AN ELECTRON-MICROSCOPIC STUDY ON MORPHOLOGICAL CHANGES OF *STAPHYLOCOCCUS AUREUS* TREATED WITH SPIRAMYCIN

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The cell wall of *Staphylococcus aureus* 209 P JC became markedly thickened with the lapse of time following exposure to spiramycin. The degree of cell wall thickening was more conspicuous when the concentration of the drug used was high. When transferred to a new medium containing no antibiotic the organism with thickened cell wall grew by way of abnormal division to resume eventually normal cell wall thickness.

Several studies on the phenomenon of cell wall thickening of bacterial cells treated with protein synthesis inhibitory antibiotics have been reported.[1,2] Among them, SHOCKMAN *et al.* showed that the cell wall of *Streptococcus faecalis* became thickened when cultivated in a medium deficient in such amino acid as threonine (Thr) or valine (Val).[3] They have been studying in detail this phenomenon in connection with the autolytic enzymes.[4~9]

As reported previously, the cell wall of sensitive strains of *Staph. aureus* becomes extremely thickened, usually with the formation of multilayers, and the electron density of the cytoplasm also increases, following exposure to protein synthesis inhibitory antibiotics as lincomycin, clindamycin and macrolides.[10~12] On the other hand, such phenomena as above mentioned are not observed with antibiotic-resistant strains.

This report deals with the relationship between the lapse of time following exposure to antibiotic and the course of cell wall thickening, and the relationship between the concentration of antibiotic used and the degree of cell wall thickening using spiramycin (SPM), one of the macrolides. In addition, the morphological changes of the organism with thickened cell wall, following transfer and incubation in a new medium containing no antibiotic, are also described.

Materials and Methods

Organism: *Staphylococcus aureus* 209 P JC was used as the test strain. The minimum inhibitory concentration (MIC) of SPM against this strain is 0.4 mcg/ml.

Antibiotic: SPM supplied by Kyowa Hakko Kogyo Co., Ltd. was used.

Culture: Cells were grown in Tryptosoya broth (Nissan) at 37°C for 18 hours. The culture was diluted 20 times with Heart infusion broth (HI, Nissan), then incubated at 37°C in shaken culture until logarithmic growth was reached.

In order to investigate the relationship between the lapse of time following exposure to the antibiotic and the course of cell wall thickening, cells were treated with 6.4 mcg/ml of SPM and at intervals of time they were collected by centrifugation and examined by electron microscopy.

To investigate the relationship between the concentration of the antibiotic and the degree of cell wall thickening, cells were treated with 0.1, 0.4, 1.6 and 6.4 mcg/ml of SPM respectively,

and at 3 and 6 hours after the addition of the antibiotic, the cells were collected and subjected to electron microscopy. The observation on the morphological changes of cells with thickened walls after transfer in a new medium free from antibiotic were made as follows:

Firstly, cells were cultured in broth containing 0.4 mcg/ml of SPM for 24 hours. The cells were then collected by centrifugation, washed thoroughly with sterilized saline to remove the antibiotic, suspended in HI medium and then incubated at 37°C in shaken culture. Cells were harvested at various intervals and subjected to electron microscopy.

Electron microscopy: The cells prepared for electron microscopy were fixed according to the method of RYTER & KELLENBERGER,[13] dehydrated with graded alcohol solution and embedded in epoxy resin according to LUFT's method.[14] Preparations were sectioned using an LKB ultra-microtome with a diamond knife, and then stained with uranyl acetate and lead citrate.[15] Ultrathin sections were examined in an Akashi S-500 electron microscope operated at an acceleration voltage of 75 kv. For the measurement of cell wall thickness, about 30 cells which seemed to have been sectioned at the central part were chosen from the negative

Fig. 1. Section of intact *Staphylococcus aureus* 209 P JC
The cytoplasm is separated in two parts by growing cross wall, and nuclear division has been performed.

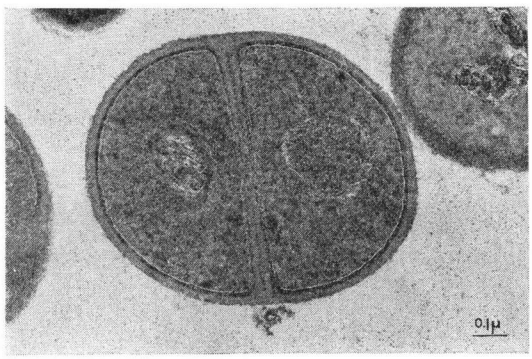


Fig. 2. Section of a cell treated with SPM for 15 minutes
Vesicular substances are observed in periplasm, and cell wall thickening is not noticed.

