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Bactericidal Action of A Quaternary Ammonium Disinfectant, Didecyldimethyl Ammonium Chloride, against *Staphylococcus aureus*

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The study was conducted using a quaternary ammonium disinfectant, didecyldimethyl ammonium chloride (DDAC), whose minimum lethal concentration (MLC) against *Staphylococcus aureus* was 32 $\mu\text{g/ml}$. No remaining viable cells were detected even after a short 20 s treatment with DDAC at the MLC. Treatment with DDAC caused to form blebs on the cell surface, and K^+ , 260 nm absorbing materials and phospholipid leaked out from the cells. The experiment using ^{14}C -labeled DDAC showed that the disinfectant was taken up in a biphasic curve to the cell regardless of the treatment temperature or time. The radioactivity was distributed significantly in the membrane fraction and the cytoplasm fraction. Tetramethyl ammonium chloride showed no biocidal activity and had no effect on the activity or uptake of DDAC.

From these results, it was deduced that DDAC, at bactericidal levels, is adsorbed physicochemically, by hydrophobic interaction, onto the cell membrane, where it can react with lipid to cause significant damage to the structure and function of the membrane, leading to death.

Keywords — bactericidal action; quaternary ammonium compound; *Staphylococcus aureus*; didecyldimethyl ammonium chloride; uptake; distribution

Introduction

Staphylococcus aureus is a major cause of suppurative infection and food-poisoning, as well as being a natural inhabitant in the mucous membrane of the healthy human nose. Treatment of the infection had been comparatively easy since the advent of methicillin usage in the 1960's. In recent years, however, methicillin-resistant strains of *S. aureus* (MRSA), which are insensitive to many antibiotics, have often been isolated from clinical environments. The control is so difficult that MRSA have given rise to a serious problem as the main causative bacteria of nosocomial infection. The incidence of *S. aureus* detection in clinical samples has exceeded that of

Pseudomonas aeruginosa in Japan since 1987.¹⁾ The best way to eliminate the nosocomial infection at present is to prevent contamination in the clinical environment by the usage of appropriate disinfectants, among which quaternary ammonium compounds (QACs) have been found to be effective in clinical and other examinations.²⁻¹⁰⁾ There are papers concerning bacterial resistance to QACs,¹¹⁻¹⁶⁾ but, only a few reports concerning their bactericidal mechanism.¹⁷⁻²¹⁾ We therefore attempted to identify this mechanism using didecyldimethyl ammonium chloride (DDAC). We chose DDAC because it is a representative disinfectant with a broad bactericidal spectrum, a cationic surfactant of simple structure having linear alkyl side chains, and also

because the ^{14}C -labeled compound most fortunately came into our hands.

Materials and Methods

Chemicals — DDAC and [^{14}C -methyl]-DDAC were generously donated by Kao Ltd. Tetramethyl ammonium chloride (TMAC), a product from Wako Pure Chemical Industries, Ltd., was used as a reference compound for DDAC.

Bacterial Strain — *S. aureus* ATCC 6538p was cultivated overnight in nutrient broth (Difco) or on nutrient agar (Difco) at 37°C before use unless otherwise specified.

Determination of Minimum Lethal Concentration (MLC) — MLC, the minimum concentration of DDAC at which recovery and growth did not occur, was assayed essentially according to the method for phenol coefficient recommended by AOAC (The Association of Official Analytical Chemists, U.S.A.).²²⁾ First, 100 μl of a serial diluted DDAC solution was poured into each glass tube. The test bacteria, which had been grown with shaking in nutrient broth (Difco) at 37°C for 24 h, were collected by centrifugation, washed twice with 50 mM sodium phosphate buffer (pH 7.0), and then suspended at OD_{600} of 1.0 (*Ca.* 10^9 colony-forming units per ml (cfu/ml)) with the same buffer. The cell suspension (900 μl) was added to the DDAC solution (100 μl), and after 10 min at 37°C , 10 μl of the reaction mixture was transferred to 3 ml of soybean-casein digest broth with lecithin and polysorbate 80 (SCDLP medium: Nihon-Seiyaku Co.), and then incubated at 37°C for 48 h. Effect of TMAC on the action of DDAC was examined at a final concentration in the reaction mixture of 500 $\mu\text{g}/\text{ml}$.

Enumeration of Viable Cells — After the cells were treated by DDAC under the same conditions as those for MLC, serial diluted solutions of the reaction mixture were applied on nutrient agar (Difco). The viable cells were counted after incubation at 37°C for 48 h.

Determination of Lethal Time by DDAC — At given times during the incubation of bac-

terial cells with 32 $\mu\text{g}/\text{ml}$ DDAC at 37°C , the reaction mixture was diluted 100 fold with nutrient broth (Difco) and incubated at 37°C for 48 h to determine the presence of survivors.

