INFECTION AND IMMUNITY, Jan. 1981, p. 21-27
0019-9567/81/010021-07$02.00/0

Vol. 31, No. 1

Eikenella corrodens Adherence to Human Buccal Epithelial Cells

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The mechanism of *Eikenella corrodens* adherence to human buccal epithelial cells in vitro was studied. Initial experiments to determine the optimal conditions for adherence of *E. corrodens* to buccal epithelial cells showed that adherence was dependent on time, temperature, bacterial concentration, and pH. Different strains of *E. corrodens* varied in their ability to adhere, and strain 1073 showed the greatest ability in adherence. Strain 1073 was selected for studies of adherence mechanisms. Trypsin treatment or heating (100°C, 10 min) of the bacterial cells abolished their capacity to adhere to buccal epithelial cells. Treatment of buccal epithelial cells with trypsin also abolished adherence of *E. corrodens* 1073, whereas neuraminidase treatment of buccal epithelial cells enhanced the adherence. The adherence was inhibited by ethylenediaminetetraacetic acid and restored by adding Ca^{2+}. The adherence was remarkably inhibited by sugars containing D-galactose and *N*-acetyl-D-galactosamine. Treatment of neuraminidase-treated epithelial cells with sodium metaperiodate or α- and β-galactosidase did not decrease the adherence. These data suggest that adherence of *E. corrodens* 1073 to human buccal epithelial cells may require the interaction of lectin-like proteins on the bacterial surface with galactose-like receptors on the surface of epithelial cells.

It is generally recognized that the colonization of bacteria on human mucosal tissues is an important step in the infectious process (17, 21). For successful colonization, selective adherence of bacteria to host mucosal surfaces is a necessity (17). Virulent group A streptococci cause pharyngitis or nasopharyngitis by the colonization on the surface of these mucosal tissues (5, 11, 12). Pathogenic strains of *Escherichia coli* cause various infectious diseases by adhering to the intestinal canals (25, 29). These pathogenic organisms can adhere to other cells as well as pharyngeal or intestinal cells (1, 2, 7, 12). It has been suggested that a portion of lipoteichoic acids on the surface of group A streptococci and a lectin-like substance on the surface of *E. coli* are involved in the adherence of these bacteria to human oral epithelial cells (4, 27).

Eikenella corrodens, a slowly growing, gram-negative, facultative anaerobic rod, is prominently isolated from deep subgingival plaque in patients with aggressive or advanced periodontitis (32, 34). It was found by some investigators that monoinfection of germfree, or gnotobiotic, rats with *E. corrodens* causes periodontal disease with severe alveolar bone loss (19, 20, 23; A.C.R. Crawford, S. S. Socransky, E. Smith, and R. Phillips, J. Dent. Res., abstr. no. 275, 1977). The colonization of periodontopathic bacteria in periodontal pockets is considered to be an important step in the etiology and pathogenesis of periodontal disease. Slots and Gibbons (33) demonstrated that *Bacteroides melaninogenicus* and other oral bacteria attached to human crevicular epithelial cells in vitro. The mechanisms of adherence of periodontopathic *E. corrodens* to oral epithelial cells, however, has not been clarified. We have been investigating the interaction of *E. corrodens* with various human cells. The study reported here focuses on the mechanism of adherence of *E. corrodens* to human buccal epithelial cells.

MATERIALS AND METHODS

Cultures and culture conditions. *E. corrodens* 1073, 1006, and 1080 were kindly provided by S. S. Socransky of Forsyth Dental Center, Boston, Mass. *E. corrodens* 205, 307, and 408 were clinical isolates from supragingival plaque in humans. The bacteria were maintained on tryptic soy agar (Difco Laboratories, Detroit, Mich.) plates supplemented with 5% sheep blood (Nippon Bio-Test Laboratories, Tokyo, Japan) and transferred weekly. Strain 1073 was used in most experiments.

