patients to colonization with these organisms. However, since seriously ill patients become colonized rapidly (20), it was possible that the observed changes in cellular adherence were the result of, rather than the cause of, colonization by gram-negative bacilli. We cannot distinguish between these possibilities in our initial studies with ARF patients. While adherence of *P. aeruginosa* to buccal cells in vitro was markedly increased in these patients, each patient was colonized in vivo. The CAB patients demonstrate clearly that cellular changes can be demonstrated before colonization with gram-negative bacilli occurs. Similarly, increased bacillary adherence to upper respiratory epithelial cells in vitro has been noted to precede colonization in patients undergoing other stressful elective surgical procedures (21). Thus, it appears likely that the cellular changes precede the acquisition of gram-negative bacilli in the upper respiratory tract. These changes cannot be attributed to the presence of endotracheal tubes, inhalational anesthetics, or other factors related to the specific surgery experienced by the CAB patients since similar changes in adherence were found in patients with a variety of operations and anesthetic regimens including spinal anesthesia (21).

Alterations in epithelial cell binding of bacteria in vitro have been proposed to underlie a variety of infections in which organisms gain access to the host via a mucosal surface. Certain bacterial factors are important determinants of adherence. For gram-negative bacilli, pili appear to be required (5). Prior incubation of epithelial cells with purified pili blocks

TABLE II

P. Aeruginosa Adherence, Buccal Cell Surface Fibronectin, and Protease Activity in Saliva from CAB Patients and Controls over a 4-d Period

	P. aeruginosa adherence‡		Cell surface fibronectin§		Protease activity‖	
Day*	Controls (8)	Patients (8)	Controls	Patients	Controls	Patients
0	2.5±0.9	3.3±0.6	3.11±0.19	2.98±0.18	2.11±0.14	2.41±0.18
1	2.4±0.9	15.6±1.4	3.10±0.22	0.58±0.09	2.11±0.18	6.28±0.65
2	2.4±0.8	17.4±1.4	3.11±0.30	0.57±0.05	2.11±0.17	5.94±0.49
3	2.3±0.9	6.5±1.2	3.10±0.18	1.71±0.17	2.11±0.15	3.19±0.29

* Day 0 equals 1 d preoperative followed by 1, 2, and 3 d postoperative.

‡ Measured by radiolabel adherence assay. Data are given as mean±SEM number of bacteria attached per epithelial cell.

§ Measured by radioimmunoassay; values represent mean±SEM counts per minute ^{125}I-antifibronectin bound to 10^4 buccal cells times 10^{-3}.

‖ Values represent mean±SEM counts per minute ^{125}I released from insoluble ^{125}I-fibrin matrix exposed to 1.0-ml secretions for 20 h at 37°C times 10^{-4}.

subsequent adherence of homologous organisms (22) and nonpilated organisms adhere poorly to epithelial cells in vitro (2). The interaction between gram-negative bacilli and epithelial cells involves sugar residues. Preincubation of *Escherichia coli* with mannose inhibits subsequent adherence to buccal epithelial cells (23). However, a variety of sugars may participate in bacillary binding to epithelial cells (21).

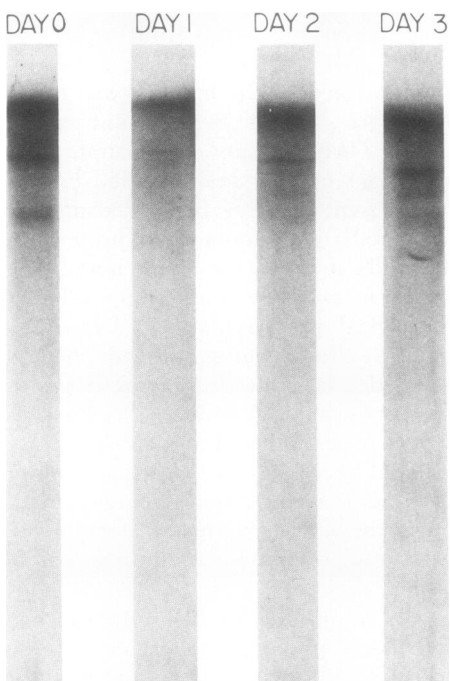

FIGURE 1 Autoradiograms of ^{125}I-labeled membrane preparations from one CAB patient's buccal epithelial cells obtained on each of the four study days and electrophoresed on a 5% SDS-polyacrylamide gel under reducing conditions.

Host factors in adherence have received less attention. Urogenital cells obtained from women subject to recurring urinary tract infection adhered greater numbers of gram-negative bacilli in vitro than cells from healthy subjects (24). This difference persisted during periods without active infection but the mechanism of the difference was not explained. Our results indicate that buccal cell adherence of bacilli may increase rapidly in an individual following major stress. Similar rapid changes in buccal cell adherence of gram-negative bacilli has been reported previously in humans (21) and experimental animals (25). The present studies suggest that alterations in cell surface proteins may mediate this change.

Fibronectin is known to be present on the surface of buccal epithelial cells (11). This protein is highly

TABLE III

Effect of Salivary Fluid Treatment of Buccal Cells on P. Aeruginosa Adherence and Cell Surface Fibronectin Levels*

Source of salivary fluid‡	*P. aeruginosa* adherence§	Cell surface fibronectin‖
Normal control	2.4±0.7	3.7±0.4
Patient	16.7±3.0¶	1.15±0.03¶

* Epithelial cells scraped from buccal mucosa of a healthy adult.

‡ Sterilized saliva (protein concentration 10 μg/ml).

§ Measured by radiolabel adherence assay. Data are given as mean±SEM of five determinations of number of bacteria attached per epithelial cell.

‖ Measured by radioimmunoassay. Values represent mean ±SEM of five determinations of counts per minute ^{125}I-antifibronectin bound to 10^4 buccal cells times 10^{-3}.

¶ Significantly different from the value for the controls ($P < 0.01$) by Student's t test.

sensitive to proteolytic enzymes such as trypsin (10). It may well be that many cell surface constituents are altered in patients with severe illness or stress and that fibronectin is only one marker of a more generalized process. However, our findings do reveal a strong association between cellular fibronectin loss and susceptibility to adherence of gram-negative bacilli in vitro. Since the exact location and nature of bacterial binding sites on the epithelial cell surface have not been defined we can only speculate on the role of fibronectin and possibly other proteins. Our data suggest that these proteins block binding sites that are normally present on the cell surface.

The cause of fibronectin loss would appear to be enhanced proteolytic activity of respiratory secretions. The source of such proteolytic enzymes is not clear. While gram-negative bacilli, especially *P. aeruginosa*, may produce copious amounts of such enzymes in vitro and in vivo (26), the sequence of changes that we observed would indicate that products of these organisms are not responsible for the loss of fibronectin. Other possible sources include inflammatory cells (27) and the epithelial cells themselves (28), or perhaps the normal flora. An additional possibility might be that protease inhibitors, normally present, were decreased in these patients. However, this was not tested.

The increase in proteolytic activity and the accompanying change in cellular adherence appear to be rapidly reversible in patients whose injury or illness is brief. Such was not the case with the ARF patients in whom altered adherence, increased protease activity, and decreased fibronectin levels persisted. Such patients are markedly predisposed to develop gram-negative bacillary pneumonias (29). The present findings suggest that prevention of such pneumonias might be possible through inhibition of protease activity in secretions, restoration of cellular surface proteins, or combinations of these approaches.

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