SENSITIVITY OF STAPHYLOCOCCI TO FATTY ACIDS: NOVEL INACTIVATION OF LINOLENIC ACID BY SERUM

R. W. LACEY AND VICKI L. LORD

Department of Bacteriology, North Cambridgeshire Hospital, Wisbech, Cambridgeshire

SUMMARY. The inhibition of coagulase-negative staphylococci and *Staphylococcus aureus* of human or animal origin by most free fatty acids was similar, but coagulase-positive staphylococci were sensitive and coagulase-negative cultures were resistant to linolenic acid. Animal strains of *S. aureus* were more sensitive to linolenic acid than were human strains. These differences were reflected in the relative abilities of the three categories of strains to survive on human skin.

The antibacterial effects of 20 mg of linolenic acid were inactivated by 1 ml of serum *in vitro*. A test organism seeded on to skin also survived better if first suspended in serum. The mechanism of the interaction between serum and linolenic acid may be due to a detergent effect of the serum and could account for colonisation of diseased skin with *S. aureus*.

Cultures of *S. aureus* seeded on to human skin were rapidly killed after the skin has been covered with linolenic acid. The possibility of therapeutic use of linolenic acid as an antibacterial agent should be explored.

INTRODUCTION

Factors that may contribute to the elimination of bacteria from intact human skin are desiccation, low pH, the presence of free fatty acids (Ricketts, Squire and Topley, 1951; Lacey, 1968; Aly *et al.*, 1976), the production of antibiotics by the resident commensal flora (Selwyn and Ellis, 1972) and shedding of squames by abrasion. However, it is not known why *Staphylococcus epidermidis* and some related species are often present in normal skin whereas *S. aureus* is infrequently found (Lacey *et al.*, 1980). It is also not clear why *S. aureus* can often be isolated from skin damaged, for example, by eczema or psoriasis, or why strains of *S. aureus* from animal sources rarely colonise man.

One of the properties of cutaneous fatty acids thought to be responsible for their antibacterial activity is their degree of unsaturation. The inhibitory capacity might increase with the degree of unsaturation so that linolenic acid (which contains three double bonds) is the most inhibitory, linoleic (two double bonds) less so, with oleic (one double bond) and stearic (saturated) with

insignificant antibacterial properties (Butcher, King and Dyke, 1976; Ko, Heczko and Pulverer, 1978; Heczko, *et al.*, 1979). However, linoleic acid can be more inhibitory to bacteria than linolenic (Bayliss, 1936; Fuller and Moore, 1967; Kabara *et al.* (1972). The amount of free linoleic acid in normal skin exceeds that of linolenic acid (Wilkinson, 1972) so the precise significance of the presence of these and other acids is not clear.

This paper reports on the relative sensitivities of coagulase-negative staphylococci, and coagulase-positive staphylococci from human and animal sources, to various fatty acids, and we also describe a novel serum activity that destroys the antibacterial effect of linolenic acid.

MATERIALS AND METHODS

Bacteria. Two hundred and sixteen strains of *S. aureus* were from a variety of animals (Lacey, 1980a), 156 strains were from infected patients at Wisbech and King's Lynn from 1975 to 1979, 49 from the Division of Hospital Infection, Central Public Health Laboratory, Colindale and 33 from the State Serum Institute, Copenhagen. Cultures were thrice purified and stored without subculture on agar slopes at room temperature. One hundred and five cultures of coagulase-negative staphylococci were obtained from the surface of forearm skin of six patients and 10 healthy laboratory staff and were allocated by the API method (API Staph., lot no. HDO-04E) (Kloos and Schleifer, 1975) into the following species: *S. epidermidis* (48 strains), *S. haemolyticus* (5), *S. haemolyticus* II (14), *S. capitis* (10), *S. warneri* (3), *S. hominis* (1), *S. saprophyticus* (2), *S. cohnii* (2) and unclassifiable (20).

Chemicals. Free fatty acids were the most purified grade available from Sigma. Purified human albumin was obtained from the Blood Products Laboratory, Elstree and contained 160 mmol Na/L. Unless otherwise stated "serum" refers to a pool obtained from about 100 human donors.

