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Determinants of the Developing Oral Flora in Normal Newborns

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The ability of *Streptococcus* species to selectively adhere to the oral epithelial cells of newborns was studied in vitro. On day 1 of life, mucosal cells from normal infants demonstrated selective attraction for the natural distribution of streptococci that would soon colonize these surfaces. *Streptococcus salivarius* and *Streptococcus mitis* adhered well in vitro to scraped cells from cheek and tongue surfaces. *Streptococcus mutans*, on the other hand, exhibited feeble or no adherence to cheek or tongue cells. Adherence of *Escherichia coli* to oral epithelial cells was also studied. The ability of strains of *E. coli* to adhere to cheek and tongue cells correlated solely with the presence of cell surface substances, probably pili. These observations, made on infants at the critical moment of their developing flora, strengthen the hypothesis that the ability of bacteria to adhere to surfaces is an important determinant of their ecological place in the oral microflora.

Until the time of birth the human infant is usually "germfree." The newborn then becomes suddenly exposed to millions of microorganisms, only a small portion of which will become part of his normal flora. Although the bacterial microflora of the mouth has been repeatedly examined (3, 5, 6, 15, 21), the mechanism of selection is not understood. Viridans streptococci are the most prominent and consistent members of this microflora. Sprunt and others have studied streptococcal interference with other organisms to account for this predominance (7, 18, 20). Gibbons et al. have observed experimentally that the adherence of streptococci to oral mucosal cells of adults in vitro and in vivo is selective and suggest that adherence determines their presence in normal flora (8, 11, 14, 22, 23). If selective adherence of oral streptococci to mucosal cells is important in determining flora, it should be demonstrable on day 1 of life when establishment of that flora begins. We tested this hypothesis in well newborn infants. Since the oropharynx of newborns is also often colonized with *Escherichia coli*, we also examined the in vitro ability of *E. coli* to adhere to oral epithelial cells.

MATERIALS AND METHODS

Bacteria. Laboratory strains of *Streptococcus salivarius* CM6, *Streptococcus mitis* 26, and *Streptococcus mutans* E49 were obtained from R. J. Gibbons at the Forsyth Dental Center, Boston, Mass. Organisms were maintained by weekly transfer on Todd-Hewitt agar (BBL). Suspensions of each organism were prepared from 24-h Trypticase soy broth (BBL) cultures incubated anaerobically at 35°C in Brewer Jars filled with 85% N_2, 10% H_2, and 5% CO_2.

Laboratory strains and clinical isolates of *E. coli* were obtained from the *Enterobacteriaceae* Laboratory Branch, Center for Disease Control, Atlanta, Ga., and George H. McCracken, Dallas, Tex. Organisms were maintained by biweekly transfer on Trypticase soy agar. Suspensions of each organism were prepared from 18-h brain heart infusion broth cultures that were aerobically incubated at 35°C.

According to the method of Gibbons and van Houte (11), organisms were harvested by centrifugation, washed once with phosphate buffer, and suspended in buffer to yield a cell suspension containing approximately 10^8 colony-forming units (CFU) per ml by optical density. (Optical density of 0.5 at 550 nm for these streptococci and 0.35 for these coliforms approximated 10^8 chains or rods per ml by direct microscopic counts.)

Selected strains of *E. coli* were depiliated as follows. After growth, centrifugation, and phosphate buffer wash, bacterial suspensions were blended at high speed in a commercial Osterizer blender at 4°C for 2 min. Bacteria were then collected by slow centrifugation at 1,400 × *g* at 26°C for 20 min (4) and resuspended in phosphate buffer to 10^8 CFU per ml. (Viability as determined by colony counts usually exceeded 90%.) For experiments involving incubation after depiliation, the blended bacteria were collected as above, suspended in brain heart infusion broth, and incubated at 35°C for 90 min. They

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were then collected by centrifugation and resuspended in phosphate buffer to 10^8 CFU per ml.

Hemagglutination tests for *E. coli*. Samples of *E. coli* cultured, modified, and modified then reincubated as described above were centrifuged at 2,000 × *g* for 10 min. The supernatant was drained off, and the deposited bacilli were mixed with the small residuum of buffer. The slide hemagglutination test of Duguid (8) was performed using 2% (vol/vol) guinea pig erythrocytes and concentrated test bacterial suspension. After mixing, the chilled slide was rocked to and fro for 10 min. Coarse clumping seen within 5 min was recorded as (+). Clumping occurring between 5 and 10 min was recorded as (±).

Electron microscopy. *E. coli* killed with formaldehyde were suspended in saline and mixed with an equal volume of 2% phosphotungstic acid at pH 7. The mixture was absorbed onto colloidin-coated grids, dried, and examined for presence of pili in an electron microscope (Siemens elmiskop 1A).