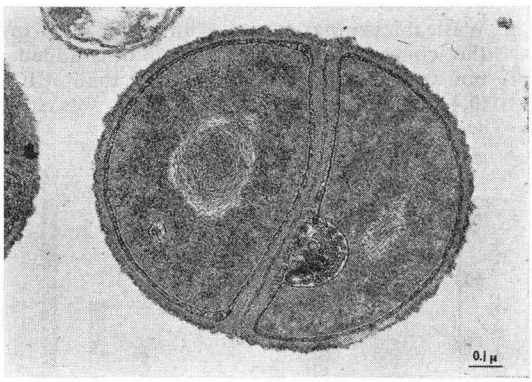


Fig. 3. Section of a cell treated with SPM for 1 hour
Cell wall thickening is obvious compared with normal intact cell. Vesicular mesosomes are observed in contact with nucleoplasm.

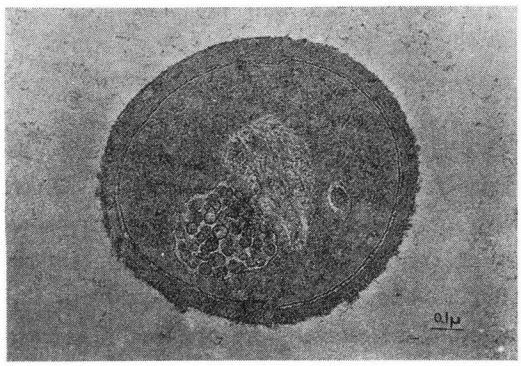


Fig. 4. Section of a cell treated with SPM for 2 hours
Elevation of electron density in cytoplasm in addition to the progress of cell wall thickening is noticed. Many tubular mesosomes are observed in contact with nucleoplasm.

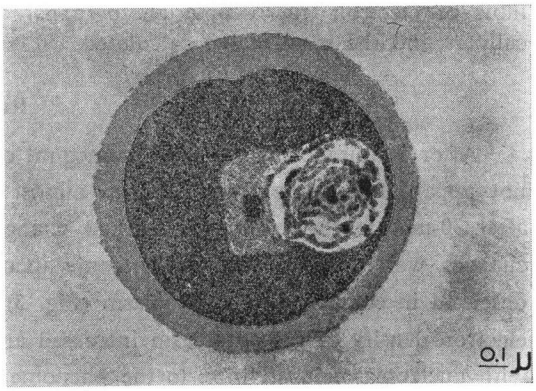


Fig. 5. Section of a cell treated with SPM for 24 hours
Thickened cell wall and dispersed nucleoplasm are observed in cytoplasm of which electron density has increased.

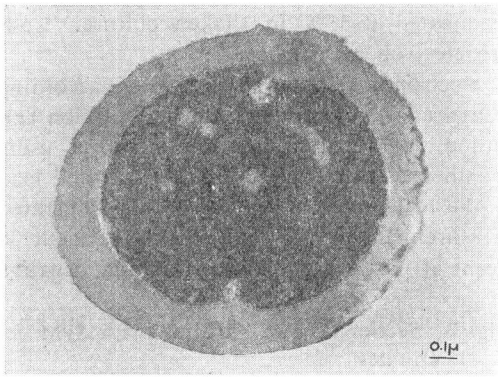


Fig. 6. Time course of cell wall thickening of *Staph. aureus* 209 P JC treated with SPM (6.4 mcg/ml)
The thickness of cell wall was measured on about 30 cells which seemed to have been sectioned at their central part.

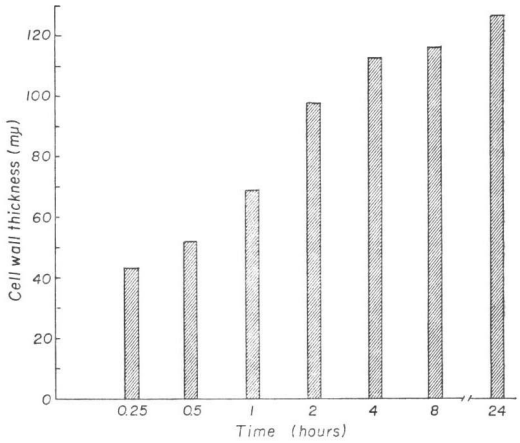


Fig. 7. Cell wall thickness of *Staph. aureus* 209 P JC treated with various concentrations of SPM
Wall thickening occurred in proportion to the concentration of the antibiotic added, but at the concentrations lower than MIC (0.4 mcg/ml), almost no thickening was observed.

