Adherence of *Haemophilus influenzae* to Monkey Respiratory Tissue in Organ Culture

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We studied the adherence of *Haemophilus influenzae* to monkey respiratory mucosa using nasal turbinates maintained in organ culture. Adherence of capsulated and rough strains was not inhibited by monosaccharides, sucrose, human albumin, foetal calf serum or polyribophosphate. However, antisera directed against surface components decreased bacterial adherence. Although variation in adherence capacity in individual strains was observed there was no correlation with capsulation, anatomical site of strain isolation or biotype. Bacterial surface structures other than capsular material appear important in effecting upper respiratory tract colonization.

INTRODUCTION

In 1908 Guyot reported studies on the adhesiveness of bacteria, yet the potential importance of this phenomenon was not recognized. The role and mechanism of adherence and its relationship to colonization and pathogenicity was stimulated by the reports of Gibbons & Van Houte (1971). They described a correlation between the ability of *Streptococcus* species to adhere to specific oral epithelial cells *in vitro* and their natural distribution on oral surfaces *in vivo*. Since then a number of studies on the adherence properties of a variety of bacteria has been described (Beachey, 1981; Craven *et al.*, 1980; Johansen *et al.*, 1979; McGee *et al.*, 1981; Reed & Williams, 1978; Salit & Morton, 1981). However, little is known about the role of adherence in *Haemophilus influenzae* infections. Recently, Lampe *et al.* (1982) compared the adhesive capacity of capsulated and unencapsulated strains isolated from patients. They found the majority of capsulated *H. influenzae* to adhere poorly to human buccal epithelial cells, while 75–90% of unrelated unencapsulated strains adhered in greater numbers to these cells.

Adherence may reflect the ability of the strain to colonize animal tissue. Moxon & Vaughn (1981) compared the ability of isogenic pairs of *H. influenzae* (the capsulated transformants and the unencapsulated parent) to colonize the infant rat. In contrast with the adherence data of Lampe, they found no relationship between possession of a capsule and the ability to colonize the nasopharynx. However, the capsulated transformants were recovered from the nasopharynx for 14 d longer than the unencapsulated parent.

The disparity between the duration of colonization *in vivo* and adherence capacity *in vitro* may be due to inherent differences between the model systems (buccal epithelial cells versus nasopharyngeal mucosa) or could indicate a lack of correlation between adherence capacity and

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Abbreviations: BHI, brain/heart infusion broth; PBSG, phosphate-buffered saline + 0.1% gelatin; PRP, polyribophosphate.

colonization by *H. influenzae*. In an attempt to distinguish between these possibilities, we examined adherence using organ cultures derived from the upper respiratory tract of monkeys. This system has the advantage of using viable intact respiratory tissue.

In this paper we report on the characteristics of the respiratory organ culture model and examine the adherence patterns of certain capsulated and unencapsulated strains of *H. influenzae*.

METHODS

Bacteria. The *H. influenzae* strains studied are described in Table 2.

Media. The medium used to grow *H. influenzae* consisted of supplemented brain/heart infusion broth (3.5% w/v; BHI) and GC medium base agar plates (Difco) supplemented with (ml⁻¹): 4.0 mg glucose; 50.0 µg glutamine; 5.0 µg ferric nitrate; 0.01 µg thiamin pyrophosphate; 20.0 µg L-histidine; 20.0 µg haemin; and 20.0 µg β-nicotinamide adenine dinucleotide (Roberts *et al.*, 1981). Medium 199 (Grand Island Biological Co., Grand Island, NY, USA) containing 10% (v/v) foetal calf serum and 2 mM-L-glutamine (M199), and M199 supplemented with 60 µg penicillin G and 100 µg streptomycin ml⁻¹ were used for organ cultures.

Organ cultures. Infant (0–5 d) and adult (1–12 years) *Macacca nemestrina* were obtained from the Regional Primate Research Center at the University of Washington. Nasal turbinates were removed from the noses of the monkeys immediately after they were killed as part of the Regional Primate Research Center Tissue Program. The tissue was placed in M199 and transported to the laboratory. Nasal turbinates from infant monkeys were cut in half yielding tissue blocks approximately 3 by 1 mm (16.0 ± 2.0 mg, SD). Nasal turbinates from adult monkeys were cut into 3 mm squares (90 ± 20 mg, SD) as determined in four different experiments. All tissue was placed in 24-well, flat bottomed tissue trays (Flow Laboratories) with a capacity of 3.5 ml. Surface area of the tissue blocks was determined by placing the tissue on a sterile, etched grid slide and measuring the perimeter microscopically.

