Competitive adherence as a mechanism of bacterial interference[1]

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To determine whether competition among bacteria for specific attachment sites on host cells can explain bacterial interference, *Staphylococcus aureus* strain 502A was tested in turn against two different nasal coryneforms, a strain of *Pseudomonas aeruginosa*, and a virulent strain of *S. aureus*, all in the presence of nasal mucosal cells. Particularly examined was the influence of sequence in which bacteria were presented to the nasal cells in comparison with initial mixtures and individual suspensions. Results paralleled those observed in clinical prophylaxis: the bacterium first to adhere to the epithelial cells was able, under uniform conditions, to interfere with the colonization of subsequently added bacteria. Secondary adherence was not eliminated but substantially reduced, and was probably related to steric blockage by the initial colonizer and its particular ability to dissociate from the host cell.

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Dans le but de vérifier si la compétition bactérienne pour des sites d'attachement spécifique sur les cellules-hôtes pouvait expliquer l'interférence bactérienne, la souche de *Staphylococcus aureus* 502A a été tour à tour étudiée contre deux souches différentes de corynéiformes d'origine nasale, une souche de *Pseudomonas aeruginosa* et une souche virulente de *S. aureus* et à chaque fois en présence de cellules de la muqueuse nasale. On a particulièrement regardé l'influence de la séquence selon laquelle les bactéries étaient présentées aux cellules nasales comparativement à des mélanges et à des suspensions pures. Les résultats obtenus vont de pair avec ceux observés en prophylaxie clinique: la première bactérie à adhérer aux cellules épithéliales était capable, dans des conditions uniformes, d'interférer avec la colonisation des bactéries ajoutées par la suite. L'adhérence secondaire n'était pas éliminée mais elle était réduite de façon importante. Cette adhérence secondaire était probablement reliée à un blocage stérique causé par la première bactérie à coloniser et sa capacité particulière à se dissocier de la cellule-hôte.

[Traduit par le journal]

Introduction

The phenomenon whereby one bacterium, inhabiting a given host tissue, prevents the colonization or pathologic expression of a second microorganism of the same or different species is known, respectively, as homologous or heterologous bacterial interference (Bibel 1982). Its application in the form of bacterioprophylaxis has successfully stemmed epidemics of virulent staphylococci among infants (Shinefield *et al.* 1963), has remedied chronic furunculosis (Maibach *et al.* 1969), and has prevented experimental infections of burned laboratory animals (Bascom and Wannamaker 1967; Wickman 1970). Possible explanations for interference include the production of specific antagonistic factors (Cybulska and Jeljaszewicz 1969), the competition for nutrients (Ribble 1967), and the selective alteration of the local physical–chemical environment (Shinefield *et al.* 1972).

Because specific attachment of bacteria to host epithelia (bacterial adherence) has recently been recognized as an important and early mechanism for microbial colonization (Beachey 1980), we have sought competition among bacteria known to bind to the same type of host cell. This report demonstrates the importance of sequence and avidity in attachment, and offers competitive adherence as another means of selective interference.

Materials and methods

Bacteria

Staphylococcus aureus strain 502A, a relatively benign bacterium long used clinically for interference against virulent staphylococci (Shinefield *et al.* 1963; Maibach *et al.* 1969), a nafcillin-resistant *S. aureus* (045005) originally isolated from a septicemic patient, and *Pseudomonas aeruginosa*, a standard test strain provided by the United States Food and Drug Administration, were grown in tryptic soy broth (Difco). Two small-colony, lipophilic, short-bacillary coryneforms were

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[1]Volunteers gave their informed consent to this study as according to the guidelines of the United States Department of Health and Human Services and that of the University of California, San Francisco, CA, U.S.A.

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isolated from the nasal mucosa of one of us on blood agar; they were subcultured in tryptic soy broth supplemented with 0.05% (v/v) Tween 80. Prior to testing, the microorganisms were incubated at 37°C for 18 h to stationary phase, centrifuged, and washed twice in 0.067 *M* phosphate-buffered saline (PBS), pH 6.8.

