Action of a Quaternary Ammonium Disinfectant on Cell Membrane of *Staphylococcus aureus*

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Treatment of *Staphylococcus aureus* cells with a quaternary ammonium disinfectant, didecylmethyl ammonium chloride (DDAC), induced increase in turbidity of the cell suspension and inhibited the cell respiration. Addition of DDAC also increased turbidity of the suspension of protoplasts, the membrane and cytoplasm fraction, and even the liposome prepared from the cell phospholipid and soybean lecithin. In addition, it enhanced membrane fluidity of the liposome prepared from the cell phospholipid.

It is proposed from findings in this and previous papers that the cationic surfactant first causes damage to the structure of the cell membrane which results in irreversible inactivation of all membrane functions.

Keywords — quaternary ammonium disinfectant; bactericidal action; cell membrane; didecylmethyl ammonium chloride; *Staphylococcus aureus*; membrane fluidity

Introduction

Much attention is now being given to the control of microorganisms in the environment, for example, to the prevention of nosocomial infection by methicillin-resistant *Staphylococcus aureus* (MRSA) in hospitals. The most practical and common means of such prevention is the use of disinfectants, especially quaternary ammonium (QA) compounds. Bactericidal actions of chemical biocides including QA salts were well reviewed by Denyer and Hugo,¹⁾ and some suggested action on the cell membrane.¹⁻³⁾ However, the mechanism has not yet been identified, and we have conducted experiments using a QA disinfectant, didecylmethyl ammonium chloride (DDAC) including its radioactive compound.

We reported earlier the bactericidal action of DDAC against *S. aureus* cells,⁴⁾ and the neutralizing effect of polysorbate 80

(PS-80) on the action,⁵⁾ suggesting the cell membrane as a first site of the QA surfactant action. Further findings supporting this are presented here.

Materials and Methods

Chemicals — DDAC was donated by Kao, Ltd. PS-80, NADH, soybean lecithin and the other reagents were purchased from Wako Pure Chemical Industries, Ltd. and other suppliers. All of the reagents were of the highest grade available.

Bacterial Strain and Enumeration of Viable Cells — Cells of *S. aureus* ATCC 6538p were cultivated in nutrient broth (Difco) at 37°C for 16–24 h. The viable cell count was determined by the conventional plate count method using soybean-casein digest agar medium supplemented with lecithin and PS-80 (Nihon Seiyaku Co., Ltd.) and expressed as colony forming units (cfu).⁴⁾

DDAC Treatment and Effect of PS-80 — The cell suspension (*Ca.* 10⁹ cfu/ml) was incubated with given concentrations of DDAC in 50 mm

sodium phosphate buffer (pH 7.0) at 37°C for 10 min, as described previously.⁴⁾

The effect of PS-80 was examined by its addition to the treatment mixture at a final concentration of 0.7%.

Determination of Turbidity Increase —

The cells (*ca.* 10⁹ cfu/ml) were suspended in 50 mM sodium phosphate buffer (pH 7.0) kept at 37°C in a cuvette for a spectrophotometer. Immediately after addition of DDAC to the suspension at a given final concentration, optical density at 600 nm (OD₆₀₀) was monitored on the spectrophotometer (Shimadzu, UV-160A). In some cases, OD₆₀₀ was determined on the suspension of protoplasts (the same in number as whole cells) and cell component fractions, which were prepared as described previously.⁴⁾ The suspension of liposome vesicles, and the protein solutions (1 mg of bovine serum albumin, ovalbumin and myoglobin in 1 ml of 50 mM sodium phosphate buffer, pH 7.0) were also used.

The occurrence of aggregates was examined in the suspension of cells and liposomes after DDAC treatment by observation under a phase contrast microscope (Olympus, BX40).

Extraction of Phospholipid from Cells —

The cells from a 3 liter culture, which had been washed twice with 50 mM sodium phosphate buffer (pH 7.0) by centrifugation and lyophilized, were suspended in 22.5 ml of a chloroform/methanol (1/2) mixture in a separatory funnel and allowed to stand for 20 min at room temperature. After addition of 7.5 ml of chloroform and the same volume of 2M KCl, the suspension was shaken well and allowed to stand. The phospholipid preparation was obtained by evaporating the organic solvent from the lower liquid.

The phospholipid fractions were obtained from soybean lecithin by silica-gel column chromatography (Wako Pure Chemicals, Wako-gel C-200) using 3 different elution systems (chloroform/methanol=95/5, 4/1, 3/2). These were identified using standard phospholipids (Funakoshi) to be phosphatidyl glycerol (PG), ethanolamine (PE), and choline (PC) by thin layer chromatography (Wako Pure Chemicals, silica-

gel 60 F254 plate) with a developing solvent (chloroform/methanol/water=70/20/4).