Attachment studies. The *H. influenzae* strains were grown to late exponential phase in BHI broth and frozen (–70 °C) in 16% (v/v) glycerol in BHI broth. On the day of use, samples were thawed and added to BHI, grown to an optical density of 0.7 at 675 nm and washed in 25 ml phosphate-buffered saline (7.5 g NaCl, 0.2 g KCl, 0.2 g KH₂PO₄, 1.15 g Na₂HPO₄ in 1.0 litre H₂O) containing 0.1% gelatin (PBSG). The bacteria were resuspended in M199 (at 37 °C) to the desired cell density and 2 ml of the bacterial suspension was added to wells containing tissue. This volume completely covered the tissue blocks. The tray was then incubated in a humidified 5% (v/v) CO₂ chamber at 36.5 °C, and tissue fragments were removed for study at the times specified. The tissue was removed aseptically, placed in a beaker, vigorously mixed in 25 ml PBSG (to remove non-adherent bacteria), weighed and homogenized in 1.0 ml PBSG with a 5 ml Potter–Elvehjem tissue grinder. Serial dilutions of the homogenate were plated on GC agar plates and incubated overnight in 5% (v/v) CO₂ at 36.5 °C, and the number of bacteria per tissue fragment was calculated. Each strain was tested five times with tissue fragments derived from different animals. Uninoculated tissue sections were homogenized, diluted and plated in duplicate; one set was incubated in an anaerobic chamber (Gas-Pak), the second in 5% (v/v) CO₂ in air, and both were incubated at 36.5 °C. This allowed determination of the number of indigenous bacteria adhering to, or present within, the tissue fragment. Indigenous flora was defined as those organisms which had a different colonial morphology than *H. influenzae* on agar plates, did not require NAD and haemin for growth and were not short or pleomorphic Gram-negative rods.

Antisera. Small donkeys (burros) were used for immunization; the protocol developed by Dr John Robbins (National Institutes of Health, Bethesda, Md., USA) was followed. This consisted of intravenous administration of an increasing dose of formalin-treated *H. influenzae* three times a week for an eight week period. One animal was immunized with a formalin-treated type b (Sb) strain E₁ and another with a type b strain no longer capable of producing detectable levels of capsule (Rb), Santo Rb (Roberts *et al.*, 1981). Both strains used were unable to agglutinate human red blood cells and did not have pili when examined by electron microscopy. Post-immunization serum was collected, heated to 56 °C for 30 min and stored at –20 °C. Antibody titre directed against somatic antigens was determined by slide agglutination, while anticapsular titres were determined by the Ouchterlony diffusion technique using purified polyribophosphate (PRP; Anderson *et al.*, 1971). Crude antisera similarly prepared have been shown to have activity against 21 *Haemophilus* antigens, including one against PRP (Buckmire, 1976). The Sb antiserum had a titre of 256 in agglutination tests and a titre of 16 in double diffusion tests. The Rb antiserum had a titre of 512 in agglutination tests and no detectable titre against PRP. Preimmune sera did not have detectable titres by these methods. No measurable bactericidal activity was found with any of the heat-inactivated sera (Anderson *et al.*, 1971); a complement source was not added.

Absorption of antisera. Santo Rb was grown in 250 ml BHI broth for 6 h and harvested by centrifugation at 5000 r.p.m. (2400 g) for 15 min. The cells were resuspended in 20 ml PBS containing 0.5% (v/v) formalin and stored at 4 °C overnight; the cells were then washed twice as above and resuspended in 5 ml PBS. Antiserum (5 ml) raised against strain Sb was mixed with 2.5 ml bacterial suspension, incubated at 37 °C for 2 h, and then stored overnight at 4 °C. The mixture was centrifuged at 5000 r.p.m. for 10 min. The top layer was removed and

recentrifuged. The resulting absorbed antiserum had a much lower agglutination titre (16) than the original untreated serum (256). In contrast, the anti-PRP titre was unaffected by the procedure and remained at 16. Antiserum (5 ml) raised against strain Rb was treated with Santo Rb bacteria. The absorbed antiserum had an agglutination titre of 32, which was also lower than that of the original untreated serum (512).

Polyribophosphate purification. PRP was prepared from strain E₁ as previously described (Anderson *et al.*, 1972, 1976).

Biotyping. This was done as previously described (Back & Oberhofer, 1978) using the Minithek method (BBL, Beckton Dickinson & Co., Cockeysville, Md., USA).

Chemical reagents. Sugars and human albumin were obtained from Sigma.

