VIRULENCE MECHANISMS OF STAPHYLOCOCCI GROWN *IN VIVO* AND *IN VITRO*

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HYGIENIC measures have proved relatively ineffective in reducing the incidence of staphylococcal infection, and the rising frequency of antibiotic resistance in *Staphylococcus aureus* poses increasingly difficult problems in therapy. Recovery from infection results in no appreciable increase in immunity and no satisfactory staphylococcal vaccine has yet been produced. An increased knowledge of the factors that determine the virulence of *S. aureus* might suggest new measures for the prevention or treatment of staphylococcal infections. However, none of the extracellular products of virulent staphylococci has been incriminated as the main determinant of pathogenicity, nor is there sufficient evidence to establish the importance of resistance to phagocytosis or phagocytic killing (Morse, 1965).

We have begun to study some of the aggressive activities by which staphylococci resist host defence mechanisms, in particular the cellular bactericidins. These deserve investigation for the following reasons. Firstly, although most of an inoculum of *S. aureus* is ingested and killed by polymorphonuclear leucocytes, a small proportion of it often appears to survive intracellularly (Rogers and Melly, 1965). Secondly, a phase of intracellular residence during invasion may be important in protecting staphylococci from extracellular antibiotic (Alexander and Good, 1968). Thirdly, persistence within the body, the recurrence of skin lesions and the reappearance of overt infection after antibiotic treatment (Simon, 1965; Worms, 1966) are reminiscent of chronic infections such as brucellosis and tuberculosis in which the key feature is intracellular parasitism. Finally, if survival within phagocytes is proved to be an essential attribute of virulent staphylococci, identification of the aggressins responsible might lead to the design of an effective vaccine.

Our approach to these problems has been guided by the considerations that virulence factors might be most readily recognised in organisms and their products isolated directly from the infected host (Smith, 1964), and that an examination of related strains of differing virulence might reveal factors of primary importance in virulence. Differences between staphylococci grown *in vivo* and *in vitro* have been reported by Gellenbeck (1962) and Beining and Kennedy (1963); organisms isolated from infected guinea-pigs differed from those grown on agar in the rate of aerobic respiration, in antigenic composition and in virulence for guinea-pigs. Staphylococci grown in implanted dialysis tubing *in vivo* produced more α -toxin than when grown *in vitro* (Gladstone

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and Glencross, 1960), but did not differ consistently in rate of elimination after subcutaneous inoculation (Taubler and Kapral, 1966).

Changes in the virulence of staphylococci after passage in animals appear to have been little studied since the work of Van de Velde (1894) and Hektoen (1906). These workers associated the increased virulence of passaged organisms with decreased susceptibility to phagocytosis and phagocytic killing. Kourilsky and Mercier (1941*a, b* and *c*) and Toshkov, Droumchev and Abrashev (1966) recorded similar observations. These findings emphasise the possible importance of staphylococcal aggressins in virulence.

In this paper we first describe the passage of a strain of *S. aureus* in rabbits to increase its virulence and the isolation of organisms that had grown *in vivo*, then we report observations on the virulence and resistance to the bactericidal action of serum and polymorphonuclear leucocyte lysates of the original and the passaged organisms. The work has been summarised in a preliminary communication (Adlam, Pearce and Smith, 1968).

MATERIALS AND METHODS

Preparation of the staphylococcal strains

The organisms used in this work were all derived from a single culture of *S. aureus*. The original strain (O) was serially passaged in rabbits, re-isolated from the pleural exudate and stored on agar slopes (strain P); strain P was injected into a rabbit, and the organisms grown *in vivo* were separated from the pleural exudate (strain V).

Unless stated otherwise, cultures on nutrient agar slopes (Oxoid Blood Agar Base no. 2) in 1 oz. (25 ml) screw-capped bottles were inoculated from mass growth and incubated overnight at 37°C with the caps loose; and the broth cultures in 100 ml Oxoid Nutrient Broth no. 2 in loosely capped 8 oz. (200 ml) bottles were incubated without aeration at 37°C for 4 hr.

Strain O. A culture of *S. aureus* (hospital no. 173/8) was received from Dr J. L. Whitby on a nutrient agar slope. The original isolation had been made from a human carbuncle 2 mth earlier. An agar slope culture had been prepared from a single colony on the primary plate; a subculture of this on to another agar slope was stored at room temperature in the dark. The 3rd subculture was sent to us.

