Infection in Mouse Peritoneal Cavity with a Pyrimidine-requiring Mutant and Naturally Occurring Staphylococcus aureus Strains

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The lethal activity of a thymineless mutant of Staphylococcus aureus Wood 46 strain has been compared with that of three naturally occurring strains: parent Wood 46, Smith, and coagulase-negative SA-13. The thymineless mutant and the parent Wood 46 strain showed a sharp decline in culturable units from the peritoneal cavity in the first 4 hr after their injection. After 6 hr, that is, 2 hr before the mice began to die, the number of culturable units of the thymineless mutant was still declining, whereas that of the parent strain increased; for both strains, the number of units was still lower than that of the inoculum. Although the thymineless mutant, unlike the parent strain, was apparently unable to multiply in mouse peritoneal cavity, it killed mice at a similar rate. The highly virulent Smith strain known to multiply rapidly and the avirulent coagulase-negative SA-13 strain were used as additional controls. Under our experimental conditions, death of mice after the injection of the thymineless mutant in the peritoneal cavity did not seem to be due to bacterial multiplication but to toxicity, death being delayed by antibiotic. The pyrimidine-requiring auxotroph we used could be better material than killed bacteria to study some aspects of the lethal activity of S. aureus.

Staphylococcus aureus strains are generally characterized as pathogenic or nonpathogenic, depending mainly on the presence or the absence of specific toxins and enzymes in their cultures. Blair (2) pointed out that among the virulence markers of pathogenic staphylococci some may damage the host because of their toxicity and others may contribute indirectly to help the cocci to grow in the animals. The importance of toxicity or of bacterial growth as lethal factors in animals infected with staphylococci is still of concern and open to investigation. Although there have been several studies (7, 10, 16) on both aspects, to our knowledge, the nutritional requirements for growth of S. aureus strains have not so far been studied with relation to pathogenicity. In this respect, Bacon et al. (1) have shown that an essential metabolite for an auxotrophic bacterial strain of Salmonella typhosa can be a virulence determinant in the intraperitoneal infection of mice with this species; thus, they were able to relate metabolism to pathogenicity in bacteria.

In the present work, we have made a comparative study in mice of the ability to grow within the peritoneal cavity and of the lethal activity of naturally occurring and nutritionally deficient S. aureus strains, especially of a thymineless auxotrophic Wood 46 strain. We have attempted to determine whether some pathogenic staphylococci, apparently not multiplying in the host, could provoke its death.

MATERIALS AND METHODS

Bacterial strains and culture media. The following S. aureus strains were used: thymineless (thy-) Wood 46 auxotrophic mutant; Smith (tryptophan-dependent); SA-13 (tryptophan-dependent); and the parent Wood 46. Smith and SA-13 strains were supplied by, or originated from, Stephen Morse and the late Romuald D. Comtois, respectively. The isolation of the thymineless Wood 46 strain and the semisynthetic culture medium have already been described (4, 5); thymine and L-tryptophan were added to this medium according to the requirements of the strains cultured. Peritoneal cavity washings were obtained by following the technique of Bacon et al. (1).

Inoculum. All strains were grown on Nutrient Agar (Difco). The bacteria were suspended in saline to give a 70% transmission at a wavelength of 500 μm with a Coleman Junior Spectrophotometer; 1.4 ml of this suspension containing approximately 1.4 × 10⁸ bacteria was added to 125 ml of culture medium.

Growth in vitro. Bacteria were grown at 37 C up to
17 hr, and growth was followed by optical density measurements and viable cell counts.

_Growth in vivo._ The basic methods of injection, harvesting of peritoneal cavity fluids, and bacterial enumeration were essentially those already described by Cohn (3).

_Lethal activity._ Groups of 10 white CF1 mice originating from Carworth Farm, weighing 20 to 22 g, were injected intraperitoneally with decimal dilutions of washed staphylococci suspensions. Death of mice within 8 to 12 hr after the injection was considered to be due to toxicity (12). In some of these experiments, groups of mice were injected intraperitoneally with overdoses of 500 units of antitoxin from _Staphylococcus_ species (Connaught Medical Research Laboratories, Toronto, Canada) 24 and 4 hr before the inoculation of bacteria.