Electron Microscopy — The cells were treated with 32 $\mu\text{g}/\text{ml}$ DDAC at 37°C for 10 min. Intact and DDAC-treated cells were fixed with 3% glutaraldehyde and dried in a critical point dryer (JEOL, JCPD-5). After the specimen was coated with gold, the scanning image of the cells was observed on an electron microscope (JEOL, 2000EX) at 40 kV.

Determination of K^+ Leaked from Cells — The cells (*Ca.* 10^9 cfu/ml) were incubated with various concentrations of DDAC at 37°C for 10 min in 50 mM sodium phosphate buffer (pH 7.0). The incubation mixture was then centrifuged at $1200 \times g$ for 20 min, and the K^+ concentration in the supernatant was measured with an atomic absorption spectrophotometer (Hitachi, Z-8100) using K^+ standard (Wako) as reference. The total amount of K^+ in the cells, 6.88 $\mu\text{g}/\text{ml}$ (5.29 $\mu\text{g}/10^9$ cells), was determined on the cell lysate obtained by digestion of the cell wall with lysostaphin.

Determination of 260 nm Absorbing Materials Leaked from Cells — The method was the same as that for determination of K^+ from cells except that a UV spectrophotometer (Shimadzu, UV-160A) was used for measurement of the concentration of materials. The control value (100%) was 1.15/ml (1.44/ 10^9 cells).

Determination of Phospholipid Released from Cells — The cells were treated with DDAC by the same method as described for determination of K^+ from cells except that 50 mM Tris HCl buffer (pH 7.0) was used instead of the phosphate buffer. The reaction mixture after the treatment was centrifuged at $1200 \times g$ for 10 min, and an aliquot of the supernatant layer was taken out and lyophilized overnight. The content of phospholipid in the lyophilized powder was estimated according to the conventional method²³⁾: 1) extraction with chloroform-methanol (1:2, v/v), 2) evaporation of the chloroform extract to dryness, 3) digestion with 70% HClO_4 around 500°C for 5 h, 4) colorimetric determination of

inorganic phosphate (Pi) after successive reactions with 5% ammonium molybdate and 1% solution of Amidol reagent (Wako), and 5) calculation of the amount of phospholipid as 25 times Pi. The control value (100%) was 37.8 $\mu\text{g}/\text{ml}$ (27.0 $\mu\text{g}/10^9$ cells).

Uptake Experiment of ^{14}C -DDAC by the Cell—The cells were placed in contact with DDAC in a similar manner to that in the assay of MLC except for the use of ^{14}C -labeled DDAC. After 10 min contact the cells were subsequently washed twice by suspending with 50 mM sodium phosphate buffer (pH 7.0) and centrifuging at $1200 \times g$ for 10 min to remove any radioactive DDAC loosely bound to the cells. The radioactivity of the precipitate (cells) was measured on a liquid scintillation counter (Aloka, LSC-903). The uptake is expressed as the ratio against that with 1 $\mu\text{g}/\text{ml}$ DDAC, 17779 cpm/ml (0.17 $\mu\text{g}/10^9$ cells).

Fractionation of Cell Components—Components of *S. aureus* cells were fractionated essentially according to the method of Kawata *et al.*²⁴⁾ as follows. The bacterial cells at logarithmic phase (cultivated for 8–10 h) were collected and suspended in 50 mM sodium phosphate buffer (pH 7.0) at OD₆₀₀ 1.0 (1.4×10^9 cfu/ml). The cell suspension was incubated with 1 $\mu\text{g}/\text{ml}$ radioactive DDAC (80813 cpm/ μg) for 10 min at 37°C, and the cells were then washed twice with the above buffer. The final resuspension buffer was changed to 10 mM Tris HCl (pH 7.8) containing 25% sucrose and 1 mM EDTA. Lysostaphin (8.5 units, Sigma) was added to the suspension at the final concentration of 20 $\mu\text{g}/\text{ml}$, and then incubated at 37°C for 45 min. The mixture was subsequently centrifuged at $1200 \times g$ for 10 min, and the supernatant layer was defined as the cell wall fraction. The residue (the protoplast) was suspended in 10 mM Tris HCl (pH 7.8) containing 1 mM EDTA but not 25% sucrose, and then incubated at 25°C for 30 min in order to burst the protoplasts. The suspension was centrifuged at $1200 \times g$ for 10 min, and the residue was the un-lysed fraction. Further, the supernatant layer was centrifuged at $10000 \times g$ for 10 min, and the residue was defined as the membrane fraction,

while the upper layer as the cytoplasm fraction. Radioactivity of each fraction was measured on a liquid scintillation counter (Aloka, LSC-903), and expressed as percentage of the whole cell fraction (57918 cpm).

