RELATIONSHIP BETWEEN ANTIBIOTIC RESISTANCE, THE PRODUCTION OF "VIRULENCE FACTORS", AND VIRULENCE FOR EXPERIMENTAL ANIMALS IN STAPHYLOCOCCUS AUREUS

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Observations on naturally occurring infections with Staphylococcus aureus, mainly in hospital patients, have led to statements that strains resistant to several antibiotics ("multiple-antibiotic-resistant strains") differed in virulence from more sensitive strains. Some observers claimed that the more resistant strains were the more virulent, others that they were the less virulent.

Few experimental investigations have yielded evidence to clarify this controversy. Krinski, Kedzia and Kaminska (1964) found an association between multiple-antibiotic resistance and greater fatality in the staphylococcal infections of children they were studying, but the LD50 in mice infected intraperitoneally with strains of S. aureus isolated from the patients was not related to the degree of antibiotic resistance that they exhibited. Hewitt and Sanderson (1974) studied the effect of methicillin therapy in guinea-pigs infected with methicillin-resistant S. aureus. In their untreated animals they found no relation between the virulence and the antibiotic-resistance pattern of the organisms tested. Lacey and Chopra (1975) examined the virulence of S. aureus for chick embryos; they compared a virulent antibiotic-sensitive strain, a multi-resistant variant derived from it by transduction and a variant of the transduced strain cured of its multi-resistance by subculture. Both the transduced strain and its cured variant were much less virulent than the parent strain.

This report is concerned with testing the hypothesis that the acquisition or loss of antibiotic-resistance determinants by S. aureus may be associated with a change in virulence, which could be related to changes in the production of extracellular enzymes and other factors that have been variously described as "virulence factors" (Abramson, 1972; Jeljaszewicz, 1972). For this purpose a multi-resistant strain of S. aureus and "cured" variants of it that had become sensitive to some antibiotics were tested for virulence by injection into the skin of guinea-pigs and mice.

Materials and methods

Strains of S. aureus

A multi-resistant strain, strain 1, phage-typing pattern 77, was isolated from a patient in a London hospital. Two other strains, PS80 (NCTC9789), phage-typing pattern 80/81, a
penicillin-resistant strain, and strain M, were kindly supplied by Professor A. A. Glynn; they were used in the mouse-virulence tests as a basis for comparison, because strain PS80 was said to be "mouse virulent" and strain M "mouse avirulent".

Temperature "curing"

Cultures of strain 1 were held in nutrient broth at 42°C for 7 days. At the end of this time variants cured of some of the resistances possessed by the original strain were isolated by replica plating.

Growth rate

Organisms were grown in Nutrient Broth (Southern Group Laboratory) and their in-vitro growth rates compared by measurement of their optical densities (OD) in nutrient broth over a period of 24 h and by taking 0.1-ml broth samples for viable counts in nutrient-agar pour-plates each time the OD was measured. The incubation temperature for this experiment and all other in-vitro experiments was 37°C.

Antibiotic sensitivity

Strains were classified as resistant or sensitive to antibiotics on the basis of the minimum inhibitory concentration (MIC) for them, by an agar dilution-method (Ericsson and Sherris, 1971), of the following antibiotics: penicillin, gentamicin, tobramycin, neomycin, streptomycin, kanamycin, erythromycin, tetracycline, methicillin and chloramphenicol.

Enzyme assay

The production of the following enzymes was assayed, as described by Abramson and Friedman (1967): coagulase, free and bound; deoxyribonuclease, measured after incubation for 24 and 48 h; lipase and phosphatase. Gelatinase production was measured with charcoal discs (Oxoid) by the method of Greene and Larks (1955).

Haemolysins

These were assayed as described by Abramson and Friedman (1967). Lysis of RBC of the rabbit was used as the indicator for α, of sheep for β and of horse and man for δ haemolysins.

Virulence tests

The bacterial growth from 18–20 h cultures in nutrient broth was spun down and resuspended in saline (0.85 NaCl w/v in water) containing 1% Todd-Hewitt broth (v/v) at the appropriate concentration for inoculation. The number of colony-forming units (c.f.u.) in each inoculum was calculated from the viable counts in three nutrient-agar pour plates.