Bacterial cells used were grown in tryptic soy broth containing 2 mg of KNO_3 per ml and 5 μg of hemin per ml at 37°C under anaerobic conditions (95% N_2 and 5% CO_2). Late-log phase cells were harvested by cen-

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trifugation and washed twice with PBS-Ca (phosphate-buffered saline [20 mM phosphate, pH 7.2 and 0.15 M NaCl] with 0.1 mM $CaCl_2$). The final concentrations of bacteria used in this study were 10^3 to 5×10^9 colony-forming units (CFU) per ml.

Oral epithelial cells. Buccal epithelial cells were collected from one of us (Y.Y.) by gently scrubbing the inner cheeks with wooden applicator sticks. Epithelial cells were washed twice in PBS-Ca and resuspended in PBS-Ca at 2×10^5 cells per ml.

Adherence assay. The adherence assay by Gibbons and van Houte (16) was used with a few modifications. All experiments were performed in duplicate. For the assay, 0.5 ml of epithelial cell suspension (2×10^5 cells per ml) and 0.5 ml of bacterial cell suspension (2×10^8 or 2×10^9 CFU per ml) were mixed in a tube and stirred by a magnetic stirrer at 37°C for 30 min. The epithelial cells were collected on 12-μ (pore size) membrane filters (Sartorius-Membranfilter GmbH, Göttingen, West Germany) and washed with 200 ml of PBS-Ca to remove nonadherent bacteria. The epithelial cells on the filter were suspended in PBS-Ca, harvested by centrifugation at $200 \times g$ for 10 min, and placed on a glass slide. After drying and fixation, the preparations were stained with gentian violet. The average number of bacterial cells that attached per epithelial cell was determined by direct light microscopic enumeration of 40 epithelial cells. Epithelial cells with more than 150 bacterial cells were assigned a value of 150. Bacterial counts of control epithelial cells incubated only with PBS-Ca were performed in a similar manner to detect the number of indigenous bacteria present before exposure to *E. corrodens*.

Trypsin treatment of bacteria. PBS-washed bacteria (5×10^9 CFU/ml) were treated for 1 h at 37°C with 0.5 mg of trypsin (Mochida Pharmaceutical Co., Tokyo, Japan) per ml in PBS (pH 7.2). Control suspensions without trypsin were incubated simultaneously under similar conditions. The trypsin-treated bacteria and control bacteria were washed three times in PBS-Ca and compared with the standard adherence assay.

Enzymatic treatment of epithelial cells. According to the experimental schedule, epithelial cells (10^5 cells/ml) were treated for 30 or 60 min at 37°C with trypsin (2 μg/ml in PBS), pronase (2 μg/ml in PBS), neuraminidase (0.2 U/ml in 20 mM acetate buffered saline, pH 5.8), or enzyme-free buffer. The epithelial cells were then washed three times and resuspended in PBS-Ca, and adherence was determined as described above. A part of the neuraminidase-treated epithelial cells (10^5 cells/ml) was in addition incubated at 37°C for 1 h with α-galactosidase (0.5 U/ml in PBS), β-galactosidase (0.5 U/ml in PBS), or PBS. The cells were then washed and resuspended in PBS-Ca, and adherence was determined. Pronase was obtained from Kaken Chemical Co., Tokyo, Japan. Neuraminidase of *Clostridium perfringens* (type VIII), α-galactosidase of green coffee beans, and β-galactosidase of *E. coli* were purchased from Sigma Chemical Co., St. Louis, Mo.

Periodate oxidation of epithelial cells. The suspension of natural or neuraminidase-treated epithelial cells (10^5 cells/ml) was incubated with 10 mM sodium metaperiodate (Wako Chemical Co., Osaka, Japan) or PBS for 10 min at room temperature by the method of Ofek et al. (28). The cells were then washed three times with PBS-Ca, resuspended in PBS-Ca, and used for the adherence assay.

