Microbial adherence to vulvar epithelial cells

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Summary. Under uniform experimental conditions, different degrees of selective attachment of *Staphylococcus aureus* and *Candida albicans* to epithelial cells of the labium majus, the labium minus, and the vagina were compared and contrasted with those found in studies with cells of the buccal and nasal mucosa and forearm skin by a novel analysis of adherence density. For both micro-organisms, the larger, rougher cells of the labium majus gave the highest adherence scores matched only by the interaction of *S. aureus* with fully keratinised nasal epithelial cells. Increasing acidity to pH 3·5 enhanced microbial adherence to vaginal cells. Menstruation also influenced attachment; highest densities were reached between the third and fourth weeks of the cycle. Autogenous ribitol teichoic acid was found to block the attachment of *S. aureus* to labium majus and labium minus cells by 76% and 81% respectively, and to vaginal cells by 66%. Adherence is considered to be an important attribute of vulvar ecology and may be a determinant of infectious disease.

Introduction

The specific attachment of bacteria and fungi to surface epithelial cells of the host is well documented (Gibbons and Van Houte, 1975; Beachey, 1980); investigators are now determining the possible functions of adhesion in various microbial ecosystems of the body (Bibel *et al.*, 1983; Beachey *et al.*, 1983; Freter and Jones, 1983). The characteristics of cellular interaction differ for a given micro-organism or host tissue. The molecular basis of this specificity varies widely, and a micro-organism may possess more than one type of adhesin for coupling with complementary receptors (Woods *et al.*, 1980; Bibel *et al.*, 1982).

Of the body regions thus far examined for adherence, the vulva has been characterised least satisfactorily. The vulva has an especially complex ecology related to several distinct tissues and influenced by hormonal cycles. Adherence studies have been limited to the vagina (Mårdh and Weström, 1976; Botta, 1979; Sobel *et al.*, 1981a and b), and investigations of the microbiology of the labia, particularly the labium majus, are rare (Aly *et al.*, 1979).

This report, focusing on the labium majus, labium minus, and vagina, demonstrates the features of microbial adherence unique to this genital area. As observed in the mouth (Gibbons and Van Houte, 1975), adjacent anatomical structures differ in adherence capability; this explains in part their carriage of particular normal flora and resistance to pathogens.

Materials and methods

Micro-organisms

Staphylococcus aureus strain 502A, *Candida albicans* isolate 57409, *Escherichia coli*, *Acinetobacter calcoaceticus*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, and an unidentified cutaneous α-haemolytic streptococcus were grown in Tryptic Soy Broth (TSB; Difco) at 37°C for 18 h. All of these micro-organisms had been subcultured in the laboratory at least ten times. Before adherence testing, the micro-organisms were centrifuged in two changes of buffer (usually 0·067 M phosphate-buffered saline, PBS; pH 6·8). In one comparative experiment, *S. aureus* was grown in Brain-Heart Infusion broth (BHI) and in MR-VP broth (Difco).

Volunteers

Seven healthy women between 22 and 40 years old gave their informed consent to these studies. They were not nasal carriers of *S. aureus*, but the labia majora of two subjects appeared to be colonised by this bacterium for the 2-month period of our observations. None was using oral contraceptives. Vaginal pH was determined by paper-strip indicator.

Epithelial cells

Cells from the mid-labium majus, labium minus, vagina, and buccal and nasal mucosa, harvested by scraping with moistened wood spatulas, were dispersed in 2 ml of buffer (PBS at pH 6·8 or, for vaginal cells, 0·1 M citric acid-phosphate, pH 3·5) and washed by filtration on a 10-μm pore-size filter (Mitex Millipore; Aly *et al.*, 1977). Volar forearm cells were collected by the detergent-scrub method (Williamson and Kligman, 1965). In this procedure, a circular sterile aluminium ring (4·15 cm²) is held against the tissue to retain 1 ml of 0·075 M phosphate buffer, pH 7·9, with Triton X-100 0·1%. After scraping for 1 min with a Teflon scrubber "policeman", the dislodged cells are obtained with a pipette and washed as above. In later studies, we found that buccal and vaginal cells, because of their larger size that impedes filtration, could be more efficiently washed by centrifugation (twice in equal volumes of buffer). We pooled two or three samples of buccal, forearm, nasal, and labial cells; vaginal specimens were tested singly. Subjects donated no more than one sample of a given tissue per week.

