Growth cycle-induced changes in sensitivity of *Staphylococcus aureus* to bactericidal lipids from abscesses

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Summary. Three *Staphylococcus aureus* strains (303, 18Z and TG), exhibiting various patterns of survival within abscesses, were significantly more sensitive to the bactericidal activity of oleic acid during the log phase of growth than at other stages of the growth cycle. Cells entering the stationary phase showed diminished sensitivity to the fatty acid. These changes were reflected by changes in the LD₅₀ and also by differences in the rate of killing by oleic acid. Additional changes were noted: the rate of killing by oleic acid declined over a 4-day period; a progressively greater proportion of the staphylococcal population became resistant to even high concentrations of oleic acid; from the fourth day onwards c. 50–55% of the cocci were totally resistant to the fatty acid. Strains 303 and 18Z became more sensitive to mono-olein during the log phase of growth, but strain TG was very resistant to mono-olein throughout the growth cycle. Growth in the presence of glycine 6% to reduce cross-links in the peptidoglycan did not alter bacterial sensitivity to oleic acid. However, all three *S. aureus* strains exhibited significant increases in membrane fluidity during the log phase of growth, but upon entering the stationary phase membrane fluidity again decreased. Concomitant changes in carotenoid content occurred during the growth cycle, but these changes did not appear to be solely responsible for the changes in sensitivity to the lipids.

Introduction

A decade ago, we first detected a novel host defence mechanism in abscesses caused by *Staphylococcus aureus*.^{1–3} This mechanism is based upon the production by the host of two types of bactericidal lipid—a pool of monoglycerides, and a pool of long-chain unsaturated fatty acids.^{3–5} These lipids are generated in abscesses by two independent mechanisms.^{3,5} Only the long-chain (C₁₄–C₂₀) unsaturated fatty acids produced in abscesses are bactericidal for *S. aureus*. The monoglycerides produced by the host are the 2-monoglycerides, but these spontaneously isomerise to form the corresponding 1-monoglycerides. Whereas the bactericidal fatty acids collectively make up c. 20% of the total lipid pool in abscesses, the monoglycerides comprise < 0.1%.

Staphylococci exhibit three basic patterns of survival within abscesses.^{6,7} With some strains, elimination begins as soon as the bactericidal lipids are mobilised and continues unabated (immediate elimination pattern), whereas other strains can persist for months (persistent pattern). Still others are eliminated, but only after a delay of c. 10 days (delayed elimination

pattern). Phagocytosis does not occur within the abscesses. There are no intact leucocytes near the core where the cocci are located, and cocci are not found at the periphery where viable leucocytes are located. However, the survival pattern of a strain is related to its sensitivity to the 2-monoglycerides.⁵

Certain factors can alter a strain's sensitivity to the bactericidal lipids. The presence of a capsule, although rare among *S. aureus* strains, can confer a two-to-four-fold increase in resistance to the bactericidal lipids.⁸ Prior growth in glucose increases a strain's resistance to monoglycerides 50-fold, but does not alter its sensitivity to the fatty acids.^{9,10} In contrast, low pigment (carotenoid) levels are associated with increased sensitivity to the fatty acids, but do not affect sensitivity to monoglycerides.¹¹ Reduced carotenoid levels are also associated with an increase in membrane fluidity and these alterations in membrane fluidity may account for the changes in sensitivity to the fatty acids.¹²

Preliminary studies suggested that staphylococci might also differ in sensitivity to these lipids at different states of the growth cycle. To evaluate this possibility, three *S. aureus* strains were harvested at different stages of the growth cycle and tested for sensitivity to mono-olein and oleic acid.

Materials and methods

Bacterial strains

S. aureus strains 303, 18Z and TG were selected for this study on the basis of their characterisation in previous studies.^{1,2,6,7} Strain 303 is immediately eliminated from abscesses, strain 18Z shows delayed elimination and strain TG is persistent in abscesses. Strains 18Z-1 and 18Z-2 are two non-pigmented mutants derived from the parent 18Z strain.¹¹

Medium

Trypticase yeast extract broth (TYB) consisting of trypticase peptone 1.7%, yeast extract 1%, NaCl 0.5% and dibasic potassium phosphate 0.25% was used throughout.

Staphylococcal suspensions

Overnight starter cultures in TYB were diluted 1 in 100 in fresh TYB and incubated at 37°C with agitation to establish the growth kinetics for each strain. Subsequently, fresh cultures were prepared, absorbance measurements at 630 nm were taken to verify the stage of the growth cycle and samples were removed at various times. The organisms were diluted in TYB-saline (saline with TYB 8%), sealed in ampoules and stored at -20°C until required.

