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Mechanisms of resistance of staphylococci grown in plasma to polymorph bactericidins

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SUMMARY

The mechanisms whereby staphylococcal strains grown in plasma assume increased resistance to polymorph bactericidins were investigated. Observations reported here showed that cultural conditions could determine the path of conversion to resistance. Staphylococcal strains and mutants lacking either free coagulase or clumping factor or both all showed enhanced resistance after 10 h incubation in plasma proteins, thus giving no clear indication that these factors were involved in the interactions. In fact, prolonged incubation in bovine serum albumin (22 h) and ordinary broth medium (24 h) also resulted in increased resistance. A distinction between staphylococcal factors interacting specifically with plasma proteins and such non-specific conversions was obtained in two different ways. Stripping of a hypothetical surface protein by treatment with trypsin or 2 M potassium bromide rendered plasma- but not 24 h-broth organisms susceptible, indicating protein coating of plasma-grown organisms. Also free coagulase-positive strains and mutants incubated in plasma for 30 min were converted while those lacking both or possessing clumping factor alone were not. It therefore appears that one of the mechanisms of acquiring resistance involves a rapid interaction between staphylococcal-free coagulase and fibrinogen, resulting in the deposition of fibrin or fibrin derivatives on the bacterial surface.

INTRODUCTION

Kolawole (1983) reported that a staphylococcal strain grown in plasma showed an increase in resistance to both intracellular and soluble bactericidin killing which was equivalent to that of organisms growth *in vivo*. Five other strains showed comparative resistance after growth (10 h incubation) in plasma while showing susceptibility after growth (4 h incubation) in Nutrient Broth No. 2, Brain Heart Infusion, Penassay or modified (Ekstedt & Bernhard, 1973) 110 broths. This indicated that the conversion in plasma, which simulated growth *in vivo*, could be a general phenomenon among coagulase-positive staphylococci. Such organisms were agglutinated within 30 min in both tube and slide agglutination tests using anti-rabbit fibrinogen antiserum while no broth-grown organisms were agglutinated. This indicated that fibrin or a related fibrinogen-derived material could be present, among other possible deposits, on the surface of the organisms grown in plasma as a result of interaction between plasma proteins and staphylococcal factors such

as clumping factor, free coagulase (Hale & Smith, 1945; Duthie, 1954) and protein A (Forsgren & Sjoquist, 1967).

This paper is a report of investigations into these possibilities.

MATERIALS AND METHODS

Preparation of staphylococcal strains

Staphylococcus aureus strains O, V8 and CN 4687 (all CF+, FC+) and the protein A-producing (Forsgren & Sjoquist, 1966) Cowan I type (CF−, FC+), were obtained from sources described previously (Kolawole, 1983). Strains Newman (CF+, FC+), Newman D_2C (CF+, FC−), Smith diffuse (CF−, FC+) and *S. epidermidis* strain Zak (CF−, FC−) as used by Hawiger, Hawiger & Koenig (1971) were kindly supplied by Dr J. Hawiger, George Hunter Laboratory, Vanderbilt University School of Medicine, Nashville, Tennessee, while strains 18ZB (CF−, FC+) and 18ZC (CF+, FC−) were provided by Professor F. A. Kapral, College of Medicine, Ohio State University, Columbus (Kapral & Li, 1960).

Strains for use in experiments were first grown in the appropriate broth media-Nutrient Broth No. 2 (NB2, Oxoid) or brain–heart infusion (BHI, Difco) as described previously (Kolawole, 1983). Growth in rabbit serum (Wellcome Reagents Ltd, prepared from clotted rabbit blood and heated at 56 °C for 30 min), normal rabbit γ-globulin (75 mg/ml), prepared by sodium sulphate fractionation and dialysis against phosphate buffered saline (PBSA) or in fibrinogen (3 :4 mg/ml, Wellcome Reagents Ltd), were as previously described for growth in plasma. Where the effect of protein A was being examined, organisms were first grown in BHI, to encourage protein A production (Forsgren & Sjoquist, 1966).

