Some Effects of Subinhibitory Concentrations of Penicillin on the Structure and Division of Staphylococci

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A strain of *Staphylococcus aureus* was planted on filter membranes placed on Trypticase soy agar (BBL). After incubation, the membranes with growing staphylococci were transferred to Trypticase soy agar containing a subinhibitory concentration of penicillin (one-third minimal inhibitory concentration) and again incubated. The membranes were then returned to drug-free agar and incubated once more. Counts of the colony-forming units and electron microscopy were carried out at several time intervals. When grown on agar containing penicillin, the staphylococci formed what appeared to be abnormally large cells with multiple and unusually thick septa. Examination of a number of sections showed that these large cells were in reality clusters of staphylococci that had divided but failed to separate. When these large cells were subsequently grown on drug-free agar, smaller cells and normal staphylococci emerged. Subinhibitory concentrations of penicillin do not kill staphylococci; they seem to inhibit lysis of cross walls, preventing the separation of otherwise divided cells.

The bactericidal effect of penicillins is due to their inhibition of cross-linking by a transpeptidase (15, 19). There is, however, biochemical evidence that the penicillins also have a second site of action. Penicillin inhibits the D-alanine carboxypeptidase of both *Bacillus subtilis* (1) and *Escherichia coli* (14). This enzyme removes the terminal D-alanine residue of pentapeptids after transpeptidation. Penicillin inhibits the carboxypeptidase in both organisms at much lower concentrations than those required to inhibit bacterial growth.

Penicillin has two distinct morphological effects on *E. coli*: at subinhibitory concentrations, the growing bacilli become elongated; whereas at inhibitory concentrations, they are killed without elongation. The same subinhibitory concentrations of penicillin that cause elongation also block *E. coli* endopeptidase (4).

Proteus mirabilis turns into filaments when grown in the presence of subinhibitory concentrations of ampicillin, and into globules when grown with subinhibitory concentrations of cephalothin. This morphological difference has been attributed to different effects of these two drugs upon D-alanine carboxypeptidase (8).

Large staphylococcal cells produced by exposure to penicillin have been observed by light microscopy (17), as well as by scanning electron microscopy (3). After a relatively short exposure of susceptible staphylococci to concentrations equal to or greater than the minimal inhibitory

concentration, the septum showed loss in density and irregularity of shape, and the peripheral cell wall was markedly thin (10). A wider septum but a normal surrounding cell wall has been observed after exposure to subinhibitory concentrations of penicillin (18).

The present paper reports changes in structure and division observed in *Staphylococcus aureus* exposed to subinhibitory concentrations of penicillin and subsequently grown on drug-free media.

MATERIALS AND METHODS

Strain 209 P of *Staphylococcus aureus* was used. The minimal inhibitory concentration (MIC) of penicillin for this strain was 0.06 µg/ml as determined with a twofold agar dilution method (13).

The *Staphylococcus* was grown at 37°C for 18 h in Trypticase soy broth (TSB) (BBL) and then diluted 1:100 in TSB. Plates with Trypticase soy agar (TSA) (BBL) and with TSA containing benzylpenicillin were prepared. Filter membranes (no. PHWP09025, Millipore Corp., Bedford, Mass.) were placed on TSA and inoculated with 0.1 ml of the diluted *Staphylococcus* culture. The inoculated membranes were first incubated on drug-free agar for 3 h and then transferred to agar containing 0.02 µg of penicillin (one-third of the MIC) per ml. After incubation on the agar with penicillin for 7 h, the membranes were transferred back to drug-free agar and incubated for 4 h. Control membranes were incubated on drug-free agar for a time equal to the total time of incubation of each experiment (Table 1).

Counts of the colony-forming units (CFU) were

made every hour. For the determination of CFU per membrane, a pair of membranes was introduced into a tube containing 10 ml of TSB, shaken on a mixer, diluted, and then planted on TSA.

