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Evaluation and use of the drug inhibition method of measuring intracellular killing in differentiating between staphylococci grown *in vivo* and *in vitro*

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SUMMARY

A *Staphylococcus aureus* strain grown once *in vivo* in rabbit pleural cavities was appreciably more resistant to killing by soluble polymorph bactericidins than its broth-grown counterpart. Parallel results were obtained using an adaptation of Solberg's drug-inhibition method of measuring intracellular killing after critical aspects of it had been evaluated and modified. Individual *in vivo* grown organisms were surrounded by a layer of less dense material which was not capsular in nature but which made the organisms clump together. This surface coating was lost on subculture *in vitro*, resulting in a reversion to broth-like susceptibility, thus indicating that the surface coating was largely responsible for the enhanced resistance and that it resulted from interactions between staphylococci and components of the rabbits' natural body fluids. Consequently, growth in plasma produced organisms which mimicked *in vivo* grown organisms in clumping, surface coating and in resistance to killing. The use of plasma-grown staphylococci in further studies of likely resistance mechanisms *in vivo* is discussed.

INTRODUCTION

Staphylococci grown *in vivo* differ from their *in vitro* grown counterparts in their α-toxin production (Gladstone & Glencross, 1960), aerobic respiration and antigenic composition (Gellenbeck, 1962) and in their virulence characteristics (Adlam, Pearce & Smith, 1970*a*; Beining & Kennedy, 1973). Adlam, Pearce & Smith, (1970*b*) also reported that staphylococci grown *in vivo* were enhanced in resistance to killing by intact rabbit polymorphonuclear (PMN) leukocytes and their crude bactericidal lysates. An *in vivo* phenotype, V, showed increased resistance over its broth-grown counterpart, P, and an original strain O, isolated from a human carbuncle and from which strain P was obtained after successive passages in rabbits.

However, Gladstone, Walton & Kay (1974) showed that strain P was relatively resistant to killing by PMN cationic proteins when compared with other broth-grown staphylococci. This was confirmed in studies, preliminary to the work reported here, in which an improved soluble bactericidin assay system-prepared by a modification of the method of Cohn & Hirsch (1960) and comparable to that of Gladstone *et al.* (1974) – was used, see Materials and Methods; V organism

suspensions contained clumps which masked an accurate assessment of its killing and, when disaggregated, V and P were similar in their resistance to killing. Comparisons between these two were therefore unsuitable for studying the differences between *in vivo* and *in vitro* organisms. Other indications from this are that the crude cell lysate preparation of Adlam *et al.* (1970*b*) might not have been sufficiently sensitive in distinguishing between *in vivo* and *in vitro* organisms and that a modification of their whole cell (phagocytic) killing system or the adoption of a more sensitive system was necessary. Since the major obstacle to accurate assessment of phagocytic killing appeared to be the extracellular attachment of organisms to PMN leukocytes (Craig & Suter, 1966; Tan, Watanakunakorn & Phair, 1971) a more sensitive method described by Solberg (1972*a*) was adopted. Essentially, Solberg used a combination of penicillin and streptomycin to inactivate extracellular organisms but since this required at least 15 min during which intracellular killing could occur to mask the results, phenylbutazone was added to block intracellular killing during this period. Viable numbers of organisms later released from the PMN leukocytes were taken as the numbers actually intracellular at the beginning of inactivation.

This paper describes, first, the results of investigations into possible mechanisms for enhancement of resistance during the growth of the more sensitive strain O *in vivo*, using soluble bactericidin killing tests and an adaptation of Solberg's method of measuring intracellular killing. In studies preparatory to the work reported here, phenylbutazone as used by Solberg (1972*a*) did not inhibit intracellular killing by rabbit PMN leukocytes but a related drug, chloroquine phosphate, did and has been used.

Second, an attempt has been made to devise cultural conditions *in vitro* which would simulate the environmental conditions *in vivo*, as this could provide a more convenient means of studying the mechanisms of acquiring resistance during growth *in vivo*.

