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Bacterioclastic Action of a Bis-Quaternary Ammonium Compound against *Escherichia coli*

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It has been reported that the bactericidal action of bis-quaternary ammonium compounds (bis-QACs) is little influenced by the molecular hydrophobicity of the drug or environmental conditions such as temperature or pH. In order to clarify the mode of bactericidal action of bis-QACs against *Escherichia coli*, the bacterioclastic action of 4,4'-(1,6-hexamethylenedithio)bis(1-octylpyridinium bromide) (4DTBP-6,8) was investigated. It was suggested that 4DTBP-6,8, which invaded the bacterial cell, induced the leakage of ATP and the inhibition of the respiratory enzymes, and resulted in cell death. Subsequently, over 2 μ g/ml of 4DTBP-6,8 caused an increase in the turbidity of the cell suspension, and the bactericidal activity of 4DTBP-6,8 was extremely increased with the increase of its concentration (over 2 μ g/ml). It indicated that 4DTBP-6,8 has an ability to induce a rapid and abundant secretion of the turbid materials from the cells and that such bacterioclastic ability is connected to its potent bactericidal activity. In addition, it was suggested that the first stage of bacterioclastic action of 4DTBP-6,8 was the leakage of magnesium ion (Mg^{2+}), and the leakage of the outer membrane pore protein E and lipopolysaccharides followed it. The formation of blebs and holes on the bacterial cell surface was revealed by scanning electron microscopic investigation. Transmission electron micrographs of the cells treated with 4DTBP-6,8 showed the destruction of peptidoglycan and large missing portions of intercellular materials. Judging from these results, as the mechanism of the bacterioclastic action of bis-QACs, it is suggested that the cationic parts of bis-QACs electrically interact with the cationic parts of Mg^{2+} on the bacterial surface, then bis-QACs invade the cell membrane by displacement reaction with Mg^{2+} , and rapidly destroy the bacterial cell surface structure.

Key words : Quaternary ammonium compound/Bacterioclastic action/Critical vesiculation concentration.

INTRODUCTION

Quaternary ammonium compounds (QACs), a variety of cationic surface-active agents, have relatively low toxicity and show bactericidal effects against a wide range of bacteria. The QACs can be classified by the number of quaternary nitrogen atoms. Of the various series of mono-QACs possessing one ammo-

nium cation in the molecule, mainly benzalkonium chloride, benzethonium chloride or cetylpyridinium chloride have been widely used as disinfectants. It has been shown that these disinfectants adsorb to the bacterial cell surface, invade the cell membrane and denature the proteins of the cell membrane. It has also been revealed that the bactericidal activity depends on the adsorbability of QACs to the bacterial cell surface (Baley et al., 1977; Devinsky et al., 1991; Kourai et al., 1980, 1994a; Okazaki et al., 1997a, 1997b). However, those studies on QACs have been performed exclusively with mono-QACs.

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Recently, it was found that bis-QACs, possessing two quaternary ammonium atoms in the molecule show a high bactericidal activity (Maeda et al., 1998, 1999; Okazaki et al., 1997c). The bactericidal activity of bis-QACs is not affected by the length of the hydrophobic alkyl chains, or the length of the methylene chain which functions as a spacer between two identical active moieties. The activity of bis-QACs is comparatively not affected by pH and temperature, unlike that of mono-QACs. In our previous report, one of the potent bactericidal actions of bis-QACs proved to be due to the quickness of their bactericidal action (Sumitomo et al., 2003). This previous report also suggested that the rate-limiting step in the bactericidal action of bis-QACs is the electrostatic adsorption on the bacterial surface. However, little is known about the mode of bactericidal action of bis-QACs.

It has been demonstrated that mono-QACs cause the formation of blebs on the cell surface, the liberation of vesicles from the cell surface and the large breaches of intracellular materials (Kourai et al., 1994a, 1994b). Recently, we found that bis-QACs at its minimum bactericidal concentration (MBC) caused a dramatic increase of turbidity in the cell suspension of *Escherichia coli*, but the bis-QACs at concentrations below MBC did not, like that of mono-QACs. It is suggested that the increase in turbidity is closely linked to the mechanisms of bactericidal action of bis-QACs.