Electron microscopy was performed on the staphylococci incubated on the agar containing penicillin, the staphylococci incubated for the same period of time on drug-free agar (control), and the staphylococci incubated for 2 h on drug-free agar after their exposure to penicillin.

Processing for electron microscopy was according to the one-step fixation staining in block method of Simionescu et al. (12). Three volumes of formaldehyde plus 5% glutaraldehyde (which has been shown to rapidly stop cell autolysis in *Streptococcus fecalis* [6]) were mixed with 2 volumes of 2% osmium tetroxide and 1 volume of a saturated solution of lead citrate. The solutions were made on 0.1 M cacodylate buffer, pH 7.2, and kept at 0°C. Eight membranes with bacteria were shaken in 10 ml of fixative on a Vortex mixer for 1 min to displace the organisms into the fixative. After 2 h of fixation at 0°C, the bacterial suspension was centrifuged for 15 min at $750 \times g$. The pellet was washed in 0.1 M veronal-acetate buffer at pH 7.2 for 15 min, and then stained in 0.5% uranyl acetate in veronal acetate buffer, pH 5, for 30 min. Without washing, the pellet was dehydrated for 25 min in graded ethanols and embedded in epon in flat molds. Sections of about 60 nm in thickness were cut with a diamond knife on a MT2-B Porter Blum ultramicrotome (Ivan Sorvall, Inc., Norwalk, Conn.). The sections were stained with uranyl acetate for 2 min and with lead citrate for 5 min. Examination was made with a Philips 300 electron microscope operated at 80 kV and provided with a 200- μ m condenser aperture and a 30- μ m objective aperture. Multiple sections were examined at final magnifications of 12,000, 30,000, and $\times 50,000$.

RESULTS

After incubation on agar containing one-third the MIC of penicillin, almost all the bacterial cells were much larger and more irregularly shaped than the control cells (compare Fig. 1A with Fig. 1B). Most cells had many wide septa (at least twice as wide as those of the control cells) without a central dense layer. A central septum divided the cells into two relatively unequal hemispheres (Fig. 2A). Each hemisphere showed two or more other septa that were, for the most part, oriented at an angle to the central septum. Some cells had two main septa, each of the three resulting segments being subdivided by further septa (Fig. 2B). The surrounding cell wall appeared to be slightly thicker in localized areas; otherwise it did not show significant abnormalities.

After growing for 2 h on drug-free agar after their exposure to penicillin, the bacterial cells still exhibited abnormal forms, but organisms of normal appearance could also be observed (Fig. 3). Many of the abnormal forms were smaller

TABLE 1. Number of CFU grown on membranes on TSA control, TSA containing penicillin, and drug-free TSA after incubation on the TSA containing penicillin

Time (h)	No. of CFU		
	Control on drug-free agar	Agar containing penicillin (0.02 μ g/ml)	Drug-free agar after exposure to penicillin
0	8.0×10^6		
0.5	6.0×10^5		
3	1.7×10^6	1.7×10^6	
4	5.4×10^6		
5	2.6×10^7	3.1×10^6	
6	3.1×10^7	4.0×10^6	
7	6.0×10^7	4.5×10^6	
8	1.1×10^8	6.5×10^6	
9	4.0×10^8	5.7×10^6	
10	7.2×10^8	5.0×10^6	5.0×10^6
11	3.0×10^9		7.5×10^6
12			1.1×10^7
13			8.8×10^7
14	5.0×10^9		4.5×10^8

and had fewer septa than those that had been observed during exposure to penicillin. Some lysis and separation of the central septum could be seen (Fig. 4).

The number of CFU per membrane increased only slightly during exposure to penicillin, but increased significantly after subsequent incubation on drug-free agar (Table 1).

DISCUSSION

An equilibrium between synthesis and lysis in the cell wall of normally growing bacteria permits division to occur without destruction of the cell (9, 11). Among the functions proposed for autolytic activity are: cleavage of bonds in the peptidoglycan network to provide new ends for synthesis and hydrolyzation of bonds in specific areas to permit cell division and separation (7).