A loopful of growth from a slope culture of the organism (the 5th or 6th subculture from the primary isolate) was inoculated into 100 ml of broth and incubated. Organisms were harvested by centrifugation at 2000*g* for 10 min. and resuspension of the pellet in fresh broth; the suspension, after the addition of a few glass beads, was vibrated for 15 s on a "Rotamixer" (Hook and Tucker Ltd) to ensure the absence of clumps. Suspensions so prepared constituted strain O.

Strain P. Organisms from the original slope culture received from Dr Whitby were inoculated into 100 ml of broth. After overnight incubation, 1 ml of culture was injected intrathoracically into a rabbit; this animal died 14 days later. At necropsy, pus was present in the pleural cavity and was found to contain viable staphylococci. It was mixed with an equal volume of Dulbecco "A" buffered saline (Oxoid, Ltd) and 1 ml of the suspension was injected intrathoracically into another rabbit; this animal died overnight with an accumulation of exudate in the pleural cavity. One ml of this exudate was injected intrathoracically into a further rabbit and after similar passages (up to a total of 5 serial passages of the organisms in pus) the final exudate was plated out. A sweep of colonies from this plate was used to inoculate an agar slope, and after incubation this growth was subcultured on ten slopes, which were sealed and stored as described for strain O.

Broth cultures from one of these slopes were prepared and harvested as described for strain O. These suspensions constituted strain P.

Strain V. 10^8 viable organisms of strain P were inoculated intrathoracically into rabbits. The pleural exudate of animals dying 24–48 hr later contained large numbers of viable organisms. The staphylococci were purified as described below, and purified preparations from single animals constituted strain V.

Purification of staphylococci grown in vivo

Pleural exudates were purified immediately after harvest; they were discarded if Gram-stained smears indicated that they contained low numbers of organisms. Exudate was chilled in ice and centrifuged at $35g$ for 20 min.; the sedimented cellular debris was discarded and the supernatant was centrifuged as before. The resulting supernatant containing the bulk of the organisms was then centrifuged at $7000g$ for 10 min., the pellet was broken up with a glass rod and made up to the volume of the previous supernatant with Dulbecco "A" buffered saline. Centrifugation at $35g$ was repeated for 10 min., the supernatant was centrifuged at $7000g$ and the pellet was washed once more in buffered saline. The final pellet of organisms was suspended in buffered saline and either used immediately or distributed in 2-ml samples and stored at -20°C .

Intrathoracic injection into rabbits

Injection was made by the lower intercostal approach along the right anterior axillary line with the aim of introducing organisms into the pleural cavity. The syringe needle was inserted in a vertical plane to a depth of about 1 in. (2.5 cm). Animals given an injection of strain P regularly showed, at necropsy, a pleural exudate containing numerous organisms. Although some of the inoculum had probably been introduced into the lung substance there was no macroscopic evidence of pulmonary abscesses. Pleural exudate was collected at necropsy; viable counts of bacteria were made on nutrient agar and total bacterial counts were made in a Thoma chamber.

Bacteriological tests

Phage-typing and antibiotic-sensitivity testing were done by Dr J. L. Whitby. Tests for sugar fermentation and production of urease, catalase, phosphatase and free coagulase were performed as described by Cowan and Steel (1965).

Assay of virulence in rabbits

"New Zealand White" rabbits of 2–3 kg were used. Groups of 6 animals received graded 1-ml doses of organisms, suspended in chilled broth, by the intrathoracic route. Deaths were recorded over a 6-wk period. In 2 control experiments no deaths occurred in 12 rabbits receiving broth alone. The data were represented graphically and values of LD₅₀ were calculated by the method of Litchfield and Wilcoxon (1949).

The bactericidal action of serum

Healthy rabbits were bled out and the blood was defibrinated by shaking with a small volume of glass beads. After centrifuging at $2000g$ for 10 min. the serum supernatant was removed; sera were used fresh or after storage at -20°C .

Bactericidal action of serum on staphylococci was measured by plate counts of samples removed at intervals after the addition of 0.1 ml suspension of organisms to 5 ml of serum in a 1 oz. screw-capped bottle and incubation at 37°C . The bottles were rotated at 30 r.p.m. in a tissue-culture roller-drum apparatus (Laboratory and Electrical Engineering Co.). Samples of 0.5-ml volume after addition of a few glass beads were vibrated for 15 s and diluted appropriately with broth before plating on Blood Agar Base no. 2. Dilution of samples was accompanied at each step by 5 seconds' vibration; suspensions were free of clumps and plate counts of replicate samples were in good agreement.