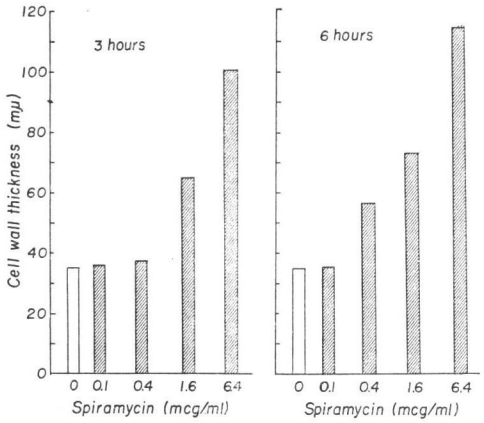
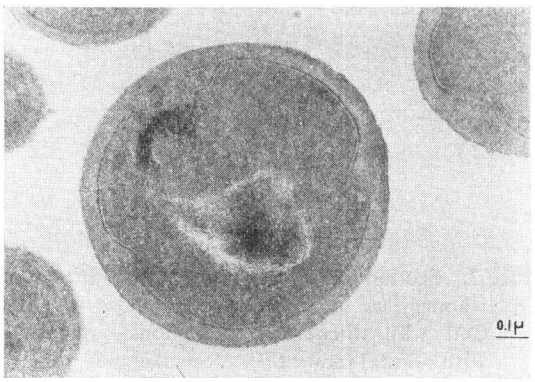


Fig. 8. Section of a cell just after transfer to a new medium
Thickened peripheral cell wall is observed.



films of electron microscopic photographs. The thickness of cell wall then measured with calipers and the mean value calculated.

Results

After 15-minute exposure to 6.4 mcg/ml of SPM (Fig. 2), thickening of the cell wall was not apparent and the thickness was almost the same as that of normal cells (Fig. 1). Even after 30-minute treatment, no marked changes were observed but after 1 hour conspicuous changes were noticed; cell wall became abnormally thickened and numerous mesosomes were observed in contact with nucleoplasm (Fig. 3). After 2~4-hour exposure to the antibiotic the electron density of the cytoplasm increased and numerous mesosomes were observed in contact with nucleoplasm in addition to the phenomenon of cell wall thickening (Fig. 4).

Fig. 9. Section of a cell cultivated in a new medium for 2 hours
A new thin cross wall has been formed and another cross wall at a right angle to the existing one is beginning to be formed.

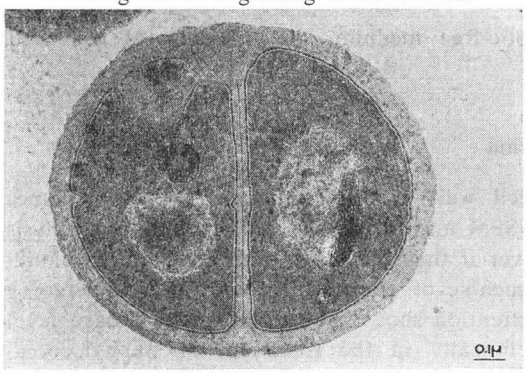


Fig. 10. Section of a cell cultivated in a new medium for 4 hours
Cytoplasm has been divided into three parts by newly formed thin cross walls. The cross wall of left hand side seems to be just about to be connected.

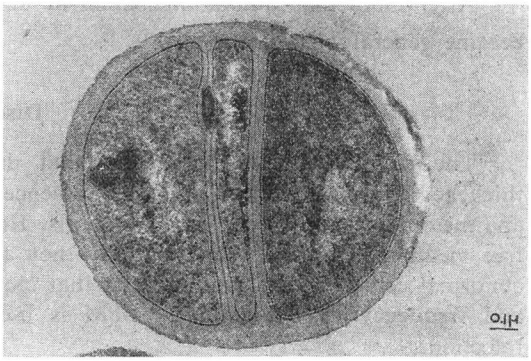


Fig. 11. Section of a cell cultivated in a new medium for 8 hours
A part divided by a thin cross wall is going to be detached. Among three parts of divided cytoplasm, nucleoplasm is not observed in one part.