In *S. fecalis*, the major site of action of the autolysin is along the leading edge and side of the growing cross wall (5), where it is of aid in separating the cross wall into the diverging peripheral walls of two new cells during division (6). Conversely, it has been shown that pneumococci with autolysin-resistant cell walls (which can be produced in a medium deprived of cholin) cannot separate after division, so that continued growth results in long coccal chains (16).

By analogy to previous suggestions concerning *B. subtilis* (1), *E. coli* (4, 14) and *P. mirabilis* (8), it is conceivable that the morphological changes produced in several organisms by subinhibitory concentrations of penicillin (2) might be the result of inhibition of whatever

FIG. 1. (A). *Staphylococci* grown on agar containing penicillin (one-third of the MIC) for 7 h. Most cells are 2 to 3 times larger than the control (Fig. 1B) and contain wide cross walls ($\times 12,000$). (B). *Staphylococci* grown on drug-free agar for 10 h (control) ($\times 1200$). Bars indicates 1 μm .

FIG. 2. (A). *Staphylococci* grown on agar containing one-third of the MIC of penicillin. (A) Wide cross walls divide the cells into two hemispheres. Insert in upper left shows control cells (×30,000). (B) Cross walls divide the cells into three hemispheres (×50,000). Bars indicates 1 μm.

FIG. 3. *Staphylococci* grown for 2 h on drug-free agar after exposure to penicillin. Smaller clusters as well as some cells of normal size are present ($\times 12,000$). Bar indicates 1 μM .

enzymes contribute to lysis of septa during cell division. Separation of the new cells could not then occur, and elongated bacilli or irregular-shaped cocci would result.

Staphylococci on drug-free media divide, separate, and, as a result of their multi-directional planes of division, form clusters. When exposed to subinhibitory concentrations of penicillin, they divide, showing numerous nuclei (17), but cannot separate.

Figure 5 is a schematic representation of a section of a three-dimensional multiplication sequence of a *Staphylococcus* grown in the presence of subinhibitory concentrations of penicillin. The first cross wall grows normally, but does not split, and the two new cells cannot separate into staphylococci A and B. Growth and division does, however, continue, and each *Staphylococcus* grows its own cross wall and divides into A₁ and A₂, and B₁ and B₂, up to some 8, 16, or 32 units. These remain crowded together within a single surrounding cell wall, which appears to be an extension of the cell wall of the original *Staphylococcus* A-B.

After 7 h on the TSA with penicillin, all staphylococci had grown into abnormal large cells (Fig. 1A), each cell consisting of several generations derived from one *Staphylococcus*. Exposure to penicillin allowed only a slight increase in CFU per membrane (Table 1). Before exposure, each organism produced one colony. During exposure, each organism divided, but many of the resulting cells remained together, so that each large cell was still one CFU. When the large cells were transferred to drug-free agar, separation into smaller clusters and even individual staphylococci of normal size took place. The number of CFU then increased significantly.

The results of the present study suggest the following conclusions: (i) subinhibitory concentrations of penicillin did not kill staphylococci and did not produce significant alterations in the peripheral cell wall. (ii) Subinhibitory concentrations of penicillin seem to inhibit lysis of the cross wall without inhibiting cell division. This resulted in the formation of what appeared to be abnormally large cells but which were in

FIG. 4. *Staphylococci* grown for 2 h on drug-free agar after exposure to penicillin. Note: lysis of central cross walls with separation (arrow) ($\times 30,000$). Bar indicates 1 μ m.

FIG. 5. Schematic representation of a section of a multiplication sequence of a *Staphylococcus* grown in the presence of subinhibitory concentrations of penicillin.

fact clusters of staphylococci, prevented from separating by the presence of many wide cross walls. (iii) The inhibition of cross-wall separation was reversible. When incubated on drug-free media, the clusters of unseparated staphylococci separated into smaller clusters and cells of normal appearance and growth rate.

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