Saccharide inhibition. The effect of saccharides on *E. corrodens* adherence to buccal epithelial cells were determined by incubating 0.25 ml of bacterial cell suspension (4×10^8 CFU/ml) with an equal volume of either sugar solution (200 mM) or PBS-Ca. After 30 min at 37°C, 0.5 ml of epithelial cell suspension (2×10^5 cells/ml) was added, and the mixture was incubated again for 30 min at 37°C. Adherence was determined as described earlier. The following saccharides were used for inhibition studies: D-glucose, D-fructose, D-galactose, D-mannose, L-fucose, *N*-acetyl-D-glucosamine (Wako), *N*-acetyl-D-galactosamine, or melibiose (Sigma).

RESULTS

General characteristics of *E. corrodens* adherence to buccal epithelial cells. Our initial studies were primarily concerned with determining the optimal conditions for adherence of *E. corrodens* to buccal epithelial cells. The optimal time of incubation was determined by incubating epithelial cell suspension (10^5 cells/ml) with bacterial cell suspension (10^9 CFU/ml) for various periods of time at 37°C (Fig. 1). Adherence was greatest after incubation for 30 min.

To determine the effect of temperature on adherence, we incubated the bacteria (10^7 to 10^9 CFU/ml) and epithelial cells (10^5 cells/ml) in PBS-Ca for 30 min at 4, 20, and 37°C (Fig. 2). Adherence of *E. corrodens* 1073 to buccal epithelial cells was greater at 4°C than at 20 or 37°C when the bacterial concentration was 10^8 or 10^9 CFU per ml of incubation mixture.

To determine the effect of pH on adherence,

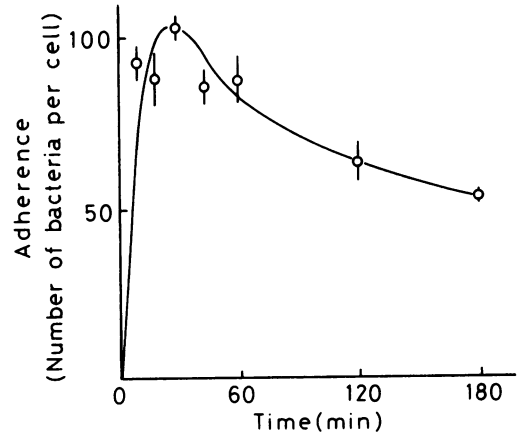

FIG. 1. *Effect of incubation time on adherence of E. corrodens 1073 to buccal epithelial cells.*

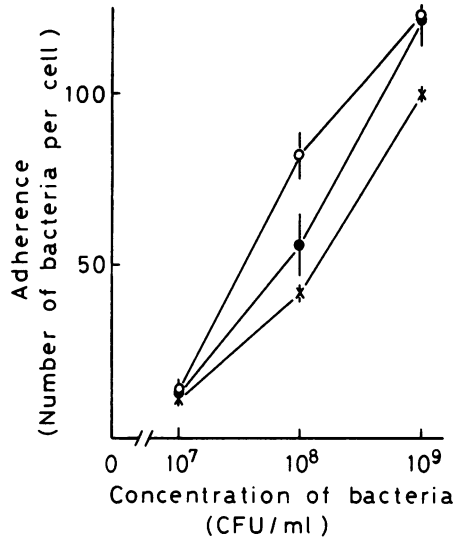

FIG. 2. *Effect of temperature on adherence of E. corrodens 1073 to buccal epithelial cells. Symbols: ○, 4°C; ●, 20°C; X, 37°C.*

the bacteria (10^8 CFU/ml) and epithelial cells (10^5 cells/ml) were incubated for 30 min at 37°C in different buffers over a pH range of 4 to 9 (Fig. 3). Adherence of *E. corrodens* 1073 to buccal epithelial cells was maximal at pH 5.0 and declined significantly at pH values below and above this pH. At a pH of 4.0 adherence could not be detected.

Adherence of different strains of *E. corrodens*. To determine whether strains of *E. corrodens* differed in their ability to adhere to buccal epithelial cells, we tested six strains (Table 1). The number of bacterial cells attached per epithelial cells ranged from 10 to 80. Strain 1073 showed the highest ability to adhere.

