THE INTERACTION OF STAPHYLOCOCCI GROWN IN VIVO AND IN VITRO WITH POLYMORPHONUCLEAR LEUCOCYTES

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ADLAM, Pearce and Smith (1970) described the production of a staphylococcal strain of increased virulence by passage in rabbits and the isolation of organisms grown in vivo after infection of rabbits with the passaged strain. The original (O) and passaged (P) strains grown in vitro and the organisms isolated in vivo (V) were ordered V>P>O in virulence (LD50) and in resistance to serum bactericidins and to polymorphonuclear leucocyte lysates.

This paper describes the resistance of strains O, P and V to ingestion and intracellular killing by intact polymorphs. Resistance to phagocytic uptake has been examined because it is a property of many pathogenic bacteria and is a special feature of certain capsulated strains of staphylococci (Koenig, 1961–62; Mudd, 1965). Van de Velde (1894) and Hektoen (1906) attributed virulence of strains passaged in animals in part to decreased susceptibility to ingestion by phagocytes. Rogers (1962) speculated that virulent staphylococci acquire in vivo a capsular surface component that confers resistance to uptake by phagocytes and is readily lost on culture in vitro.

The resistance of strains O, P and V to intracellular killing by intact polymorphs has been examined to confirm the work with cell lysates and to assess the level of intracellular survival in view of its possible role in the pathogenicity of staphylococci.

MATERIALS AND METHODS

Staphylococcal strains

Strains O, P and V were prepared as described previously (Adlam et al.). Suspensions of strain V in nutrient broth consisted of washed organisms that had been separated from rabbit pleural exudate; suspensions of O and P were washed organisms in fresh nutrient broth that had been harvested after 4 hours' incubation in broth (Nutrient Broth no. 2, Oxoid Ltd) at 37°C.

Peritoneal polymorphonuclear leucocytes

Polymorphs were obtained from rabbits by the method of Cohn and Morse (1959). Cells (>95 per cent. polymorphs) were centrifuged from peritoneal fluid and resuspended in heated serum (see below) for experiments on phagocytic uptake or killing.

Serum

This was prepared from defibrinated rabbit blood as described previously (Adlam et al.). Sera for experiments with peritoneal polymorphs were pooled from 6 animals and preparations were heated at 56°C for 1 hr to inactivate bactericidins.

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Concentration of rabbit blood leucocytes

Defibrinated blood (100 ml) was centrifuged at 1200g for 10 min. and the serum supernatant was removed. The buffy coat of about 5 ml of sedimented cells was removed and resuspended in 5 ml of serum supernatant. After centrifugation at 750g for 10 min. the upper 2-ml layer of sedimented cells was removed and resuspended in serum supernatant for use. The yield of blood leucocytes was about 50 per cent.; preparations contained c. 80 erythrocytes per leucocyte; about 50 per cent. of leucocytes were polymorphs.

Measurement of phagocytic uptake

Peritoneal polymorphs were suspended in 10 ml of heated serum in waxed 1 oz. (25 ml) screw-capped bottles at a concentration of 10⁷ cells per ml and 0.1 ml of a suspension of organisms was added to a final concentration of 3 x 10⁷ or 10⁸ staphylococci per ml by total count (in a Thoma chamber). The mixed suspension was incubated at 37°C with rotation at 30 r.p.m. in a tissue-culture roller-drum apparatus (Laboratory and Electrical Engineering Co.). Samples of 1-ml volume were taken after 15 or 25 min. and centrifuged at 100g for 10 min.; the cell pellets were resuspended in heated serum, smears on slides, and stained with Giemsa.

Uptake was assessed by microscopic count (x 1200 magnification) of the total number of organisms in cells. In all experiments 4 smears were surveyed for each staphylococcal strain and 400 cells were examined per smear; the average number of organisms per cell was calculated.

Measurement of phagocytic killing

The viability of staphylococci exposed to blood leucocytes or peritoneal polymorphs was investigated by a modification of methods used for the study of intracellular growth of Brucella (Pearce et al., 1962). Plate-counts of suspensions were made as described by Adlam et al.

Leucocytes in rabbit blood. Experiments were designed to simulate conditions in blood, and concentrated preparations of leucocytes were suspended in serum from the same cell donor. 10-ml volumes of leucocytes, at 10⁷ cells per ml of serum, were placed in waxed 1 oz. bottles and 0.1 ml of a suspension of organisms was added to a final concentration of about 10⁵ viable staphylococci per ml. The mixed suspension was incubated at 37°C with rotation at 30 r.p.m. on a roller drum. Samples of 1-ml volume were removed after 1 or 3 hr and 0.1 ml of streptomycin sulphate (Glaxo, Ltd) in Dulbecco 'A' buffered saline (Oxoid, Ltd) was added to the remaining suspension to a final concentration of 5 µg per ml; incubation and rotation were continued and further samples were removed at intervals. Control suspensions of organisms in serum alone were similarly prepared; these were sampled before and after the addition of antibiotic in parallel with the mixed suspensions of organisms and leucocytes.