Since *S. aureus* strain 502A was tested in turn against the other four bacteria, we adjusted the cultures to approximately equal populations by averaging the tallies in three high-power microscopic fields obtained from 0.01-mL portions and diluting accordingly to about 10^7 colony-forming units/mL.

Nasal cells

The procedures of harvesting nasal epithelial cells by scraping with a wooden spatula and of subsequent washing in PBS by filtration have been published in detail (Bibel *et al.* 1982; Aly *et al.* 1977). For each test, we pooled the cells from seven to eight volunteers known not to carry *S. aureus*. Nasal cells numbered about 10^5/mL.

Adherence testing

An experiment consisted of five tubes, each containing 1 mL of a suspension of nasal cells. Tubes 1, 2, and 3 received also 1 mL of *S. aureus* strain 502A; to tubes 3, 4, and 5 were added 1 mL of the second bacterium. After 90 min of incubation at 37°C in a reciprocal-shaking water bath, 1 mL of PBS was introduced to tubes 1 and 5; 1 mL of the second bacterium was mixed with strain 502A in tube 2; and 1 mL of the staphylococcus joined the suspension of tube 4. All tubes were then incubated for another 90 min. Thus, we examined the influence of the order in which bacteria come in contact with nasal cells in comparison with an initial mixture and individual bacterial suspensions.

Staining and scoring

After incubation, each preparation was washed by filtration (Bibel *et al.* 1982; Aly *et al.* 1977) and then fixed to slides by heating. We manually counted the bacteria attached to each nasal cell as observed by light microscopy. Nasal cells were placed into four categories based on epidermal layer or degree of keratinization, which we previously found to strongly influence adherence (Bibel *et al.* 1982): maximal adherence and the development of a secondary receptor was correlated with the fully keratinized cell. Some 15 to 24 cells of each type were examined, and the mean bacterial counts, standard deviations, and where appropriate, Student *t*-test values were calculated. This adherence assay is a reproducible technique which has been used repetitively and successfully over the past 6 years. The staphylococcus–coryneform series was stained with crystal violet, and the bacteria were differentiated on the nasal cells by their shape and form of aggregation. Although the staphylococcus–pseudomonad slides could be similarly treated, we used the Gram-staining procedure to facilitate scoring.

The problem of microscopically differentiating the two staphylococci was solved by first prestaining the washed suspension of strain 502A in a 0.1% (w/v) solution of acridine orange and then filtering the bacterium onto a membrane of 0.22-μm pore size (Millipore Corp., Bedford, MA). Once the residual stain had been washed away with PBS, the staphylococcus was dislodged into buffer by scraping with a pipette tip and by agitation. Even 2 weeks over the time required for the experiment, the dye did not leach into solution. Incubation with nasal cells and fixation to slides proceeded as mentioned above. Preliminary studies demonstrated no difference in adherence ability between prestained and control staphylococci.

We stained the control slides (those having a single strain) with crystal violet for inspection under a bright-field microscope. The slides with mixed bacteria required the use of an incident-light fluorescent microscope with simple switching, conventional bright-field capability. These preparations were first rinsed in distilled water to remove dried salts. By viewing each entire slide in a systematic fashion, we constructed a map recording the relative position of each nasal cell and its tally of fluorescent cocci. After the slides were subsequently stained with crystal violet, which masks the acridine orange, the plotted nasal cells were examined by bright-field microscopy. For each cell, subtraction of the number of fluorescent bacteria from the total now observed yielded the population of nafcillin-resistant staphylococci.

Results and discussion

All four tests demonstrated the importance of sequence in which microorganisms are presented to a receptive epithelium. Although the first bacterium interfered with the attachement of the second, the degree and mechanism varied with each test system.

Figure 1 displays the results of the first staphylococcal–coryneform competition. The same pattern of relationships was found with each of the four fundamental layers of nasal cells (Bibel *et al.* 1982). Interference is apparent upon comparing the sum of bacteria incubated alone with that of each mixture. The lower amounts noted with the mixtures suggest a lack of independence in adherence with bacteria vying for at least some of the same receptors. This concept is similarly supported by the only slightly higher combined counts of mixed bacteria compared with the tally of the lone coryneform (Figs. 1 and 2).