_Microscopical examination of the peritoneal cavity fluids of the infected mice._ Fluorochroming with acridine orange, examination under the fluorescence microscope, and microphotography of staphylococci obtained in the different cultures have already been described (4, 6), but, in the present work, fluorochroming was carried out at pH 8.0. Under these conditions, fluorescence microscopy gives more precise and more differentiated color images than those obtained with ordinary light microscopy and staining methods. The capsule of the Smith strain could not be observed in these conditions.

**RESULTS**

The rates of growth of the four different strains in the culture media and in the washings of mouse peritoneal cavity are presented in Table 1. They show that thymine is not available at this site for growth since the thymineless Wood 46 mutant did not grow at all in the mouse peritoneal cavity washings unless thymine was added. We had already found in other experiments that the Smith and SA-13 strains are tryptophan-dependent. Tryptophan was present in the washings but not in a sufficient amount to support full growth. In the same table are also presented results on the growth of the four strains in the peritoneal cavity of mice. The number of bacteria injected was $10^9$. The culturable units of the thymineless strain fell to $2.3 \times 10^9$ within 6 hr after the injection, thus behaving differently from the parent Wood 46 strain which, after a decline to $1.0 \times 10^7$ culturable units 4 hr after the injection, showed an increase and reached $1.5 \times 10^7$ culturable units. There was a difference of approximately almost one decade in culturable units between the parent Wood 46 strain and its thymineless mutant. Some growth seemed to take place between 4 and 6 hr after thymine had been injected with the thymineless staphylococci. For the Smith strain, which is well known for its virulence in mice when injected intraperitoneally, the culturable unit count increased to $5.0 \times 10^9$. There was considerable and steady decrease for the SA-13 strain, in a way similar to that of the thymineless mutant.

Table 2 shows results on the changes of the bacterial population in mouse peritoneal cavity and the mortality in mice within 6 and 8 hr, respectively, after the injection of all the strains used in this investigation. When $10^9$ bacteria were injected, the percentages of mice mortality for the parent Wood 46 strain and its thymineless mutant were 25 and 5%, respectively; to kill 90 and 85% of the mice with these strains, $10^6$ or more bacteria had to be injected. With this greater challenge dose, there was, when compared with the inoculum, a reduction in culturable units to 64% for the thymineless Wood 46 strain and to 80% for the parent Wood 46 strain. With the thymineless strain, when $10^9$ bacteria were injected, death of the mice occurred in 8 hr without

**Table 1. Growth of the Wood 46 thymineless mutant and three other _Staphylococcus aureus_ strains in culture media and in mouse peritoneal cavity washings as compared with that of culturable units from the peritoneal cavity of mice after the intraperitoneal injection of these strains**

<table>
<thead>
<tr>
<th>Strains inoculated</th>
<th>Metabolites added</th>
<th>Growth after 10 hr in culture media</th>
<th>Growth after 17 hr in peritoneal cavity washings</th>
<th>Culturable units from mice injected intraperitoneally with $10^9$ bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OD</td>
<td>Culturable units</td>
<td>OD</td>
<td>Culturable units</td>
</tr>
<tr>
<td>Wood 46 thy-</td>
<td>None</td>
<td>0.02</td>
<td>—</td>
<td>0.02</td>
</tr>
<tr>
<td>Parent Wood 46</td>
<td>Thymine</td>
<td>0.45</td>
<td>$1.1 \times 10^9$</td>
<td>0.61</td>
</tr>
<tr>
<td>Smith</td>
<td>None</td>
<td>0.57</td>
<td>$1.6 \times 10^9$</td>
<td>0.55</td>
</tr>
<tr>
<td>SA-13, coagulase-negative</td>
<td>None</td>
<td>0.02</td>
<td>—</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>Tryptophan</td>
<td>0.61</td>
<td>$1.8 \times 10^9$</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>0.02</td>
<td>—</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>Tryptophan</td>
<td>0.46</td>
<td>$1.2 \times 10^9$</td>
<td>0.45</td>
</tr>
</tbody>
</table>