Determination of sensitivity to fatty acids. Free fatty acids that were oils at 56°C were incorporated into molten Blood Agar Base (Oxoid) to final concentrations of 1.0, 0.5, 0.25, 0.1, 0.05, 0.025, 0.01 and 0.005% (v/v). Stearic and palmitic acid were dissolved in 74% (v/v) alcohol before addition to molten agar at 56°C. The agar was then shaken for ~20 sec. and cooled under running cold water with rotation until the temperature was ~40°C. Of the resultant emulsions, 20 ml was poured into 9-cm petri dishes. The plates were dried for 3 h at 37°C and then inoculated with cultures diluted sufficiently to give isolated colonies. The end point (MIC) was defined as the concentration of fatty acid required to inhibit the growth of 95% of the inoculated cocci after incubation at 37°C for 24 h.

Survival of staphylococci on the skin. All the test organisms were first "marked" by a selection for mutants resistant to rifampicin, and the media for viable counts of the original inoculum and the bacteria recovered from the skin contained rifampicin 100 µg/ml. Overnight broth cultures of each test culture were centrifuged, resuspended in physiological saline and then diluted 1 in 100 with physiological saline or serum, and 10-µL portions were spread over 44 mm diameter circular areas of unprepared skin on the forearms of healthy volunteers. Subjects were asked not to use antibacterial soap during the course of the experiments. The inoculated arms were immediately covered by a loose polyethylene tube fastened by elastic bands at the elbow and wrist. The volunteers continued normal technical activities for 6 h then each inoculated area was sampled: a plastic ring was firmly placed in contact with the skin, 1.0 ml of physiological saline was pipetted into the ring and the area was vigorously irrigated with a pipette for 30 s. The fluid was withdrawn and viable counts obtained after further dilution in physiological saline. Preliminary experiments showed that ~50% of the inoculum was lost during the initial application of the culture. Twelve replicates gave a coefficient of variation of 63%. A control, uninoculated area on each arm was sampled to exclude the possibility of pre-existing rifampicin-resistant *S. aureus* and coagulase-negative staphylococci. In some experiments 10 µL of pure linolenic acid was spread over each test area immediately before the application of *c.* 10⁶ bacteria

suspended in 10 μ L of serum. For these experiments the recovery and dilution fluid contained 0.01M calcium chloride to inactivate the antibacterial effects of linolenic acid. A proportion (2%) of cultures of *S. aureus* recovered from the skin was checked for identity by bacteriophage typing.

Culture media, mutagenesis with nitrosoguanidine, and bacteriophage typing have been described previously (Lacey and Stokes, 1979).

Interaction between linolenic acid and serum. Linolenic acid was dissolved in 74% (v/v) ethanol and added either to nutrient agar (Mast) or to distilled water containing 1% (v/v) pure agar, pH 6.0. Serum or its components (100 μ L) were diluted in physiological saline and replicates were placed in randomly allocated 9-mm wells. The resultant clearing of the fatty-acid emulsion around each well was measured after incubation for 20 h at 30°C, unless otherwise stated.

RESULTS

Sensitivity of staphylococci to fatty acids

Initially, six strains of *S. aureus* of human origin (from non-hospitalised patients), six of animal origin (four cattle, two poultry) and six coagulase-negative staphylococci were tested for sensitivity to several fatty acids, excluding linoleic and linolenic acids. Little difference in the susceptibility of different strains was seen, but there were considerable differences in bactericidal potency among the different fatty acids (table I).

Sensitivity of staphylococci to linoleic and linolenic acids

Some variation in the inhibitory capacities of fatty-acid emulsions prepared on different occasions might be anticipated. This was assessed by tabulating the end point obtained on 20 different days for two reference strains, 1030 and 1030(55) (Lacey, 1980b). For linolenic acid, the median MIC for

TABLE I

Sensitivity of coagulase-negative staphylococci and Staphylococcus aureus from human and animal sources to fatty acids