Observation of organism suspensions during incubation in plasma (whether 30 min, 4 h or 10 h) showed that they contained clots of organisms. These clots were broken up as far as possible by vigorous vibration with sterile glass balls and any persisting large clumps removed by low-speed centrifugation, leaving small clumps of two to four organisms among organisms dispersed singly. Washed organisms suspended in fresh broth were held at 0 °C until required (usually overnight) by immersing the tubes in ice. Similarly, phase-contrast examination of organisms incubated for 10 h in serum, γ-globulin or fibrinogen showed that they were present either singly or in groups of two and four and some (except Zak) appeared to be surrounded by a thin layer of less-dense material. No large organism clumps were, however, present.

Growth of organisms in bovine serum albumin (BSA)

Approximately 4×10^5 viable organisms in 0·1 ml volume from a broth suspension already held overnight at 0 °C were inoculated into a 25 ml screw-capped bottle containing 3·9 ml of BSA solution at a concentration of 40 mg (equivalent to its level in plasma) per ml in phosphate buffer (pH 6·8). This was incubated at 37 °C for 22 h, to achieve growth of organisms equivalent to that in other protein solutions (i.e. 10^3 to 10^4-fold increase). Such organism suspensions contained no clumps and individual organisms had no surface covering throughout as judged by phase-contrast microscopic examination at intervals during the incubation.

Ultrasonic treatment of organisms

Samples of organism suspensions were ultrasonicated in a MSE ultrasonic disintegrator operated at 1·2 A for 1 min at 0 °C to disaggregate clumps of organisms. Viable counts of ultrasonic-treated samples at the start and at the end of experiments were compared with actual experimental viable counts to obtain a factor of increase and hence the extent to which clumping could affect results.

Trypsin treatment of organisms

Organisms (10^9 by viable count) in 0·9 ml broth were mixed with 0·1 ml trypsin, Sigma London (1 mg/ml in phosphate buffer, pH 6·8) and incubated at 37 °C for 45 min. Soya-bean trypsin inhibitor, Sigma London (0·1 ml from 1 mg per ml solution in phosphate buffer, pH 6·8) was then added and allowed to act for 1 min. The organisms were centrifuged (7000 ***g*** for 10 min) and after washing three times, the organism pellet was finally re-suspended in 1 ml ice-cold broth. The viability of organisms so treated, as judged by plate counts before and after treatment, was $> 95\%$ of the initial viable numbers.

Treatment of organisms with 2 M *potassium bromide*

Organisms (5×10^9 by viable count) in 0·5 ml were mixed with 0·5 ml 4 M potassium bromide (KBr) to give final concentration of 10^9 organisms/ml and 2M-KBr respectively. The bottle was immersed in ice and kept in the cold for 2 h. The organisms were centrifuged and treated as described for trypsin treatment. This treatment also resulted in $< 5\%$ loss in viability as judged by plate counts before and after treatment.

Preparation and measurement of the activity of soluble bactericidins

PMN leukocytes were homogenized and extracted for soluble bactericidins, and their activity was measured as previously described (Kolawole, 1983).

Killing of organisms was calculated from the ratio of the number of viable organisms at the start of incubation with soluble bactericidins to the viable numbers at the end and is expressed in folds of reduction (or depression) denoted by an 'X' after the value. Organisms showing 15-fold reductions and below are considered resistant (i.e. 6–100% survival) while organisms showing greater than 40-fold reductions are susceptible (i.e. $< 2{\cdot}5\%$ survival). All depression values falling in between these two levels have been referred to as indicating 'intermediate' resistance.

RESULTS

Effect of coagulase, clumping factor or protein A interactions with plasma proteins on resistance

In soluble bactericidin killing tests (Table 1), strains O-serum, Newman serum and Cowan serum showed a resistance to killing which was intermediate between the usual plasma- (range 5- to 14-fold reduction) and broth-grown organisms (40-fold reduction and above). Strain Zak-serum, however, showed a much greater susceptibility to killing. Organisms grown in γ-globulin also showed intermediate resistance between plasma-grown organisms and their counterparts grown in

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Table 1: *Killing of organisms after growth in various plasma proteins or in brain heart infusion broth (BHI)*

(Each value is a mean of depression* values from two different experiments and the standard errors are shown in parentheses.)