* Made with minimal medium and clarified by centrifugation before inoculating with bacteria.
TABLE 2. Changes in culturable units from the peritoneal cavity and mortality after intraperitoneal injection in mice of Wood 46 thymineless mutant and three other Staphylococcus aureus strains

<table>
<thead>
<tr>
<th>Strains inoculated</th>
<th>No. of bacteria</th>
<th>Changes (%) in culturable units 6 hr after injection</th>
<th>Mortality (%) in mice 8 hr after challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Un-treated</td>
<td>Treated with antitoxin</td>
</tr>
<tr>
<td>Wood 46 thy⁻</td>
<td>10⁸</td>
<td>2.3</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>10⁹</td>
<td>64</td>
<td>85</td>
</tr>
<tr>
<td>Wood 46 thy⁻ (with thymine)</td>
<td>10⁸</td>
<td>1.5</td>
<td>0</td>
</tr>
<tr>
<td>Parent Wood 46</td>
<td>10⁸</td>
<td>43</td>
<td>100</td>
</tr>
<tr>
<td>Smith</td>
<td>10⁹</td>
<td>15</td>
<td>25</td>
</tr>
<tr>
<td>SA-13, coagulase-negative</td>
<td>10⁹</td>
<td>80</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>10⁹</td>
<td>500</td>
<td>100</td>
</tr>
</tbody>
</table>

* Data represent averages from three replicates.

* Calculation: [culturable units 6 hr after challenge]/(number of bacteria inoculated) × 100.

* Figures in parentheses represent percentage of mortality 48 hr after injection of challenge.

any indication of bacterial growth in the host.

SA-13 strain was coagulase-negative and almost nonlethal for mice when compared with the Smith strain, although both strains were tryptophan-dependent. The only strain among those we have studied which killed the mouse while multiplying at a rapid rate was the Smith strain. The highest challenge dose used for this strain was 10⁹ cells, which was lethal.

The thymineless Wood 46 mutant, the parent Wood 46, and the Smith strains, 6 hr after having been injected (in doses of 10⁹ cells for the mutant and the parent strains and of 10⁸ for the Smith strain), showed changes of 64, 80, and 500%, respectively, in culturable units when compared to the inoculum, and also gave high mortality rates in untreated mice of 85, 90, and 100%, respectively. When the mice were pretreated with antitoxin, the mouse mortality rates 8 hr after the injection fell to 12, 25, and 0%, respectively. Even when only a few culturable units could be recovered from the peritoneal cavity 24 hr after the injection of bacteria, the mouse mortality rates increased to 40, 50, and 15%, respectively, 48 hr after the injection of the challenge dose.

Figure 1 consists of fluorescence micrographs of staphylococci (seen as white dots) from the peritoneal cavity of mice. These bacteria are in microscopical fields of smears made from samples also used for counting culturable units (see Table 1). When the thymineless Wood 46 auxotrophic strain was injected in a sublethal dose of 10⁸ bacteria, only a very few staphylococci were outside the leukocytes in the samples taken 6 hr after the injection (Fig. 1A and B). When the challenge dose was increased to 10⁹ bacteria to kill the mice, the number of bacterial cells in a microscopical field did not seem to have increased 6 hr after the injection (Fig. 1C and D). Conversely, a comparison of the last two micrographs makes it evident that the tryptophan-dependent Smith strain grew extensively in the peritoneal cavity. For the Smith strain, the cells were dispersed and outside the leukocytes (Fig. 1E and F), whereas, for the thymineless Wood 46 strain (and similarly the parent Wood 46 strain), they were clumped or phagocytized (Fig. 1B and D).