Cell samples were centrifuged at 100g for 10 min., the pellets were resuspended in 0.9 ml of 0.002 per cent. tryptose (Oxoid, Ltd) in water with a few glass beads and vibrated for 1 min. on a "Rotamixer" (Hook and Tucker Ltd) to disrupt the cells. Disrupted cell suspensions were plated out directly or after storage in ice for up to 15 min.; if dilution in nutrient broth was needed this was done immediately before plating out. Control samples of organisms in serum were treated similarly to disrupted cell suspensions.

Peritoneal polymorphs. These were suspended in heated serum and killing was measured as described above with the following differences: streptomycin was added to suspensions after 30 minutes' incubation; supernatants from centrifuged cell samples were plated out; the behaviour of organisms in serum alone was not examined.

Control experiments. The following observations established the validity of procedures described above: recovery of cells in pellets was >95 per cent. by haemacytometer count; pellets were contaminated with extracellular organisms to <5 per cent. of viable numbers in supernatants as judged by plate counts of washings from resuspended pellets.
Disruption of cells in tryptose water appeared complete when stained preparations were examined under the microscope. The plate count of organisms added to cells just before disruption did not differ appreciably from counts on organisms after equivalent dilution in tryptose water; this indicated that clumping or death of organisms was negligible on disruption.

Organisms chilled for 30 min. in tryptose water or heated serum and streptomycin did not differ appreciably in viability from the original suspension plated out in broth.

Duplicate counts on cell suspensions agreed to within ±10 per cent.

RESULTS

Resistance of staphylococcal strains to uptake by polymorphs

Uptake was rapid for all 3 strains at the concentration of organisms present and measurement was made at one time-point only (table I). The lack of significant difference between strains was confirmed in two other experiments by the finding that supernatants from phagocytosis mixtures had comparable numbers of strains O, P and V by total count.

Determining only the total number of intracellular organisms in cells masked the possible influence on uptake of heterogeneity in phagocytic capacity. This was assessed in one experiment by scoring cells with given numbers of intracellular organisms (table II). No marked difference in distribution of strains was apparent.

Resistance of staphylococcal strains to phagocytic killing

Blood leucocytes. In these experiments the interval before addition of streptomycin was 1 or 3 hr, so that extracellular multiplication of strain V or extracellular killing of strain O might have affected appreciably the number of viable organisms available for phagocytosis. To assess such changes in extracellular number, survival of organisms in serum was examined in parallel with survival in cell suspensions.

In two experiments (one of which is shown in table III), with 1 hour's incubation before the addition of streptomycin, the viable number in serum of
strain V rose and that of strain O fell; this made it difficult to assess the extent of intracellular killing during the 1st hr. In other experiments, extracellular killing of strain O and multiplication of strain V was increased when the addition of streptomycin was delayed for a further 2 hr. Once the extracellular

**TABLE II**

*Distribution of staphylococcal strains in peritoneal polymorphs*

<table>
<thead>
<tr>
<th>Number of bacteria per cell</th>
<th>Percentage of cells containing the stated number of bacteria per cell, for strain</th>
<th>Percentage* of total ingested organisms present in cells containing the stated number per cell, for strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O</td>
<td>P</td>
</tr>
<tr>
<td>----------------------------</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>0</td>
<td>47</td>
<td>46</td>
</tr>
<tr>
<td>1–5</td>
<td>25</td>
<td>28</td>
</tr>
<tr>
<td>6–10</td>
<td>11</td>
<td>15</td>
</tr>
<tr>
<td>11–15</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>16–20</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>21–40</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

* E.g., 18 per cent. of the total strain O organisms ingested were observed in cells containing between 1 and 5 organisms.

The data are from experiment no. 4 (table I).

organisms had been reduced in numbers by the antibiotic the intracellular numbers of both strains V and O declined; this occurred in all experiments, but the numbers of strain V surviving in leucocytes after 3–6 hr exceeded the numbers of strain O surviving 20- to 100-fold.

**TABLE III**

*Survival of staphylococcal strains in blood leucocytes*

<table>
<thead>
<tr>
<th>Staphylococcal strain (in cell suspension, or in serum alone)</th>
<th>Viable count (hr 1–6) on</th>
<th>Number of viable organisms per ml at hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0*</td>
</tr>
<tr>
<td>O (cells)</td>
<td>lysed cells</td>
<td>$3\times10^4$</td>
</tr>
<tr>
<td>V (cells)</td>
<td>lysed cells</td>
<td>$7\times10^4$</td>
</tr>
<tr>
<td>O (serum)</td>
<td>fluid</td>
<td>$3\times10^4$</td>
</tr>
<tr>
<td>V (serum)</td>
<td>fluid</td>
<td>$7\times10^4$</td>
</tr>
</tbody>
</table>

* Viable number added at 0 hr to blood leucocytes ($10^7$ cells per ml serum) or serum alone.
† Streptomycin added to organisms in cell suspensions or in serum after removal of samples for counting at 1 hr.