MATERIALS AND METHODS

Preparation of staphylococcal strains

The staphylococcal strain O was derived by Adlam *et al.* (1970*a*). *Staphylococcus aureus* strain Newman was supplied by Dr J. Hawiger, George Hunter Laboratory, Vanderbilt University School of Medicine, Nashville Tenessee, while the Cowan I type, known to produce protein A, was provided by Mr G. Stewart, Queen Elizabeth Hospital, Birmingham. The *S. aureus* strains V8, CN55 and CN4687 were kindly donated by Dr C. Adlam, Wellcome Research Laboratories, Beckenham, Kent. All strains were clumping factor and free coagulase positive.

Growth of organisms in broth

Organisms incubated for 3–4 h in Nutrient Broth (NB2, Oxoid Ltd) were harvested by centrifugation at 1800 *g* for 15 min, washed and finally suspended in fresh, ice-cold broth. Suspensions so prepared were called strain-O broth, Newman broth, etc., respectively.

Growth of organisms in vivo

Overnight broth cultures containing 1×10^9 viable organisms, were injected intrathoracically into rabbits (New Zealand White, Oxfordshire Laboratory Animal Colonies, Northern, Ltd). The pleural exudate of animals dying 24–48 h later were transferred directly into sterile bottles kept in ice and purified as described by Adlam *et al.* (1970*a*). The purified organisms, called strain O-vivo, were finally suspended in fresh broth, distributed in 0·2 ml amounts and used immediately or stored at −20 °C until required, without further culture *in vitro*, except where stated. Phase contrast examination under the microscope showed that such organisms were clumped together and individual organisms were surrounded by a layer of less dense material which was not capsular in nature, as judged by microscope observation of samples after India ink staining. For some experiments designed to study the role of this surface covering, the organisms were subjected to different treatments, as enumerated below.

Unclumped (single) organisms were separated from populations containing clumps by low speed centrifugation (150 ***g*** for 15 min) and have been called 'separated single O-vivo' organisms.

In vivo grown organisms first incubated overnight on an agar slope and then for 3–4 h in broth have been called '*in vitro* subcultured O-vivo' organisms. Phase contrast examination showed that these no longer possessed a surface covering.

'Ultrasonic-treated O-vivo' were *in vivo* organism suspensions ultrasonicated in a MSE ultrasonic disintegrator (operated at 1·2 A for 1 min at 0 °C) to disrupt the clumps without appreciable loss in viability (< 5 %), as confirmed by microscope examination under phase contrast and subsequent plate counts.

Growth of organisms in plasma

Approximately 4×10^5 viable broth-grown organisms in 0·1 ml volume were inoculated into 3·9 ml of sterile rabbit plasma (citrated blood plasma sterilized by membrane filtration) and incubated with rotation for 10 h, determined by the time required to achieve equivalent growth to broth organisms as well as reasonable break-up of solid clots. Organisms harvested by centrifugation were washed and finally suspended in fresh, ice-cold broth by shaking vigorously for 1 min with sterile glass balls on a 'Rotamixer' (Hook and Tucker Ltd) to disperse clumps. The suspension, called strain-O plasma, Newman plasma, etc., was distributed in 0·2 ml amounts and used immediately or stored at −20 °C until required. Phase-contrast examination of plasma-grown organisms showed that they were in clumps and had a less-dense surface material similar to that of *in vivo* grown organisms.

Agglutination of plasma-grown organisms

Doubling dilutions of reconstituted sheep anti-rabbit fibrinogen serum (Wellcome) were made in sterile (0·85 %) saline and put into test tubes in a water bath at 37 °C. Samples (0·5 ml) from serial dilutions of plasma-grown organisms were added to the serum dilutions and the tubes incubated for 30 min. The tubes were then examined for agglutination, both visually and by microscope observation after transferring loopfuls of the mixture on to slides. These were compared with

control tubes containing similar concentrations of anti-fibrinogen serum and either a fibrinogen standard which should show agglutination or O-broth organisms, which do not possess a similar surface material.

Bacterial counts

Total bacterial counts were made in a Thoma chamber under phase contrast while viable counts were made by plating out known dilutions of bacterial suspensions on horse blood agar. Each dilution was vibrated for 5 s on a Rotamixer to discourage clumping and ensure uniform distribution before plating out. Plate counts of replicate samples were in good agreement (coefficient of variation $< 5\%$).