Fatty acid (number of carbon atoms:double bonds)	Mean MIC (% v/v) against		
	coagulase-negative staphylococci (6 strains)*	<i>S. aureus</i> (6 human strains)	<i>S. aureus</i> (6 animal strains)
Capric (C _{10:0})	0.1	0.1	0.1
Lauric (C _{12:0})	0.1	0.1	0.1
Myristic (C _{14:0})	0.05-0.1	0.1-0.5	0.1-0.5
Palmitic (C _{16:0})	1.0	2.5	2.5
Stearic (C _{18:0})	0.5	0.5	0.5
Oleic (C _{18:1})	> 1.0	> 1.0	> 1.0
Elaidic (C _{18:1})	1.0	1.0	1.0
Linoelaidic (C _{18:2})	1.0	1.0	1.0
Arachidonic (C _{20:4})	0.25	0.01-0.25	0.01-0.25

* Three were *S. epidermidis* and three *S. haemolyticus*.

both strains was 0.025% (v/v) with a range from 0.01 to 0.05%. For linoleic acid the median was 0.5 with a range 0.1–1.0%. About 60% of each of the end points were at the medians. Because linolenic acid decomposes in aqueous emulsions (Gutteridge, Lamport and Dormandy, 1976), some experiments were performed in which a 10% (v/v) emulsion of linolenic acid in physiological saline was incubated with agitation for 72 h at 37°C at 150 rpm before inclusion into nutrient agar. The antibacterial activity as judged by 18 test bacteria (see above) was the same as that for medium containing freshly incorporated linolenic acid.

The sensitivity of a large number of staphylococci to free linolenic acid was measured. Most coagulase-negative staphylococci were at least 50 times more resistant than *S. aureus* of human origin, and these, in turn, were slightly more resistant than *S. aureus* strains from animals (table II). Of the 14 coagulase-negative staphylococci inhibited by $\leq 1.0\%$ (v/v) linolenic acid, eight were *S. capitis*. Although the coagulase-negative staphylococci were resistant to linoleic acid, the sensitivity of *S. aureus* was variable, regardless of source (table II). Furthermore, eight (36%) strains of *S. aureus* showed the phenomenon that some lower concentrations of acid (e.g., 0.05% v/v) were inhibitory, but higher concentrations (e.g., 0.1% v/v) permitted growth over a narrow range. Such an effect was seen reproducibly in replicate experiments on different occasions for all these eight strains. The end points in table II are the concentrations of linoleic acid finally inhibiting growth.

TABLE II

Sensitivity of coagulase-negative staphylococci and Staphylococcus aureus from human and animal sources to linolenic and linoleic acids

Fatty acid	MIC (% v/v)	Number of strains with stated MIC among		
		coagulase-negative staphylococci (105 strains)	<i>S. aureus</i> (238 human strains)	<i>S. aureus</i> (216 animal strains)
Linolenic	0.005	0	0	16
	0.01	0	6	80
	0.025	0	185	98
	0.05	0	47	22
	0.1	0	0	0
	0.25	1	0	0
	0.5	1	0	0
	1.0	12	0	0
	> 1.0	91	0	0
Linoleic	0.01	0	0	1
	0.025	2	35	54
	0.05	0	9	4
	0.1	0	19	38
	0.25	0	85	5
	0.5	0	17	113
	> 0.5	103	73	1

Survival of staphylococci on human skin

There were considerable but reproducible differences in the survival of individual strains in different volunteers, but within each group of strains the levels of survival were fairly consistent with respect to each volunteer. *S. aureus* strains of animal origin on average survived poorly compared with *S. aureus* strains from humans, and these in turn were poor survivors compared with the coagulase-negative staphylococci (see table III). Thus, the relative levels of survival of these groups of strains resembled their relative resistances to linolenic acid.

Rifampicin-resistant strains of *S. aureus* were recovered from the uninoculated area on only one subject on two occasions. They were identical in typing pattern to a culture seeded on the arm 4 days previously.

TABLE III

Survival of coagulase-negative staphylococci and Staphylococcus aureus from human and animal sources on the forearm skin of volunteers

Staphylococci (origin)	Number of strains tested	Number of experiments (volunteers)	Percentage of inoculum recovered at 6 h (range)
Coagulase-negative (healthy human skin)	11	44 (6)	72.0 (7.0-75.0)
<i>S. aureus</i> (skin lesions of non-hospitalised patients)	6	36 (6)	6.2 (0.2-18.0)
<i>S. aureus</i> (animal: cattle 11 strains, pigs 3, poultry 2)	16	52 (6)	0.1 (0.01-28.0)

Effect of suspension in serum on survival of S. aureus on human skin

The effect of serum on the survival of the animal strain of *S. aureus* (CIX 76) was studied in six volunteers. Apart from one subject (no. 2) whose skin was highly bactericidal in the presence of saline or serum, suspension of the test organism in serum markedly increased the survival of the test culture (table IV).