Histopathology. Selected tissue fragments were washed in 25 ml PBSG, fixed in formaldehyde, embedded, sectioned and stained with haematoxylin and eosin for light microscopy. Other samples were washed and fixed in 6.25% (v/v) glutaraldehyde in 0.1 M-cacodylate buffer, pH 7.3. The specimens were post-fixed with 2% (v/v) osmium tetroxide in sodium collidine, pH 7.4, dehydrated and embedded in Epon, and examined with an electron microscope (Zeiss model EM-9).

Statistical analysis. This was done using the F-test (Armitage, 1974).

RESULTS

Organ culture properties

Uninfected tissue was examined after various incubation times in M199 to assess cell damage and loss of cilia over a period of time. No detectable tissue changes were found in the first 36 h by microscopic and histologic examination. Incubation in M199 supplemented with penicillin G and streptomycin for 10–30 min caused no visible tissue damage; however, loss of cytoplasmic detail was observed after 24 h in this medium.

The presence of indigenous flora was determined. Infant animals (0–5 d old) had undetectable colonization (< 10 bacteria ml⁻¹), while older animals of (> 5 d old) had a small population (10–100 bacteria ml⁻¹) of facultative Gram-negative anaerobes and a few Gram-positive species. These did not appear to affect *H. influenzae* growth and were not a problem for the study.

Conditions for adhesion

Strains E₁ and Santo Rb were chosen for the pilot experiments because they have been well characterized in *in vivo* models and have been shown to be capable of colonizing the infant rat and monkey after intranasal inoculation (Moxon & Vaughn, 1981; Roberts *et al.*, 1981; Scheifele *et al.*, 1980).

Identical tissue fragments were incubated together in the same bacterial suspension, rinsed in 25 ml PBSG once, twice or three times, and the colony counts compared with unrinsed tissue. After rinsing $1.2\text{--}1.9 \times 10^6$ bacteria were recovered regardless of the number of rinses in PBSG. In contrast, the tissue incubated without rinsing had a consistently greater number of bacteria ($2.1\text{--}4.2 \times 10^6$). Since one rinse of the tissue in PBSG appeared to remove non-adherent bacteria, and further rinsing had little effect on the number of organisms associated with the tissue fragments, we inferred that these residual bacteria were 'adherent'.

The effect of tissue size on the number of adhering bacteria was examined. Adult tissue was cut into different-sized blocks (10 to 220 mg) and incubated with 10^8 c.f.u. of strain E₁ for 60 min. The number of adherent bacteria per fragment increased from 0.3×10^6 to 1.6×10^6 as the tissue fragments increased from 30 to 220 mg. Fragments weighing 90 mg were easy to manipulate and were selected for use in this study. On any one day the weight of the fragments varied by < 1.0 mg while the ciliated surface areas remained the same; fragments prepared on different days varied by < 20 mg. The effect of the cut edge was examined by comparing blocks with similar weights and surface areas but having one, two or three cut edges. No significant differences in the number of adherent bacteria were observed. Electron microscopic examination revealed no bacteria adhering to the cut edges of the tissue fragments, while bacteria were seen to be associated with the cilia, suggesting that the bacteria preferentially bind to the ciliated surface. This correlates with observations in other studies (Denny, 1974).

The effect of inoculum size on the ability of strain E₁ to adhere to adult respiratory mucosa is illustrated in Fig. 1. With adult tissue, bacterial adherence increased linearly with bacterial

Fig. 1

Fig. 2

Fig. 1. Numbers of *H. influenzae* strain E₁ adhering to adult tissue blocks at different bacterial concentrations. ▲, Infant tissue; ●, adult tissue. The means of three experiments after 60 min incubation are shown.

Fig. 2. Adherence of *H. influenzae* strain E₁ to adult nasal tissue with increasing incubation time. Starting concentrations of 10¹⁰ (●), 10⁹ (▲) and 10⁸ (■) bacteria were used. The means of two experiments are shown.

concentration in the range 10²–10¹⁰ ml⁻¹. In contrast, the binding capacity of the infant tissue appeared to be saturated at 10⁶ bacteria per fragment. Animals three weeks old, or older, had larger turbinates that could be cut into blocks 50–90 mg net weight with surface areas similar to adult fragments; these had more adherent bacteria. However, when large fragments from young animals (3 weeks to 6 months old) were examined they consistently had fewer adherent bacteria than adult tissue incubated in the same bacterial suspension.

The influence of incubation time on the adherence of bacteria to adult tissue was examined at three inocula (10¹⁰, 10⁹ and 10⁸ c.f.u.). The majority of adherence occurred within the first 30 min (Fig. 2); similar results were found with infant tissue. Sections of tissue incubated with strains E₁ or Santo Sb for 30 min were washed and prepared for electron microscopic examination. After 30 min incubation bacteria were associated primarily with cilia (Fig. 3). After incubation for 60 min bacteria were associated with cell surfaces, and internal vacuoles which appeared to contain bacteria were evident for E₁. Longer incubation periods (> 90 min) were required for Santo Rb to become internalized (Fig. 4). We found no significant differences in adherence patterns between early exponential phase organisms and organisms harvested in the early stationary stage (data not shown).