Adherence testing

One ml of a microbial suspension containing 10^7–10^8 cfu/ml was mixed with 1 ml of host epithelial-cell suspension and incubated for 90 min at 37°C in a shaking water bath (Aly *et al.*, 1977). Bacterial suspensions were then washed by membrane (Millipore) filtration, but candida-cell mixtures were more effectively separated by dilution in buffer. Smears of the test suspensions on glass slides were fixed by heat and stained with crystal violet. By microscopy, we tallied the number of adhering micro-organisms on each of 18–21 cells/class and calculated the average.

In later studies, we used a micrometer eyepiece to measure the size of at least 20 epithelial cells and counted their respective adhering micro-organisms; we then calculated the average density of adherence. Because the various host cells differ in area, especially between classes, a standard of comparison was thereby provided. Adherence scores were analysed by Student's *t* test.

Teichoic acid blockage of adherence

Using our previously described procedures (Aly *et al.*, 1980; Bibel *et al.*, 1982) we tested the ability of staphylococcal ribitol teichoic acid to prevent the attachment of subsequently added *S. aureus* to vulvar epithelial cells. Briefly, a suspension of host cells and teichoic acid 2 mg/ml was incubated at 37°C for 90 min. *S. aureus* was then added and the tubes were again incubated for 90 min. The preparations were washed and observed as above. We evaluated scores by comparing them with those for tests with untreated cells. Labium majus cells were tested in PBS, pH 6·8; vaginal cells were suspended in citric acid-phosphate buffer. pH 3·5.

Results

Two general types of cells from the labium majus could be distinguished: those of medium size with smooth texture and fairly even perimeter and those of large size, rough consistency, and irregular shape. Small fragments of the larger keratinised cells were plentiful but were not scored. Transitional cells were also common, making differentiation difficult; the stage of enucleation was not a helpful index.

Staphylococcal adherence was tested simultaneously with labium majus and nasal cells. In a preliminary study, labial cells from two *S. aureus* nasal carriers and two non-carriers were also compared. The nasal cell system, being well characterised and familiar (Aly *et al.*, 1977; Bibel *et al.*, 1982), served as an inoculum and buffer control. In all five tests, adherence differed significantly between labial cell types (p < 0·001) and their nasal-cell equivalent (upper granular and fully keratinised layers, p < 0·001 and p < 0·001). Adherence scores on medium and large labial cells averaged respectively 72% and 63% less than those of nasal cells. No significant differences were found in adherence of *S. aureus* to labial cells of nasal carriers and non-carriers.

Table I, listing the average population of given micro-organisms per labial cell, demonstrates the superior adherence of *S. aureus*. Streptococci attained intermediate levels, 40–50% of staphylococcal counts. *C. albicans* adherence was poor. *E. coli* did not attach to this tissue. Uninoculated control preparations of labium majus, labium minus, and vaginal cells displayed an average background of less than three staphylococci (range 0·3–3·05) and no candida organisms per cell.

TSB has been the routine culture medium. The effect of medium on adherence was ascertained by

Table I. Adherence of various micro-organisms to labium majus cells

Micro-organism	Index of adherence*	
	Medium, smooth cell	Large, rough cell
S. aureus	47·28 ± 14·52	85·03 ± 22·35
Str. pyogenes	23·20 ± 9·29	37·20 ± 8·36
α-haemolytic streptococcus	24·56 ± 14·13	28·89 ± 12·57
E. coli	1·11 ± 1·36	1·14 ± 1·21
P. aeruginosa	16·58 ± 7·41	28·17 ± 19·20
A. calcoaceticus	8·21 ± 6·53	11·43 ± 6·61
C. albicans	9·31 ± 4·87	24·54 ± 9·03

* Number of micro-organisms per cell of stated type expressed as mean of 18–21 cells ± SD.