Interaction between serum and linolenic acid

This was studied by incorporating pooled serum 1% (v/v) linolenic-acid emulsion 0.01-1.0% (v/v) into nutrient agar and noting the effect on the MIC of the 18 test staphylococci (see above). All the bacteria became more resistant to linolenic acid in the presence of serum so that 1 ml of serum neutralised the antibacterial effect of 20 mg of linolenic acid.

TABLE IV
Effect of serum on the survival of Staphylococcus aureus (strain CIX76) on human skin

Subject no.	Percentage* of inoculum† recovered from skin after 6 h suspended in	
	serum	saline
1	36.0	0.62
2	0.06	0.05
3	71.0	0.02
4	88.0	0.06
5	2.5	0.4
6	10.0	0.07

* Mean of three experiments.

† c. 10^6 bacteria in $10\ \mu\text{L}$.

The interaction between linolenic acid and serum was also studied by observing the clearing of an emulsion of 0.1% (v/v) linolenic acid in pure agar around wells containing serum. The diameter of the zones of clearing followed closely those of neutralisation of the antibacterial activity of linolenic acid with different dilutions of serum (data not shown).

The nature of the linolenic neutralising capacity of serum has proved difficult to establish. The activity is not associated with purified human albumin or globulin, it is stable for 24 h at 56°C or 1 h at 100°C , it is not extractable with chloroform, methanol or methanol/chloroform, suggesting that it is not a phospholipid or protein. It disappears during dialysis through a membrane (mol. wt. $< 125\ 000$) and is not associated with calcium or magnesium ions because serum collected into containers with EDTA has unchanged activity. The effect of dilution of serum and a detergent (Triton X) or the inhibitory power of linolenic acid was similar. Thus, serum and Triton X cleared a linolenic-acid suspension to a similar extent as shown by the relationship between the log concentration of each substance and the size of the resultant zones. For both substances the relationship was not linear, with an unexpectedly large effect at low dilutions. This suggests that the linolenic-acid antagonism effect of serum may be due to "detergent" activity of a protein, polysaccharide or glycoprotein. Relevant to this was our finding that pooled serum, and serum from each of six healthy volunteers, inhibited the growth of four out of four (three coagulase-negative and one coagulase-positive) strains tested. The inhibition occurred optimally at a final dilution of 2% serum in nutrient agar and subinhibitory concentrations of linolenic acid reversed this inhibition. Thus serum and linolenic acid can be individually inhibitory, but a mixture loses this capacity.

Effect of applied linolenic acid on the bactericidal activity of human skin

In each of six volunteers, the application of neat linolenic acid to the skin caused a bactericidal effect on staphylococci subsequently inoculated (table V)

TABLE V

Effect of addition of linolenic acid on the antibacterial properties of human skin

Subject no.	Percentage of inoculum* recovered after 6 h from	
	untreated skin	skin treated with 10 μ L of linolenic acid
1	77.8	<0.01
2	56.6	<0.01
3	93.0	<0.01
4	8.4	<0.01
5	69.1	0.02
6	73.5	0.16

* Approximately 10^6 cells of *S. aureus* strain 1030 in 10 μ L of serum.

and the bacteria that survived produced smaller-than-usual colonies of phage-typing pattern similar to that of the inoculated strain 1030. The destruction of the bacteria probably occurred on the skin surface because the recovery fluid contained 0.01M calcium chloride to inactivate any residual fatty acid. However, we cannot exclude the possibility that linolenic acid is taken up by bacteria on the skin surface, and its destructive effect occurs subsequently on nutrient agar.