	Killing by soluble bactericidins after 10 h growth in				
Staphylococci	Serum	δ-globulin	Fibrinogen	Fibrinogen + δ-globulin	BHI
0	25 × (±0·2)	23 × (±0·5)	7·8 × (±1·2)	5·2 × (±1·6)	210 × (±6·0)
Newman	33 × (±4·6)	33 × (±3·0)	12·4 × (±0·3)	22 × (±2·4)	2,300 × (±100)
Cowan I	24 × (±0·5)	20 × (±2·2)	—	—	200 × (±10)
Zak	74 × (±10)	48 × (±2·5)	47 × (±4·2)	—	210 × (±5·0)
CN 4687	—	—	4·2 × (±0·7)	2·7 × (±0·2)	2,050 × (±50)
V8	—	30 × (±4·5)	6·3 × (±0·3)	20 × (±0·8)	552 × (±20)

—, Experiments not done.

* Depression values are folds of reduction in viable numbers and have been calculated from viable number at 0 h/viable numbers at 3 h.

Table 2. *Killing of staphylococcal variants after growth in plasma, fibrinogen or broth*

(Means of depression values from two different experiments are shown with standard errors in parenthesis.)

Staphylococcal	Killing by soluble bactericidins after growth* in Plasma	Fibrinogen	Broth
Newman D_2C (FC−, CF+)	6·2 × (±0·8)	43 × (±1·8)	155 × (±5·5)
18ZC (FC−, CF+)	12·4 × (±0·8)	15 × (±0·9)	128 × (±4·0)
18ZB (FC+, CF−)	11·6 × (±0·3)	22 × (±0·4)	138 × (±3·0)
Smith-diffuse (FC+, CF−)	16·2 × (±0·8)	21 × (±1·1)	120 × (±3·0)
Zak (FC−, CF−)	8·7 × (±1·2)	47·4 × (±4·1)	159 × (±4·0)

* The variants were incubated for 10 h in plasma or fibrinogen and 4 h in broth.

brain–heart infusion (BHI) except, again, the Zak strain which was susceptible. Growth in fibrinogen conferred a plasma-like resistance on strains O, Newman, V8 and CN 4687 but not on Zak. The additive effect expected by growing organisms in a mixture of fibrinogen and γ-globulin was not confirmed since only strains O and CN 4687 showed resistance while Newman and V8 still showed intermediate resistance. All strains grown in BHI showed the usual susceptibility.

Staphylococcal strains lacking in clumping factor (CF), free coagulase (FC) or both (called variants), grown in either plasma or fibrinogen, were compared in soluble bactericidin killing tests. All variants, including the Zak strain (CF−, FC−), were enhanced in resistance to killing after growth in plasma, the Smith diffuse (CF−, FC+) being rather less enhanced (Table 2). Strains D_2C and Zak grown in fibrinogen were significantly suceptible, while the others showed intermediate (18ZB and Smith) to full (18ZC) conversion to resistance.

Microscopic examination, under phase contrast, of organisms withdrawn at intervals during incubation in plasma showed that clumping and coating as reported earlier for strain O-plasma (Kolawole, 1983) were apparent within 3 h of commencement in all variant suspensions except the Zak and Smith diffuse strains. Clumping became apparent in suspensions of these two only after 6 h incubation. The Zak organisms were, however, not agglutinated by anti-rabbit fibrinogen antiserum. In fibrinogen solutions, slight clumping was observed in the suspensions of clumping factor positive strains (D_2C and 18ZC) after 6 h incubation, but there was none in suspensions containing Zak, 18ZB or Smith diffuse strains throughout. Consequently, Zak organisms taken out at 6 h showed broth-like susceptibility (128-fold reduction) while those taken out after 10 h incubation still showed resistance (9-fold reduction).

Effect of length of incubation on conversion to resistance

Following from the results of tests on Zak organisms grown for 6 and 10 h in plasma, organisms incubated for varying lengths of time in bovine serum albumin (BSA), NB2 (neither of which is known to interact with coagulase factor or protein A) or in plasma were compared in soluble bactericidin killing tests (Table 3). Strain O withdrawn from the BSA solution at 30 min was susceptible while the sample at 22 h showed intermediate resistance. Strains O and CN 4687 withdrawn from

Table 3. *Killing of organisms after growth in bovine serum albumim (BSA), broth or plasma for varying lengths of time*

(Each value is a mean of at least two experiments; standard errors are shown in parentheses.)