It has been shown that QACs have strong bacteriolytic activity against the exponential phase cells of *E. coli* (Kourai et al., 1994b). Bis-QACs at low concentrations also have rapidly lysed the exponentially growing cells (unpublished data). In order to clarify the bactericidal mechanism of bis-QACs, it is important to examine the initial stage of the bacterioclastic action of bis-QACs. In the present study, as the indices which indicate the mode of the bacterioclastic action of bis-QACs, the minimum concentration to inhibit bacterial respiratory function and the minimum concentration to induce leakage of intracellular materials (ATP, LPS, Mg^{2+} and protein) by treatment with bis-QAC, 4,4'-(1,6-Hexamethylenedithio)bis(1-octylpyridinium bromide) (4DTBP-6,8) were examined. In addition, cells injured by treatment with 4DTBP-6,8 were observed by using scanning and transmission electron microscopes.

MATERIALS AND METHODS

Chemicals

4,4'-(1, 6-Hexamethylenedithio) bis (1-octylpyridinium bromide) was synthesized in our laboratory and is abbreviated as 4DTBP-6,8 (Fig.1).

FIG. 1. Chemical structure of 4,4'-(1,6-hexamethylenedithio)bis(1-octylpyridinium bromide) (4DTBP-6,8).

The procedures for its synthesis and its chemical properties and purity were previously described (Okazaki et al., 1997c). Other chemicals for this study were of commercially available reagent grade and used without further purification.

Bacterial strain and preparation of cell suspension

Escherichia coli IFO 12713 strain was used through all experiments. The cell suspensions were prepared according to a previous report (Sumitomo et al., 2003). *E. coli* was incubated at 37°C in nutrient broth (NB, BECTON DICKINSON, Sparks, MD, USA) until the middle exponential phase was reached. The grown cells were harvested by centrifugation and washed with sterile ice-cooled distilled water, and the cells were suspended and adjusted to a concentration of 2×10^7 cells/ml with sterile ice-cooled distilled water.

Measurement of inhibition of respiration

Respiration inhibitory activity of QACs was determined from the reduction in the amount of dissolved oxygen consumed as monitored by a Galvani-type oxygen electrode (DO8F, Horiba). A cell suspension (10^7 cells/ml) containing 0.2% (w/v) glucose as a substrate was poured into 100ml branching flask. The sample flask equipped with a dissolved oxygen electrode was incubated while being agitated in a water bath at 25°C, and dissolved oxygen levels were monitored to estimate the basal metabolism as a control. The sample was prepared once again as described above, and the 4DTBP-6,8 was added when the decreasing rate of dissolved oxygen became steady. After 0, 5, 10, 15, 20, 25, 30 and 35min, the remaining dissolved oxygen was measured.

Analysis of materials leaked from cells

The bacterioclastic activity was measured in terms of the increase of turbidity of the cell suspensions according to the method described in the previous report (Kourai et al., 1994a, 1994b).

4DTBP-6,8 was dissolved in sterile distilled water. The cell suspension (10^7 cells / ml) and the antimicrobial solutions ($0.01\text{--}5 \mu\text{g / ml}$) were

respectively incubated in a water bath shaker at 25 °C for 5min. After the cell suspension was treated with the antimicrobial solution at 25°C for 2min, the treated cells were rapidly filtered through a membrane filter (ADVANTEC, Toyo Roshi Kaisha, Japan, pore size: 0.2 μ m). The filtrate was used for the analysis of materials leaked from cells.