Attempts to select variants of S. aureus resistant to linolenic acid

All the derivatives of strain 1030 containing plasmids or bacteriophages (Lacey 1980b) were tested for sensitivity to linolenic acid, and the MIC for each derivative was the same as for the wild strain. Heavy inocula ($\sim 10^{11}$) of the pigmented cultures of strains 6936, 609 and 1030 were then plated on medium containing increasing concentrations of linolenic acid and the plates were incubated for 72 h. Each culture yielded (at a frequency of $\sim 10^{-9}$) some non-pigmented resistant variants at critical concentrations of fatty acid, usually 0.05–0.10%(v/v). These variants grew slowly but on subculture reverted rapidly to normal-growing sensitive pigmented cells. Similar results were obtained after mutagenesis with nitrosoguanidine. Thus it was not possible to isolate stable variants of *S. aureus* resistant to linolenic acid.

Sensitivity of other bacteria to linolenic acid

Twenty-five strains of *Escherichia coli*, 25 *Pseudomonas aeruginosa*, 16 *Proteus* spp. and 25 *Klebsiella* spp. were all resistant to 0.25%(v/v) linolenic acid. Streptococci showed variable sensitivity with MICs from 0.01 to 0.025% for five of group A, 0.025% for three group B, 0.025% for two group C and $\geq 0.25\%$ for nine strains of *Streptococcus faecalis*.

DISCUSSION

Although there is uncertainty as to whether linolenic or linoleic acid is the fatty acid most inhibitory to bacteria in general, linolenic is shown here to be more inhibitory than linoleic acid to many strains of *S. aureus*. Sensitivity to linoleic acid is difficult to define because many cultures show a paradoxical effect whereby a high concentration is less inhibitory than a low one. Although sensitivity tests to linolenic acid separated the coagulase-positive staphylococci (sensitive) from the coagulase-negative strains (resistant), other unsaturated fatty acids showed little such effect.

Differences in sensitivity of staphylococci to linolenic acid *in vitro* may explain the variation in their survival on skin under the above experimental conditions. Cultures of *S. aureus* from animal sources survived less well on the skin than those from human material and the latter had a reduced survival compared with coagulase-negative strains. The poor survival of animal staphylococci on skin may be one of the factors explaining why there is apparently little parasitism of man by these bacteria.

Linolenic acid, although present in small quantities in the skin (Wilkinson, 1972) may well be an important naturally occurring antibacterial agent. Its presence could explain why pathogenic staphylococci are rarely found on intact skin. It will be interesting to examine individuals with a predisposition to recurrent skin sepsis, notably those suffering from diabetes mellitus, to determine whether there is a shortage of linolenic acid in their skin. Although *S. aureus* is rarely found on normal skin, patients with a variety of skin diseases are well known to harbour large numbers of pathogenic staphylococci. It is possible that the inhibitory property of linolenic acid is neutralised by serum, because most dermatoses are associated with a serous exudate. Similarly the skin around wounds created by surgical incision may be bathed in serum and might lose its natural antibacterial properties. In support of this postulated interaction between skin and serum *in vivo*, we have found that staphylococci applied to the skin surface suspended in serum survive better than those suspended in saline.

The nature of the interaction between linolenic acid and serum is not clear. Previously the only serum component known to react with free fatty acids is albumin (Spector, 1975); but albumin is not involved here because purified albumin has insignificant effect, and prolonged heating or extraction with organic solvents had no effect on the capacity of serum to inactivate linolenic acid. However, the neutralising capacity of the serum correlates with its ability to dissolve emulsions of linolenic acid in water, i.e., a "detergent" effect.

The addition of pure linolenic acid to the skin surface resulted in a dramatic enhancement of the bactericidal effect of the skin, even when the test bacteria were applied in serum. Presumably adequate free linolenic acid was still present. Pathogenic staphylococci and also cultures of *Str. pyogenes* are predictably sensitive to linolenic acid, although Enterobacteriaceae and *Str. faecalis* are resistant. Linolenic acid would, therefore, possess all the attributes of an ideal antibacterial agent and it could have specific indications, i.e., to

control staphylococcal skin sepsis. It is naturally occurring and hence untoward reactions would be not anticipated, it should not destroy the commensal flora, resistance to it would not be expected to develop, and there would apparently be no risk of resistance to antibiotics developing during its use.

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