Results

MLC of DDAC against *S. aureus* and Effects of TMAC

When the cells of *S. aureus* (1.3×10^9 cfu/ml) were incubated in serial dilutions of DDAC, viable cells were reduced with increasing concentrations of DDAC, as shown in Fig. 1. MLC of DDAC on 10 min contact at 37°C was determined to be 32 $\mu\text{g}/\text{ml}$, while TMAC showed no bactericidal activity itself and no effect on the activity of DDAC (Table I).

Lethal Time by DDAC

When the bacterial cells were exposed with DDAC at the MLC of 32 $\mu\text{g}/\text{ml}$, they were completely killed within 20 s, as shown in Table II.

Surface Structure of *S. aureus* Cells Treated with DDAC

It was observed that many blebs were formed on the cell surface when the cells were treated with DDAC (Fig. 2-B). In contrast, the surface of the intact cells was relatively smooth (Fig. 2-A).

Leakage of K^+ from Cells

Treatment of the cells with DDAC for 10 min released K^+ from the cells, and the amount of leakage was dependent on the concentration of

Fig. 1. Viability after DDAC Treatment

The cells (1.3×10^9 cfu/ml) were treated with a given concentration of DDAC at 37°C for 10 min in 50 mM sodium phosphate buffer (pH 7.0). Arrows indicate no viable cells.

TABLE I. Bactericidal Activity of DDAC and TMAC and Effect of TMAC

Treatment	DDAC or TMAC concentration ($\mu\text{g/ml}$)							
	1	8	16	32	64	125	250	500
DDAC	+++	+++	+++	---	---	---	---	---
+500 $\mu\text{g/ml}$ TMAC	+++	+++	++-	---	---	---	---	---
TMAC	+++	+++	+++	+++	+++	+++	+++	+--

The cells were incubated with a given concentration of DDAC or TMAC at 37°C for 10 min. After dilution with SCDLP medium, they were incubated for 48 h to determine the viability. Growth or no growth after that is expressed as + or -, respectively.

TABLE II. Lethal Time during DDAC Treatment

Treatment	Time (s) of DDAC treatment							
	0	10	20	30	40	60	90	120
DDAC	+++	++-	---	---	---	---	---	---

During treatment with 32 $\mu\text{g/ml}$ DDAC, nutrient broth was added to the cell suspension at a given time (s), and incubation was followed for 48 h to see the viability. Growth or no growth after that is expressed as + or -, respectively.

Fig. 2. Surface Structure of the Intact Cell (A) and the DDAC-treated Cell (B)

The cells were treated with 32 $\mu\text{g/ml}$ DDAC under the same conditions as in Fig. 1 (See text for details). Bar represents 100 nm.

DDAC (Fig. 3). About 80% of the intracellular K^+ was leaked out into the medium by the DDAC treatment at 4 $\mu\text{g/ml}$ and almost 100% at 8 $\mu\text{g/ml}$.

Leakage of 260 nm Absorbing Materials from Cells

The leakage of 260 nm absorbing materials by the DDAC treatment was also found to follow a similar pattern to K^+ ion (Fig. 4). The leakage began from 2 $\mu\text{g/ml}$ and reached a plateau at 8 $\mu\text{g/ml}$.

Phospholipid Release from Cells

Figure 5 shows that phospholipid was also

Fig. 3. Leakage of K^+ from Cells by DDAC Treatment

The cells were treated with DDAC under the same conditions except using Tris-HCl buffer as in Fig. 1 (See text for details).

Fig. 4. Leakage of 260 nm Absorbing Materials from Cells by DDAC Treatment

The cells were treated with DDAC under the same conditions as in Fig. 1 (See text for details).

Fig. 5. Release of Phospholipid from Cells by DDAC Treatment

The cells were treated with DDAC under the same conditions as in Fig. 1 (See text for details).

Fig. 6. Uptake of DDAC by Cells

The cells were treated with DDAC under the same conditions as in Fig. 1 (See text for details).

TABLE III. Effect of Treatment Time and Temperature on Uptake of ^{14}C -DDACA by Cells

Time	Uptake (%)
3min	103
5	128
10	100
15	93

Temperature	Uptake (%)
4°C	102
37	100

The cells were treated with 1 $\mu\text{g/ml}$ ^{14}C -DDAC at 37°C for a given time (left), or at a given temperature for 10 min (right), and the radioactivity was determined. The uptake is expressed as percentage of that at 37°C for 10 min.

released from the cells dependently on the concentration of DDAC. Significant release was found even from the cells treated at 4 $\mu\text{g/ml}$, where more than 90% of cells survived, and it reached a plateau at the MLC level, 32 $\mu\text{g/ml}$.