The reproducibility of results from different animals was examined by testing the adherence of 10⁸ c.f.u. of strain E₁ with tissue from six adult and six infant monkeys. One animal was examined per day and four or six tissue blocks were prepared from each animal and assayed. The adherence of E₁ for the six different animals, expressed as number of organisms per block, was calculated as 1.8 ± 0.5 (SD) $\times 10^6$ c.f.u. using adult tissue and 0.19 ± 0.09 (SD) $\times 10^6$ c.f.u. using infant tissue. Variation between different tissue blocks from the same animal was less than with tissue from different animals; SD $\pm 0.2 \times 10^6$ with the same adult animal and SD $\pm 0.5 \times 10^6$ between different adult animals.

Bacteria were incubated in M199 with 10% foetal calf serum and in M199 without serum, to rule out the possibility of bacterial adherence to foetal calf serum components. No difference was observed with either Sb or Rb cells when the test was done without serum in the media.

Fig. 3. Electron micrograph of nasal tissue after 30 min incubation with strain E₁. The arrows indicate bacterial forms in association with cilia. Bar, 2 μ m.

Fig. 4. Electron micrograph of tissue incubated with Santo Rb for 180 min. Internalized cells are indicated by the arrow. Bar, 1 μ m.

Table 1. *Effect of preincubation with antisera prior to adherence testing*

The number of bacteria adhering was determined after incubation. The PBS control values (10^6 c.f.u.) were defined as 100%; the other values were simultaneously compared to the PBS control. The mean of four different experiments is shown.

Preincubation conditions	Adherent bacteria (%)
E ₁ + PBS	100
E ₁ + Preimmune sera	90
E ₁ + Antisera Sb	33
E ₁ + Antisera Rb	14
E ₁ + Antisera Sb, absorbed with Rb	67
Santo Rb + PBS	100
Santo Rb + preimmune sera	95
Santo Rb + antisera Sb	30
Santo Rb + antisera Rb	10
Santo Rb + antisera Rb, absorbed with Rb	95

Inhibition of adherence

D-Glucose, D-mannose, D-galactose, ribitol, L-rhamnose, D-raffinose, D-arabinose, D-fructose, D-fucose, D-xylose, D-ribose, sucrose or human albumin, all at $500 \mu\text{g ml}^{-1}$, or PRP at $200 \mu\text{g ml}^{-1}$ were incubated with either strain Rb or Sb at 36.5°C for 30 min prior to exposure to tissue. None of the 12 sugars tested blocked adherence, and neither albumin nor PRP altered the ability of bacteria to adhere. Antisera at a 1/75 dilution in M199 were incubated with selected bacterial strains for 60 min at 36.5°C . At this dilution, clumping of the cells was not visually apparent, and the number of c.f.u. was not reduced in comparison to bacteria incubated without antisera. This concentration of antisera was added to the bacteria prior to incubation with the tissue. Preimmune sera had little effect on adherence; in contrast, antisera reduced the ability of the bacteria to adhere. Antiserum raised against the Rb strain was more effective than either the Sb antiserum or antiserum absorbed with strain Rb (Table 1).

Adherence of capsulated and unencapsulated H. influenzae

Nasal tissue from adult animals was chosen for the study because of the ready availability of these tissue specimens. Tissue fragments were inoculated with 10^8 bacteria and incubated for 60 min at 36.5°C to examine the ability of selected capsulated and unencapsulated *H. influenzae* strains to adhere (Table 2). The variability in the size of the inoculum between experiments was $< 5.0 \times 10^5$ c.f.u. Twenty strains (six unencapsulated, nine type b and five of other capsule types) adhered at $< 2.0 \times 10^6$ bacteria per tissue block. Nine strains (four unencapsulated, one type b and four of other capsule types) adhered at $> 2.0 \times 10^6$ bacteria per tissue block. The adherence values ranged from 0.3×10^6 to 11.4×10^6 bacteria per tissue block. DC33, DC15 (type b), GL-14 (type e), the three type f strains and C500 and C501 (unencapsulated) were the least adherent strains ($< 1.0 \times 10^6$) while C668 (type b), C188 (type c) and C546, GL-8 and C800 (unencapsulated) were the most adherent ($> 5.0 \times 10^6$). However, as a group, the unencapsulated strains were not significantly more adherent ($P > 0.1$) than either the type b or types a, c, d, e or f. We found no relationship between adherence and site of strain isolation for either capsulated or unencapsulated strains (Fig. 5a). There was no correlation between biotype and adherence (Fig. 5b).