The amounts of lipopolysaccharides (LPS) in the filtrate were measured as endotoxins by using a kit, the Toxinometer (Wako Pure Chemicals, Osaka), according to the manufacturer's manual. The amounts of magnesium ion (Mg^{2+}) in the filtrate were measured by enzyme assay with a magnesium assay kit (DENKA SEIKEN Co., Ltd., Tokyo) using magnesium chloride hexahydrate (KANTO CHEMICAL Co., Inc., Tokyo) as a standard. The amount of ATP in the filtrate was determined by using the ATP assay reagent (Lucifeal HS set, KIKKOMAN, Tokyo) in a luminescence reader (BLR-201, Aloka, Tokyo) according to the manufacturer's methods. The bacterial subcellular fractions were prepared according to the methods of Heppel (Anraku et al., 1967; Nossal et al., 1966) and Nakajima et al. (Nakajima et al., 1998) with a slight modification. The leaked proteins in the filtrate were dissolved in the Laemmli's sample buffer (20%, w/v, glycerol (Wako Pure Chemicals, Osaka), 4.6%, w/v, sodium dodecyl sulfate (Wako Pure Chemicals, Osaka), 1.6%, w/v, 2-amino-2-hydroxymethyl-1,3-propanediol (Wako Pure Chemicals, Osaka), 0.01%, w/v, bromophenol blue (Sigma-Aldrich Co., U.S.A.), and 10%, v/v, β -mercaptoethanol (Wako Pure Chemicals, Osaka), pH 6.8) and separated by so-

dium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% (w/v) acrylamide separation gels according to the method described by Laemmli (Laemmli, 1970). The electrophoresed gel was stained with Coomassie brilliant blue R250. Furthermore, the electrophoresed gel was also used to prepare samples for amino acid sequencing of the N-termini of the proteins. Proteins were transferred to a polyvinylidene difluoride membrane (PVDF membrane, Nihon Millipore Ltd., Japan, pore size: 0.45 μ m) for 1h at 100mA. The proteins were visualized by staining with Amido Black [0.3% (v/v) amido black 10B (Wako Pure Chemicals, Osaka) in 40% (v/v) methanol, 10% (v/v) acetic acid] for 10min, and the bands of interest were excised with a scalpel and were air-dried. The sample was subjected to automated Edman degradation using a PPSQ-10 protein sequencer (Shimadzu Co., Kyoto).

Electron microscopy

The middle exponential phase cell suspension of *E. coli* was treated with 4DTBP-6,8 (0.05, 0.5 and 2 μ g/ml) for 2min at 25°C. The treated cells were observed with a HITACHI S-4700 scanning electron microscope (SEM) and a JEM-1010 transmission electron microscope (TEM) (JEOL) according to a method previously reported (Kourai et al., 1994a, 1994b).

RESULTS

Inhibition of respiration

FIG. 2. (A) Respiratory inhibition by 4DTBP-6,8 against *E. coli*. Symbols: ●, 0 μ g/ml (control); ○, 0.1 μ g/ml; ■, 0.2 μ g/ml; □, 0.5 μ g/ml; ◆, 1 μ g/ml; ◇, 1.5 μ g/ml. The arrow indicates the addition of 4DTBP-6,8. (B) The oxygen consumption of *E. coli* treated with 4DTBP-6,8 for 2min. Symbols: ●, oxygen consumption. The arrow indicates the critical concentration to inhibit respiratory function (0.1 μ g/ml).

FIG. 3. (A) Effect of 4DTBP-6,8 on the turbidity increments (ΔOD at 660nm for 2min at 25°C) of the cell suspension of *E. coli* and the viable cell number. Symbols: ●, the turbidity increments; ○, viable cell number. The arrow indicates the critical vesiculation-causing concentration (CVC= $2\mu\text{g/ml}$). (B) Effect of 4DTBP-6,8 on the leakage of ATP from *E. coli* cells. *E. coli* cells were treated with 4DTBP-6,8 for 2min at 25°C. The arrow indicates the critical concentration to cause ATP-leakage ($0.2\mu\text{g/ml}$). (C) Effect of 4DTBP-6,8 on the leakage of LPS from *E. coli* cells. *E. coli* cells were treated with 4DTBP-6,8 for 2min at 25°C. The arrow indicates the critical concentration to cause LPS leakage ($0.6\mu\text{g/ml}$). (D) Effect of 4DTBP-6,8 on the leakage of Mg^{2+} from *E. coli* cells. *E. coli* cells were treated with 4DTBP-6,8 for 2min at 25°C. The arrow indicates the critical concentration to cause Mg^{2+} leakage ($0.04\mu\text{g/ml}$).