Effect of EDTA and divalent cations on adherence. The adherence of *E. corrodens* 1073 to buccal epithelial cells was almost inhibited by 0.1 mM ethylenediaminetetraacetic acid (EDTA) (Table 2). The addition of 0.2 mM Ca^{2+} to the incubation mixture not only protected the adherence from EDTA inhibition but also enhanced the adherence 2.5 times. At this concentration, Mg^{2+} or Mn^{2+} did not protect the adherence from EDTA inhibition.

Effect of trypsin treatment and heating of *E. corrodens* cells on adherence. To determine which bacterial surface components might be important in adherence, we conducted two treatments of the bacterial cells (Table 3). Heating (100°C, 10 min) and trypsin treatment of the bacteria before incubation with buccal epithelial cells each caused a 90% decrease in adherence.

Effect of enzymatic treatment of buccal epithelial cells on adherence. *E. corrodens* 1073 did not adhere to the epithelial cells pretreated with trypsin, whereas a large number of the bacterial cells (250% of control) adhered to the epithelial cells pretreated with neuraminidase (Table 4). Pronase treatment also caused 50% decrease in adherence.

Effect of saccharides on adherence. Various investigators have demonstrated that bacterial adherence to animal cells can be inhibited by certain saccharides (3, 14, 21, 22, 28). Experiments were carried out to see whether sugar inhibition could be observed in *E. corrodens* 1073 and the human buccal epithelial cell system (Table 5). D-Galactose and *N*-acetyl-D-galactosamine remarkably inhibited adherence of the

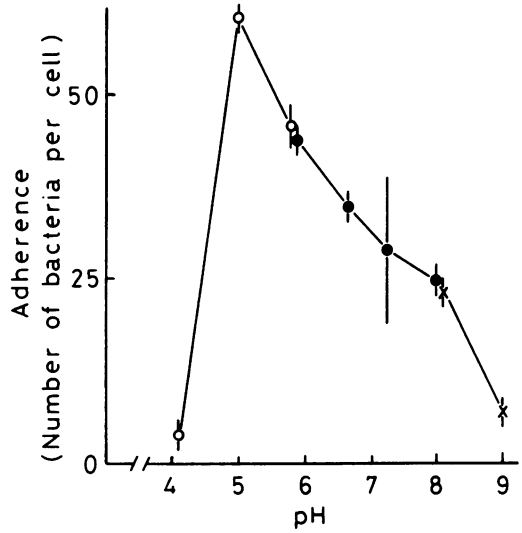

FIG. 3. *Effect of pH on adherence of E. corrodens 1073 to buccal epithelial cells. Symbols: ○, 20 mM acetate buffer with 0.1 mM $CaCl_2$; ●, 20 mM phosphate buffer with 0.1 mM $CaCl_2$; X, 20 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer with 0.1 mM $CaCl_2$.*

TABLE 1. *Adherence of different strains of E. corrodens to buccal epithelial cells*[a]

Strain	No. of bacterial cells attached per epithelial cell ± SE[b]
1073	80 ± 6
1006	24 ± 7
1080	10 ± 2
205	66 ± 9
307	22 ± 2
408	44 ± 5

[a] Bacterial cells (10^9 CFU/ml) were incubated for 30 min at 37°C with buccal epithelial cells (10^5 cells/ml) in PBS-Ca, and adherence was determined as described in the text.

[b] Mean of duplicate determinations. SE, Standard error of the mean.

TABLE 2. *Effect of EDTA and divalent cations on the adherence of E. corrodens 1073 to buccal epithelial cells*[a]

EDTA (mM)	Divalent cation (0.2 mM)	No. of bacterial cells attached per epithelial cell ± SE[b]
None	None	42 ± 7
0.1	None	11 ± 3
0.1	Ca^{2+}	104 ± 16
0.1	Mg^{2+}	10 ± 0
0.1	Mn^{2+}	18 ± 4

[a] Bacterial cells (10^9 CFU/ml) were incubated for 30 min at 37°C with buccal epithelial cells (10^5 cells/ml) in the absence and presence of EDTA at pH 7.2, and adherence was determined as described in the text.

