SHORT ARTICLES

ADHERENCE OF ENTEROBACTERIACEAE TO HUMAN BUCCAL CELLS

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ADHERENCE of bacteria to mammalian cells is an important initial step in certain mucosal infections of the genitourinary tract (Stamey *et al.*, 1978; Ward and Watt, 1972), gastrointestinal tract (McNeish *et al.*, 1975), and respiratory tract (Ellen and Gibbons, 1974; LaForce *et al.*, 1976; Powell *et al.*, 1976). We have studied the possible role of adherence of gram-negative bacteria to oral mucosal cells as an initial phase in their colonisation and infection of the respiratory tract of hospital patients.

MATERIALS AND METHODS

Bacteria. Strains of Enterobacteriaceae were obtained from the clinical microbiology laboratory of the University of Iowa Hospitals and Clinics. Each specimen represented at least 50% of the total growth on culture plates of expectorated sputa. They were stored at 4°C in pure culture on nutrient-agar slants and used within 30 days without passage. Clinical histories and chest radiographs of the patients from whom the sputa were obtained were without evidence of respiratory-tract infections.

Radiolabelling. Bacteria were grown in 5 ml of minimal essential medium at 37°C in the presence of 25 µCi of (methyl-³H)-thymidine (New England Nuclear) for 16 h. The bacterial suspensions were then centrifuged for 10 min. at 12 300 *g* and washed thrice in phosphate-buffered saline, 0.05M, pH 7.4 (PBS). Bacteria were resuspended in PBS to the required concentrations by optical density, verified by total radioactivity of bacterial suspensions and quantitative pour plates.

Buccal cells were obtained by scraping the buccal mucous membranes of one of the investigators with wooden applicators. These cells were collected in PBS, centrifuged for 10 min at 1100 *g* and washed thrice in PBS. The final concentration was determined by haemocytometry, and adjusted as necessary with additional PBS. The buccal cells were typical of squamous epithelium, flat and discoid, 61 ± 11 µm in maximum diameter.

Radio-adherence assay. On 3 separate days 1-ml suspensions of bacteria and buccal cells, 1×10^8 and 1×10^5 /ml respectively unless otherwise indicated, were incubated in duplicate in 10-ml plastic vials for 45 min. at 37°C in a shaking apparatus. Control bacterial suspensions were incubated with PBS. After incubation, the mixtures were filtered through 12-µm membrane filters (Nuclepore), or 0.2-µm membrane filters to determine total bacterial counts. The filters were then washed with PBS, dried and placed in scintillation vials containing Omnifluor (New England Nuclear) in a toluene base, and radioactivity (counts per minute, c.p.m.) was then determined in a liquid scintillation counter. Conversion to number of adherent bacteria was performed by dividing ³H c.p.m. (less control counts of bacteria alone) by the average c.p.m. per bacterium.

For labelled Enterobacteriaceae, 1×10^8 cells yielded counts of 2×10^5 – 5×10^5 c.p.m. For each of six studied strains, the c.p.m. of 1×10^8 bacteria displayed a standard deviation of $\leq 1 \times 10^4$ c.p.m. when tested from three separate 16-h incubations. Control counts of 1×10^8 of each Enterobacteriaceae strain incubated in PBS for 45 min. and washed over 12-µm filter paper,

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to detect bacterial aggregation, were all less than 2×10^2 c.p.m. Controls centrifuged after incubation for 45 min. yielded ^3H activity in the supernates of $\leq 0.6 \pm 0.2\%$ of the initial bacterial counts, indicating no significant leakage of the ^3H label from the bacteria. Adherence of free ^3H -thymidine to buccal cells could not be demonstrated with the incubation system employed.

Light microscopy assay. With the same incubation and washing procedures as described above, the filters were mounted on glass slides and were Gram stained. The average number of bacteria on 100 randomly selected buccal cells at each incubation concentration was determined.

Statistical methods. Standard t-value analyses were employed.

RESULTS

Adherence of gram-negative rods

Strains of *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Escherichia coli*, and *Proteus mirabilis* isolated from sputa were found to display markedly different abilities to adhere to the same buccal cells (fig. 1). The attachment abilities ranged from statistically no greater than control ("nonadherent", e.g., *K. pneumoniae* strain 3) to more than 20 times greater than control ("very adherent", e.g., strains 1, 2, 4, and 5).

FIG. 1.—Adherence of different gram-negative bacilli to human buccal cells. Strain numbers 1–3 represent different strains of *Klebsiella pneumoniae*, and numbers 4–6 represent strains of *Enterobacter aerogenes*, *Proteus mirabilis*, and *Escherichia coli*, respectively. I = standard deviation.

Effect of varying bacteria: cell ratio, and correlation with light microscopy

When the number of buccal cells was kept constant, increasing the number of bacteria over a 1-log range from 1×10^7 to 1×10^8 led to linear increase in the number of bacteria binding to

FIG. 2.—Results of incubating 1×10^5 buccal cells with varying numbers of either *K. pneumoniae* (strain 1 of fig 1) or *P. mirabilis* (strain 5 of fig 1), measured by radioadherence (●—●) and light microscopy (○---○). $\bar{x}$ and $\bar{s}$ =standard deviation.

buccal cells (fig. 2). Similarly, increasing the number of buccal cells while maintaining a constant number of bacteria led to a linear increase in the number of bacteria bound. The results of the radioadherence assay were found to correlate well with those obtained by light microscopy (fig. 2).