Rabbit polymorphonuclear (PMN) leukocytes

PMN leukocytes were induced with glycogen and collected from the peritoneum of rabbits (under local anaesthesia by injection with lignocaine) by the method of Cohn & Morse (1959) with slight modifications. Exudates containing $> 95\%$ polymorphs and which were $> 99\%$ viable were considered satisfactory. Viability was estimated from the proportion of cells excluding trypan blue revector (Hopkins and Williams Ltd, 0·5% (w/v) in PBS) after a 2 min period. Cells were centrifuged from peritoneal fluid and resuspended in heated serum (56 °C) if required for phagocytic killing experiments or immediately homogenized and extracted overnight for soluble bactericidins using a modification of the method described by Cohn & Hirsch (1960) described below.

Preparation of soluble bactericidins

Cells centrifuged from peritoneal fluid were washed once in sucrose and finally resuspended in ice-cold, 0·34 M sucrose (Fisons Scientific Apparatus Ltd) to a known concentration per ml and homogenized for 3–5 min in a tissue grinder (Gallenkamp and Co. Ltd), before extracting (16 h) for soluble bactericidins in 0·01 M citric acid (BDH chemicals Ltd). The supernatant from the 15000 *g* centrifugal fraction contained the bulk of active bactericidins and has been called 'soluble bactericidins' throughout.

Measurement of the activity of soluble bactericidins

Test suspensions containing 10^5 viable organisms and 10^7 PMN equivalents of bactericidins in phosphate buffer at a pH of 6·75 and in final volumes of 1·0 ml (Adlam *et al.* 1970*b*) were incubated at 37 °C with rotation at 30 r.p.m. Samples were removed at intervals, diluted appropriately in ice-cold broth and plated out for viable numbers.

Control suspensions for these contained an equivalent number of organisms in a mixture of buffer (pH 6·75 as in test suspension), and citric acid and sucrose in the same proportions as in soluble bactericidin preparations but without the bactericidins. Organisms incubated in such control mixtures showed no viability reductions throughout.

Measurement of phagocytic uptake and killing of staphylococci

A phagocytic mixture containing 10^5 viable O-broth organisms and 10^7 PMN leukocytes per ml. (Adlam *et al.* 1970*b*) in 50% (v/v) serum in Hanks' solution was

incubated at 37 °C with rotation (30 r.p.m.) for 10 min to allow phagocytosis. One ml samples were then withdrawn for the determination of viable intracellular bacteria at 10 min and for control experiments. Streptomycin (10 μg/ml, Dista Products Ltd) was added to the remaining test suspension and incubation continued.

Measurement of the 10 min intracellular number involved the addition, to one of the samples, of 10^{-3} M chloroquine phosphate (CQP; Imperial Chemical Industries Ltd) to halt intracellular killing, and streptomycin (10 μg/ml), to kill extracellular bacteria during a 20 min incubation period. The cells were then withdrawn by differential centrifugation (100 ***g*** for 10 min), resuspended in dilute tryptose (0·02 %, w/v, Oxoid) to the original volume and disrupted by vigorous vibration with sterile glass balls to release intracellular organisms. These and the supernatants were plated out for viable counts and the number of viable bacteria from the cell pellet was taken as the starting intracellular number in all measurements of the rate of intracellular killing. Meanwhile, samples were withdrawn at predetermined intervals from the main test suspension, the cell pellets centrifuged out, resuspended and finally disrupted in tryptose as usual. Both cell pellet and supernatants were then plated out for viable counts. The difference between these values and the viable number at 10 min represented the extent of killing, at each stage.

The centrifugation procedure resulted in the recovery of > 95 % of cells as judged by haemocytometer count and the supernatants contained < 50 viable organisms per ml after incubation with streptomycin for 20 min as judged by their plate counts.

RESULTS

Preliminary observations

Fig. 1 shows the interrelationships of the various strains O, P, V and O-vivo as a result of which comparisons between V and P were considered unsuitable for studying the differences between *in vivo* and *in vitro* organisms (see Introduction).

Control experiments to validate the drug – inhibition method of measuring intracellular numbers

Fig. 2 shows that CQP only partially inhibited intracellular killing at 5×10^{-5} M and 10^{-4} M concentrations but showed complete inhibition at 10^{-3} M, especially during the first 20 min, which is the period in which it is required to act while antibiotics inactivate extra-cellular organisms. It was neither toxic to PMN leukocytes as judged by the ratio of the number of cells which excluded trypan blue stain at the end to that at the start of experiments (> 99 %), nor to the organisms, as judged by viable counts after 1 h incubation with the drug (> 90 %). Pellets of PMN leukocytes previously incubated with CQP before introduction of organisms (10^5) into the suspension contained numbers of organisms ($1{\cdot}03 \times 10^3$ per ml) similar to those with which organisms were mixed right from the start of experiments ($9{\cdot}6 \times 10^2$ per ml) showing that CQP did not prevent phagocytic uptake.