	Killing by soluble bactericidins after (h) incubation in								
	BSA			Broth			Plasma		
Staphylococci	½ h	10 h	22 h	4 h	10 h	24 h	½ h	4 h	10 h
O (FC+, CF+)	155 × (±8·5)	99 × (±5)	22 × (±1·2)	128 × (±5·2)	28 × (±2·5)	16·5 × (±0·9)	*6 × (±0·2)	*7·2 × (±0·4)	9·5 × (±1·5)
CN4687 (FC+, CF+)	—	—	—	168 × (±6·2)	23 × (±1·8)	10 × (±0·5)	*5·2 × (±0·4)	*5·7 × (±0·3)	6·2 × (±0·4)
18ZB (FC+, CF−)	—	—	—	109 × (±4·0)	—	—	*10 × (±1·6)	*2·2 × (±0·1)	10·5 × (±1·1)
NEWMAN D_2C (FC−, CF+)	—	—	—	148 × (±5·3)	—	—	180 × (±12)	193 × (±9·0)	6·0 × (±0·4)
ZAK (FC−, CF−)	—	—	—	127 × (±6·2)	—	—	121 × (±9)	115 × (±5·0)	8·7 × (±1·8)

—, Experiments not done.

* Values corrected for the presence of clumps.

Table 4. *Killing of organisms after treatment designed to remove surface proteins*

(Each value is a mean of two different experiments; standard errors shown in parentheses.)

	Killing by soluble bactericidin of		
		Organisms treated with	
Staphylococcal strain-medium (incubation time)	Untreated organisms	Trypsin	2m-KBr
O-plasma (30 min)	6 × (±0·2)	368 × (±14·0)	301 × (±16·0)
O-plasma (10 h)	9·6 × (±0·9)	516 × (±27·0)	228 × (±17·0)
18ZB-plasma (30 min)	12 × (±20·0)	402 × (±20·0)	144 × (±5·0)
O-broth (24 h)	18·8 × (±1·7)	28 × (±3·0)	14·7 × (±1·2)

NB2 at 4, 10 and 24 h showed correspondingly increasing resistance. All wild strains and variants were resistant after 10 h incubation in plasma. However, while strains O and CN 4687 (both CF+, FC+) and 18ZB (CF−, FC+) showed resistance after 30 min or 4 h incubation, strains Zak (CF−, FC−) and Newman D_2C (CF+, FC−) showed broth-like susceptibility.

Effect of the presence of clumps of organisms on the observed resistance

After correction of experimental viable counts by appropriate factors of increase (both at the start and at the end of experiments), the depression in viable numbers of such organisms (figures with asterisks, Table 3) were still in the range of resistant organisms.

Effect of removal of surface coating on the observed resistance to killing

After exposure to trypsin digestion or physical dissociation using 2 M potassium bromide, strains O-plasma (30 min and 10 h) and 18ZB-plasma (30 min) became markedly susceptible to killing by soluble bactericidins (Table 4). However, strain O-broth (24 h), used as control here since it does not possess a similar surface covering, remained resistant.

DISCUSSION

The intermediate resistance conferred on *S. aureus* cells grown either in γ-globulin or serum suggests that while Protein A–IgG interactions may be playing a part in enhancement of resistance to killing, they do not appear to be responsible for the main plasma effect. Therefore only growth in media conferring equivalent resistance to that of *in vivo*-and plasma-grown organisms were used in further studies. Also, the expansion in viable numbers due to ultrasonic disaggregation of clumps did not eliminate the differences observed in the resistance of plasma-grown organisms and their broth-grown counterparts, indicating that clumping could not account for the observed enchanced resistance. It therefore appeared that fibrin coating of individual organisms, as indicated by agglutination by anti-rabbit fibrinogen antiserum, was responsible.