[b] Mean of duplicate determinations. SE, Standard error of the mean.

TABLE 3. *Effect of trypsin and heat treatment of bacterial cells on adherence of E. corrodens 1073 to buccal epithelial cells*[a]

Treatment	No. of bacterial cells attached per epithelial cell ± SE[b]
None	92 ± 7
Heat (100°C, 10 min)	9 ± 1
Trypsin (0.5 mg/ml, 37°C, 60 min)	9 ± 1

[a] Treated and untreated bacterial cells (10^9 CFU/ml) were incubated for 30 min at 37°C with buccal epithelial cells (10^5 cells/ml) in PBS-Ca, and adherence was determined as described in the text.

[b] Mean of duplicate determinations. SE, Standard error of the mean.

TABLE 4. *Effect of enzymatic treatment of buccal epithelial cells on adherence of E. corrodens 1073*[a]

Treatment	Relative adherence[b] (%)
None	100 ± 10
Trypsin (2 μg/ml)	0
Pronase (2 μg/ml)	53 ± 3
Neuraminidase (0.2 U/ml)	249 ± 2

[a] Bacterial cells (10^9 CFU/ml) were incubated for 30 min at 37°C with treated and untreated buccal epithelial cells (10^5 cells/ml) in PBS-Ca, and adherence was determined as described in the text.

[b] (Treated/control) × 100. Mean of duplicate determinations ± standard error.

bacteria to both native epithelial cells and neuraminidase-treated epithelial cells. Melibiose, which contains D-galactose residue at the nonreducing terminal, was also a potent inhibitor. D-Mannose, which is known to be a specific inhibitor of *E. coli* adherence to epithelial cells (13, 28), was a moderate inhibitor. Other saccharides tested did not result in any significant inhibition of adherence.

Periodate oxidation and galactosidase treatment of buccal epithelial cells. Inasmuch as neuraminidase treatment of epithelial cells enhanced adherence of *E. corrodens* 1073, experiments were performed to determine whether receptor sites on human buccal epithelial cells for *E. corrodens* adherence were galactose-like sugars linked to sialic acids or those located inside of the sugar chains. The oxidation of native and neuraminidase-treated epithelial cells by sodium metaperiodate, which is known to cleave the C-C bond between vicinal hydroxyl groups of sugar, did not reduce the adherence, but enhanced it (Table 6). Furthermore, adherence of *E. corrodens* 1073 to periodate-treated epithelial cells was inhibited by D-galactose as well as that in native epithelial cells without oxidation.

Table 7 shows the effect of α- and β-galactosidase treatment of neuraminidase-treated epithelial cells on *E. corrodens* adherence. The treatment with α- or β-galactosidase did not affect the adherence. Results of Tables 6 and 7 suggest that receptor sites on buccal epithelial cell for *E. corrodens* adherence may be galactose-like substances located inside of the sugar chains.

DISCUSSION

Three strains (1073, 1006, and 1080) of *E. corrodens* employed in this study were isolated from patients with periodontal disease, and the other three strains (205, 307, 408) used were isolated from dental plaque of healthy subjects. Of the strains used, strain 1073 was shown to be periodontopathic in animals (19, 20, 23), but the others have not been examined for their ability

TABLE 5. *Inhibition of adherence of E. corrodens 1073 to native and neuraminidase-treated buccal epithelial cells by carbohydrates*[a]

Carbohydrate (50 mM)	No. of bacterial cells attached per epithelial cell ± SE[b]	
	Native cell	Neuraminidase-treated cell
None	36 ± 3	89 ± 1
D-Glucose	31 ± 4	77 ± 3
D-Fructose	31 ± 1	89 ± 9
D-Galactose	9 ± 1	11 ± 0
D-Mannose	16 ± 2	33 ± 1
L-Fucose	31 ± 5	77 ± 15
N-Acetyl-D-glucosamine	31 ± 0	93 ± 4
N-Acetyl-D-galactosamine	8 ± 2	13 ± 1
Melibiose [α-D-Gal-(1 → 6)-D-Glu]	7 ± 1	14 ± 3

[a] Experimental procedures as described in the text.