Table II. Comparison of *S. aureus* adherence to various epithelial cells*

Cell source and type	Area (μm^2)†	Number of cocci	Density‡
Nose			
spinous	294·7 ± 105·3	4·70 ± 3·83	0·019 ± 0·016
low granular	541·9 ± 140·2	18·70 ± 4·45	0·037 ± 0·015
high granular	882·3 ± 129·6	59·67 ± 14·86	0·068 ± 0·017
keratinised	1095·0 ± 217·3	119·73 ± 31·10	0·113 ± 0·036
Forearm	1196·7 ± 340·9	29·87 ± 14·19	0·026 ± 0·012
Buccal mucosa	2075·3 ± 640·9	63·27 ± 28·29	0·031 ± 0·013
Labium majus			
medium, smooth	708·5 ± 276·9	37·47 ± 9·78	0·059 ± 0·026
large, rough	832·5 ± 345·8	87·75 ± 28·00	0·119 ± 0·052
Labium minus	1468·2 ± 351·6	20·26 ± 7·34	0·015 ± 0·001
Vagina	1406·2 ± 575·5	13·10 ± 7·68	0·011 ± 0·009

* Standard buffer: 0·067 M PBS, *p*H 6·8.
† Mean of 20 cells ± SD.
‡ Cocci/μm^2, calculated per cell then averaged.

Table III. Comparison of *C. albicans* adherence to various epithelial cells*

Cell source and type	Area (μm^2)†	Number of candida cells	Density‡
Nose			
spinous	249·1 ± 179·3	0·78 ± 0·83	0·0035 ± 0·0049
low granular	526·7 ± 137·5	2·33 ± 2·12	0·0048 ± 0·0048
high granular	900·0 ± 138·9	1·56 ± 1·33	0·0018 ± 0·0015
keratinised	1051·1 ± 94·4	2·67 ± 1·66	0·0025 ± 0·0014
Forearm	1203·0 ± 333·2	5·50 ± 3·56	0·0046 ± 0·0021
Buccal mucosa	2480·0 ± 481·9	13·65 ± 4·67	0·0056 ± 0·0018
Labium majus			
medium, smooth	756·2 ± 248·7	9·31 ± 4·87	0·0129 ± 0·0075
large, rough	855·8 ± 312·7	24·54 ± 9·03	0·0305 ± 0·0134
Labium minus	1364·8 ± 254·7	6·80 ± 2·58	0·0049 ± 0·0009
Vagina	1512·1 ± 200·2	9·50 ± 4·87	0·0065 ± 0·0036

* Standard buffer: 0·067 M PBS, *p*H 6·8.
† Mean of 20 cells ± SD.
‡ Candida cells/μm^2, calculated per cell then averaged.

growing *S. aureus* in simple MR-VP broth (consisting of peptone, dextrose, and phosphate) and in complex broth (BHI). Recentrifuged in buffer, the staphylococci from BHI broth did not form a pellet like those from MR-VP broth. Instead, the bacteria clung to the side of the test tube but were easily dispersed. They adhered twice as well as staphylococci grown in MR-VP broth (p < 0·001 for both types of labial cell). BHI broth cultures gave the same adhesion scores as organisms grown in TSB.

Table II compares the average cellular area, tally of attached *S. aureus*, and calculated density of adherence of various epithelial cells. The largest cells examined were from the buccal mucosa, but the density of attached staphylococci, despite their moderately large number, was similar to our findings with forearm and lower granular layer nasal cells. Fully keratinised nasal cells and the large, rough cells of the labium majus had superior and nearly equivalent adherence characteristics respect-
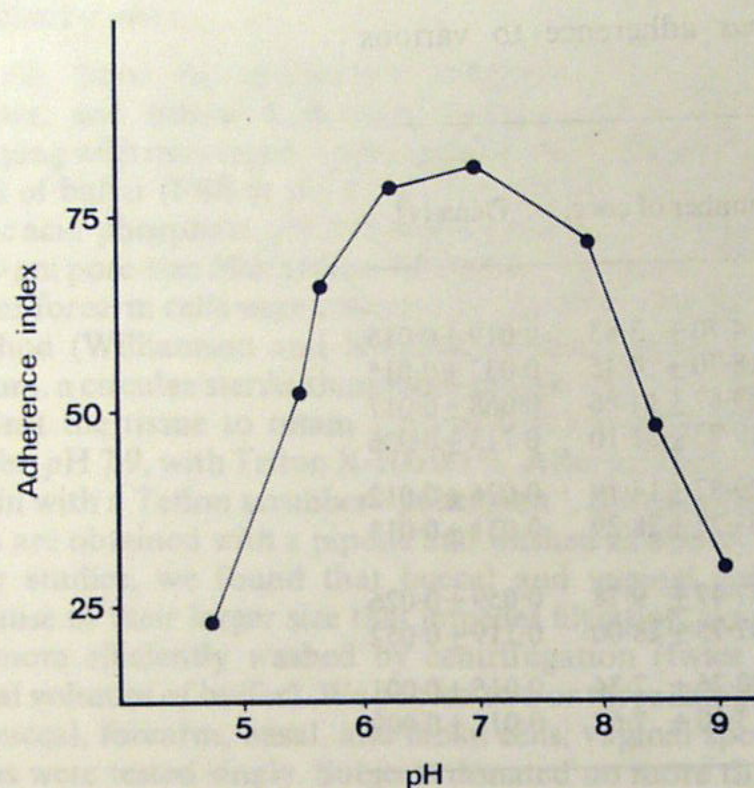
Fig. 1. Effect of *p*H on adherence of *S. aureus* to nasal cells. Average number of staphylococci per cell. See *Methods*.