In guinea-pigs. The skin of the trunk was clipped, and dense coccal suspensions selected to give lesions in guinea-pig skin greater than 10 mm in diameter, and 1 in 5 dilutions of these, were injected intracutaneously in 0.1-ml volumes; eight inoculations, representing four strains each at two dilutions, were made at partially randomised sites on the skin of the trunk in each guinea-pig. Each dose of cocci was tested in a total of 21 animals. After 18–24 h the clipped area of skin was depilated and the diameters of the lesions were recorded (Miles, Miles and Burke, 1957).

In mice. Male AG2 mice, 25–40 g in weight, were used. Dense suspensions, concentrated some threefold from overnight broth cultures and selected to give lesions greater than 10 mm in mouse skin, and 1 in 3 dilutions of these, were injected subcutaneously in 0.1-ml volumes, two to each mouse, midway down on each side of the back. After 18–24 h the animals were killed and the skin dissected to expose the under-surface of the lesion. The diameters of the areas of inflammation with induration were measured. Each dose of cocci was tested in six animals.
VIRULENCE OF STAPHYLOCOCCUS AUREUS

RESULTS

Curing and antibiotic resistance

To detect any changes in the staphylococci caused by growth at elevated temperature but not reflected in a difference in the antibiogram, 20 heat-treated but "uncured" isolates were compared with the untreated strain 1. None of them differed from the original in guinea-pig and mouse virulence, in enzyme and haemolysin production, or in in-vitro growth rate.

Three of the cured variants, designated strains 2, 3 and 4, were chosen for study as they differed from strain 1 in antibiogram. For all antibiotics an MIC of >5 mg/litre was taken as evidence of resistance (table I). Strain 1 was resistant to penicillin, methicillin, tetracycline and to the three aminoglycosides, gentamicin, tobramycin and kanamycin. Strains 2 and 3 had lost resistance to penicillin only and strain 4 to penicillin and the three aminoglycosides. Though strains 2 and 3 had similar antibiotic sensitivities, they were chosen from many other penicillin-sensitive variants derived from strain 1 because they displayed differences in enzyme and haemolysin production which could possibly affect virulence.

Cure rates were calculated as the number of c.f.u. exhibiting a change in antibiogram isolated per thousand c.f.u. replicated. The highest individual cure rate (4%) was of penicillin resistance in strain 3, but the gentamicin cure rate for strain 4 was only 0.3%.

Enzymes. Strain 2 was the most enzymically active, with strains 3, 4 and 1 successively less so in that order (table II).

Haemolysins. None of the strains produced much haemolysin (table III) in comparison, for instance, with the production of α toxin by strain Wood 46 (no. NCTC7428), the rabbit RBC haemolysin titres of which exceeded 5000. The highest α-toxin titres (8) were given by strains 2 and 4, strain 1 being again the least active strain.

Table I
Antibiotic-sensitivity patterns of a multiple-antibiotic-resistant strain of Staphylococcus aureus (strain 1) and of three variants of it (strains 2-4)

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Minimum inhibitory concentration (mg/litre) of the stated antibiotic for strain 1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td>32 (R) 0.25 (S)</td>
<td>0.25 (S)</td>
<td>0.25 (S)</td>
<td>0.25 (S)</td>
</tr>
<tr>
<td>Methicillin</td>
<td>64 (R) 32 (R)</td>
<td>32 (R)</td>
<td>32 (R)</td>
<td>32 (R)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>64 (R) 64 (R)</td>
<td>64 (R)</td>
<td>64 (R)</td>
<td>64 (R)</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>16 (R) 16 (R)</td>
<td>16 (R)</td>
<td>16 (R)</td>
<td>16 (R)</td>
</tr>
<tr>
<td>Neomycin</td>
<td>0.5 (S)</td>
<td>0.5 (S)</td>
<td>0.5 (S)</td>
<td>0.5 (S)</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>0.5 (S)</td>
<td>0.5 (S)</td>
<td>0.5 (S)</td>
<td>0.5 (S)</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>40 (R)</td>
<td>40 (R)</td>
<td>40 (R)</td>
<td>40 (R)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0.1 (S)</td>
<td>0.1 (S)</td>
<td>0.1 (S)</td>
<td>0.1 (S)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>0.1 (S)</td>
<td>0.1 (S)</td>
<td>0.1 (S)</td>
<td>0.1 (S)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>200 (R)</td>
<td>200 (R)</td>
<td>200 (R)</td>
<td>200 (R)</td>
</tr>
</tbody>
</table>