The use of isogenic strains eliminates other bacterial characteristics which could effect adherence. To further test the correlation between adherence capacity and the presence of capsule, two unencapsulated laboratory strains, Santo Rb and Good Rd, and their capsulated transformants (Santo Sa, Santo Sb, Good Sa, Good Sb, Good Sd), and E₁ and the unencapsulated mutant S₂, were compared (Roberts *et al.*, 1981). No significant difference ($P > 0.1$) in the number of bacteria adhering to a fragment was observed between isogenic capsulated and unencapsulated derivatives. Piliated and non-piliated isogenic pairs of E₁ also showed no significant differences in *in vitro* adherence.

Table 2. Adherence of *Haemophilus influenzae* to nasal turbinates in culture

Clinical strains:	Capsule type*	Biotype	Isolation site†	$10^{-6} \times$ Adherent bacteria per tissue block‡ ($\pm$ SD)	Source§
C121	b	I	CSF	1.6 $\pm$ 0.6	CHMC
C616	b	I	CSF	1.4 $\pm$ 0.4	COHMC
C668	b	I	CSF	5.9 $\pm$ 0.3	COHMC
C735	b	I	CSF	1.2 $\pm$ 0.2	COHMC
Rab	b	IV	CSF	2.0 $\pm$ 0.1	G. Leidy
C654	b	I	CSF	1.8 $\pm$ 0.3	COHMC
R375	b	II	NP	1.3 $\pm$ 0.4	M. Glode
DC37	b	II	NP	2.0 $\pm$ 0.5	D. Scheifele
DC33	b	I	NP	0.5 $\pm$ 0.2	D. Scheifele
DC15	b	IV	NP	0.3 $\pm$ 0.1	D. Scheifele
R421	a	II	unknown	2.9 $\pm$ 0.5	J. Connor
C188	c	IV	B	5.5 $\pm$ 2.0	CHMC
Garf	d	IV	NP	1.1 $\pm$ 0.5	G. Leidy
GL-16	e	IV	B	4.4 $\pm$ 0.5	G. Leidy
GL-14	e	IV	CSF	0.3 $\pm$ 0.1	G. Leidy
R600	e	III	NP	3.2 $\pm$ 1.0	D. Scheifele
C909	f	I	CSF	0.53 $\pm$ 0.1	COHMC
GL-7	f	I	B	0.82 $\pm$ 0.2	G. Leidy
R731	f	IV	ear	0.3 $\pm$ 0.1	S. H. Sell
C546	u	V	CSF	9.9 $\pm$ 0.3	COHMC
GL-8	u	III	CSF	6.7 $\pm$ 0.2	G. Leidy
Ramirez	u	II	CSF	2.6 $\pm$ 0.6	G. Leidy
C501	u	III	NP	0.72 $\pm$ 0.5	COHMC
DC45	u	III	NP	1.8 $\pm$ 0.2	D. Scheifele
C453	u	II	NP	3.4 $\pm$ 0.1	COHMC
C500	u	II	NP	0.37 $\pm$ 0.2	COHMC
C488	u	II	NP	1.2 $\pm$ 0.2	COHMC
C763	u	I	S	1.4 $\pm$ 0.2	COHMC
C793	u	I	S	1.5 $\pm$ 0.2	COHMC
C800	u	III	S	11.4 $\pm$ 2.0	COHMC
Laboratory-derived strains:					
E ₁	b		CSF	1.8 $\pm$ 0.51	Anderson <i>et al.</i> (1976)
S ₂	(b)¶			0.69 $\pm$ 0.5	Anderson <i>et al.</i> (1976)
Santo Sb	b		CSF	1.4 $\pm$ 0.2	Roberts <i>et al.</i> (1981)
Santo Sa	a		V	1.5 $\pm$ 0.2	Roberts <i>et al.</i> (1981)
Santo Rb	u		V	2.0 $\pm$ 0.5	Roberts <i>et al.</i> (1981)
Good Rd	u		V	0.46 $\pm$ 0.4	Roberts <i>et al.</i> (1981)
Good Sb	b		V	0.52 $\pm$ 0.3	Roberts <i>et al.</i> (1981)
Good Sa	a		V	0.54 $\pm$ 0.1	Roberts <i>et al.</i> (1981)
Good Sd	d		V	0.46 $\pm$ 0.1	Roberts <i>et al.</i> (1981)

* Typed by using Difco antisera for types a-f; u indicates unreactive with this antiserum.