Preparation of Liposome — Small unilamellar vesicles were prepared from phospholipid extract from the cells, soybean lecithin and purified phospholipids (PG, PE, and PC) essentially according to the following method of Sunamoto and Iwamoto.⁶⁾ Each phospholipid preparation (30 mg) was solubilized with 1 ml of chloroform, to which 0.5 mg 1,6-diphenyl-1,3,5-hexatriene (DPH; Wako Pure Chemicals) was added for measurement of the fluidity. The solution in a flask was evaporated to dryness under reduced pressure at 50°C to make a thin layer and kept in a desiccator overnight. The thin film was then peeled off into 4 ml of 50 mM sodium phosphate buffer (pH 7.0) by vibrating with 3 polyethylene beads on a Vortex mixer, and was subjected to sonication (Branson, Sonifier 250) at 60W for 20 min (1 min × 20 times) in an ice-water bath. The resultant emulsion was centrifuged at 800 × *g* for 10 min, and the supernatant was used as the liposome preparation after storage at 4°C in a sealed tube filled with nitrogen gas.

Measurement of Membrane Fluidity —

Change of the membrane fluidity was estimated from the decrease in fluorescence polarization. From immediately after the addition of DDAC at a given concentration to the liposome preparation labeled by the fluorescent probe, DPH, the fluorescence in a vertical direction (*I_v*) and a horizontal direction (*I_h*) was monitored with a fluorescence spectrometer (excitation: 360 nm, emission: 430 nm; Hitachi, F-4010). As the fluorescence was stabilized within 1 min, the *P* value was calculated from the values at 1 min by the following equation:

$$P = (I_h - I_v) / (I_h + I_v)$$

The ratio of *P* to *P*₀ is plotted in Figs. 2 and 3: *P*₀ means the *P* value without DDAC. A lowering of the ratio represents an increase in the membrane fluidity.

Determination of Oxygen Consumption by Cells — Oxygen uptake was determined by measuring dissolved oxygen concentration of the cell suspension (*Ca.* 10⁹ cfu/ml) at 37°C on a

biological oxygen monitor (Yellow Spring Instrument, Model 5300).

Measurement of NADH Dehydrogenase Activity in Membrane Fraction — Successively the membrane fraction was prepared by cell wall digestion with lysostaphin, the bursting of protoplasts by osmotic shock, differential centrifugation, and sonication.⁴⁾ The preparation was incubated in 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 mM NADH at 37°C, and after 10 min incubation the mixture was centrifuged at $10000 \times g$ for 10 min. Absorbance at 340 nm of the supernatant was measured on a spectrophotometer (Shimadzu, UV-160A). The activity of NADH dehydrogenase was calculated from the decrease in absorbance between before and after incubation.

Results

Phenomena Caused by DDAC Treatment^{4,5)}

Our previous findings on the action of DDAC on the cells of *S. aureus* are summarized in Table I. Briefly, minimum lethal concentration (MLC) of DDAC was 32, 250, and 32 $\mu\text{g/ml}$ in the presence of none, 0.7% PS-80, and 500 $\mu\text{g/ml}$ tetramethyl ammonium chloride (TMAC), respectively. None of the viable cells was detected even after 20 s treatment with DDAC at MLC, whereas survivors were detected when PS-80 was added to the treatment mixture within 60 s. The uptake isotherm was biphasic: the first ended around MLC and the second started around MLC. The DDAC treatment caused the fol-

lowing phenomena: the formation of blebs on the cell surface, and leakage of intracellular components (K^+ , 260 nm absorbing material, and phospholipid). These phenomena including the uptake of DDAC by the cell were suppressed by the addition of PS-80, but not by that of TMAC; the bactericidal activity was also suppressed.

Turbidity Increase of Cell Suspension by DDAC

As shown in Fig. 1, the treatment with DDAC induced an increase in OD_{600} of the cell suspension within 20 s. The extent was dependent on the DDAC concentration although a significant increase started from near MLC (32 $\mu\text{g/ml}$). Such induced increase was also observed when the protoplast suspension was

Fig. 1. Turbidity Increase of the Cell Suspension by DDAC Treatment

The cells were incubated with given concentrations of DDAC at 37°C, and OD_{600} was monitored.

TABLE II. Turbidity Increase of the Cell and Protoplast Suspension by DDAC Treatment with or without Polysorbate 80 (PS-80)

DDAC ($\mu\text{g/ml}$)	OD ₆₀₀ increase (%)			
	Whole cell		Protoplast	
	None	+PS-80	None	+PS-80
1	0	0	0	0
32	11	0	14	—
500	49	23	170	0

Turbidity was determined on the suspension of whole cells and protoplasts before and after incubation with DDAC at 37°C for 10 min in the presence and the absence of 0.7% PS-80, and the increased percent to the initial OD was calculated.