Plate counts of supernatants showed that streptomycin at the concentration employed (10 μg/ml) killed > 99 % of all extracellular bacteria (approximately

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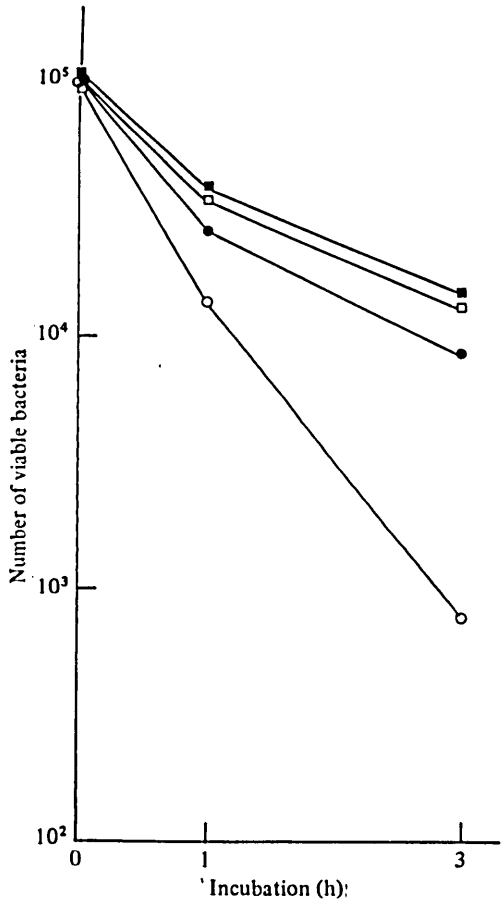

Fig. 1. The interrelationships of the resistance of strains V (■), P (□), O-vivo (●) and O-broth (○) to killing by soluble bactericidins.

75–90 % of starting numbers). The absence of reduction in viable numbers during 1 h incubation of 10 min samples with both CQP and streptomycin (Fig. 3) also showed that it did not penetrate PMN leukocytes to kill intracellular bacteria. Organisms released from intracellular residence, whether previously incubated with both CQP and streptomycin or streptomycin alone were drastically reduced in numbers on exposure to further streptomycin showing that while intracellular, the organisms had not been altered to a form giving resistance to the antibiotic but were just being protected by the PMN leukocytes from streptomycin in the external medium. Viable numbers obtained at intervals from the main test suspension, to which only streptomycin had been added, could therefore be regarded as the true intracellular numbers at the times of withdrawal.

In all experiments, phagocytic killing was initially rapid before fading off. Usually, active killing occurred only during the first 2–3 h and so sample withdrawal has been limited to this period.

Phagocytic killing of O-vivo, O-plasma and O-broth

The number of viable intracellular organisms after the initial 10 min phagocytosis

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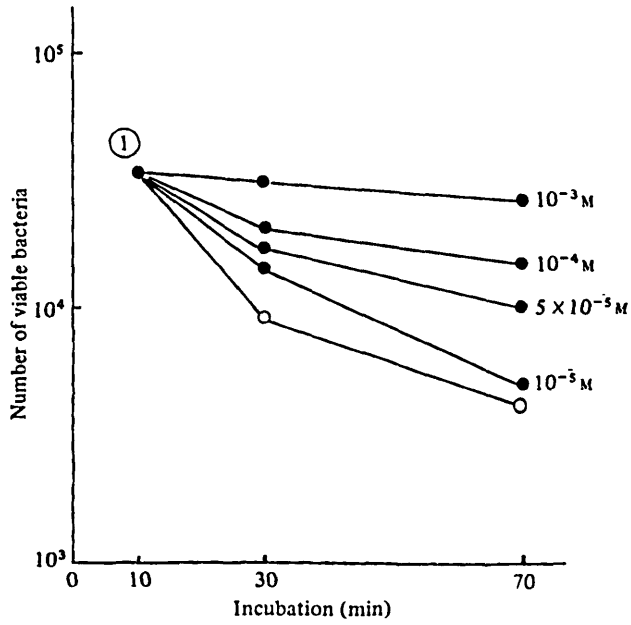