The bactericidal action of polymorphonuclear leucocyte lysates

Polymorphonuclear leucocytes were obtained from rabbits by the method of Cohn and Morse (1959). The polymorphs (>95 per cent. pure) were separated from peritoneal fluid by centrifugation at 100*g* for 10 min., resuspended in Dulbecco "A" buffered saline, counted in a haemocytometer and adjusted to a concentration of 10^7 cells per ml. They were lysed by freezing and thawing 6 times in ethanol-solid carbon dioxide freezing mixture with vigorous resuspension at each step by adding glass beads and vibrating for 1 min. Lysates were used fresh or after storage at -20°C .

The bactericidal action of the lysates was measured by the same technique as that used in the serum bactericidin test.

RESULTS

Animal passage and isolation of staphylococci grown in vivo

Some 10^{10} organisms were recovered from the majority of the pleural exudates (volume 10–20 ml) in animals that died or were killed 24–48 hr after infection, indicating multiplication of the inoculum at least 100- to 200-fold. A comparison of the total and viable counts of staphylococci indicated that the viability of the organisms in the exudate was initially 10–20 per cent., but slowly declined after purification and storage at -20°C ; preparations with a viability less than 2 per cent. were discarded. In 2 preparations of strain V used in assays of virulence, the proportions of the cocci that were living were respectively 7 per cent. and 12 per cent.

Practically no debris was evident microscopically in purified isolates of strain V examined under phase contrast or in stained films. Water-washed preparations pooled from 4 animals contained $(6.1-9.6) \times 10^9$ organisms per mg of dry preparation (60°C , constant weight) in comparison with values for late log-phase cultures of strains O and P of $(0.8-1.3) \times 10^9$ and $(1.7-3.3) \times 10^9$ organisms per mg dry preparation respectively. Thus, by dry weight determination there was no evidence of impurity in preparations of strain V.

Comparison of strains by bacteriological tests

Bacteriological tests were carried out on strains O, P and V to detect any contamination during passage or isolation from rabbits.

All strains were lysed by phage 42E; all were resistant to penicillin and sensitive to tetracycline, chloramphenicol, erythromycin, cloxacillin, neomycin and fucidin. All produced free coagulase, catalase, phosphatase and urease; all fermented glucose, lactose, mannitol, maltose and sucrose with production of acid but no gas; they did not ferment arabinose, dulcitol, salicin or sorbitol.

Assay of virulence of staphylococcal strains

The lethality of strains O, P and V on intrathoracic injection into rabbits is shown in fig. 1. Values of LD₅₀ were as follows: for strain O, 4.0×10^8 (95 per cent. confidence limits 9.3×10^7 to 1.7×10^9); for strain P, 1.6×10^6 (4.5×10^5 to 5.7×10^6); for strain V, 6.4×10^4 (1.8×10^4 to 2.3×10^5). On this

basis strain V was 25 times more virulent than strain P and 6000 times more virulent than strain O.

Resistance of staphylococcal strains to serum bactericidins

In preliminary experiments on phagocytosis in defibrinated blood, marked differences in initial killing of the strains were apparent. Further study showed

FIG. 1.—Lethality of staphylococcal strains for rabbits. Animals were observed over a 6-wk period; 80 per cent. of deaths occurred in the 1st wk. The data (pooled from 2 experiments) are represented according to Litchfield and Wilcoxon (1949); log dose is plotted against percentage of deaths per group on a probability scale. By χ^2 test the lines are of good fit.

- △ Strain O (original).
- Strain P (passaged).
- Strain V (grown *in vivo*).
- △*, ○*, ●* Values for 0 or 100 per cent. death corrected by the method.

that differential killing occurred in serum alone; typical results are shown in fig. 2. In the first 3 hr, viable counts of strains O and P declined whilst those of strain V increased; thereafter growth of all 3 strains was apparent. The recovery of strains O and P appeared to be due to decay in bactericidin rather than emergence of a resistant population, because transfer of the growing O organisms to a fresh sample of serum led to a similar fall in viability. Although sera from different animals varied in their bactericidal power, the pattern of strain resistance to killing was always maintained; of 15 sera tested 3 only showed no bactericidal activity.

Resistance of staphylococcal strains to bactericidins of polymorphonuclear leucocyte lysates

To assess resistance to killing by polymorphs most simply and without complications due to differential phagocytosis, the 3 strains were subjected to bactericidins released by lysis of polymorphs (fig. 3). In these experiments the viability of the preparations of strain V (5 to 20 per cent.) was lower than

FIG. 2.—Bactericidal action of serum on staphylococcal strains.