† CSF, cerebrospinal fluid; B, blood; NP, nasopharynx; S, sputum; V, variant.

‡ Means of five separate experiments.

§ COHMC, Children's Orthopedic Hospital and Medical Center, Seattle, Wash., USA; CHMC, Children's Hospital and Medical Center, Boston, Mass., USA; G. Leidy, Columbia University College of Physicians and Surgeons, New York, NY, USA; M. Glode, Children's Hospital, Denver, Colo., USA; D. Scheifele, Children's Hospital, Vancouver, BC, Canada; J. Connor, University of California, San Diego, Calif., USA; S. H. Sell, Vanderbilt University, Nashville, Tenn., USA.

¶ Mean of six separate experiments.

¶ Produces <1/1000 the amount of capsule compared to the parent E₁.

Penetration of nasal tissue in culture

Initial experiments with strain E₁ and Santo Rb indicated that *H. influenzae* could penetrate the epithelial layer of the tissue fragment. Isogenic strains E₁, S₂, Santo Sb, Santo Sa and Santo Rb were chosen for further study. No difference in penetration capacity was found when capsulated organisms were compared with unencapsulated isogenic derivatives. However,

Fig. 5. *In vitro* adherence (see Table 2) is plotted versus source of isolation (a) or biotype (b). ●, *H. influenzae* type b; ■, types a, c, d, e, f; ▲, unencapsulated strains. CSF, cerebrospinal fluid; NP, nasopharynx; Other, blood or sputum isolation site.

Fig. 6. Electron micrograph of tissue containing bacteria dividing in the submucosa (arrowed). Bar, 2 μm.

strain-to-strain variation in the time required for penetration as assayed by electron microscopic examination was evident. Both E_1 and its unencapsulated derivative S_2 penetrated the tissue in a short (30–60 min) incubation period. Structures which appeared to be bacteria enclosed within vacuoles near the cell surfaces were found in tissue samples after 30 min and 60 min incubation. After 60 min bacteria were found in the submucosa some distance from the cell surface. Occasionally organisms in these locations were seen to be dividing (Fig. 6). In contrast, the Santo derivatives required longer incubation times (3–4 h) before penetration was observed.

DISCUSSION

Two groups have examined the *in vitro* adherence of *H. influenzae* (Lewis & Dajani, 1980; Lampe *et al.*, 1982). Both found capsulated type b strains to adhere poorly to buccal epithelial cells or established cell lines, while unrelated unencapsulated strains had increased adherence capacity. However, unencapsulated *H. influenzae*, like Gram-negative enteric bacteria, did not adhere in large numbers to buccal epithelial cells (Lampe *et al.*, 1982). They suggested that differential adherence between capsulated and unencapsulated strains could explain differences in colonization, pathogenesis and types of infection caused by these strains. Our findings differ from their results. Less adherent strains were identified in both capsulated and unencapsulated groups. However, we found no correlation between capsulation and the ability of bacteria to adhere to organ cultures. Nor could adherence be correlated with biotype or original anatomic site of isolation.

Recent studies have demonstrated that the attachment of *Neisseria meningitidis* to pharyngeal cells was better than their attachment to buccal epithelial cells (Craven *et al.*, 1980; Salit & Morton, 1981). This suggests that the cells present in the tissue normally colonized by the organism under study should be used for adherence studies. Neither buccal epithelial cells nor established cell lines resemble the ciliated nasopharyngeal tissue usually colonized by *H. influenzae*, and experiments with such cells may not accurately represent the *in vivo* environment. For this reason we used nasal mucosa for adherence assays. Inherent differences in the cells used could greatly influence the apparent adherence patterns of *H. influenzae*. Nasopharyngeal tissue derived from subhuman primates may bind *H. influenzae* differently than human buccal epithelial cells; this also could explain the differences between our results and those obtained by Lampe *et al.* (1982).

Guerina *et al.* (1982) found that non-piliated and poorly piliated *H. influenzae* adhered in lower numbers to human oropharyngeal cells as compared to moderate-to-heavily piliated bacteria. Our initial findings suggested that the presence of pili did not greatly influence adherence; however, we did not determine the grade of piliation of the bacteria. Therefore, piliation can not be ruled out as a potential bacterial adhesin.

With similarly-sized tissue fragments adherence to adult monkey tissue was greater than to tissue from infants. Therefore, it is possible that the number and distribution of tissue receptors for *H. influenzae* changes with age.