TABLE I. Phenomena Caused by DDAC Treatment to Cells²⁾

Minimum lethal concentration (10^9 cells/ml, 37°C, 10min)	32 $\mu\text{g/ml}$
Lethal time at MLC	20 s
Surface structure	Bleb formation
Uptake isotherm	Biphasic (below and above MLC)
Leakage from cell	Phospholipid, K^+ , and 260 nm absorbing materials

used instead of the whole cell suspension (Table II).

The turbidity increase of the whole cell and the protoplast suspension by DDAC treatment were also depressed by the presence of PS-80 (Table II), as well as the other phenomena. The increase in turbidity of the cell suspension was reduced to some extent even when PS-80 was added 5 and 10 min later where no viable cells were counted (data not shown). These results allowed us to deduce that the detectable increase occurred following death of the cells.

The addition of DDAC at 100 $\mu\text{g/ml}$ or higher also increased turbidity of the membrane fraction, the cytoplasm fraction, the liposome preparations from cell and soybean phospholipids (except PC), and even typical protein solutions (Table III).

Change in Membrane Fluidity by DDAC

Figure 2 shows the increase in membrane fluidity of the liposome prepared from the cell extract of phospholipid according to the concentration of DDAC. Similar increase was observed on the liposome prepared from PG and PE, but not on that from PC (Fig. 3).

Inhibition of Respiration by DDAC

The cells began to consume the dissolved oxygen when glucose was added to the cell

suspension, and stopped immediately after addition of DDAC over 16 $\mu\text{g/ml}$ (Fig. 4). The NADH dehydrogenase activity in the membrane fraction was also inhibited by DDAC at 64 $\mu\text{g/ml}$ and above, but the activity was retained by the presence of PS-80 (Fig. 5).

Discussion

The uptake isotherm of DDAC by the cells was biphasic. The first uptake below MLC was deduced to be physicochemical adsorption on the hydrophobic site of the cell

Fig. 2. Increase of Membrane Fluidity by DDAC: Liposome of Cell Phospholipid

The liposome labeled with DPH was incubated with DDAC and the fluorescence polarization was measured. See text for details.

Fig. 3. Increase of Membrane Fluidity by DDAC: Liposome of Phospholipid in Soybean Lecithin

See legend to Fig. 2.

TABLE III. Change in Turbidity of Cell and Other Fractions by DDAC Addition

Suspension or solution	Turbidity with DDAC
Membrane fraction	Increase
Cytoplasm fraction	Increase
Liposome	
cell phospholipid	Increase
soybean lecithin	Increase
phosphatidyl ethanolamine	Increase
phosphatidyl glycerol	Increase
phosphatidyl choline	No increase
Bovine serum albumin	Increase
Ovalbumin	Increase
Myoglobin	Increase

Turbidity was determined on the suspensions and the solution before and after incubation with 100 $\mu\text{g/ml}$ DDAC or higher at 37 $^{\circ}\text{C}$ for 10 min. See text for details.

Fig. 4. Inhibition of Oxygen Consumption by DDAC

Dissolved O₂ in the cell suspension was monitored during incubation at 37°C with glucose and DDAC. See text for details.

Fig. 5. Inhibition of NADH Dehydrogenase Activity by DDAC

The membrane fraction was incubated with DDAC at 37°C for 10 min, and the enzyme activity was determined after another 10 min incubation with NADH. See text for details.

surface, possibly the cell membrane phospholipid, from the following facts: DDAC was taken up rapidly and independently of the treatment temperature, and it was distributed mainly in the membrane and the cytoplasm fraction.⁴⁾ Further, the uptake was depressed by the addition of PS-80, the non-ionic surfactant, but not by TMAC, the QC compound without hydrophobic alkyl chains.^{4,5)} Our interpretation of these findings is that DDAC taken up at the first phase is more important for the bactericidal action and that the first target must be phospholipid in the cell membrane.

A second large uptake was observed at

higher concentrations of MLC, where no viable cells were recovered. This uptake is interpreted as uptake by killed cells, for example, the complex formation with intracellular macromolecules.