Figure 2A shows that how 4DTBP-6,8 affected the respiratory function of *E. coli* IFO 12713 when the QAC was added to the cell suspension containing 0.2% glucose as a respiratory substrate. In the presence of $0.1\mu\text{g/ml}$ 4DTBP-6,8, the dissolved oxygen was decreased as well as in the control sample with the progress in the time. However, the decreasing rate of dissolved oxygen concentration became less severe with an increase in the drug concentration ($0.2\mu\text{g/ml}$). In addition, the decrease of dissolved oxygen was completely stopped at the concentrations above $0.5\mu\text{g/ml}$. On the basis of this results, the oxygen consumptions of *E. coli* treated with 4DTBP-6,8 (0.1 – $1.5\mu\text{g/ml}$) for 2 min were calculated, and plotted against the concentrations of 4DTBP-6,8 (Fig.2B). It was confirmed that the respiratory function of *E. coli* was inhibited by over $0.1\mu\text{g/ml}$ 4DTBP-6,8, and completely stopped by $0.5\mu\text{g/ml}$.

Bacterioclastic action

Turbidity of the cell suspension of *E. coli* was continuously measured after the addition of 0 – $10\mu\text{g/ml}$ of 4DTBP-6,8 for 2min at 25°C in a temperature-controlled cell holder. 4DTBP-6,8 at concentrations greater than $2\mu\text{g/ml}$ also caused an increase in the turbidity of the cell suspension, but not at lower concentrations. Turbidity reached rapidly the maximum value within the first 10–30s, and the height of each curve seem to be proportional to the concentrations of added 4DTBP-6,8 from 2 to $10\mu\text{g/ml}$ (data not shown). A plot of the net increase (ΔOD_{660} for 2min) of the turbidity against the concentration of 4DTBP-6,8 gave two straight lines (Fig.3A). The critical vesiculation concentration (CVC) determined from the point of intersection of the two straight lines was $2\mu\text{g/ml}$. The relation between the viable cell number with treatment of 4DTBP-6,8 for 2min and

concentration of 4DTBP-6,8 is also shown in Fig.3A. The bactericidal activity of 4DTBP-6,8 increased greatly with the increase in the concentration of 4DTBP-6,8 (over $2 \mu\text{g/ml}$).

Leakage of ATP, LPS and magnesium ion

We examined the leakage of ATP from cells treated with 4DTBP-6,8 at concentrations of 0.05 – $10 \mu\text{g/ml}$ (Fig.3B). The basal ATP content per cell was 1.1×10^{-19} mol (data not shown). Slight leakage of ATP from the cells could be seen with low concentrations of 4DTBP-6,8 (0.05 – $0.2 \mu\text{g/ml}$) as well as in the control sample without adding the drug solution. However, at concentrations over $0.2 \mu\text{g/ml}$, 4DTBP-6,8 clearly induced the leakage of ATP, and the amount of the leakage seems to be proportional to the concentration of 4DTBP-6,8. When the amount of cell-leaked ATP was plotted against the concentration of 4DTBP-6,8, the plots gave two straight lines. The minimum concentration of 4DTBP-6,8 for ATP leakage was $0.2 \mu\text{g/ml}$.

The amounts of endotoxins (LPS) leaked from bacterial cells treated with 4DTBP-6,8 were measured using the limulus amoebocyte lysate (LAL) assay (Fig.3C). Slight leakage of endotoxins from cells could be seen with low concentrations of 4DTBP-6,8 (0.1 – $0.7 \mu\text{g/ml}$) as well as in the control sample without adding 4DTBP-6,8. However, at concentrations over $0.7 \mu\text{g/ml}$, 4DTBP-6,8 clearly induced the leakage of endotoxins. A plot of the amount of endotoxins leaked against the concentration of 4DTBP-6,8 gave two straight lines. The point of intersection of the two straight lines was $0.6 \mu\text{g/ml}$, which was defined as the minimum concentration to cause LPS leakage by the bactericidal action of 4DTBP-6,8.

We measured the amounts of Mg^{2+} leaked from bacterial cells treated with 4DTBP-6,8 (Fig.3D). At concentrations over $0.06 \mu\text{g/ml}$, 4DTBP-6,8 caused an increase in the leakage of Mg^{2+} from cells, but low concentrations (0 – $0.06 \mu\text{g/ml}$) did not. A plot of the amount of Mg^{2+} leaked against the concentration of 4DTBP-6,8 gave two straight lines. The point of intersection of the two straight lines was $0.04 \mu\text{g/ml}$, which defined the critical concentration of 4DTBP-6,8 to cause Mg^{2+} leakage.

