QUANTITATIVE STUDIES ON COMPETITIVE ACTIVITIES OF SKIN BACTERIA GROWING ON SOLID MEDIA

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PLATE XXXVI

INTERACTIONS among skin micro-organisms have begun to receive attention in recent years, but detailed quantitative work has apparently been restricted to growth studies in liquid media (Marsh and Selwyn, 1977). However, such culture systems bear little or no relationship to the skin surface. As a first step towards a more realistic approach, we have devised a quantitative method to study pure and mixed cultures growing on the surface of agar media, and experiments were carried out on antagonistic activities between skin bacteria by means of this procedure. The method, and representative results obtained with it, are described in this paper.

MATERIALS AND METHODS

Bacterial strains. The three commensal organisms used were an antibiotic-producing strain of *Staphylococcus epidermidis* biotype 4 (Baird-Parker, 1974) referred to as $S6^+$, a penicillin-resistant non-antibiotic-producing strain of *Staph. epidermidis* of biotype 4 referred to as $S6^-$, a strain of *Micrococcus* sp. classified as M7 according to Baird-Parker (1965), and a group-D diphtheroid strain (Evans, 1968). These were all isolated from normal skin. The three pathogenic organisms used were a penicillin-resistant strain of *Staphylococcus aureus* (bacteriophage group III) and a strain of *Streptococcus pyogenes*, both from infected skin lesions, and a strain of *Corynebacterium diphtheriae* var. *mitis* from a stock culture. The *Staph. aureus* strain showed relatively small inhibition zones in direct antagonism tests with $S6^+$ when compared with all but one of the other bacteria studied. The exception was the non-antibiotic-producing strain, $S6^-$, which was totally resistant to the action of $S6^+$ (Marsh and Selwyn, 1977).

Media. Nutrient agar and blood agar were used as growth media, and stock cultures were kept in nutrient broth at 4°C. The media were prepared as described by Cruickshank (1965) with Proteose Peptone and Lab-Lemco (Oxoid), New Zealand Agar and Defibrinated Horse Blood (Tissue Culture Services), and, in addition, 0.3% (w/v) Liver Digest and 0.3% (w/v) Yeast Extract (Oxoid). The selective media devised by Marsh and Selwyn (1977) made it possible to obtain differential counts of the bacteria in mixtures (table I).

Sampling method. The quantitative study of bacteria growing on a solid surface was carried out with special Petri dishes (Sterilin, code 504 S) which were originally designed for direct contact culture of contaminated surfaces. Each dish had a surface area of 24 cm² and a grid divided into centimetre squares.

The inoculum was prepared by diluting a 24-h culture to give approximately 10⁶ colony-forming units (c.f.u.) per ml. For pure culture studies, a 0.2-ml volume was placed on the surface of the medium after it had dried for 20 min. Each plate was gently rotated to distribute the inoculum evenly over the surface (8 × 10³ c.f.u. per cm²). In mixed-culture studies, 1-ml volumes containing 1 × 10⁶ c.f.u. of each strain were mixed and 0.2 ml of the mixture was used as the inoculum. Readings of viable counts taken at intervals permitted the construction of growth curves from the mean results of three plates.

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TABLE I
Summary of viable counts in mixed cultures on solid media

Organisms present in mixed cultures	Growth medium	Selective medium	Log ₁₀ viable count (c.f.u. per cm ²) at		
			0.25 h	12 h	28 h
{ S6 ⁻ M7	NA	NA+B+P	3.4	9.4	9.4
		NA+F+K	4.5	8.4	9.0
{ S6 ⁺ M7	NA	NA+B	3.9	9.8	9.8
		NA+F	3.4	5.8	3.8
{ S6 ⁺ Group-D diphtheroid	NA	NA+B	4.5	10.1	9.8
		NA+F	4.2	5.1	0
{ S6 ⁺ <i>C. diphtheriae</i>	BA	NA+B	4.4	10.1	10.3
		Tell.	<3.0	6.3	0
{ S6 ⁺ <i>Strep. pyogenes</i>	BA	MacC.	4.5	10.0	9.8
		BA+CV	3.4	6.2	0
{ S6 ⁺ <i>Staph. aureus</i>	NA	NA	4.9	10.8	10.3
		NA+G	4.4	9.9	10.3

c.f.u. = Colony-forming units.