△—△ Strain O (original).
○—○ Strain P (passaged).
●—● Strain V (grown *in vivo*).

that of suspensions of strains O and P (30 to 70 per cent.) and experiments were done to test the possibility that the resistance of strain V was due to its content of dead organisms or non-bacterial products. A sample of strain O was incubated with lysate in the presence of the standard dose of V organisms previously sterilised by ultraviolet irradiation {15 W, 10 cm from 10 ml of suspension at 10⁷ organisms per ml in a 3½ in. (9 cm) diameter petri dish}. The presence of killed V organisms did not affect the bactericidal action of lysate on strain O (fig. 3).

The survival of strain V varied between experiments from a 1·5- to 2-fold increase to a 2- to 3-fold decrease over 3 hr. The order of resistance of the 3 strains was maintained in all experiments and in no instance was growth of strains O and P observed in lysate during incubation for up to 5 hr.

DISCUSSION

The correlation between increased virulence and resistance to serum and polymorph bactericidins strongly suggests that resistance to these defence mechanisms is an important attribute of the virulence of staphylococci. Measurement of virulence by lethality in rabbits appears valid in this context, since production of sufficient α -toxin to kill animals (if this is the cause of death)

FIG. 3.—Bactericidal action of polymorph lysates (10^7 cells per ml) on staphylococcal strains.

- △—△ Strain O (original).
- Strain P (passed).
- Strain V (grown *in vivo*).
- ▲—▲ Strain O in the presence of killed strain V.
- △---△ Strain O in the absence of lysate.
- ▲---▲ Strain O in the presence of killed strain V and absence of lysate.

must depend on bacterial growth following the outcome of initial interaction between host defences and bacterium. It seems unlikely that deaths were due merely to preformed toxin released from inocula; this would require strain V to have contained 6000 times more toxin than strain O and is inconsistent with the observation, in preliminary experiments, that the time of death was delayed when antibiotic was given shortly after inoculation.

The serum bactericidin, presumed to be β -lysin (Skarnes and Watson, 1957), was active only in the first 3 hr of incubation; this may be a matter of initial concentration, but the period corresponds to the critical time for the establishment of infection (Miles, Miles and Burke, 1957). Resistance to polymorph bactericidins is probably of greater importance for virulence, especially

in view of the uncertain role of β -lysin *in vivo* (Hirsch, 1960), although the same bacterial components may be responsible for resistance to both types of bactericidin.

The fact that organisms grown *in vivo* were markedly more resistant to killing than the parent population grown *in vitro* indicates that the substances responsible are qualitatively different or quantitatively increased on growth *in vivo*. In view of the passage series by which strain P was derived from strain O, strain V is probably a phenotypic variant of strain P rather than a selected population differing in genotype. Proof of this (Meynell, 1961) would require the demonstration that the LD50 of the population derived by subculture of strain V *in vitro* was the same as that for strain P.

The identity of the components determining increased resistance to serum and cell bactericidins is unknown. The phenomena may be due to increased production of extracellular components such as α -toxin, leucocidin or coagulase. Ekstedt (1956, 1965) claimed that coagulase-positive strains of staphylococci are less susceptible to serum killing than coagulase-negative strains, but this has been disputed by Myrvik (1956) and Cybulska and Jeljaszewicz (1966). The factors responsible are more likely to be on the cell surface, possibly associated with the cell-wall components described by Fisher (1963, 1965) and by Hill (1968); adsorbed extracellular products or "bound" coagulase cannot be excluded. The behaviour of the strains in phagocytosis and their susceptibility to killing by intact polymorphs is described in a subsequent paper.

SUMMARY

A coagulase-positive strain of *Staphylococcus aureus* grown *in vitro* (strain O) was passaged in rabbits to increase virulence. The passaged organisms grown *in vitro* (strain P) were used to infect rabbits from which organisms grown *in vivo* (strain V) were isolated.

By LD50 measurement in rabbits, strain V was 25 times more virulent than strain P and 6000 times more virulent than strain O. On incubation in rabbit serum, strain V multiplied whilst strains P and O decreased in viable number (strain O the more rapidly) during the first 3 hr, recovering subsequently. In lysates of polymorphonuclear leucocytes, strain V multiplied or was slightly reduced in number; strains P and O decreased in number (strain O the more rapidly).

The findings suggest that resistance to serum and polymorph bactericidins is an important attribute of the virulence of staphylococci and that the substances responsible are qualitatively different or quantitatively increased on growth *in vivo*.

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