Analysis of the leaked proteins

SDS-PAGE patterns of proteins in the filtrates from *E. coli* cells treated with 4DTBP-6,8 (0.25 , 0.5 and $2 \mu\text{g/ml}$) are shown together with those of the outer membrane, periplasm, cytoplasmic membrane and cytoplasm (Fig.4). The amount of the proteins leaked from cells increased with increasing concentrations of 4DTBP-6,8. In particular, a band corresponding to

one of the major outer membrane proteins, having an apparent molecular weight of 39kDa , was found among the leaked proteins. Then, the N-terminal amino acid sequencing of the 39kDa protein leaked from cells was carried out and the 9 residues (AEIYNKDG N) were determined. This sequence was identical to the N-terminal sequence of an outer membrane pore protein E (OmpE) of *E. coli*.

In addition, we further examined the relationship between the drug concentration (0 – $0.5 \mu\text{g/ml}$) and

FIG. 4. SDS-PAGE patterns of proteins in the filtrate from *E. coli* cells treated with 4DTBP-6,8 (0.25 , 0.5 and $2 \mu\text{g/ml}$) for 2min. Lanes: 1, molecular weight marker proteins; 2, outer membrane; 3, periplasmic proteins; 4, cytoplasmic membrane; 5, cytoplasm; 6, filtrate from *E. coli* cells treated with $0.25 \mu\text{g/ml}$ of 4DTBP-6,8; 7, filtrate from *E. coli* cells treated with $0.5 \mu\text{g/ml}$ of 4DTBP-6,8; 8, filtrate from *E. coli* cells treated with $2 \mu\text{g/ml}$ of 4DTBP-6,8.

FIG. 5. Effect of 4DTBP-6,8 on the leakage of OmpE from *E. coli* cells. *E. coli* cells were treated with 4DTBP-6,8 for 2min at 25°C . The arrow indicates the critical concentration to cause OmpE leakage ($0.1 \mu\text{g/ml}$).

FIG. 6. Scanning electron micrograph of the 4DTBP-6,8-treated cell surface of *E. coli* at (A) 0 $\mu\text{g/ml}$, (B) 0.05 $\mu\text{g/ml}$, (C) 0.5 $\mu\text{g/ml}$ or (D) 2 $\mu\text{g/ml}$ for 2min at 25°C. Arrow (a) indicates the formation of a bleb, arrow (b) a vesicle, arrow (c) the formation of a hole and arrow (d) the destruction of cell surface structure.

FIG. 7. Transmission electron micrograph of the 4DTBP-6,8-treated cell of *E. coli* at (A) 0 $\mu\text{g/ml}$, (B) 0.05 $\mu\text{g/ml}$, (C) 0.5 $\mu\text{g/ml}$ or (D) 2 $\mu\text{g/ml}$ for 2min at 25°C. Arrow (a) indicates the formation of a bleb, arrow (b) the destruction of peptidoglycans and the cytoplasmic membrane and arrow (c) a missing portion of intracellular materials.

the amount of OmpE leaked from cells (data not shown). The amount of the leaked OmpE increased with increasing concentrations of 4DTBP-6,8. We also analyzed the digital image of each band of the leaked OmpE by the NIH Image program (Section), and plotted the leaked OmpE against the concentration of 4DTBP-6,8 (Fig.5). As shown in Fig.5, there was a sharp increase in the amount of the leaked OmpE with concentrations above $0.1 \mu\text{g/ml}$ of 4DTBP-6,8, and the plots gave two straight lines.