NA = Nutrient agar; BA = blood agar; B = bacitracin (1.25 mg per litre); P = benzylpenicillin (0.1 mg per litre); F = furazolidone (7.5 mg per litre); K = kanamycin (0.156 mg per litre); Tell. = tellurite agar; MacC. = MacConkey's agar; CV = crystal violet (1 in 666 666, w/v); G = benzylpenicillin (3 mg per litre).

Viable counts were obtained by cutting a centimetre square of agar from each inoculated plate by means of a sterile disposable scalpel (fig. 1). These squares were then transferred to a sterile screw-capped container, and washed with 5 ml of 0.05% Triton X-100 in 0.0375M phosphate buffer, pH 7.9 (B.D.H. Chemicals Ltd); this efficiently washed organisms from the surface of the plates, and large clusters of bacterial cells were dispersed by subjecting the resulting suspension to mechanical agitation in a Rotamixer. To ensure the removal of the greatest possible number of bacteria, the surfaces of the squares immersed in dispersing fluid were scraped with a scalpel before agitating the container. Viable counts were derived from this suspension by the method of Miles, Misra and Irwin (1938), with 4-strength Ringer's solution as diluent.

When assessing the reproducibility of the method, 10 replicate cultures were sampled in strict sequence. The sampling procedure was not sensitive enough for the accurate determination of viable counts below 10³ c.f.u. per cm². Below this level, however, the presence or absence of significant numbers of bacteria was established by homogenising the agar squares in dispersing fluid. All the fluid from the homogenate was then passed through a Millipore filter (0.45-µm porosity), after which the bacteria on the filter disk were cultured on the appropriate media.

The numbers of residual bacteria were estimated in representative instances by sampling several squares in parallel from a single plate. After rinsing gently in dispersing fluid, the squares were pooled together, mixed with an equal volume of fresh dispersing fluid, and homogenised. The fluid was harvested as completely as possible, and viable counts were determined as above.

Studies of interactions. The qualitative techniques adopted were those described by Marsh and Selwyn (1977). The quantitative studies were carried out as described above. Each strain was grown and studied in pure culture. Mixed cultures were then studied under

similar conditions, and growth curves were constructed. All the cultures described were incubated at 37°C, but a number of experiments were duplicated at 33°C and no significant difference was noted.

RESULTS

Reproducibility of the method. Table II shows the range of viable counts and statistical data for the staphylococcal strain S6⁺ growing on nutrient agar plates after simultaneous inoculation with 8×10^3 c.f.u. per cm². The viable counts of the first three plates in the series of ten showed remarkable uniformity, varying only by a maximum of 0.25 of a logarithm.

The proportion of the culture not liberated from the agar was found to be negligible. It was less than 0.1% of the total calculated from the initial viable count when the population was greater than 10^5 c.f.u. per cm², and was between

TABLE II

Variability of the viable counts of the staphylococcal strain S6⁺ obtained by the quantitative solid surface method

Time (h)	Number of plates	Mean log ₁₀ viable count	Range of counts (log ₁₀)	Standard deviation (SD)	Standard error
1	All 10	4.24	4.00-4.40	0.148	0.047
	First 3	4.09	4.00-4.18	0.090	0.052
7	All 10	7.92	7.62-8.19	0.195	0.062
	First 3	7.70	7.62-7.79	0.086	0.050
28	All 10	9.13	8.87-9.44	0.207	0.065
	First 3	9.04	8.87-9.14	0.146	0.084

TABLE III

Summary of mixed-culture results for the antibiotic-producing staphylococcal strain S6⁺ and Staph. aureus on nutrient agar with inocula of different sizes