[b] Mean of duplicate determinations. SE, Standard error of the mean.

TABLE 6. *Effect of enzymatic treatment and periodate oxidation of buccal epithelial cells on adherence of E. corrodens 1073*[a]

Treatment	No. of bacterial cells attached per epithelial cell ± SE[b]		
	None	Glucose[c]	Galactose[c]
None	27 ± 5	23 ± 3	7 ± 1
Neuraminidase	82 ± 1	71 ± 3	10 ± 0
$NaIO_4$	47 ± 10	44 ± 1	5 ± 1
Neuraminidase + $NaIO_4$[d]	137 ± 4	ND[e]	13 ± 2

[a] Bacterial cells (10^8 CFU/ml) were incubated for 30 min at 37°C with treated and untreated buccal epithelial cells (10^5 cells/ml) in PBS-Ca. Saccharides inhibition studies were performed as described in the text.

[b] Mean of duplicate determinations. SE, Standard error of the mean.

[c] Carbohydrate added (50 mM).

[d] Buccal epithelial cells were treated with 0.2 U of neuraminidase per ml for 1 h at 37°C and then washed, resuspended in 10 mM sodium metaperiodate, and incubated for 10 min at room temperature.

[e] ND, Not determined.

TABLE 7. *Effect of galactosidase treatment of neuraminidase-treated buccal epithelial cells on adherence of E. corrodens 1073*[a]

Treatment	No. of bacterial cells attached per epithelial cell ± SE[b]
None	19 ± 1
Neuraminidase	71 ± 2
Neuraminidase + α-galactosidase[c]	75 ± 13
Neuraminidase + β-galactosidase[c]	76 ± 1

[a] Bacterial cells (10^8 CFU/ml) were incubated for 30 min at 37°C with treated and untreated buccal epithelial cells (10^5 cells/ml) in PBS-Ca, and adherence was determined as described in the text.

[b] Mean of duplicate determinations. SE, standard error of the mean.

[c] Buccal epithelial cells were treated with 0.2 U of neuraminidase per ml for 1 h at 37°C and then washed, resuspended in 0.5 U of α- or β-galactosidase per ml, and incubated for 1 h at 37°C.

to induce periodontal disease. Strain 1073 showed the highest ability to adhere to buccal epithelial cells (Table 1). It is not known at this time whether there is a corelationship between the ability of *E. corrodens* to adhere to epithelial cells and the capability of the bacteria to induce periodontal disease in animals.

Adherence of *E. corrodens* 1073 to buccal epithelial cells appeared to be pH and temperature dependent. Adherence peaked at pH 5.0 and declined at pH values below and above 5 (Fig. 3). Adherence of *Klebsiella pneumoniae* to rat bladder epithelial cells was reported to be maximum at pH 5.0 (14), and optimal pH of group B streptococcal adherence to human vaginal epithelial cells was shown to be 5.5 (35). The data that the number of adherent *E. corrodens* cells was lower at 37°C than at 4°C suggested that enzymatic processes or the mobility of epithelial cell membrane components might not be involved in the adherence of *E. corrodens* 1073 to buccal epithelial cells. It was reported that the adherence of several gram-positive bacteria was maximum at 37°C (3, 35) and that there was no differences in adherence of certain gram-negative bacteria at incubation temperatures of 4 and 37°C (14, 31). It is interesting that the ability of several plant lectins to bind to their specific sugars is higher at 2°C than at 20 or 37°C (9, 18, 24).