Electron microscopy

The bacterial cell surface and intracellular changes that occur during the treatment with 4DTBP-6,8 at concentrations of 0 (A), 0.05 (B), 0.5 (C) or $2 \mu\text{g/ml}$ (D) were investigated by scanning electron microscopy (Fig.6) and transmission electron microscopy (Fig.7), respectively. Fig.6 shows the changes of the bacterial cell surface from smooth to rough, the formation of blebs (arrow a), vesicles (arrow b), hole (arrow c) and the destruction (arrow d) by treatment with $0.05 \mu\text{g/ml}$ of 4DTBP-6,8. The blebs are very small (*ca.* $0.1\text{--}0.2 \mu\text{m}$ in diameter). In addition, as the transmission electron micrograph (Fig.7B and C) showed expansion of the periplasmic space of the cells damaged by 4DTBP-6,8, it seems that the blebs (arrow a) and vesicles contained periplasmic materials. The micrograph of cells treated with $2 \mu\text{g/ml}$ of 4DTBP-6,8 shows the destruction of peptidoglycan and cytoplasmic membrane (arrow b), and a large missing portion (arrow c) of intracellular materials (Fig.7D).

DISCUSSION

Quaternary ammonium compounds (QACs), which have an ammonium or pyridinium cation moiety in their molecules, have been widely used as general disinfectants. It has been proposed that the QACs rapidly destroy the bacterial cell structure and that turbid materials are formed from outer membrane and/or intracellular materials (Kourai et al., 1994a, 1994b). The above critical concentration of 4DTBP-6,8 causing vesiculation also caused an increase in the turbidity of the cell suspension, and the drug-susceptibility of *E. coli* against 4DTBP-6,8 was dependent on the amount of the turbid materials. The mirror image relationship shown in Fig.3A indicated that 4DTBP-6,8 has the ability to cause a rapid and abundant leakage of the turbid materials from cells, and such a bacterioclastic ability leads to a potent bactericidal activity. The change in the outer membrane has already been suggested from our previous results indicating that the *E. coli* treated with 4DTBP-

6,8 induces blebbing and vesiculation of their surfaces (Maeda et al., 1998; Okazaki et al., 1997). Morphological change of the *E. coli* cells was observed by a SEM (Fig.6) and a TEM (Fig.7). Blebbing and vesiculation on the bacterial cell surface was observed in a study on heat-treated cells of *E. coli* (Katsui et al., 1982). However, the size of blebs induced by heat treatment was very large (*ca.* $0.2\text{--}0.3 \mu\text{m}$). Consequently, the size of the blebs formed by 4DTBP-6,8 apparently differs from those caused by heat treatment. The blebbing caused by 4DTBP-6,8 on the fission site of the bacterial cells surface (Fig.6) suggested that the 4DTBP-6,8 preferentially attacks the fission site of the cells, which is much more hydrophobic, and that the electro-negative charge is probably localized within the cell surface. The transmission electron micrographs of the *E. coli* treated with 4DTBP-6,8 show the destruction of peptidoglycans and cytoplasmic membrane, and a large missing portion of intracellular materials (Fig.7). It has been reported that cell division in most bacteria calls for the simultaneous constriction of both the cytoplasmic membrane and the peptidoglycans. In gram-negative bacteria, the outer membrane is also invaginated concomitantly (Nanninga, 1998). In cell separation, lytic transglycosylases, endopeptidases and amidases can act as powerful autolysins in the presence of peptidoglycan synthesis inhibitors (Kitano et al., 1986; Kohlrausch et al., 1991). However, the destruction of peptidoglycans and cytoplasmic membrane by 4DTBP-6,8 would not depend on the action of these autolysins, because this destruction by 4DTBP-6,8 is very rapid. It is reasonable to suppose that the blebs on the bacterial surface might reflect the weakening of associations between the outer membrane and peptidoglycans, and represent envelope sites vulnerable to the progress of injury from the outer to the cytoplasmic membrane.

The relationship between the concentration of 4DTBP-6,8 and the destruction of cell structure treated with 4DTBP-6,8 is shown in Fig.8. 4DTBP-6,8

FIG. 8. Schematic representation of the stepwise destruction of cell structure by the bacterioclastic action of 4DTBP-6,8.