**DISCUSSION**

Auxotrophic strains of a number of pathogenic bacterial species had already been used to study virulence of bacteria for animals but not pyrimidineless strains (14). The *S. aureus* thymineless strain used in the present work, unlike the parent strain, apparently cannot multiply in the host, although most of its cells are still living when injected and the macromolecular determinants associated with virulence are likely to be intact or even may be still actively synthesized. These cells can be considered as a better material to study some of the factors possibly responsible for the death of animals in the experimental infection than staphylococci killed by chemical or physical means which are known to have denaturing effects. Nevertheless, the thymineless strain cells may still undergo a thymineless death in the peritoneal cavity of the host because of the unavailability of thymine, their specific growth factor, since the number of culturable units from the peritoneal cavity did show a steady decline. For the parent strain after a similar initial decline, there was an increase probably beginning about 4 hr after the injection. The number of culturable units found after 6 hr was higher than that found after 4 hr; it also exceeded the number found for the thymineless strain. The lethal activity for mice 8 hr after the injection of the challenge dose was about the same for both strains. Obviously, it is difficult to quantitate in numbers of culturable units the total *S. aureus* population in the mouse peritoneal cavity, because clumping and phagocytosis interfere. Therefore, microscopic techniques were used in conjunction with culturable unit counts for the thymineless and the control strains. Among the *S. aureus* strains which have been used as additional controls, the Smith strain...
FIG. 1. Fluorescence micrographs showing staphylococci in smears of mouse peritoneal cavity fluid. (A) Sample taken 15 min after the injection of $10^8$ cells of thymineless Wood 46 strain. (B) Same as in A, but 6 hr after the injection, the cells are phagocytized. (C) Sample taken 15 min after the injection of $10^8$ cells of thymineless Wood 46 strain; the cells are already clumped. (D) Same as in C, but 6 hr after the injection. (E) Sample taken 15 min after the injection of $10^8$ cells of Smith tryptophan-dependent strain; the cells are not clumped. (F) Same as in E, but 6 hr after the injection.
described by Morse (13) as encapsulated and resistant to phagocytosis, with an inoculum of 10⁸, killed the mice when the culturable units in the peritoneal cavity attained a level which was not far from the fatal dose for the thymineless strain.

We made many unsuccessful attempts to obtain a thymineless auxotroph of the Smith strain which would have been very useful in this investigation. The avirulent coagulase-negative SA-13 strain followed a similar pattern in the decline of culturable units, but had a much lower lethal activity when compared with the thymineless mutant strain.

Under the fluorescence microscope, the thymineless Wood 46 mutant cells from the mouse peritoneal cavity fluid were similar in numbers and also in dimensions of the clumps to the parent Wood 46 strain cells when there were comparable numbers of culturable bacteria; thus, the proportion of dead cells in these bacterial populations should not be large. The clumping phenomenon in mouse peritoneal cavity, studied by Kapral with conventional coagulase-positive strains (11), occurs probably very quickly, so that the effects of the possible thymineless death of the cells do not interfere with it. The fluorescence micrograph (Fig. 1C) shows that the clumping was well under way 15 min after the injection of 10⁸ thymineless cells.

The Smith strain also used as a nonclumping control in these microscopical observations gave, as expected, a very different picture, which is in accordance with Kapral’s concept of events for this strain in the mouse peritoneal cavity (11). The growth of this strain was extensive, as determined by culturable units (Table 1) and as can be seen by comparing Fig. 1E and F. The cells did not clump and remained clearly visible outside the leukocytes.

In a situation in which no growth is likely to take place, toxic material present in the injected bacteria or due to some metabolic activity seems to be the factor responsible for death in mice after the injection of the thymineless mutant. It seems possible that for some staphylococci toxin is released from bacterial cells in the mouse peritoneal cavity without simultaneous bacterial multiplication, as has been shown in vitro for Corynebacterium diphtheriae (15).

The protection afforded by antitoxin in mice up to 8 hr and more after the inoculation of thymineless staphylococci also suggests that death in mice after the injection of this strain is mainly due to toxins, considering the number of bacteria and of antitoxin units injected (8, 9).

To conclude, our work suggests that death of the mice after the injection of a S. aureus pyrimidineless mutant in the peritoneal cavity is due to toxic materials either already present in the bacterial dose or deriving from some metabolic activity or growth undetected by our techniques. This assumption is based on our observations with the Wood 46 thymineless strain as compared to those with its parent strain: (i) apparent inability of the mutant strain to multiply in mouse peritoneal cavity; (ii) similar lethal activity for both strains after intraperitoneal challenge in mice which can be delayed by antitoxin.

ACKNOWLEDGMENT

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LITERATURE CITED