Fig. 2. Inhibition of PMN killing by chloroquine phosphate (CQP). Test suspensions containing 10^5 viable O-broth organisms and 10^7 PMN leukocytes per ml in 50% (v/v) serum/Hanks' solution were incubated for 10 min to allow phagocytosis. Free extracellular bacteria were removed by centrifugation, the cell pellet was washed twice in Hanks' and then re-suspended to original volume. The initial number of viable cell-associated bacteria was measured (①) and the remaining suspension divided into 5 portions. Graded concentrations of CQP in Hanks' (0·1 ml volumes) were added to the first 4 (●) and Hanks' alone to the fifth (○). The cell-associated viable numbers were assessed after 20 and 60 min incubation.

period were $3{\cdot}3 \times 10^3$, $3{\cdot}42 \times 10^3$ and $6{\cdot}62 \times 10^3$ for O-vivo, O-plasma and O-broth respectively (Fig. 4) from an inoculum concentration of 10^5 viable organisms each. At the end of 3 h incubation, O-vivo had been reduced to $6{\cdot}24 \times 10^2$, O-plasma to $5{\cdot}25 \times 10^2$ and O-broth to $1{\cdot}32 \times 10^2$, representing 5·5-, 6- and 50-fold reductions in viable intracellular numbers respectively.

A comparison of these results with those of soluble bactericidin killing (see Fig. 1) shows that, although the reductions in viable numbers were not identical, killing in the two systems appeared to run in parallel and are in agreement on the difference in the resistant levels of O-vivo and O-broth organisms.

Mechanisms of resistance of staphylococci grown in vivo to polymorph bactericidins

Fig. 5 shows that in soluble bactericidin killing tests, broth-grown O-vivo organisms reverted to broth-like susceptibility (showing less than 2% survival) while the clumped, single and sonicated samples showed appreciable resistance to killing (ranging from 6 to 10% survival). Organisms (O-broth and O-vivo) in a buffer-citric acid-sucrose control mixture (not shown) were not reduced in numbers but actually grew in the absence of soluble bactericidins.

Strain O staphylococci, together with five other coagulase-positive strains, grown in plasma and four different peptone media were then compared for

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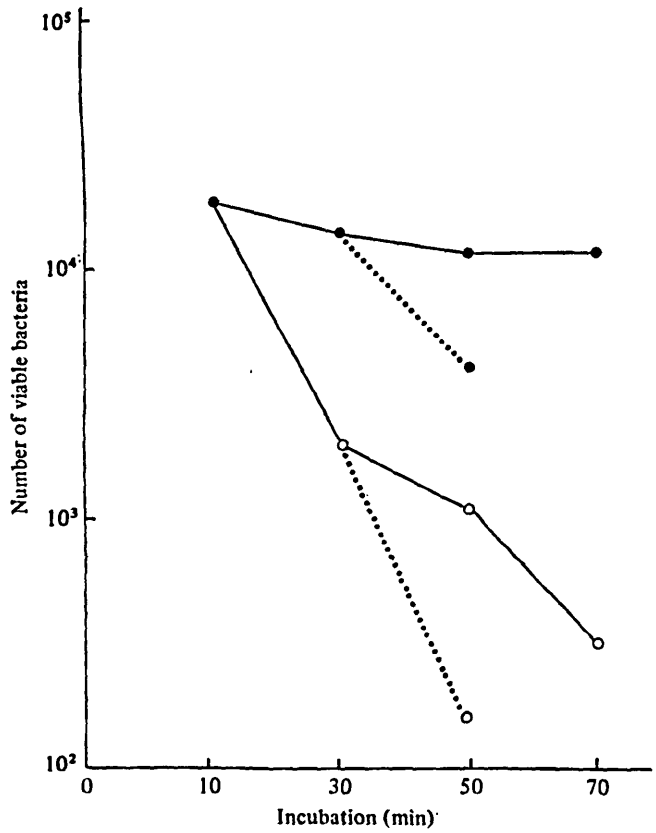