could cause the leakage of Mg^{2+} , protein and LPS from cells below CVC. LPS are located in the external leaflet of the outer membrane of gram-negative bacteria, and their interactions with cations are important for the barrier function of the membrane (Leive, 1974; Sanderson et al., 1974). LPS are constructed of a variety of ionic groups with acidic phosphates and carboxyl moieties concentrated within the core and lipid A regions (Nikaido, 1985). The LPS molecules are anionic and are stabilized within the outer membrane by associated cations, primarily the magnesium ion (Mg^{2+}) and calcium ion (Ca^{2+}) (Coughlin et al., 1983). Therefore, a strong assumption can be proposed that the ammonium salts react with anionic site of LPS and destabilize or destroy the outer membrane structure. Leive found that such chelating agents EDTA-treated cells became hypersensitive to a number of other hydrophobic agents, including dyes and detergents (Leive, 1965, 1974). Removal of divalent cations that neutralized the electrostatic repulsion between LPS molecules resulted in the destabilization of the LPS monolayer of the outer membrane. Several polycationic compounds such as polymyxin B are also known to permeate the outer membrane using pathways other than those of porins. Although the detailed mechanism of the outer membrane permeability barrier disruption remains undetermined, complex formation by polycationic compounds with LPS is expected to be the first stage (Katsu et al., 1984; Vaara, 1992; Srima et al., 1996; Thomas et al., 1998). Being bulkier than the inorganic divalent cations that it displaces, polycationic compounds change the packing order of LPS and increases the permeability of the outer membrane. 4DTBP-6,8 might also access their targets by this pathway.

Judging from all results obtained, it seems that the mechanism of destruction of the membrane structure by 4DTBP-6,8 can be summarized as follows: the first step of the bactericidal action is the approach of 4DTBP-6,8 to the hydrophobic and electronegative sites of the bacterial cell surface and the invasion of 4DTBP-6,8 into the cell membrane by a displacement reaction with the boundary Mg^{2+} on the bacterial surface which causes the Mg^{2+} to leak from the outer membrane. The 4DTBP-6,8 replacing the Mg^{2+} makes the cell membrane bulge since the size of 4DTBP-6,8 is much larger than that of Mg^{2+} , and blebs are formed on the cell surface. The second step is the inhibition of respiratory enzymes and the leakage of cell membrane components such as LPS and OmpE. The third step causing the fatal deathblow is the leakage of intracellular materials through the formation of holes caused by damage to the cell surface due to

the vesiculation and destruction of the peptidoglycans. It is likely that this hypothesis may be applied to other bis-QACs. To clarify the detailed mechanism of bleb and vesicle formation due to the bactericidal action of bis-QACs, further investigation is now in progress.

REFERENCES

- Anraku, Y., and Leon, A. Heppel. (1967) On the nature of the changes induced in *Escherichia coli* by osmotic shock. *J. Biol. Chem.*, **242**, 2561-2569.
- Baley, G. J., Peck, G. E., and Banker, G. S. (1977) Bactericidal properties of quaternary ammonium compounds in dispersed systems. *J. Pharm. Sci.*, **66**, 696-699.
- Coughlin, R. T., Tonsager, S., and McGroarty, E. J. (1983) Quantitation of metal cations bound to membrane and extracted lipopolysaccharide of *Escherichia coli*. *Biochemistry*, **22**, 2002-2007.
- Devinsky, F., Masarova, L., Lacko, I., and Mlynarcik, D. (1991) Structure-activity relationships of "soft" quaternary ammonium amphiphiles. *J. Biopharm. Sci.*, **2**, 1-10.
- Katsu, T., Yoshimura, A., Tsuchiya, T., and Fujita, Y. (1984) Temperature dependence of action of Polymyxin B on *Escherichia coli*. *J. Biochem.*, **95**, 1645-1653.
- Katsui, N., Tsuchido, T., Hiramatsu, R., Fujikawa, S., Takano, M., and Shibasaki, I. (1982) Heat-induced blebbing and vesiculation of the outer membrane of *Escherichia coli*. *J. Bacteriol.*, **151**, 1523-1531.
- Kitano, K., Tuononen, E., and Tomasz, A. (1986) Transglycosylase and endopeptidase participate in the degradation of murein during autolysis of *Escherichia coli*. *J. Bacteriol.*, **167**, 759-765.
- Kohlrausch, U., and Holtje, J. -V. (1991) Analysis of murein and murein precursors during antibiotic-induced lysis of *Escherichia coli*. *J. Bacteriol.*, **173**, 3425-3431.
- Kourai, H., Horie, T., Takechi, K., and Shibasaki, I. (1980) The antimicrobial characteristics of quaternary ammonium salts and their alkyl chain length (in Japanese). *Bokin Bobai*, **8**, 191-199.
- Kourai, H., Hasegawa, Y., Goto, S., and Nakagawa, K. (1994a) Bacterioclastic action of dodecylpyridinium iodide against *Escherichia coli* K12 W3110. *J. Antibact. Antifung. Agents*, **22**, 461-468.
- Kourai, H., Oda, K., Takechi, H., and Nakagawa, K. (1994b) The antimicrobial characteristics of poly [dimethyliminio (polymethylene) chloride] s. *J. Antibact. Antifung. Agents*, **22**, 519-530.
- Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680-685.
- Leive, L. (1965) Release of lipopolysaccharide by EDTA treatment of *E. coli*. *Biochem. Biophys. Res. Commun.*, **21**, 290-296.
- Leive, L. (1974) The barrier function of the gram-negative envelope. *Ann. N.Y. Acad. Sci.*, **235**, 109-129.
- Maeda, T., Okazaki, K., Nagamune, H., Manabe, Y., and Kourai, H. (1998) Bactericidal action of 4,4'-(α, ω -polymethylenedithio)bis(1-alkylpyridinium iodide)s. *Biol. Pharm. Bull.*, **21**, 1057-1061.
- Maeda, T., Manabe, Y., Yamamoto, M., Yoshida, M., Okazaki, K., Nagamune, H., and Kourai, H. (1999) Synthesis and antibacterial characteristics of novel biocides, 4,4'-(1,6-hexamethylenedioxydicarbonyl)bis(1-