Adherence was neither inhibited nor enhanced by the 12 sugars tested or by albumin or foetal calf serum. This correlates with observations by Wideback & Kronvall (1982) who found that the four strains of *H. influenzae* they examined did not adhere to albumin. Antiserum directed against noncapsular antigens (Rb antiserum) inhibited adherence better than either Sb antiserum or antiserum to strain Sb absorbed with strain Rb. This suggests that the bacterial adhesin(s) of *H. influenzae* are more likely to be surface molecules other than capsule.

When compared with other type b *H. influenzae*, strain E₁ is more virulent in animals (Roberts *et al.*, 1981 and unpublished observations). Similarly, our preliminary data suggest that strain E₁ and its unencapsulated derivatives are able to penetrate the organ culture faster (30 to 60 min) than other strains tested (> 90 min). Therefore, it is possible that the adherence assay underestimates the number of adherent bacteria or that the value represents both internal and external bacteria for this strain.

The stability of bacterial binding was examined by removing the bacterial suspension and replacing it with fresh M199. The loss of adherent bacteria was not significant, suggesting that bacteria-tissue interaction is more complex than the steady state between binding and release of bacteria. The possibility that there is more than one adhesin-receptor interaction has been suggested in other systems (Gibbons *et al.*, 1983) and must be considered with *H. influenzae*.

The tissue used in these studies was colonized by a small population of indigenous bacteria, which could have decreased or enhanced adherence of *H. influenzae*. The actual number of bacteria in this system was less than that found on buccal or pharyngeal cells used in other adherence studies (Gibbons & Van Houte, 1971; Salit & Morton, 1981). Normal flora organisms were not seen in cultures containing isolated colonies of *H. influenzae*, presumably because of the

low numbers, but the use of a non-sterile system more closely resembles the normal *in vivo* conditions for *H. influenzae* colonization.

Previous data obtained *in vivo* from rats indicated that submucosal colonies of *H. influenzae* are evident 48 h after inoculation in nasal turbinates (Smith *et al.*, 1982). The penetration studies reported here suggest that the presence or absence of the capsule does not appear to influence the ability of the organism to penetrate the tissue in culture. It is possible that the differences in the time required for penetration may play a role in virulence.

The data presented here suggest that, as a group, unencapsulated *H. influenzae* are not more adherent than capsulated strains. Our data also suggest that the distribution of *H. influenzae* in the human population may not be attributed solely to the presence or absence of a capsule as suggested by Lampe *et al.* (1982).

Organ models have been used successfully to study and gain information about other bacteria (Gabridge *et al.*, 1978; McGee *et al.*, 1976; Ramphal & Pyle, 1983). The model described here should contribute to the study of adherence and its relationship to colonization and disease. Such work may lead to better ways of controlling, treating, and preventing Haemophilus disease.