Fig. 3. The effect of streptomycin on intracellular organisms. The usual phagocytic mixture was incubated for 10 min to allow uptake, the number of viable, cell-associated organisms determined as described under Fig. 2 and the remaining suspension divided into 2 portions. These were incubated either with both CQP and streptomycin (●) or with streptomycin alone (○) and samples removed at intervals to determine viable intracellular numbers. After streptomycin had been allowed to act for 20 min, samples were withdrawn from each portion and washed. The organisms were then released from the cells into streptomycin and plated out after a further 20 min incubation (dotted lines). Supernatants, not shown, for clarity, contained less than 50 viable organisms per ml at the end of incubation with streptomycin.

resistance to soluble bactericidin killing. Table 1 shows that all strains were relatively resistant after growth in plasma and susceptible after growth in NB 2, brain heart infusion (BHI) penassay (Pen) or modified 110 (Ekstedt & Bernhard, 1973) broths. The differences in resistant levels were similar to that between O-vivo and O-broth organisms.

Like O-vivo, India ink staining failed to show a capsular material on O-plasma organisms. However, strain O-plasma organisms and the other strains listed in Table 1 were agglutinated by anti-rabbit fibrinogen antiserum after growth in plasma while their broth-grown counterparts were not.

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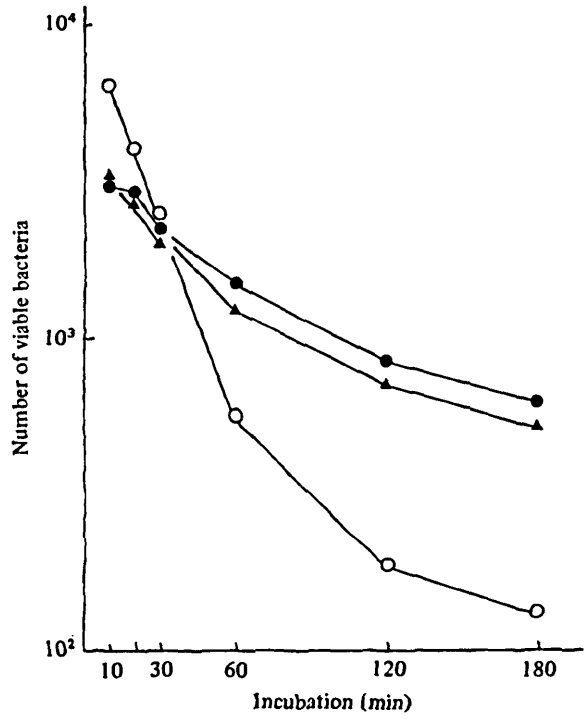

Fig. 4. Intracellular killing of staphylococcal strains O-broth (○), O-vivo (●) and O-plasma (▲).

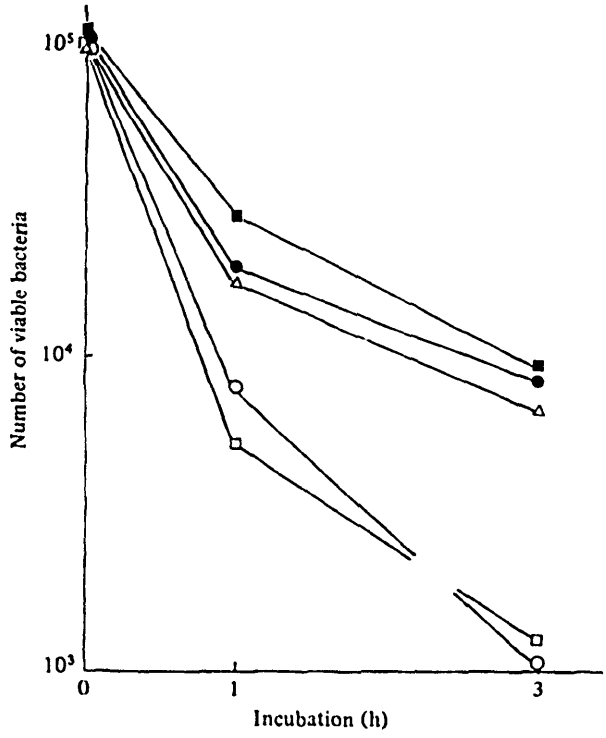

Fig. 5. Soluble bactericidin killing of strain O-vivo (●), separated single O-vivo (■), ultrasonic-treated O-vivo (△), in vitro subcultured O-vivo (□) and O-broth (○) organisms.