Table IV. Blockage of labium majus and vaginal cell receptors to *S. aureus* by ribitol teichoic acid

Cell source and type	Number of adherent cocci/cell		Signifi-cance
	Treated	Untreated	
Labium majus*			
Medium, smooth	11.47 ± 10.12‡	48.14 ± 11.52	$p < 0.001$
Large, rough	16.48 ± 11.37	87.38 ± 16.79	$p < 0.001$
Vagina†	8.70 ± 3.21	25.43 ± 6.08	$p < 0.001$

* Phosphate-buffered saline, *p*H 6·8.
† Citrate-phosphate buffer, *p*H 3·5.
‡ Mean ± SD of 20 cells.

ively. The densities of *S. aureus* on labium minus and vaginal cells were the lowest of the group.

Table III similarly summarises the variation in cell-candida coupling. In repeated experiments with two isolates of the micro-organism that differed in virulence, *C. albicans* failed to attach to nasal cells and clung poorly to forearm and labium minus cells. Cells of the labium majus, however, generally supported colonisation. In scores but not in calculated density, vaginal cells provided the same degree of adherence as the smaller cells of the labium majus.

The vagina presented two unique ecological variables: variation in hydrogen ion concentration and menstruation-related cellular changes. Routine adherence testing was done with buffers at *p*H 6·8, in accordance with results from our early studies on nasal cells (fig. 1). This acidity reflects normal in-vivo conditions on buccal mucosa also, and is compatible with the environment of labial and forearm cells. (The skin environment is moderately acidic, *p*H *c*. 5·5.) Adherence indices of *S. aureus* on labium majus cells tested at *p*H 5·5 and 6·8 were nearly identical. However, the healthy vagina had a *p*H of 4·0–4·7 during the 4-week sampling period; the standard test at *p*H 6·8, although useful for cellular comparison, proved suboptimal for adherence.

Figs. 2 and 3 show the improved attachment of *C. albicans* and especially *S. aureus* to vaginal cells in increasingly acidic buffers. Centrifugation of these micro-organisms in buffer at *p*H < 5·0 produced a thin layer of organisms against the test tube wall

rather than the usual pellet; dispersion, nevertheless, was easily attained with a vortex mixer. When subjects were examined individually, or as a group after individual mean base levels were in effect standardised, differences in adherence scores for both *C. albicans* and *S. aureus* between *p*H 6·8 and 3·5 were significant $(0.001 < p < 0.005)$. These graphs further show that menstruation also affects adherence. The extent of this influence and the ranking of microbial densities by week varied among the individuals. Averaged data suggest that highest levels are reached between the third and fourth weeks of the cycle and that vaginal cells collected during the first or second week give the lowest densities for each of the test micro-organisms. The plots of *S. aureus* cover a broader range than those of *C. albicans* and show greater weekly and individual differences.

We previously demonstrated that autogenous ribitol teichoic acid could partially block (65% reduction) the attachment of *S. aureus* to fully keratinised nasal cells, suggesting that the wall component is an adhesin for these host cells. Table IV shows that bacterial receptors on the two types of cell of the labium majus, and on vaginal cells were also blocked in this way with reductions of 76%, 81%, and 66% respectively.