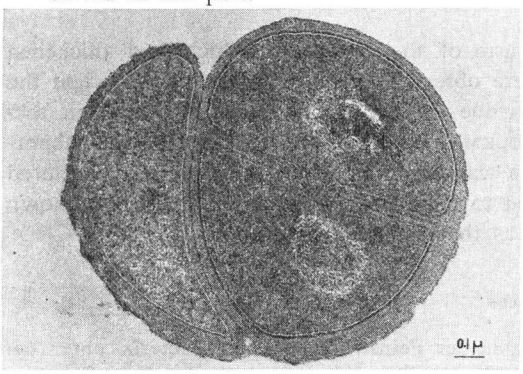
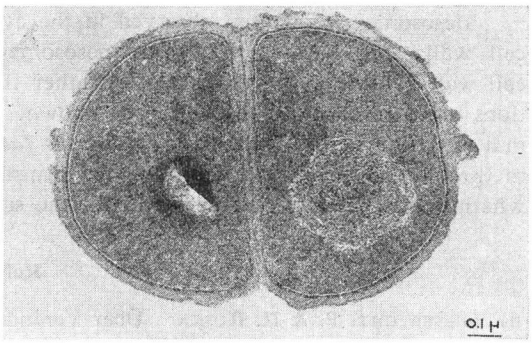


Fig. 12. Section of a cell cultivated in a new medium for 8 hours
It seems cell wall thickness has reverted to normal and at some places of peripheral cell wall, the fragments of old cell wall are observed.



The progress of cell wall thickening was still apparent after 24 hours (Fig. 5). In the cytoplasm of bacteria with thickened cell walls, numerous mesosomes were often observed in contact with the nucleoplasm, but in some cases mesosomes were not observed in the cytoplasm even though the cell wall was greatly thickened. After 24-hour exposure to the antibiotic, only the extremely thickened cell wall and shrunken cytoplasmic membrane remained and the cytoplasmic contents flowed out. The time course of cell wall thickening is shown in Fig. 6.

The thickening of the cell wall occurred in proportion to the concentration of the antibiotic added, but at concentrations lower than the MIC, almost no thickening of the cell wall was observed (Fig. 7).

Immediately after transfer to antibiotic free medium, the organism treated with SPM for 24 hours revealed the thickened peripheral cell wall (Fig. 8).

After 1-hour cultivation in antibiotic-free medium the formation of a thin cross wall was completed, and another cross wall, at a right angle to the existing one, was beginning to be formed (Fig. 9). After 4~6-hour incubation, an abnormal cell division occurred, with the

cytoplasm divided into three parts by two thin cross walls (Fig. 10). Fig. 11 shows a cell after 8-hour incubation in which part of the cell has been separated off by a thin wall and this part is about to become detached. It can be seen that in this cell the old wall is quite thick, whereas the new wall formed in the absence of antibiotic is thin.

After more than 8-hour incubation in antibiotic-free medium, the thickness of cell wall became generally normal (Fig. 12).

Discussion

The results reported here show that the cell wall of *Staph. aureus* 209P JC becomes thickened as a result of growth in the presence of SPM and finally the cells lyse by plasmolysis and the outflow of the cytoplasmic contents. However if these cells are transferred to antibiotic-free medium before they lose viability they are capable of continued growth after undergoing abnormal cell division. This suggests that close attention should be given to the dosage level and frequency of dosage when SPM is used clinically in the treatment of staphylococcal infection.

In previous studies dealt with the morphological changes in *Staph. aureus* treated with lincomycin, clindamycin and macrolides,[10,11] multilayers were often noticed in the thickened cell wall, but in the experiments reported here such structures were not noticed even when the cell wall became thickened. This may be due to differences in culture conditions; in the previous experiments the cells were cultivated on agar medium whereas in this study the cells were grown in liquid medium in shaken culture.

Mesosomes were often observed in the cytoplasm of the organisms which had thickened cell walls, but in some cases no mesosomes were observed in the cytoplasm even when the cell wall became very thickened. Whether this is due to the cutting angle of the thin sections or to some other reason is not known. SHOCKMAN *et al.* have also reported that abnormal septation of the cells of *Streptococcus faecalis* was observed when they were precultured in threonine-deficient medium and then transferred to complete medium.[16] It is not known whether this phenomenon is essentially the same as that reported here or not.

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