Figure 2 depicts the results obtained earlier with a different coryneform. Since spinous cells were few in number and carried small bacterial populations (Fig. 1), differences were not significant. We have not depicted the data for simplicity. When the staphylococcus had a head start in adherence, this coryneform appeared to be less competitive than coryneform B. The means for keratinized cells differed by 75% versus 21%. However, upon comparing adherence scores after their respective delayed inoculation, coryneform A still reached higher levels than *S. aureus*. The pattern held for the various types of nasal cells.

The pseudomonad offered an improved but unique situation. It did not form aggregates, but it was motile. This bacterium, whose ability to adhere to nasal cells was previously demonstrated (Aly *et al.* 1977), is not a normal resident of the nasal mucosa. Nevertheless, it attached in large numbers when alone and could prevent the binding of subsequently added *S. aureus* (Fig. 3) (*p*

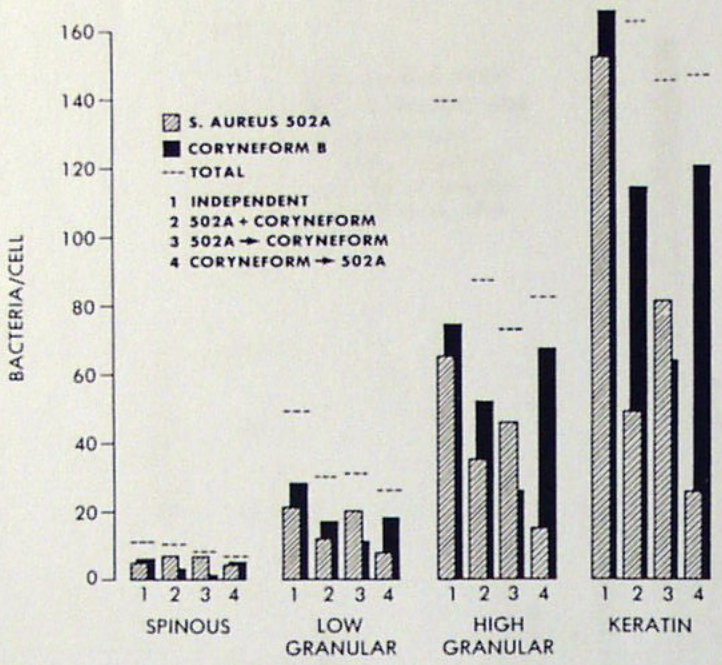

FIG. 1. Adherence of *Staphylococcus aureus* and a nasal coryneform to human cells from various layers. Bacteria incubated alone, in simultaneous mixture, and in succession.

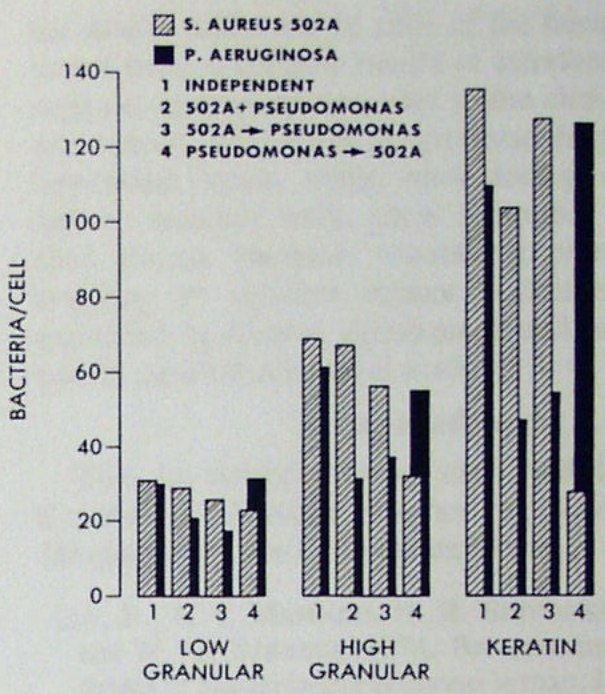

FIG. 3. Competitive adherence of *S. aureus* and *Pseudomonas aeruginosa* to nasal cells from various layers. Bacteria incubated alone, in simultaneous mixture, and in succession.