Bactericidal assay

Oleic acid and mono-olein were selected as representative bactericidal lipids because they are the most prominent components of the fatty acid and monoglyceride pools which accumulate in abscesses.^{2,5} Bactericidal activity was assayed as described previously.² Briefly, a stock solution of either lipid in ethanol was prepared and diluted 1 in 10 in diluent (2 M NaCl with 2 mM EDTA) just before use. Additional serial two-fold dilutions of the suspensions were made in diluent and a standard number of the organisms being tested (in diluent) was added to each dilution. After incubation of the tubes in a water bath at 37°C for 1 h with shaking, a 0.5-ml sample was removed from each tube and plated on trypticase soy agar (TSA) to count the number of surviving cocci. Several control tubes containing organisms and diluent only were included in each assay. All assays were done in duplicate. The LD50 was determined by the method of probits¹³ and was expressed as µg/ml. Statistical evaluation of the different strains was done by Student's *t* test.

Killing rates

Staphylococcal suspensions were appropriately diluted in diluent (2 M NaCl with 2 mM EDTA) as for the bactericidal assay. The diluted suspension was incubated at 37°C with shaking, and a pre-determined amount of oleic acid was added. At zero time and every 10 min thereafter, samples were removed and

plated on TSA to count the number of surviving organisms. Suspensions incubated without the fatty acid served as controls.

Fluorescence polarisation values

Staphylococci harvested at different stages of the growth cycle were washed once in saline, resuspended in saline with glycerol 20%, and stored at -70°C. At time of use the suspensions were suspended in saline containing 2 µM 1,6-diphenyl-1,3,5-hexatriene to give an $A_{600\text{nm}}$ of 0.300 (SD 0.005), and then incubated at 37°C for at least 30 min. Polarisation values were obtained in an SLM Aminco 8000C fluorimeter (excitation 365 nm; emission 425 nm). Data were acquired at 37°C and polarisation values were calculated by software provided by SLM.¹²

Statistical analysis for comparing the difference in mean polarisation value was performed by one-way analysis of variance (ANOVA) to determine differences in the group and Bonferroni's post test to determine where the differences in the group occurred.¹⁴

Carotenoid extraction and quantification

Staphylococcal carotenoids (pigments) were extracted and quantified as previously described.¹² In some cases carotenoids were separated by thin-layer chromatography and individually quantified spectrophotometrically at their absorption maximum as described by Marshall and Wilmoth.¹⁵

Alteration of cell wall structure

Studies by Hammes *et al.*¹⁶ have shown that growth of *S. aureus* in the presence of high concentrations of glycine leads to a more loosely cross-linked peptidoglycan. Accordingly, *S. aureus* strains 303, 18Z and TG were grown in media with different concentrations of glycine. The effect of glycine was assessed by the presence of swollen cells and changes in $A_{630\text{nm}}$ measurements. Subsequently, the organisms were grown in medium with 0.8 M glycine and harvested after 45 min, 2 h, and 48 h. Organisms grown similarly in medium without added glycine served as controls. The organisms were assayed for sensitivity to the lipids as described previously.

Results

Sensitivity to bactericidal lipids

S. aureus strains 303, 18Z and TG became more sensitive to oleic acid as they entered the log phase, but upon entering the stationary phase they again became more resistant (table I). The ratio between maximum sensitivity (log phase) and minimum sensitivity (stationary phase) was at least 260-fold for strain 303 and 18–20-fold for strains 18Z and TG.

S. aureus strains 303, 18Z and TG were also examined for their sensitivity to mono-olein. Strain

Table I. Sensitivity of *S. aureus* strains to oleic acid at different stages of growth cycle

Stage of growth cycle	Oleic acid LD50 (SE) for strain		
	303	18Z	TG
0 h	0.58 (0.63)	0.81 (0.44)	3.45 (0.49)
Early log (0.5 h)	0.14 (0.11)	0.32 (0.12)	1.12 (0.72)
Early log (1.0 h)	0.095 (0.035)	0.23 (0.095)	0.34 (0.26)
Mid-log (1.5 h)	0.099 (0.076)	0.18 (0.074)	0.22 (0.07)
Late log (2 h)	—	0.21	—
Early stationary (4 h)	—	0.39	—
Early stationary (6 h)	0.29	1.23	0.44
Stationary (10 h)	—	3.3	2.1
Stationary (12 h)	1.83	15.1	3.5
Stationary (24 h)	> 25	> 25	4.5
Late stationary (48 h)	—	> 25	—

LD50, $\mu\text{g/ml}$ (average of three-to-five assays).**Table II.** Sensitivity of *S. aureus* strains to mono-olein at different stages of growth cycle

Stage of growth cycle	Mono-olein LD50 for strain		
	303	18Z	TG
Mid-log (1.5 h)	0.42	9.8	> 25
Early stationary (6 h)	2.9	> 25	> 25
Stationary (10 h)	> 25	—	> 25
Stationary (12 h)	> 25	> 25	> 25
Stationary (24 h)	> 25	> 25	> 25
Late stationary (48 h)	> 25	> 25	> 25

LD50, $\mu\text{g/ml}$ (average of two-to-four assays).