Epithelial cell suspensions. Thirty-two healthy term infants less than 12 h old were the subjects of this study. Epithelial cells were collected by gently scraping oral mucosal surfaces with a wooden applicator stick. The applicators were then agitated in saline. Suspensions were used singly or those of three to four infants were pooled. The cells were rendered free of unattached bacteria by repeated saline washes through membrane filters (pore size, 14 μm) (11), and then were suspended in saline to contain approximately 10^5 cells per ml as determined by direct microscopy.

Adherence tests. The ability of bacteria to adhere to epithelial cells was studied by mixing 1 ml of the standardized bacterial suspensions in buffer and 1 ml of standardized epithelial cells in saline. A control tube containing 1 ml of epithelial cells and 1 ml of phosphate buffer established the number of naturally adhering bacteria at the time of collection. The text mixtures were incubated at 35°C for 30 min in a shaking water bath. Epithelial cells were then washed free of unattached bacteria by membrane filtration as before. Each filter, with its collected epithelial cells, was then turned onto a clean labeled microscope slide as in making "touch preparations." Slides were dried, heat fixed, and stained for 15 s with Gram crystal violet. The number of bacteria adherent to epithelial cells was determined by direct microscopy at ×900 magnification. For the streptococci, chains of two to five cocci sometimes occurred. In that case, chains were counted. For the coliforms, individual rods were counted. For simplicity these counts are expressed as the number of bacteria per cell. Considerable variation in number of attached bacteria occurred from cell to cell in the same sample. Consequently, 50 cells were counted for each sample, and an average was obtained.

RESULTS

Selective adherence of *S. salivarius*, *S. mitis*, and *S. mutans*. Preliminary studies indicated that after 24 h of life all infants had bacteria naturally attached to mouth epithelial cells. In 3-day-old infants, this averaged about 50 bacteria per cell in samples from the cheek and about 60 bacteria per cell in samples from the tongue. In contrast, infants less than 12 h of age predictably had few, if any, naturally adherent bacteria (2, 2, 0, 0, and 0 bacteria/cell) (Tables 1-3). When *S. salivarius* and *S. mitis* were incubated with cheek and tongue cells from these newborns, significant and similar attachment occurred (57 and 54 bacteria/cell, and 44 and 65 bacteria/cell, respectively) (Table 1). When *S. mutans* was tested, it was only weakly adherent (9 bacteria/cell and 10 bacteria/cell, respectively) (Table 1).

Piliation of cultured and modified *E. coli*. Strains of *E. coli* that were similarly cultured differed in their ability to agglutinate guinea pig erythorcytes (Table 2). Hemagglutination was associated with presence of multiple pili protruding from bacteria cell surfaces. Blender modification resulted in loss of pili and inability to hemagglutinate (Table 3). Mechanically depiliated *E. coli* reincubated for 90 min in brain heart infusion broth regained both pili and the ability to hemagglutinate guinea pig

TABLE 1. *Adherence of streptococci to oral epithelial cells of the newborn (5 patients or pools studied)*

Organism	Cheek (bacteria/cell)[a]	Tongue (bacteria/cell)
Background control	2 ± 1[b]	2 ± 1
Streptococcus salivarius	57 ± 14	44 ± 7
S. mitis	54 ± 11	65 ± 14
S. mutans	9 ± 2	10 ± 4

[a] Bacteria per cell are chains per cell.
[b] Mean plus or minus standard error.

TABLE 2. *Adherence of E. coli to oral epithelial cells of the newborn*

Organism	HA[a]	Cheek (bacteria/cell)[b] (5 patients or pools studied)	Tongue (bacteria/cell) (3 patients or pools studied)
Background control		0 ± 0.2	0 ± 0.3
Streptococcus mitis control		58 ± 6	65 ± 12
Escherichia coli 016:K1:H6	−	3 ± 1	3 ± 1
E. coli 016:K1:NM	−	2 ± 0.2	1 ± 0.2
E. coli K-12 (B380)	−	0	0
E. coli 06:K2:H1	+	27 ± 4	25 ± 8
E. coli 03:K2:H2	+	65 ± 8	78 ± 20
E. coli K-12 (AB311)	+	39 ± 3	36 ± 4

[a] HA, Hemagglutination of 2% guinea pig erythrocytes.
[b] Bacteria per cell are chains per cell for the streptococci.

TABLE 3. *Adherence of E. coli to cheek cells*

Organism	HA[a]	Organisms/cell (2 patients or pools studied)
Background		0
Cultured		
Escherichia coli 03:K2:H1	+	58
E. coli 018ac:K1:H7	+	20
E. coli K-12	+	45
Depiliated		
E. coli 03:K2:H1	−	0
E. coli 018ac:K1:H7	−	0
E. coli K-12	±	1
Depiliated and reincubated		
E. coli 03:K2:H1	+	71
E. coli 018ac:K1:H7	+	12
E. coli K-12	+	67

[a] HA, Hemagglutination of 2% guinea pig erythrocytes.

cells (Table 3). Thus, hemagglutination correlated with presence of pili.