Uptake of DDAC into the Bacterial Cells

Figure 6 shows the biphasic curve of ^{14}C -DDAC uptake by the cells dependent on the concentration. The first significant uptake was observed in the range of 1 to 8 $\mu\text{g/ml}$ where most of the cells survived, and the second, large uptake of DDAC started from around the MLC, (32 $\mu\text{g/ml}$) meaning that this uptake was by the killed cells. Therefore, the first uptake could be involved in the action of DDAC. In the first uptake using 1.0 $\mu\text{g/ml}$ DDAC, the radioactivity at 4°C was at the same level as that at 37°C, and also it did not vary with the incubation time after 3 min (Table III). The uptake of DDAC was not influenced by the presence of TMAC and the DDAC taken up was also not released by addition of TMAC during the

treatment (data not shown).

Distribution of DDAC in the Cell

The cellular distribution of the radioactivity was examined on the cells treated with 1 $\mu\text{g/ml}$ DDAC (Fig. 7). The radioactivity was distributed at 36% in the membrane fraction and 30% in the cytoplasm fraction, and the remainder was in the cell wall and the un-lysed fraction.

Discussion

Although there have been some reports concerning the resistance mechanism of *S. aureus* cells, including MRSA, against QACs,^{11,13-15} there have been only a few papers describing the mechanism of bactericidal action of these agents including their distribution, metabolism and physiological action in the cells.^{19,20} Therefore, we sought to collect evidence on the action of a QAC by experiments using DDAC including the labeled compound.

Fig. 7. Distribution of DDAC in Cell Fractions

The cells were treated with 1 $\mu\text{g/ml}$ ^{14}C -DDAC at 37°C for 10 min, and fractionated, to determine the radioactivity, using the method described in the text.

The bactericidal activity of DDAC was dependent on the concentration applied, and the MLC was 32 $\mu\text{g/ml}$ at which concentration no viable cells were detected even after 20 s contact. We observed the following phenomena caused by treatment with DDAC: the formation of blebs on the cell surface, the leakage of intracellular substances, *i.e.* K^+ and 260 nm absorbing materials, and the release of membrane components, *i.e.* phospholipid. Similar phenomena were reported on *Escherichia coli* when treated with other QACs.^{18,20,21}

To understand the mechanism of DDAC, we have to know how it is taken up by the cell before expression of the bactericidal action. The uptake isotherm was biphasic: the first phase (see the insert in Fig. 6) ended around the MLC level, and thereafter the second phase was observed. The large uptake in the second phase could be interpreted as the adsorption to the intracellular components which were thrown out by the DDAC action at higher concentrations than MLC, meaning that this second uptake would not be involved in the bactericidal activity. Therefore, subsequent uptake and distribution experiments were carried out using 1 $\mu\text{g/ml}$ DDAC. The intracellular distribution of ^{14}C -labeled DDAC revealed that the majority of the QAC taken up was distributed in the membrane and the cytoplasm fraction. However, re-distribution

to the membrane fraction is more unlikely than that to the cytoplasmic fraction during the fractionation procedure, meaning that at least the cytoplasmic membrane must be the first uptake site of DDAC.

QACs, cationic surfactants, are believed to interact with both negatively charged groups and hydrophobic sites of cell components. Since the rapid uptake of DDAC by the cell was independent of the temperature and contact time, it is judged to have arisen by physicochemical adsorption to the cell components rather than transport through the membrane. Another QAC, TMAC, carrying no hydrophobic alkyl chains in the moiety exhibited no biocidal activity, nor was the biocidal activity and the uptake of DDAC influenced by TMAC. These indicate that DDAC is more reactive in hydrophobic interaction with the cell component.

These findings were interpreted as evidence for action on the membrane, strongly suggesting the membrane lipid as the target site. Such interaction must cause damage to the membrane permeability by increasing the fluidability, allowing the formation of blebs composed of the cytoplasmic membrane through the cell wall, and leakage of the intracellular materials from the cells. Release of K^+ was almost total, but that of phospholipid and 260 nm absorbing materials (nucleic acid and protein) was not, perhaps because holes in the damaged membrane were not large enough to permit these macromolecular compounds to pass through and they made a large complex with DDAC and precipitated in the inside of cells.

The following mechanism of action of DDAC is proposed on the basis of these results: DDAC at bactericidal levels reaches hydrophobic regions of the cell membrane and adsorbs by physicochemical binding, possibly by interaction with the membrane lipid, causing disturbance of the membrane structure and damage to membrane function including permeability. At low concentrations of DDAC

and brief contact, the structure of the membrane may be maintained by self-restoration, but exposure beyond a certain concentration of DDAC for a longer time leads to irreversible damage and cell death.

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