Time (h)	Organisms	Mean log ₁₀ viable counts (c.f.u. per cm ²) in experiment			
		A	B	C	D
0	S6 ⁺	4.9	6.3	7.2	4.0
	Staph. aureus	4.4	5.6	3.8	<3.0
12	S6 ⁺	10.8	10.1	10.2	9.9
	Staph. aureus	9.9	7.6	4.0	8.0
24	S6 ⁺	10.7	10.0	10.1	10.1
	Staph. aureus	10.3	5.4	0	7.8
72	S6 ⁺	10.2	10.0	10.0	9.3
	Staph. aureus	9.6	5.0	0	7.8

0.1 and 0.2% when the initial count was between 10^4 and 10^5 . The proportion of residual organisms rose to approximately 1% when the initial count was between 10^3 and 10^4 c.f.u. per cm^2 .

Bacterial antagonism. The results are summarised in tables I and III. When $S6^-$ was mixed with M7 on nutrient agar, no inhibitory effect was produced, but when $S6^+$ was mixed with M7 on the same medium, M7 was antagonised and its viable count fell by more than six logarithms (fig. 2). $S6^+$ showed an even stronger inhibitory effect when it was cultured with comparable inocula of the group-D diphtheroid, *C. diphtheriae* var. *mitis* or of the group-A streptococcus. These strains were completely inhibited within approximately 15 h. As a typical example of this unequivocal effect, fig. 3 shows the results obtained with *Strep. pyogenes*.

In contrast, as shown in table III and, in greater detail, in figs. 4 and 5, the outcome of interaction between $S6^+$ and *Staph. aureus* depended on the initial inoculum size of both organisms. When the two organisms were inoculated at

FIG. 2.—Growth curves of pure and mixed cultures of strains $S6^+$ and M7 on nutrient agar at 37°C . — = $S6^+$ (pure); = M7 (pure); ——— = $S6^+$ (mixed); - - - - = M7 (mixed).

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FIG. 1.—View of mixed culture of strains S6⁺ and M7 after incubation for 12 h. Note retraction of agar after excision of the square. $\times 4$.

FIG. 3.—Growth curves of pure and mixed cultures of strains S6⁺ and *Strep. pyogenes* on blood agar at 37°C. — = S6⁺ (pure); --- = S6⁺ (mixed); = *Strep. pyogenes* (pure); -.-.- = *Strep. pyogenes* (mixed).

the same population level *Staph. aureus* was not inhibited. However, when the initial inoculum of S6⁺ (10^6 c.f.u. per cm²) was only one logarithm higher than that of *Staph. aureus*, the viable count of *Staph. aureus*, after 60 h, was six logarithms lower than the corresponding count in pure culture. Moreover, when the original population level of S6⁺ was 1000-fold higher than that of *Staph. aureus*, the latter was completely inhibited within 24 h. In another experiment (D), the original inoculum of S6⁺ was 10^4 c.f.u. per cm² and that of *Staph. aureus* was less than 10^3 c.f.u. per cm². Despite this difference, *Staph. aureus* had reached a viable count of 10^8 c.f.u. per cm² after incubation for 12 h, and after a slight decline maintained a population level of 6×10^7 c.f.u. per cm² for up to 72 h. In all the experiments S6⁺ showed the same pattern of growth as in pure culture.

FIG. 4.—Growth curves of mixed cultures of strain S6⁺ and *Staph. aureus* on nutrient agar at 37°C (experiments A and B). = S6⁺ (mixed), experiment A; ——— = S6⁺ (mixed), experiment B; — = *Staph. aureus* (mixed), experiment A; - - - = *Staph. aureus* (mixed), experiment B.

DISCUSSION

The quantitative method devised, based on the use of a solid surface, was found to give reproducible results, and it provided an in-vitro model for studying the interactions of skin micro-organisms. Where more than three replicate plates were sampled, the viable counts of the earlier cultures in the set were noticeably lower than those of the later cultures, due to significant bacterial growth during the extended sampling procedure.