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Table 1. *Killing of staphylococcal strains after growth in plasma or various peptone media*

Staphylococcal strains	Killing by soluble bactericidins after growth in: Plasma	NB2	BHI	Pen-broth	110-broth
0	7·2×	128×	216×	1080×	825×
Newman	6·1×	170×	2400×	1027×	655×
V8	9·5×	135×	2690×	1132×	213×
CN55	8·7×	173×	552×	1780×	155×
CN4687	3·0×	108×	2100×	197×	199×
Cowan I	10·5×	159×	190×	745×	202×

* Killing is expressed in folds of reduction (depression value) in viable numbers, calculated from $\frac{\text{viable numbers at 0 h}}{\text{viable numbers at 3 h}}$.

Viability depression values here are from one experiment but subsequent results with individual strains were similar.

DISCUSSION

In preliminary experiments, it was observed that chloroquine phosphate effectively inhibited intracellular killing during the period required by streptomycin to eliminate extracellular organisms and that the latter did not penetrate PMN leukocytes to influence intracellular killing of engulfed organisms. These observations are in agreement with those of Holmes *et al.* (1966), Alexander & Good (1968) and Solberg (1972*b*). The method was therefore considered valid for accurate determination of intracellular viable numbers of staphylococci and hence, the rate of intracellular killing. This point has been further strengthened by the striking parallelism in the resistance-susceptibility patterns of organisms in both this and the soluble bactericidin assay system which indicated that the two test systems were now comparable. As in the case of phagocytic killing, soluble bactericidins were active only in the first 3 h. The depression values are not identical probably because of environmental differences in the test suspensions. In soluble bactericidin killing tests the organisms were exposed directed to bactericidal factors right from the start and throughout incubation and this might have eliminated complications that could arise from differential phagocytosis.

The differences observed in the reductions of *in vivo* and *in vitro* grown organisms, whether in phagocytic or soluble bactericidin killing systems, showed that strain O-vivo was markedly more resistant than strain O-broth. Although O-vivo suspensions, like strain V, contained clumps, the increase in viable numbers on exposure to ultrasonic disruption could not eliminate the observed greater resistance, which was at least 10 fold greater than that of strain O-broth. Also, O-vivo organisms disaggregated by ultrasonic treatment were not appreciably more susceptible to killing by soluble bactericidins than the original suspension. It thus appeared that the resistance of O-vivo organisms was not dependent on the presence of clumps, but might be due to an acquired greater ability than strain O-broth, to resist killing by polymorph bactericidins. This resistance was lost after subculture *in vitro* in broth, indicating that O-vivo organisms were phenotypic variants of O-broth and not organisms selected for resistance *in vivo*.

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At this stage, it appeared that the surface material, which is the only other observed difference between *in-vivo* and *in-vitro* grown organisms and which was lost upon subculture *in vitro*, might be responsible for the added resistance. This could only have been deposited on the organisms as a result of their interactions with the hosts' body fluids. Organisms were therefore grown in each of rabbit serum, pleural exudate and plasma and tested for resistance. Of these, only growth in plasma conferred a resistance equivalent to that of *in vivo* grown organisms in preliminary investigations and is therefore the only aspect reported in this paper. The conversion of five other strains to resistance after growth in plasma while still showing susceptibility after growth in various broth media showed that strain O was not unique in showing such resistance and that the broth in routine use was not atypical of other peptone media in conferring susceptibility. The similarity between strain O-vivo and plasma-grown organisms suggested that plasma contains the components which take part in the interaction, *in vivo*, between staphylococci and their hosts and that these gave full conversion to an in vivo – like phenotype. Thereafter, all strains grown in plasma were agglutinated by anti-rabbit fibrinogen antiserum, using a method adapted from both the coagulase test and the assay of fibrinogen and its degradation products, as recommended by Wellcome Research Laboratories. The results indicated the presence of fibrin or a related fibrinogen-derived substance on the organisms and that staphylococcal components such as free coagulase and/or clumping factor (Duthie, 1954) might have been involved in the interaction with plasma. Investigations into the roles of these factors in the conversion in plasma should therefore provide information on the nature of the enhancement *in vivo* and is the subject of a subsequent paper.

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