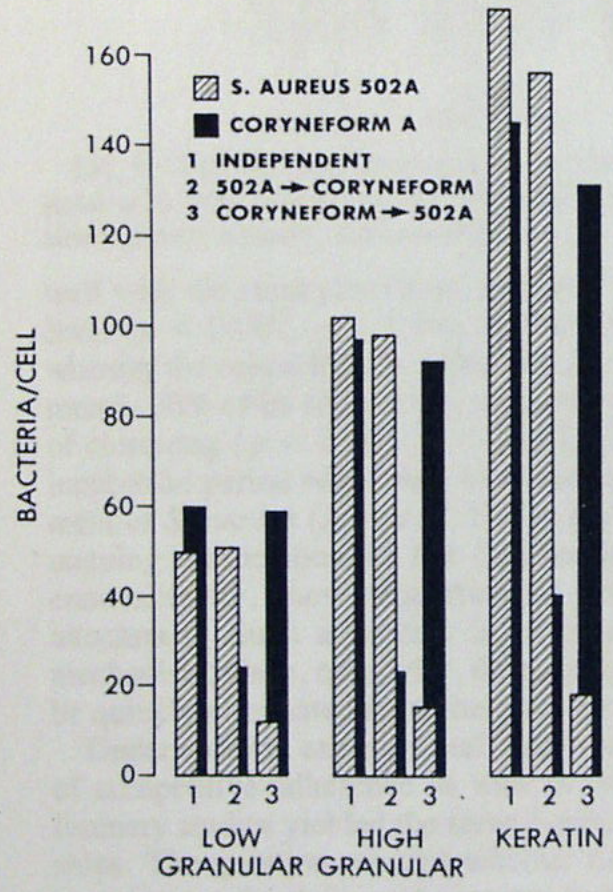

FIG. 2. Competitive adherence of *S. aureus* and another nasal coryneform to nasal cells from various layers. Bacteria incubated alone and in succession.

< 0.006 for high granular, and $p < 0.001$ for keratin-layer cells). As opposed to coryneform B, the pseudomonad was unable to compete with the staphylococcus when both were present at the outset ($p < 0.001$; $p < 0.001$). These bacteria differ in at least one adhesin-receptor system. The sums of bacteria in mixtures are over the tally of either individually incubated bacterium yet far below the combination of these two controls.

The incubation of *S. aureus* strain 502A with *S. aureus* 045005 examined the role of adherence in homologous interference. Figure 4 shows that, when tested singly, the virulent staphylococcus adhered in slightly greater numbers than strain 502A ($p < 0.02$ for high granular and $p < 0.05$ for keratin-layer cells). This has been a consistant phenomenon in three previous tests. The total of each mixture was essentially equal to the figure of isolate 045005 alone. Since the same staphylococcal adhesins and nasal-cell receptors are presumably involved, the difference is probably due to the number and distribution of adhesins and the strength and duration of bonding, factors also important with the previous experiments. The nafcillin-resistant staphylococcus also reached higher levels in the initial mixture ($p < 0.3$; $p < 0.02$), but again chronological order governed dominance in the sequential preparations ($p < 0.001$ for granular and keratinized cells). Strain 502A could block the adherence of isolate 045005 only when the low virulent strain already had attached to the nasal cells.

Crowding or steric blockage is a significant factor not to be disregarded in comparisons, since the differential is less pronounced among the younger nasal cells which have fewer receptors per unit area. Another consideration is that the coryneforms characteristically tend to form aggregates of some five to eight cells in contrast to the staphylocci, which are found mainly as diplococci. Attachment of only one coryneform in a cluster may cause the occlusion of adjacent receptors and thereby reduce the tally of adhering staphylococci.