Discussion

Vulvar skin supports a unique microbial flora. This warm and moist region is composed of various specialised epithelia whose micro-environments and associated microbiota are influenced by the successive folds of protective tissue. Assorted facultative, microaerophilic and anaerobic gram-positive and gram-negative bacteria are sheltered in the vagina (see Bartlett *et al.*, 1977) whereas the
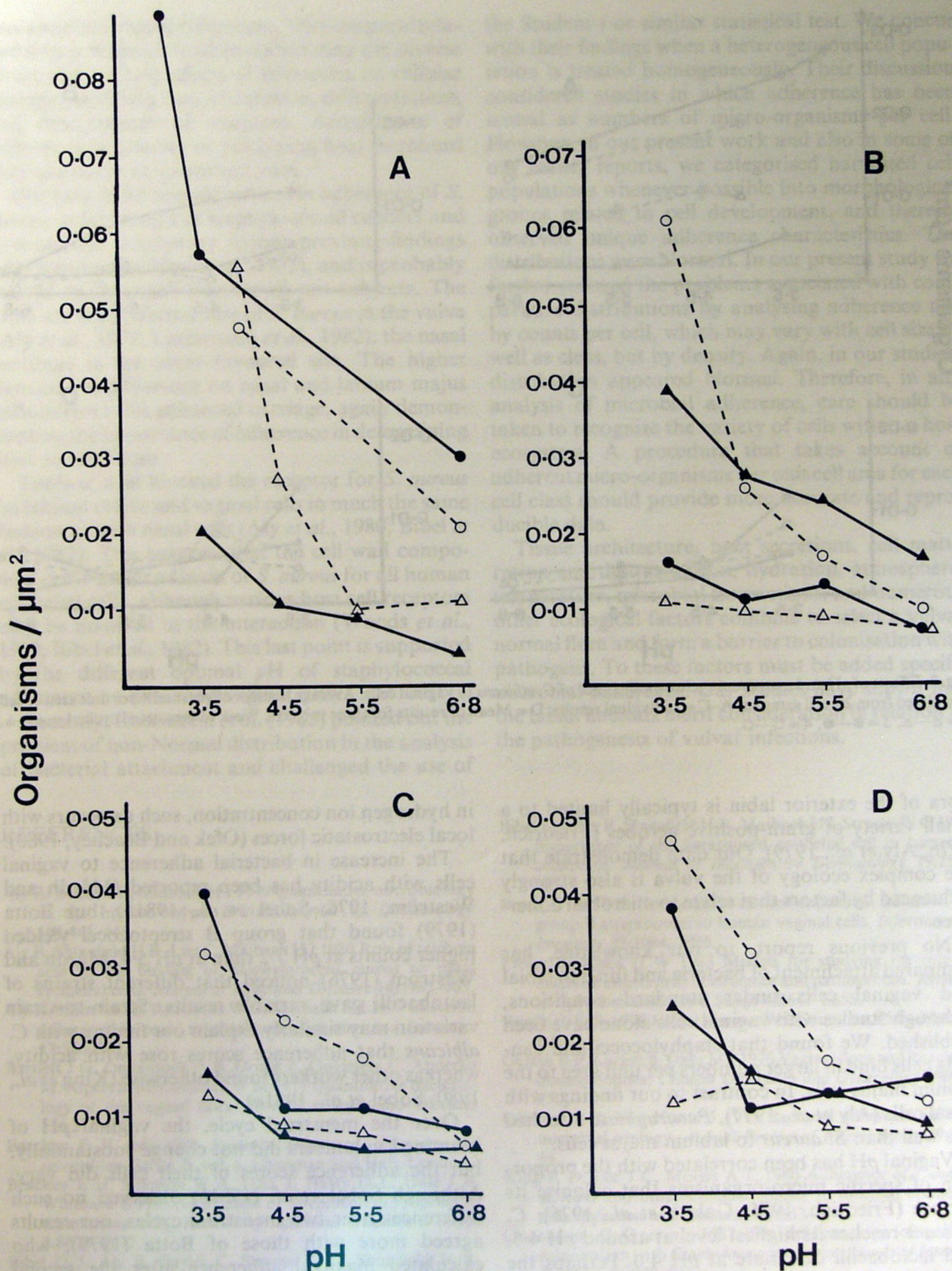
Fig. 2. Effects of *p*H and menstrual cycle on adherence of *S. aureus* to vaginal cells. Average