(R) = resistant; (S) = sensitive.
TABLE II
Titres of enzymes produced in liquid culture* by strains 1–4

<table>
<thead>
<tr>
<th>Strain number</th>
<th>Coagulase titre (Free Bound)</th>
<th>Deoxyribonuclease titre (24 h 48 h)</th>
<th>Lipase</th>
<th>Gelatinase</th>
<th>Phosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16 &gt; 256</td>
<td>0 8</td>
<td>2 1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>128 &gt; 256</td>
<td>&gt; 512 &gt; 512</td>
<td>1 8</td>
<td>&gt; 512</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>64 &gt; 256</td>
<td>16 &gt; 512</td>
<td>2 4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>64 &gt; 256</td>
<td>8 8</td>
<td>2 1</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

* Unless otherwise stated, incubation was for 24 h at 37°C.

TABLE III
Haemolytic titres of liquid cultures* of strains 1–4

<table>
<thead>
<tr>
<th>Strain number</th>
<th>Titres of haemolysin of RBC of man sheep horse rabbit</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1(w) 1(w) 1(w) 2</td>
</tr>
<tr>
<td>2</td>
<td>1(w) 4 1(w) 8</td>
</tr>
<tr>
<td>3</td>
<td>1(w) 4 1(w) 2</td>
</tr>
<tr>
<td>4</td>
<td>1(w) 2 1(w) 8</td>
</tr>
</tbody>
</table>

(w) = Weak positive reaction.
* After incubation overnight at 37°C.

Growth rates. No difference in growth rate between strain 1 and the three "cured" variants was observed.

Virulence

In guinea-pigs. Preliminary three-dose assays of strains 1–4 showed that the regression of lesion diameter upon log dose was linear between diameters of 5 and 15 mm. Accordingly, for the full assays, two doses producing with each strain lesions between these diameters were tested and the linearity of the response was assumed. Batches of three guinea-pigs were tested on seven occasions and the results averaged for each of the four strains. For statistical analysis, arbitrarily chosen lesion sizes of 7 and 12 mm were plotted against the log dose required by each strain to produce such lesion sizes (fig. 1). The average slopes for each strain were significantly parallel (p = 0.001) and the results correspondingly subjected to an analysis of variance. The doses required by each strain to produce 7 and 12 mm lesions (fig. 1) were significantly different (p = 0.001), and for an arbitrary 9.5-mm lesion size the mean log doses (as c.f.u. in 0.1 ml) for strains 1, 2, 3 and 4 were respectively 7.8, 8.3, 8.38 and 8.95 (average standard error ±0.042). Strain 1 was most virulent and strains 2, 3 and
4 successively less so. The individual animal variation was insignificant
(p = 0.202).

In mice. These assays were made not only to determine whether the order of
virulence of the four strains, as determined in the guinea-pig, would hold true
for another species of test animal, but also to provide a basis for examination of
the strains in terms of cell-wall constituents, said by Hill (1968) to be deter-
minants of mouse virulence in *S. aureus*. The diameters of lesions made by
relatively large doses of cocci were preferred, as a measure of virulence, to the
scoring method of Hill (1968) which was applied to lesions produced by much
smaller subcutaneous doses. Two doses were used, and linearity assumed from
the results of some three-dose tests in which the responses were substantially
linear to log dose.

From the combined results of titrations in which each dose was measured,
definite comparison of virulence was not possible, because the response lines
were far from statistically parallel (fig. 2) (p = 0.999). Nevertheless it appears
that the order of virulence at the arbitrarily chosen level of 12 mm was strains 1, 4,
3, 2. All four strains appeared to be more virulent than the "mouse virulent"
strain PS80 which, in confirmation of Hill's (1968) results, was more virulent than
strain M.