The inhibitory action of S6⁺ on the strains tested on solid media was comparable to, but more pronounced than, that demonstrated in liquid media (Marsh and Selwyn, 1977). For example, in liquid medium the viable count of M7 was reduced only 300-fold, whereas on solid medium it had decreased by almost seven logarithms. Again, in liquid media S6⁺ was unable to inhibit *C. diphtheriae* var. *mitis* or *Strep. pyogenes*, but on solid media both organisms were totally inhibited and indeed killed. This may be attributable to the dilution of antibiotic as a result of its dispersal throughout the liquid culture; on solid surfaces the antibiotic is held locally in the medium in a concentration sufficiently high to reach not only bacteristatic but also bactericidal levels.

FIG. 5.—Growth curves of mixed cultures of strain S6⁺ and *Staph. aureus* on nutrient agar at 37°C (experiments C and D). — — — = S6⁺ (mixed), experiment C; - - - - = S6⁺ (mixed), experiment D; — = *Staph. aureus* (mixed), experiment D; - · - · = *Staph. aureus* (mixed), experiment C.

The results obtained from experiments with mixed cultures of S6⁺ and *Staph. aureus* are in contrast to those obtained in conventional direct and deferred tests of antagonism, in which the zones of inhibition of the *Staph. aureus* strain were consistently produced by S6⁺, although they were significantly smaller than those of, for example, the group-D diphtheroid. The lack of consistent inhibition is also in contrast to the results of in-vivo work on rabbits (T. N. Sethna, personal communication). A possible explanation of these discrepancies is the relatively large inoculum of S6⁺ used in the conventional tests for antagonism. This is confirmed by the results (table III). Clearly, S6⁺ had to be present not only in a count of at least a logarithm higher than this particular strain of *Staph. aureus* to inhibit it, but also at an absolute level of at least 10⁶ c.f.u. per cm². Presumably, at lower population levels, insufficient antibiotic was produced in the early stages of the mixed culture to prevent the relatively resistant *Staph. aureus* strain from proliferating to population levels too high to be affected by S6⁺.

A further interrelated factor to be considered, particularly in instances of relative resistance, is the spatial relationships of the two organisms seeded on the solid medium. The physical distribution of the antibiotic-producing organism is clearly all-important. At the earliest stages of micro-colony

formation, there will be an inadequate effect of antibiotic if the producer organism is comparatively widely dispersed. At this critical stage, the second organism is then able to multiply unimpeded by any significant amount of antibiotic, as was well seen when *Staph. aureus* and S6⁺ were mixed in relatively low numbers.

There are obvious potential applications of antagonistic skin commensals in the prevention and treatment of infections of burns, wounds and other skin lesions. The possible protective role of antibiotic-producing organisms in dermatological lesions was indicated in a recent epidemiological study (Selwyn, 1975). A limited amount of experimental work in animals has been carried out by Pryjma, Heczko and Wilburg (1972), who used *Staph. epidermidis* to protect against *Staph. aureus* infections, but no mention was made of antibiotic production. Our current work on the application of antibiotic-producing organisms both to experimental animals and human volunteers is yielding promising results in the prevention of colonisation and infection with pathogenic bacteria (unpublished experiments).

SUMMARY

Earlier quantitative investigations of antagonism between skin bacteria were based on the use of liquid cultures, but a more realistic model has now been devised, based on the use of the surfaces of solid media. Pure or mixed inocula were spread evenly over suitable agar media in Petri dishes marked out with a standard grid. Growth curves were constructed from viable counts of the surface bacteria after they had been removed from excised squares of the agar media and dispersed. The method was highly reproducible, and competitive interactions were revealed more clearly than in studies with liquid media. An antibiotic-producing strain of *Staphylococcus epidermidis* (S6⁺) readily suppressed strains of *Micrococcus*, *Corynebacterium* and *Streptococcus* species. However, a *Staphylococcus aureus* strain which was less sensitive to the antibiotic effect of S6⁺ interacted in a complex manner, depending on the absolute and relative size of the S6⁺ inoculum.

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