This effect may explain the superior counts of the coryneform B over *S. aureus* recorded from the initially mixed preparation ($p < 0.02$ for high granular and $p < 0.001$ for keratin-layer cells). Even when its introduction was delayed for 90 min, coryneform B competed

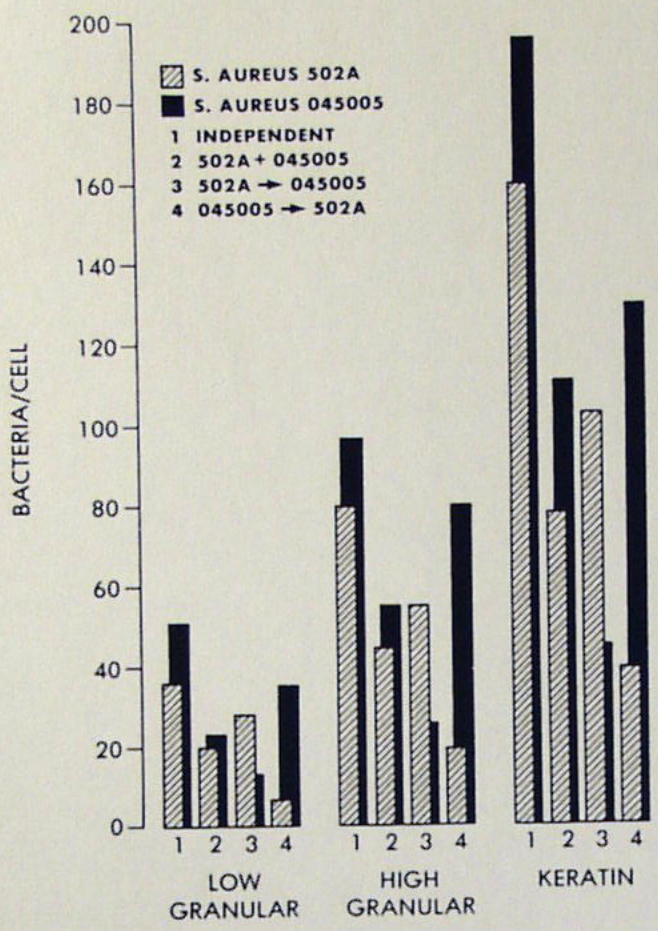

FIG. 4. Competitive adherence of two strains of *S. aureus* to nasal cells from various layers. Bacteria incubated alone, in simultaneous mixture, and in succession.

well with the staphylococcus, attaining 60–75% of its level ($p < 0.001$; $p < 0.09$). In the reverse sequence whereby the coryneform is added first, *S. aureus* reached merely 20% of its competitor, again the probable result of clustering ($p < 0.001$; $p < 0.001$). Since a 90-min incubation period was found to afford maximal attachment of *S. aureus* (Aly *et al.* 1977), the data imply the ongoing dissociation of the coupled bacterium and, concomitantly, new opportunities for competitive attachment. Such a process is an important survival mechanism, since, otherwise, the microorganism would be quickly eliminated from the body by desquamation.

Under uniform experimental conditions, all four tests of competitive adherence as well as unpublished preliminary studies yielded the same fundamental relationships. The question remains whether such competition explains, at least in part, interference *in vivo*. The clinical use of *S. aureus* strain 502A is limited to prophylaxis. The bacterium cannot displace already established virulent strains, and indeed, it is unable to colonize such a host tissue unless huge doses are applied (Shinefield *et al.* 1966; Aly *et al.* 1974). Application is performed on newborn infants, whose normal flora is sparse and in wide flux, or on adults only after their indigenous microbial population has been reduced by antibiotics (Shinefield *et al.* 1963, 1966; Aly *et al.* 1974). Interference is an inherently sequence-based phenomenon, and hence our results of competitive adherence echo those of the clinic. Our *in vitro* assay is the first to make use of cells of the human habitat and seems to be a suitable model of interference. The data suggests an explanation why in the clinical experience one cannot, within limits, overdose the patient with an interfering strain while underdosing could lead to failure: receptor sites, once saturated, would simply shed excess bacteria; vacant receptors may permit coupling of virulent strains. Adherence is thereby expanded in clinical significance and in its regulatory role in medical microbial ecology.

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