![Graph showing lesion diameters](image)
Fig. 2.—Diameters of lesions produced by subcutaneous injections of different doses of six strains of Staphylococcus aureus (strains 1–4, PS80 and M) into mice. Each point represents a mean diameter from lesions in six mice.

DISCUSSION

The view of Hill (1968) and Mitsuhashi et al. (1976) that the acquisition of multi-resistance is associated with a decrease in virulence is not supported by my findings with guinea-pigs, in which a decline in virulence in association with a loss of antibiotic-resistance determinants was established to a high degree of statistical significance. Although the work of Lacey and Chopra (1975) suggested that the acquisition of antibiotic-resistance determinants resulted in a decrease of virulence for chick embryos—a change in the opposite direction from that demonstrated here in guinea-pigs—they found that subsequent loss of the determinants was not associated with a return of virulence.

The results obtained in mice are difficult to interpret, and suggest that further studies of this model are necessary. The slope of the dose-response lines given by various strains differed widely. Strains 1, 2 and 3 gave steep slopes and strains 4, PS80 and M gave shallow slopes. I have also obtained shallow slopes with other S. aureus strains that appeared to be of low virulence for the guinea-pig. The significance of variations in the slope of dose-response lines is uncertain, but might be related to differing modes of attachment of the cocci to animal tissues or to differences in the production by these bacteria of toxic materials or
aggressins. However, if a shallow slope did correspond to low virulence, the order of virulence among strains 1–4 would not be 1, 4, 3, 2, as suggested by comparing the size of the dose of cocci needed to produce a 12-mm lesion, but would be 1, 3, 2, 4. This, again, would suggest that loss of resistance determinants was associated with decrease in virulence, and would correspond rather more closely with the results obtained in guinea-pigs.

Strain PS80 was reported by Noble (1965) and Hill (1968) to be "virulent" for mice when injected subcutaneously, and strain M was considered by Hill (1968) to be "avirulent"; I found, however, that strains 1–4 were more virulent than both, though I confirmed the difference in virulence between strains PS80 and M. These workers used experimental models different from mine; the former injected the cocci in much smaller doses with a foreign body, and both used an entirely different method of grading the lesions.

Some authors (Abramson, 1972; Jeljaszewicz, 1972) have reported changes in enzyme and haemolysin production related to changes in antibiogram. No such association was found in the strains that I studied. There was no positive association between enzyme and haemolysin production and virulence, as the most virulent organism produced the lowest titres. With strains 2 and 3, however, where the antibiograms are the same, strain 2 was the more virulent of the pair in guinea-pigs and a more active producer of haemolsins and enzymes than strain 3. The lack of correlation between enzyme production and virulence is in accord with the findings of Noble (1966) and Chaudhuri and Chakrabarty (1969).

There are some indications of the way in which changes in staphylococci, known to accompany changes in antibiogram, might affect virulence. Thus, penicillin-resistant staphylococci have a greater content of cell-wall lipid and different immunoelectrophoretic mobilities than penicillin-sensitive variants of the same strain (Jagicza, Varosi and Sztankov, 1975); penicillin resistance and cell-wall thickness are associated (Murray, Francombe and Mayall, 1959), and gentamicin resistance may produce morphological changes in the cell wall (Beesley and Klouda, 1976).

Cell-wall factors have already been shown to be determinants of virulence for staphylococci, for example, Fisher's (1965) cell-wall aggressin and Hill's (1968) cell-wall deoxycholate-citrate residue. It is possible that changes in the cell wall that may be related to the antibiogram could affect the virulence of S. aureus, producing, in some strains at least, a more virulent form of bacterium.

SUMMARY

Variants that had lost some of their antibiotic-resistance determinants were selected from a multiple-antibiotic-resistant strain of Staphylococcus aureus. When tested by subcutaneous injection into guinea-pigs, and measured as the number of cocci needed to produce a skin lesion of an arbitrarily chosen diameter, the virulence of strains fell progressively with loss of resistance determinants. When the staphylococci were injected intracutaneously into mice, however, the results were less easy to interpret, but loss of resistance appeared to be associated with a reduction of the slope of the dose-response line.
There was no association between the antibiogram of the strains and their production of certain enzymes and haemolysins.

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REFERENCES