number of bacteria per unit area of cell calculated from 20-cell samples. A–C = Individual results; D = Mean of results for three subjects. Week of menstrual cycle: 1 = ▲—▲, 2 = △—△, 3 = ●—●, 4 = ○—○.
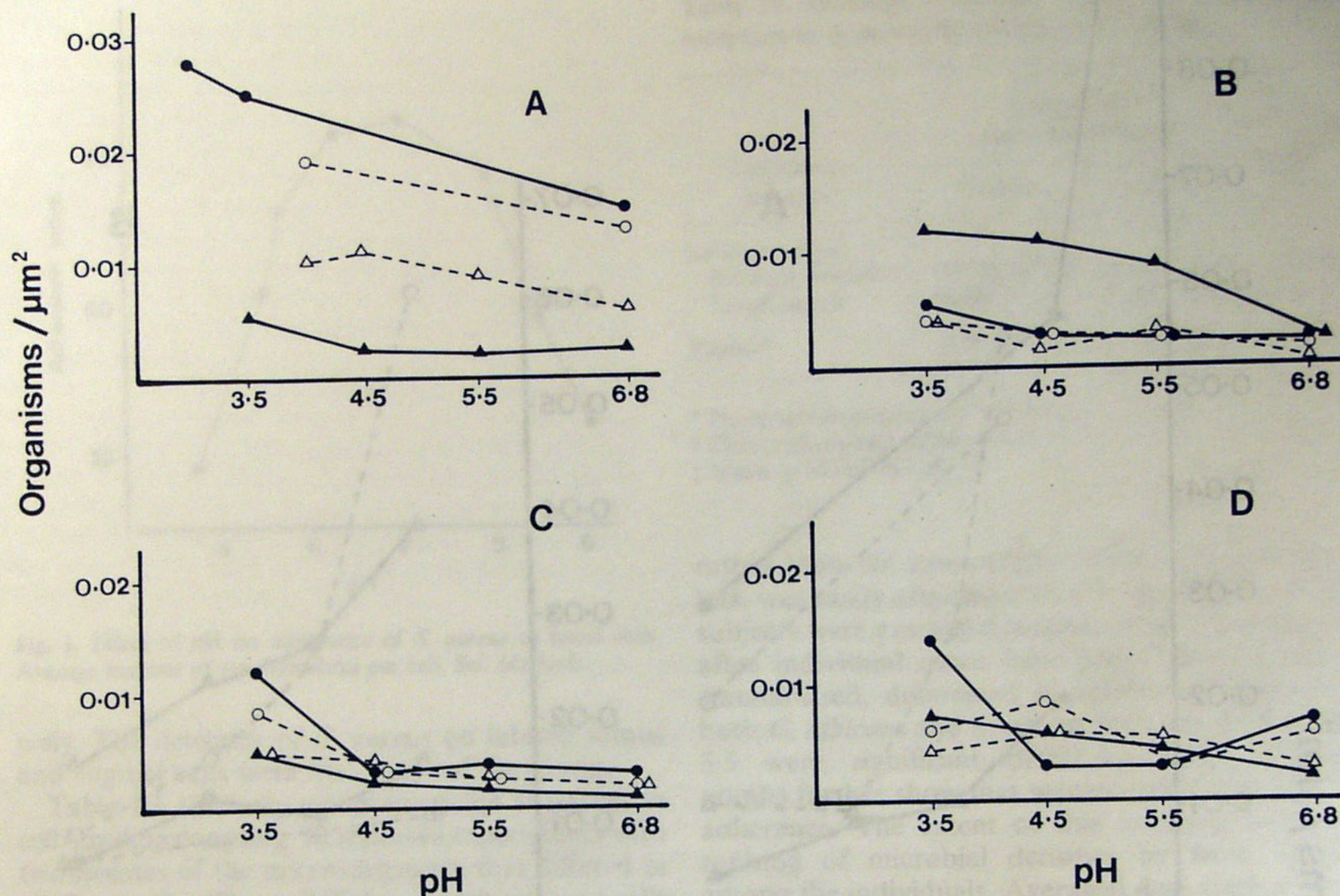
Fig. 3. Effects of pH and menstrual cycle on adherence of *C. albicans* to vaginal cells. Average number of yeast cells per unit area of cell calculated from 20-cell samples. A–C = Individual results; D = Mean of results for three subjects. Week of menstrual cycle: 1 = ▲—▲, 2 = △—△, 3 = ●—●, 4 = ○—○.