Similar results were obtained in one other experiment.

**Peritoneal polymorphs.** In these experiments normal serum was replaced by heated serum; streptomycin was added after 30 minutes’ incubation so that appreciable extracellular multiplication was prevented. With the short
interval before removal of the first sample it was possible that the ratio of extracellular to intracellular organisms in such samples would be high. This might have led to an erroneously large estimate in intracellular numbers through contamination of cell pellets with sedimented extracellular organisms. Therefore numbers of organisms in both supernatants and cell pellets were counted; this established that at no sampling time were extracellular organisms sufficiently numerous to have materially contributed to counts of intracellular organisms (contamination of a cell pellet by extracellular organisms was <5 per cent. of the organisms in the supernatant; see Methods).

**Figure.**—Phagocytic killing of staphylococcal strains by peritoneal polymorphs (10⁷ cells per ml). Viable numbers of organisms are shown for both cell pellets and supernatants of centrifuged suspensions. Streptomycin was added to all suspensions after removal of samples at 30 min. The results are typical of 5 experiments.

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

It became apparent that there were two phases of intracellular killing (see the figure). In the 1st hr of incubation, killing was rapid and fall of intracellular numbers of strains O and P was faster than of strain V; thereafter the rate of killing of all 3 strains decreased; survival levels of strain V were 10- to 100-fold above those of strains O or P from the 2nd to the 5th hr. In general strain P was not markedly more resistant to killing than strain O. There appeared to be some variation in cellular bactericidal activity, for in 3 other experiments out of a total of 8 neither strain O nor strain P could be detected in cells after 2 hours’ incubation; strain V maintained a consistent pattern of resistance in all experiments.
DISCUSSION

The similarity of strains O, P and V in uptake by polymorphs indicates that resistance to ingestion by phagocytes is not responsible for increased virulence and is not conferred by growth in vivo. Since uptake of the organisms was rapid (17–80 per cent. in 15–25 min.; calculated from data in tables I and II), it is unlikely that differences in uptake would have been apparent in the more efficient conditions of surface phagocytosis that predominate in vivo (Wood, Smith and Watson, 1946). Examination of India ink films of the 3 strains did not reveal the presence of the surface material that Mudd (1965) states is typical of capsulated staphylococci.

The substantially greater resistance of strain V to polymorph killing, both initially and after prolonged incubation, is in general agreement with the earlier observations on resistance to cell lysates. No information is available on the significance of the two-phase process of intracellular killing for pathogenicity of staphylococci. Release of organisms from cells with subsequent extracellular multiplication may occur in vivo; this could not be detected in our experiments because of the streptomycin present.

The phagocytic killing of strains in fresh and heated serum appeared to be comparable; this accords with the minor role ascribed to heat-labile components of rabbit serum in the digestion of staphylococci by polymorphs (Shayegani, Kapral and Mudd, 1964).

The survival of strains O, P and V in polymorphs differed in two respects from their survival in cell lysates: the degree of killing of strain V appeared greater, and strain P was not consistently more resistant to killing than strain O. This divergence in behaviour is probably due to the much higher concentration of bactericidins present in intact cells. Recent experiments with cell lysates support this view: at high lysate concentration, the fall in viability of strain V was similar to that in intact cells and differences in susceptibility of strains O and P to killing were reduced. However, if polymorph killing in vitro is representative of cellular action in the host and strains O and P are equally susceptible in vivo, some factor additional to resistance to phagocytic killing may be responsible for differences in virulence (because strain V is 25 times more virulent than strain P, and strain P is 240 times more virulent than strain O). Such a factor might be resistance to serum bactericidins (Adlam, Pearce and Smith, 1970). Alternatively, the LD50 values may reflect primarily the behaviour of organisms that have undergone phenotypic conversion in vivo and have increased in resistance to host defence mechanisms; this has been observed for a number of bacteria (Meynell, 1961). Thus, in measurements of LD50, the "O" population arising in vivo from the infecting O inoculum may be appreciably more susceptible to cell bactericidins than the population (V) arising by conversion of the infecting P inoculum. The small difference in LD50 between strains P and V could be accounted for by the advantage to V inocula of all infecting organisms being in the altered form. These possibilities are being investigated.
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SUMMARY

The resistance to phagocytic uptake and intracellular killing of staphylococci grown *in vivo* and *in vitro* has been compared. Strains O (original) and P (rabbit passaged) grown *in vitro* and strain V (isolated directly from rabbits infected with strain P) did not differ in their susceptibility to ingestion by polymorphs. Intracellular killing was most marked in the 1st hr after phagocytosis; survival of strain V was substantially greater than that of strains O and P. This is in agreement with earlier observations on the resistance of these strains to the bactericidins of polymorph lysates. In contrast to behaviour in lysates, strain P was not significantly different from strain O in resistance to phagocytic killing. It is suggested that the virulence difference between strains P and O depends on the properties of the phenotypic form that each assumes *in vivo*.

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REFERENCES