The resistance subsequently conferred on *S. aureus* strains after incubation in fibrinogen while the Zak (*S. epidermidis*) strain remained susceptible, further suggested that the mechanism of conversion to resistance was by direct interaction

of clumping factor to cause surface fibrin coating. However, the resistance of variants grown in either plasma or fibrinogen which should have been useful in confirming this did not give a clear correlation with the possession of either free coagulase or clumping factor. For example, strain Newman D_2C (CF+, FC−) was appreciably susceptible while the Smith diffuse (CF−, FC+) showed intermediate resistance after growth in fibrinogen. Several explanations may be advanced for these inconclusive results. First, it could be due to the production, by these *S. aureus* variants, of a cell wall polysaccharide known as the Compact Colony Forming Active Substance (CCFAS, Yoshida, Ohtomo & Minegishi, 1975), which can cause a clotting reaction with fibrinogen (Yoshida, Ohtomo & Minegishi, 1977, 1980) and which has been extracted from at least one *S. epidermidis* strain (Yoshida, Ohtomo & Usui, 1978), resulting in conversion irrespective of which of clumping factor or free coagulase they lacked. Other reasons could be that plasma components reacted through the capsular substance of the Smith diffuse strain, that the fibrinogen (and indeed the other plasma components and serum) solutions used might not have been 100% pure or that a time-dependent mechanism which is unrelated to a coagulase or clumping factor–fibrinogen interaction was also involved. For example, the observation that strain Zak-plasma at 6 h was susceptible while that at 10 h was resistant supports the last explanation. Zak-plasma at 10 h was, however, not agglutinated by antifibrinogen antiserum, suggesting that its resistance, slight surface coating and clumping were not due to fibrin formation on the surface. It is not known whether the strain Zak used here produces CCFAS-like *S. aureus* strains, but it is known to produce a protamine-like substance which is capable of paracoagulation with soluble fibrin monomers (Lipinski, Hawiger & Jeljaszewicz, 1967; Hawiger, Collins & Horn, 1969; Horn, Hawiger & Collins, 1969) and which could be responsible for the observed resistance. Also, the observed resistance of organisms after prolonged incubation in BSA (22 h) or broth (24 h), both of which are not known to interact with coagulase or clumping factor, could only result from different, time-dependent mechanisms since organisms taken out at earlier times were susceptible. These observations would appear to conform with those of Gladstone, Walton & Kay (1974), who reported a time-dependent resistance of staphylococci grown in peptone media supplemented with glucose against killing by cationic proteins from rabbit PMN. These workers had suggested that the resistance was the result of a shift from a usually aerobic respiration to an anaerobic one, in response to changing requirements in the growth media. This way, the NADH oxidase enzyme system on the bacterial cytoplasmic membrane, which they propose is the normal target of cationic proteins, is by-passed. The development of resistance outside coagulase or clumping factor–fibrinogen interactions in this work could be by a similar mechanism or it could be due to purely chemical interactions between organisms and the various by-products of metabolism which accumulate during incubation (Lipinski *et al* 1967).

In view of all the above, prolonged incubation may not be suitable for suitable for studying specific interactions. Moreover, it is known from tests for coagulase that clotting could occur any time from within a few seconds to 30 min, suggesting that growth was probably not necessary for conversion due to specific interaction to occur. However, the demonstration of resistance in only the free

coagulase-positive strains (strains O, 4687 and 18ZB) after only 30 min incubation in plasma suggests that one of the mechanisms at play is a rapid plasma conversion which depends on interactions of free coagulase with plasma proteins, resulting in the deposition of fibrin or fibrin derivatives on the surface of the organisms. Organisms possessing clumping factor alone appear to require a longer time for conversion.

The reversion to susceptibility, of plasma- and not broth-grown organisms, after treatment with trypsin or 2 M potassium bromide (procedures known to remove proteins from membrane surfaces) further supports the idea of a protective role for the surface deposits. This could be achieved either by blocking possible sites of bactericidin action on the bacterial surface (Pennial, Hollbrook & Zeya, 1972; Hibbit & Benians, 1973) including the covering up of NADH oxidase enzyme sites on the cytoplasmic membrane, or by forming complexes with basic components of soluble bactericidins, as it does with protamines, thus rendering them ineffective (Hawiger *et al.* 1969).

If such a rapid conversion and surface coating occurs readily *in vivo*, it could account at least in part, for the intracellular survival of organisms (Rogers & Tompsett, 1952; Adlam, Pearce & Smith, 1970; Kolawole, 1983) and is therefore likely to be an important virulence mechanism. The virulence of organisms grown with and without plasma is currently being investigated.

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