flora of the exterior labia is typically limited to a small variety of gram-positive aerobes (Friedrich, 1976; Aly *et al.*, 1979). Our data demonstrate that the complex ecology of the vulva is also strongly influenced by factors that relate to microbial adherence.

No previous report, to our knowledge, has compared attachment of bacteria and fungi to labial and vaginal cells under standard conditions, although studies with vaginal cells alone have been published. We found that staphylococci and candida cells bind in larger numbers per unit area to the labium majus cells. In contrast to our findings with nasal cells (Aly *et al.*, 1977), *P. aeruginosa* attached less well than *S. aureus* to labium majus cells.

Vaginal pH has been correlated with the proportion of specific micro-organisms that colonise its surface (Friedrich, 1976; Galask *et al.*, 1976); *C. albicans* reaches its highest level at around pH 4·5, and lactobacilli dominate at pH 4·0. Perhaps the incidence of particular vaginal flora is influenced by the alteration of adherence stemming from changes

in hydrogen ion concentration, such as occurs with local electrostatic forces (Ofek and Beachey, 1980).

The increase in bacterial adherence to vaginal cells with acidity has been reported (Mårdh and Weström, 1976; Sobel *et al.*, 1981b), but Botta (1979) found that group B streptococci yielded higher counts at pH 7·2 than at pH 5·4. Mårdh and Weström (1976) noticed that different strains of lactobacilli gave variable results. Strain-to-strain variation may similarly explain our finding with *C. albicans* that adherence scores rose with acidity, whereas other workers found otherwise (King *et al.*, 1980; Sobel *et al.*, 1981a).

Over the menstrual cycle, the vaginal pH of examined volunteers did not change substantially, but the adherence scores of their cells did vary. Although Sobel *et al.* (1981b) observed no such differences over two menstrual cycles, our results agreed more with those of Botta (1979), who calculated maximal adherence after the second week of menses. However, we found the highest levels after the third week instead and noted more

profound individual differences. This temporal relationship is reasonable when considering the diverse direct and indirect effects of hormones on cellular biology, including size, maturation, differentiation, and development of receptors. Acquisitions of adherence-interfering or enhancing host secretions may also have an important role.

Our lack of finding differences in adherence of *S. aureus* to labial cells of staphylococcal carriers and non-carriers is contrary to our previous findings with nasal cells (Bibel *et al.*, 1982), and is probably related to the small number of test subjects. The labia are the preferred sites of *S. aureus* in the vulva (Aly *et al.*, 1979; Linnemann *et al.*, 1982); the nasal vestibule is the other favoured site. The higher densities of adherence on nasal and labium majus cells mirrors this enhanced carriage, again demonstrating the importance of adherence in determining host normal flora.

Teichoic acid blocked the receptor for *S. aureus* on labium majus and vaginal cells in much the same fashion as with nasal cells (Aly *et al.*, 1980; Bibel *et al.*, 1982). This suggests that the cell wall component is the major adhesin of *S. aureus* for all human epithelial cells, although various host cell receptors may be involved in the interaction (Woods *et al.*, 1980; Bibel *et al.*, 1982). This last point is supported by the different optimal *p*H of staphylococcal adherence on nasal and vaginal cells.

Recently, Rosenstein *et al.* (1985) pointed out the problem of non-Normal distribution in the analysis of bacterial attachment and challenged the use of the Student *t* or similar statistical test. We concur with their findings when a heterogeneous cell population is treated homogeneously. Their discussion considered studies in which adherence has been scored as numbers of micro-organisms per cell. However, in our present work and also in some of our earlier reports, we categorised harvested cell populations whenever possible into morphological groups related to cell development, and thereby observed unique adherence characteristics. The distributions were Normal. In our present study we further reduced the problems associated with comparative distributions by analysing adherence not by counts per cell, which may vary with cell size as well as class, but by density. Again, in our studies, distribution appeared Normal. Therefore, in any analysis of microbial adherence, care should be taken to recognise the variety of cells within a host ecosystem. A procedure that takes account of adherent micro-organisms per unit cell area for each cell class should provide more accurate and reproducible data.

Tissue architecture, host secretions, cell maturation and desquamation, hydration, atmosphere, temperature, microbial interactions, and numerous other ecological factors combine to select a vulvar normal flora and form a barrier to colonisation with pathogens. To these factors must be added specific microbial adherence. Our studies have shown that the labial habitats merit consideration in relation to the pathogenesis of vulvar infections.

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