A significant increase of turbidity was also induced above MLC. Such turbidity increases by other disinfectants have been reported by many researchers: *Escherichia coli* with phenols by Beckett *et al.*,⁷⁾ *E. coli* and *S. aureus* with chlorhexidine by Hugo and Longworth,⁸⁾ *E. coli* with cetyltrimethyl ammonium salts by Salt's group,^{9,10)} *S. aureus* with Triton X45 and X100 by Lamikanra and Allwood,¹¹⁾ *Klebsiella pneumoniae* and *E. coli* by Salt and Wiseman,¹²⁾ and *E. coli* with alkyl pyridinium salts by Kourai *et al.*¹²⁾

The increase of turbidity is assumed to be due to (1) increase of the cell size, (2) insolubilization of released cellular materials in the medium, (3) precipitation of cellular macromolecules inside the cells, and (4) change in refractive properties of cell surface.

Cause (1) was suggested in *K. pneumoniae* and *E. coli* cells by Salt and Wiseman,¹²⁾ but it is unlikely in *S. aureus* because the cell size distribution analysis using a laser particle analyzer (Horiba; LA-700) on another *S. aureus* strain showed that the cell, in fact, shrank from 0.7–0.8 µm to 0.6–0.7 µm in diameter rather than being enlarged by the DDAC treatment around MLC level; the size did, however, return to an intact level by treatment at 125 µg/ml and above (data not shown).

Cause (2) is supported by the following facts. Intracellular macromolecules such as phospholipid, protein and nucleic acid were leaked out from the cells by the treatment with disinfectants,^{1,4,7,13,14)} and they could be precipitated with cationic surfactants (this study and refs.1, 14, 15). Kourai *et al.*¹²⁾ recently, succeeded in isolating from the supernatant of the treatment mixture small vesicles, possibly liberated blebs, which could exhibit a higher density. We occasionally

observed aggregates in the DDAC-treated samples, at much higher concentration of MLC, of cells and liposomes prepared from soybean phospholipid under a phase-contrast microscope (data not shown), which suggested a change of surface charge and/or hydrophobicity. We also sometimes found that OD_{600} went down after the increase when the cells were incubated with higher concentrations of DDAC for a longer time (data not shown), probably due to the precipitation and/or resolubilization of the complex of cellular components, as well as sedimentation of the cell aggregates.

Cause (3) was supported by the insolubilization of intracellular macromolecules, as described above, after penetration of DDAC into the cytoplasm; that is, DDAC taken up at the second phase could insolubilize the components and the precipitation could raise the density of the cell body. This cause would mean postmortem changes in the cells following permeability damage in the cell membrane. This interpretation was also suggested in the case of the other QA salts,^{1,6,9,10,14} and of chlorhexidine.⁸⁾

Lamikanra and Allwood¹¹⁾ suggested the last cause in terms of alterations to the re-

fractive properties of cell walls based on the interaction of alkyl-phenols with isolated cell walls of *S. aureus* with a large OD increase and with spheroplasts with a small increase. DDAC, however, induced significant increase of the protoplast suspension, the membrane fraction and also the liposome suspension of phospholipids (except PC) although we did not examine the cell wall suspension. Accordingly, these suggest alterations in the refractive properties of the cell wall and the membrane, resulting in turbidity increase of the cell suspension.

Whatever the cause is for the increase in turbidity, and even though it happens after the death of the cell, the measurement of turbidity of the cell suspensions is a useful tool for screening biocidal compounds which can interact with the cell components.

Matsumura *et al.*¹⁶⁾ reported that alkyltrimethyl ammonium salts were adsorbed largely by hydrophobic interaction and secondarily by electrostatic interaction onto the phospholipid monolayer and vesicles, encouraging us to examine the fluidity of liposome of the cell phospholipids, PE, PG, and PC. DDAC induced increase in the membrane fluidity of all liposomes except PC. Turbidity

Fig. 6. Proposed Mode of Action of DDAC on *S. aureus* Cells

increase was also observed on the liposome suspensions except PC. In contrast, bacterial cells contained PE and PG, but not PC.¹⁷⁾ Thus, DDAC can increase the membrane fluidity of bacterial cells. Disorganization of the membrane structure causes damage to all functions of the membrane including transport and respiration, as shown here. Mlynarcik *et al.*¹⁸⁾ also reported on the modification of respiration by bisquaternary ammonium salts on *S. aureus* cells, as well as the proton uptake increase, K⁺ leakage and inhibition of ATP synthesis.

The proposed mode of action of DDAC is illustrated in Fig.6 on the basis of these

results. Namely, DDAC adsorbs initially to the hydrophobic region of the cell membrane mainly by hydrophobic interaction. It is a reversible interaction at a lower concentration of DDAC, but the QA salt at a higher concentration induces greater increase in membrane fluidity and causes irreversible disorganization to the membrane structure. This results in damage to the membrane functions such as loss of semi-permeability and inhibition of respiration. This finally leads to cell death as well as to penetration of the disinfectant into the cytoplasm to denature and inactivate intracellular macromolecules accompanied by the increase in turbidity.

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