303 was decidedly more sensitive to mono-olein during the log phase than during the stationary phase (table II). The ratio between maximum and minimum sensitivity was at least 60-fold. Strain 18Z was moderately sensitive at mid-log phase, but by 6 h was fully resistant

to mono-olein and remained so for the remainder of the growth cycle. No such differences were detected with strain TG which was quite resistant to mono-olein throughout.

Changes in killing rates

S. aureus 303 harvested during the log phase of growth was killed rapidly by oleic acid ($6.25 \mu\text{g/ml}$) and < 0.5 % of the population survived after 10 min (fig. 1). With cocci recovered during the late stationary phase (24 h), the rate of killing was markedly reduced, but c. 99 % of the cocci were still destroyed within 1 h. However, no further reductions were seen with additional incubation. Bacteria harvested later (2–7 days), were killed even more slowly (fig. 1), but a progressively greater proportion of the population was resistant to this and even greater concentrations of oleic acid (fig. 2). Similar results were obtained with *S. aureus* strains 18Z and TG.

Alteration of cell walls and sensitivity to lipids

To see whether the differences in sensitivity might be due to changes in cell-wall integrity, we attempted to weaken the cell walls by growing the staphylococci in the presence of high glycine levels. With all three strains, growth was altered by glycine 4–6 %. With glycine 6 %, growth proceeded normally for 3–4 h, but then the $A_{630\text{nm}}$ decreased until 18–24 h. During this period swollen cells were evident. Thereafter, growth resumed and continued until the usual maximum cell density was attained at 48 h. Consequently, organisms were harvested after 45 min, 2 h and 48 h and assayed for their lipid sensitivity. Although organisms harvested after 45 min and 2 h were more sensitive than

Fig. 1. The rate of killing by oleic acid $6.25 \mu\text{g/ml}$ of *S. aureus* 303 harvested at different times during the growth cycle. Only representative times are shown: 1.5 h (●), 24 h (■) and 96 h (◆). No further killing occurred after incubation for 1 h.

Fig. 2. Proportion of *S. aureus* 303 resistant to oleic acid 6.25 µg/ml when harvested at different times during the growth cycle. The cocci surviving incubation for 1 h were resistant to this and to higher concentrations of oleic acid.

Table III. Membrane fluidity in *S. aureus*

Stage of growth cycle	Polarisation value (SD) for strain		
	303	18Z	TG
Mid-log (1.5 h)	0.367 (0.008)	0.3522 (0.0065)	0.384 (0.002)
Early stationary (6 h)	0.346 (0.004)*	0.3162 (0.0040)*	0.364 (0.006)†
Stationary (12 h)	0.368 (0.005)	0.3556 (0.0072)	0.356 (0.005)†
Stationary (24 h)	0.365 (0.003)	0.3747 (0.0053)	0.388 (0.005)
Stationary (24 h)	0.367 (0.008)	0.3774 (0.0053)	0.384 (0.002)

* Significantly different from time 0 and 6 h ($p = 0.01$).

† Significantly different from time 0 and 12 h ($p = 0.001$), but not different from each other.

organisms harvested at 48 h, there was no difference in sensitivity between organisms grown in the presence and absence of glycine.

Changes in membrane fluidity

Since previous studies had indicated that an increase in membrane fluidity was associated with increased sensitivity to the bactericidal fatty acids,¹² the strains were examined for any changes in membrane fluidity during the different stages of the growth cycle. *S. aureus* strains 303, 18Z and TG all exhibited an increase in membrane fluidity while growing exponentially (90 min), but fluidity returned to baseline values thereafter (table III). In the case of strains 303 and 18Z, the polarisation values evident at 90 min were significantly different from values obtained at zero time or 6 h ($p = 0.01$). With strain TG, the values at 90 min and 6 h were significantly different from those at zero time and 12 h ($p = 0.001$) but were not different from each other.

Changes in carotenoid levels

Because reduced levels of carotenoids are associated with increased membrane fluidity and increased sen-

Table IV. Carotenoid content* of *S. aureus* strains 303, 18Z and TG when harvested at various times of the growth cycle

Stage of growth cycle (h)	A_{464} ($\pm$ SD) for strain		
	303	18Z	TG
0	0.174 (0.058)†	0.184 (0.10)†	0.152 (0.049)†
1.5	0.094 (0.0096)	0.044 (0.012)	0.039 (0.0033)
6	0.159 (0.092)	0.148 (0.013)†	0.121 (0.063)†
12	0.305 (0.095)†	0.416 (0.199)†	0.182 (0.064)†
24	0.411 (0.087)†	0.892 (0.151)†	0.227 (0.109)†

* In 0.25 g wet weight of cells.