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REFERENCES

- ANDERSON, P., JOHNSTON, R. B. & SMITH, D. H. (1971). Human serum activities against *H. influenzae* type b. *Journal of Clinical Investigation* **52**, 31–38.
- ANDERSON, P., JOHNSTON, R. B., JR, WETTERLOW, L. H. & SMITH, D. (1972). Immunization of humans with polyribophosphate, the capsular antigen of *Haemophilus influenzae*, type b. *Journal of Clinical Investigation* **51**, 39–44.
- ANDERSON, P., PITT, J. & SMITH, D. H. (1976). Synthesis and release of polyribophosphate by *Haemophilus influenzae* type b *in vitro*. *Infection and Immunity* **13**, 581–589.
- ARMITAGE, P. (1974). *Statistical Methods in Medical Research*, pp. 131–138. New York: John Wiley and Sons.
- BACK, A. E. & OBERHOFER, T. R. (1978). Use of the minitek system for biotyping *Haemophilus* species. *Journal of Clinical Microbiology* **7**, 312–313.
- BEACHEY, E. H. (1981). Bacterial adherence: adhesion-receptor interactions mediating the attachment of bacteria to mucosal surfaces. *Journal of Infectious Diseases* **143**, 325–345.
- BUCKMIRE, F. L. A. (1976). Identification and quantification of capsular antigen in capsulated and non-capsulated strains of *H. influenzae* type b by crossed-immunoelectrophoresis. *Infection and Immunity* **13**, 1733–1742.
- CRAVEN, D. E., PEPPLER, M. S., FRASCH, C. E., MOCCA, L. F., McGRATH, P. O. & WASHINGTON, G. (1980). Adherence of isolates of *Neisseria meningitidis* from patients and carriers to human buccal epithelial cells. *Journal of Infectious Diseases* **142**, 556–568.
- DENNY, F. W. (1974). Effect of a toxin produced by *Haemophilus influenzae* on ciliated respiratory epithelium. *Journal of Infectious Diseases* **129**, 93–100.
- GABRIDGE, M. G., GUNDERSON, H., SCHAEFFER, S. L. & BURDEN-STAHLL, Y. D. (1978). Ciliated respiratory epithelial monolayers: new model for *Mycoplasma pneumoniae* infection. *Infection and Immunity* **21**, 333–336.
- GIBBONS, R. S. & VAN HOUTE, J. (1971). Selective bacterial adherence to oral epithelial surfaces and its role as an ecological determinant. *Infection and Immunity* **3**, 567–573.
- GIBBONS, R. S., MORENO, E. C. & ETHERDEN, I. (1983). Concentration-dependent multiple binding sites on saliva-treated hydroxyapatite for *Streptococcus sanguis*. *Infection and Immunity* **39**, 280–289.
- GUERINA, N. G., LANGERMAN, S., CLEGG, H. W., KESSLER, T. W., GOLDMAN, D. A. & GILSDORF, J. R. (1982). Adherence of piliated *Haemophilus influenzae* type b to human oropharyngeal cells. *Journal of Infectious Diseases* **146**, 564.
- GUYOT, G. (1908). Über die bakterielle Hamagglutination. *Zentralblatt für Bakteriologie* **47**, 604–653.
- JOHANSON, W. G., JR, WOODS, D. E. & CHAUDHARI, T. (1979). Association of respiratory tract colonization with adherence of Gram-negative bacilli to epithelial cells. *Journal of Infectious Diseases* **139**, 667–673.
- LAMPE, R. M., MASON, E. O., KAPLAN, S. L., UMSTEAD, C. L., YOW, M. D. & FEIGIN, R. D. (1982). Adherence of *Haemophilus influenzae* to buccal epithelial cells. *Infection and Immunity* **35**, 166–172.
- LEWIS, J. M. & DAJANI, A. S. (1980). Adherence of *Haemophilus influenzae* to human epithelial cells. In *Abstracts of the Annual Meeting of the American Society for Microbiology*, p. 26. Washington, DC: American Society for Microbiology.
- MCGEE, Z. A., JOHNSON, A. P. & TAYLOR-ROBINSON, D. (1976). Human fallopian tubes in organ culture: preparation, maintenance, and quantitation of damage by pathogenic microorganisms. *Infection and Immunity* **13**, 608–618.
- MCGEE, Z. A., JOHNSON, A. P. & TAYLOR-ROBINSON, D. (1981). Pathogenic mechanisms of *Neisseria*

- gonorrhoeae*: observations on damage to human fallopian tubes in organ culture by gonococci of colony type 1 or type 4. *Journal of Infectious Diseases* **143**, 413–422.
- MOXON, E. R. & VAUGHN, K. A. (1981). The type b capsular polysaccharide as a virulence determinant of *Haemophilus influenzae*: studies using clinical isolates and laboratory transformants. *Journal of Infectious Diseases* **143**, 517–524.
- RAMPHAL, R. & PYLE, M. (1983). Adherence of mucoid and non-mucoid *Pseudomonas aeruginosa* to acid-injured tracheal epithelium. *Infection and Immunity* **41**, 345–351.
- REED, W. P. & WILLIAMS, R. C., JR. (1978). Bacterial adherence: first step on pathogenesis of certain infections. *Journal of Chronic Diseases* **31**, 67–72.
- ROBERTS, M., STULL, T. L. & SMITH, A. L. (1981). Comparative virulence of *Haemophilus influenzae* with a type b or type d capsule. *Infection and Immunity* **32**, 518–524.
- SALIT, I. E. & MORTON, G. (1981). Adherence of *Neisseria meningitidis* to human epithelial cells. *Infection and Immunity* **31**, 430–435.
- SCHEIFELE, D. W., DAWN, R. S., SYRIOPOULOU, V. P., AVERILL, D. R. & SMITH, A. L. (1980). *Haemophilus influenzae*, bacteria and meningitis in infant primates. *Journal of Laboratory and Clinical Medicine* **95**, 450–462.
- SMITH, A. L., DAUM, R. S., SCHEIFELE, D., SYRIOPOULOU, V., AVERILL, D. R., ROBERTS, M. C. & STULL, T. L. (1982). Pathogenesis of *Haemophilus influenzae* meningitis. In *Haemophilus influenzae: Epidemiology, Immunology and Prevention of Disease*, pp. 89–109. Edited by S. H. Sell & P. F. Wright. Amsterdam: Elsevier Science Publishing.
- WIDEBACK, K. & KRONVALL, G. (1982). Surface receptors for serum albumin in group C and G Streptococci show three different types of albumin specificity. *Infection and Immunity* **38**, 1154–1163.