The present study indicated that lectin-like protein(s) on the bacterial surface might be responsible for adherence of *E. corrodens* 1073 to buccal epithelial cells. The adherence was abolished by trypsin treatment or heating (100°C, 10 min) of bacterial cells (Table 3). Calcium ion was required for adherence (Table 2), and adherence was inhibited specifically by D-galactose or *N*-acetyl-D-galactosamine (Table 5). Several studies have shown that pili on the bacterial surface was responsible for adherence of certain gram-negative bacteria to mammalian cell surfaces (14, 26, 30, 31). A mannose-specific lectin, which agglutinated yeast cells, human epithelial cells, and mouse lymphocytes, has been isolated from *E. coli* (13). However, pilus-like structures were not found on the cell surface of *E. corrodens* 1073 by electron microscopy using the negative-stain method, and it was observed that the surface of unwashed cells of *E. corrodens* 1073 was covered with a slime layer (M. Nakao and S. Ebisu, manuscript in preparation). Further work on the characterization of the lectin-like substances is in progress.

The adherence of *E. corrodens* 1073 to buccal epithelial cells was inhibited by D-galactose (Table 5), and the addition of D-galactose to epithelial cells to which *E. corrodens* was preadhered caused displacement of the bacteria from the epithelial cells (data not shown). Ofek et al. (28) reported that the adherence of *E. coli* to human buccal epithelial cells was inhibited specifically by D-mannose. The adherence of *K. pneumoniae* to rat bladder epithelial cells was also inhibited by D-mannose (14). Sialic acid was a specific inhibitor of the interaction of *Mycoplasma pneumoniae* with human lung fibroblasts (15), and L-fucose inhibited adherence of *Vibrio cholerae* to rabbit brush border membrane (22). Smoot et al. demonstrated that adherence of

Fusobacterium nucleatum to human buccal epithelial cells was inhibited by D-galactose (C.N. Smoot, J.R. Mongiello, and W.A. Falker, Jr., J. Dent. Res., abstr. no 637, 1979). These observations indicate that mammalian cell surfaces have some different receptor sites for adherence of various microorganisms.

Treatment of buccal epithelial cells with neuraminidase enhanced the adherence of *E. corrodens* 1073 to the cells (Table 4). Two possible mechanisms might be proposed to explain this observation: (i) many of the epithelial cell surface receptors for adherence might be masked by sialic acids, and (ii) negative charges of sialic acids might have an influence on the adherence. In the case of *Actinomyces viscosus* and *Actinomyces naeslundii* hemagglutination, Costello et al. (6) and Ellen et al. (10) demonstrated that *Actinomyces* hemagglutination proceeds via a two-step mechanism: (i) neuraminidase removal of terminal sialic acid on the erythrocytes and (ii) lectin-like binding to exposed β-galactoside receptors on the erythrocytes. If epithelial cell surface receptors for *E. corrodens* adherence are galactose-like sugars linked to sialic acid, they should be destroyed by neuraminidase treatment and periodate oxidation. However, periodate oxidation of neuraminidase-treated epithelial cells enhanced galactose inhibitable adherence of *E. corrodens* 1073 (Table 6). These results suggested that negative charges of sialic acids at the nonreducing terminal of the sugar chains on the buccal epithelial cell surface might have an influence on the adherence of *E. corrodens* 1073 to sugar receptors on epithelial cells. The influence of the surface potential, charge, and topography of animal cells on adherence of bacteria to the cells has been reviewed (21). In connection with this, it may be noted that Ebisu et al. (8) demonstrated that the presence of charged amino group in an antigen molecule influenced antigen-antibody interaction.

The findings of the present investigation on *E. corrodens* adherence to galactose-like receptors on the buccal epithelial cell surface suggests that the bacteria may adhere to other cell surfaces which possess galactose-like substances. Indeed, human erythrocytes were agglutinated by *E. corrodens* cells irrespective of the ABO blood group, and hemagglutination was inhibited by D-galactose or *N*-acetyl-D-galactosamine (S. Ebisu, Y. Yamazaki, and H. Okada, J. Jpn. Assoc. Periodontol., abstr. no. 10, 1979).

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