† Statistically significant from 1.5-h values ($p = < 0.05$).

sitivity,¹² the total amounts of carotenoids present throughout the life cycle were measured to see whether this could explain the changes in sensitivity to oleic acid. All three strains had significant reductions in carotenoid content during the exponential phase, but as the organisms entered the stationary phase the carotenoid levels again increased (table IV).

If changes in the amounts of carotenoids were solely responsible for the changes in sensitivity to oleic acid, carotenoid-negative mutants should show little or no

Table V. Sensitivity of carotenoid-negative mutants to bactericidal lipids in abscesses

Stage of growth cycle (h)	Oleic acid LD50 for strain		Mono-olein LD50 for strain	
	18Z-1	18Z-2	18Z-1	18Z-2
1.5	0.094	0.054	1.36	0.52
6	1.36	0.15	> 25	16.4
12	14.2	0.31	> 25	14.1
24	17.0	2.1	> 25	> 25
48	1.70	0.32	> 25	10.6

LD50, *µg/ml (average of two-to-four assays).

change in sensitivity to oleic acid throughout the growth cycle. Consequently, two colourless mutants of *S. aureus* 18Z (colour index 0) which had been isolated in another study¹¹ were examined. Carotenoids were collected, extracted and quantified from *c.* 20 g of each organism. Strain 18Z-1 contained only 1.02% as much carotenoid as the parent strain 18Z and strain 18Z-2 only 0.79% as much. In both cases, the only carotenoid present was the colourless 4,4'-diapophytofluene, which is the initial triterpenoid intermediate in the biosynthetic pathway.¹⁷ Subsequently these mutants were harvested at different times in the growth cycle and assayed for sensitivity to oleic acid and mono-olein.

These mutants were both more sensitive to oleic acid during the log phase than during the stationary phase (table V). Maximum resistance occurred at 24 h, but by 48 h the organisms were becoming more sensitive again. The ratio between maximum sensitivity (log phase) and minimum sensitivity (24 h) was 181 for strain 18Z-1 and 39 for strain 18Z-2.

Discussion

Three *S. aureus* strains were decidedly more sensitive to oleic acid during the exponential phase than at any other time in the growth cycle. This increased sensitivity was reflected by changes in both the LD50 and the rate of killing (table I, fig. 1). Concomitant increases in membrane fluidity and decreases in carotenoid levels were also seen at this time (table III). Previous studies had already noted a relationship between sensitivity to fatty acids and carotenoid

levels.¹¹ In this case, *S. aureus* mutants deficient in carotenoid production or pigmented strains grown under conditions minimising pigment content were found to be more sensitive to oleic acid and also showed an increase in membrane fluidity. Again, these changes were manifest through changes in both the LD50 and the killing rate.^{11,12}

While it may be argued that the increased sensitivity to the lipids during the log phase is due to an increase in membrane fluidity resulting from a reduction in the carotenoid content, our data suggest that this is not the only explanation. The carotenoid-negative mutants, 18Z-1 and 18Z-2, possessing only *c.* 1% of the carotenoid content of the parent strain, still showed substantial differences in sensitivity to the lipids at various stages of the growth cycle (table V). As was expected, the mutants were somewhat more sensitive to oleic acid than the parent strain 18Z; nevertheless, additional growth cycle-related changes were evident. It is possible that the remaining 1% of 4,4'-diapophytofluene could be further reduced during the log phase and thus account for the changes in sensitivity. However, this does not seem likely because the ratio of sensitivity between non-pigmented mutants and fully pigmented parent strains was only about five-fold,¹¹ whereas the ratio of maximum sensitivity and minimum sensitivity during the growth cycle was in the range 39–181 (table V).

Upon entering the stationary phase *S. aureus* survives for long periods without dying. Nevertheless, when harvested at various times during the stationary phase the organisms continued to change in their response to oleic acid, as evidenced by further reductions in the rate of killing and the fact that an increasing fraction of the population became resistant to high concentrations of the fatty acid (figs. 1 and 2). These responses appear unrelated to membrane fluidity, but the basis for this population change is not known. In any case, the stationary phase should not be treated as a period of metabolic inactivity. Numerous studies have shown that during the stationary phase gram-negative bacteria can undergo changes in membrane composition and cell-wall structure, and thereby become more resistant to different environmental stresses;¹⁸ similar changes might also occur in gram-positive organisms.

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