Saturation of binding sites

Addition of more than one strain of Enterobacteriaceae, tested over a 4.5-log concentration range, resulted in an increased average number of bacteria adherent per cell until a ratio of 10^3

FIG. 3.—Saturation of binding sites. The average number of bacteria bound per cell was determined after incubation of 1×10^5 buccal cells with 1×10^5 – 5×10^9 *K. pneumoniae* (strain 1 of fig 1) and *P. mirabilis* (strain 5 of fig 1).

bacteria per cell was reached. No more than 10^2 bacteria adhered per cell at higher bacteria:cell ratios (fig. 3). Results with other gram-negative bacilli tested were similar.

Specificity of binding

The ability of bacteria to interfere with binding of other gram-negative bacilli to buccal cells was studied. Fig. 4 illustrates that adherent bacteria (strains 1 and 4) of various genera significantly ($p < 0.05$) interfered with binding whereas “non-adherent” (strain 3) bacteria did not.

DISCUSSION

Colonisation of the human respiratory tract with gram-negative bacilli frequently occurs in hospitalised patients, and can be the initiation of significant infection with these organisms (Johanson, *et al.*, 1972; Pierce and Sanford, 1974). Light-microscopy assay of adherence was used in most reported studies of adherence (Gibbons and van Houte, 1971; Ward and Watt, 1972; Ellen and Gibbons, 1974; McNeish *et al.*, 1975; Aly *et al.*, 1977; Eden, Eriksson and Hanson, 1977) and reports of the use of radiolabelled organisms in the assay of adherence have also been published (Gibbons, van Houte and Liljemmark, 1972; Gould *et al.*, 1975; Hu, Collier and Baseman, 1975; Powell *et al.*, 1976; Salit and Gotschlich, 1977; Bartelt and Duncan, 1978). The results of our studies demonstrate a good correlation between the two assay systems.

FIG. 4.—Interference with binding; 1×10^5 buccal cells were incubated for 15 min. with various unlabelled suspensions (1–4) before an additional 45-min. incubation with 1×10^8 labelled *E. aerogenes* (strain 4 of fig 1). 1 = PBS (control), 2 = *K. pneumoniae* “nonadherent” strain 2, 3 = *Enterobacter aerogenes*, same strain as labelled *E. aerogenes*, 4 = *K. pneumoniae* “very adherent” strain 1.

The buccal cells assayed averaged $61 \mu\text{m}$ in maximum diameter and were flat and discoid. Thus, the total surface area of both sides is approximately $5000 \mu\text{m}^2$ per cell. Most Enterobacteriaceae average $0.5 \times 3 \mu\text{m}^2$ (Joklik and Willett, 1976), and thus might occupy about $1.5 \mu\text{m}^2$ if closely adherent to a buccal cell. Saturation of the binding sites on the buccal-cell surface occurred when less than 10% of the available surface was occupied. That radioactivity per cell did not continue to increase at high bacteria:cell ratios implies that bacterial aggregation was not significant; control counts of bacteria incubated alone for 45 min. were also noted not to be significant. The results of the saturation study therefore indicate that either the buccal-cell surface is nonhomogeneous in its distribution of bacterial receptor sites, or that repulsive forces or non-cooperativity prevented more bacteria from adhering. The ability of preincubation with “very adherent” bacteria to decrease subsequent adherence of the same and different genera, and lack of such effect with “nonadherent” bacteria, supports the conclusion that receptor sites do not cover the whole of the buccal-cell surface.

Preliminary results from the same bacteria and different mammalian tissue-cultured cell lines of epithelial and adrenal origin are similar. There appears to be a limited number of receptor sites on mammalian cells to which Enterobacteriaceae adhere, and interference with them can be demonstrated *in vitro*. It remains to be determined whether the receptor sites for all Enterobacteriaceae and other bacillary types on mammalian cells are the same, or whether multiple sites exist. However, the present findings that adherence of one species diminishes adherence of others indicate that the receptor sites for different species are either identical or are close together so that steric interference occurs. These observations suggest that use of innocuous bacteria or chemical agents to prevent, or perhaps even reverse, colonisation of mucous membranes with gram-negative bacilli might prove helpful in the control of some mucosal infections.

SUMMARY

A preliminary examination has been made of the adherence to human buccal cells of ^3H -thymidine labelled strains of Enterobacteriaceae isolated from sputa. The radioadherence

method was found to correlate well with the conventional light-microscope adherence technique. Saturation of buccal-cell binding sites with bacteria occurred when less than 10% of the buccal-cell surface was occupied. The adherence of *Enterobacter aerogenes* to buccal cells was impaired by the prior adherence of bacilli of either the same strain, or of a strain of *Klebsiella pneumoniae*.

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