Adherence of cultured and modified *E. coli*. To investigate whether pili were important in adherence to oral epithelial cells, hemagglutination-positive and -negative strains of *E. coli* were compared. Hemagglutination-positive strains of *E. coli* adhered as avidly to cheek and tongue cells (27, 65, and 39 bacteria/cell, and 25, 78, and 36 bacteria/cell, respectively) as *S. mitis* (58 and 65 bacteria/cell) (Table 2). Strains of hemagglutination-negative *E. coli* similarly cultured and incubated with cheek and tongue cells did not adhere (3, 2, and 0 bacteria/cell, and 3, 1, and 0 bacteria/cell, respectively) (Table 2).

Table 3 illustrates the adherence of mechanically depiliated *E. coli* to infant cheek cells in vitro. Cultured, hemagglutination-positive strains adhered as expected (58, 20, and 45 bacteria/cell). The same strains, blender modified, became hemagglutination negative and did not adhere (0, 0, and 1 bacteria/cell). These mechanically depiliated strains when reincubated for 90 min became hemagglutination positive again and adhered avidly (71, 12, and 67 bacteria/cell).

DISCUSSION

Within 24 h after birth the oropharynx of the newborn becomes rapidly colonized with bacteria. The early flora is simple and is composed of *Staphylococcus epidermidis*, viridans streptococci, gram-negative bacilli occasionally, and a small group of variable transients (3). The early establishment of streptococci as constant members of the oral microflora is maintained in health throughout life. As established in adults, different species of the genus *Streptococcus* are naturally found at specific sites in the mouth. *Streptococcus sanguis* and *S. mutans* predictably occur in highest proportions on the tooth surface. *S. salivarius* is predominant on the tongue, and *S. mitis* predominates on the vestibular mucosa (11, 14, 22). Furthermore, labeled strains introduced into the mouths of adults are not distributed randomly but attach primarily to the surfaces where they are naturally found (i.e., *S. salivarius* to tongue, *S. mutans* to teeth) (11). Scraped epithelial cells from these surfaces in adults behave similarly in vitro (11, 14, 22).

The data observed in the present investigation indicate that this mechanism is found to be operative on day 1 of life.

For the oral streptococci, indirect evidence thus exists that adherence is a "regulator" mechanism that allows organisms to survive and colonize specific sites within the mouth. The mechanism for this selective adherence is unknown. Investigations have focused on the role of specific antibody as directing bacterial attachment (13) and degree of keratinization of epithelial cells as allowing variability of attachment of a single organism from one mouth site to another. Alteration of epithelial cell by lipolytic enzymes reduces adherence and suggests that the binding site may be a phospholipid component of the epithelial cell membrane (12). Properties of bacterial cell surface are also important since Liljemark, van Houte, and Gibbons observed an electron-dense protein "fuzzy" layer on all the gram-positive cocci that normally adhere to mucosa of the human cheek (11, 14). Disruption of this surface by trypsin impairs adherence (12). Specific secretory antibody protects against *Vibrio* infection by preventing mucosal adherence (10).

The oropharynx of approximately 16% of normal 3-day-old infants in our nursery included gram-negative bacilli (unpublished data). The data in this study suggest that adherence of *E. coli* to the epithelial cells of newborns is strain specific in vitro and is associated with the presence of pili extending from the bacterial cell surface. Incubation of oral epithelial cells with strains of *E. coli* that hemagglutinated guinea pig erythrocytes and that demonstrated pili on electron microscopy resulted in adherence that was unaltered by vigorous washings. Incubation of oral epithelial cells with *E. coli* that did not possess pili or that were mechanically depiliated resulted in total inability to adhere. Ninety-minute incubation of mechanically depiliated strains restored pili and strong adher-

ence of the bacteria to oral epithelial cells of the newborns. Strains demonstrating pili and adherence had the same degree of affinity for cheek as for tongue cells.

For *E. coli*, our data suggest that piliation may be the mechanism for adherence to mouth surfaces. Okuda and Takazoe have demonstrated that approximately one-half of *Bacteroides melaninogenicus* found in human gingival crevices are piliated (16). It has been suggested that adherence is important in initiating *E. coli* gastroenteritis in piglets (1, 2, 19) and in humans (9) and that pili are related to the virulence of *Neisseria gonorrhoeae* (17). However, the role, if any, of pili and adherence in the pathogenesis of neonatal gram-negative infections remains unexplored. Further studies will elucidate whether the ability of certain piliated *E. coli* to adhere to oral mucosa is the first step to invasion and disease or harmless colonization and immunity.

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