- alkylpyridinium iodide)s. *Chem. Pharm. Bull.*, **47**, 1020-1023.
- Nakajima, A., Hoshikawa, M., and Nakae, T. (1998) Antibiotic stress induces a large amount of outer membrane protein in *Pseudomonas aeruginosa*. *FEMS Microbiol. Lett.*, **165**, 261-265.
- Nossal, N. G., and Heppel, L. A. (1966) The release of enzymes by osmotic shock from *Escherichia coli* in exponential phase. *J. Biol. Chem.*, **241**, 3055-3062.
- Nanninga, N. (1998) Morphogenesis of *Escherichia coli*. *Microbiol. Mol. Biol. Rev.*, **62**, 110-129.
- Nikaido, H., and Varti, M. (1985) Molecular basis of bacterial outer membrane permeability. *Microbiol. Rev.*, **49**, 1-32.
- Okazaki, K., Maeda, T., Nagamune, H., and Kourai, H. (1997a) Synthesis and antibacterial characteristics of *N*-alkyl-4-alkylthiopyridinium bromides. *Biocontrol Sci.*, **2**, 39-42.
- Okazaki, K., Maeda, T., Nagamune, H., and Kourai, H. (1997b) Antibacterial characteristics of *N*-alkyl-2-alkylthiopyridinium and *N*-alkyl-4-alkylthiopyridinium salts. *Jpn. J. Toxicol. Environ. Health.*, **43**, 251-260.
- Okazaki, K., Maeda, T., Nagamune, H., Manabe, Y., and Kourai, H. (1997c) Synthesis and antibacterial characteristics of 4,4'-(α, ω -polymethylenedithio)bis(1-alkylpyridinium iodide)s. *Chem. Pharm. Bull.*, **45**, 1970-1974.
- Sanderson, K. E., MacAlister, T. J., and Costerton, J. W. (1974) Permeability of lipopolysaccharide-deficient (rough) mutants of *Salmonella typhimurium* to antibiotics, lysozyme, and other agents. *Can. J. Microbiol.*, **20**, 1135-1145.
- Srimal, S., Surolia, N., Balasubramanian, S., and Surolia, A. (1996) Titration calorimetric studies to elucidate the specificity of the interactions of polymyxin B with lipopolysaccharide and lipid A. *Biochem. J.*, **315**, 679-686.
- Sumitomo, T., Maeda, T., Nagamune, H., and Kourai, H. (2003) Kinetic analysis of the bactericidal action of a bis-quaternary ammonium compound against *Escherichia coli*. *Biocontrol Sci.*, **8**, 145-149.
- Thomas, C. J., Gangadhar, B. P., Surolia, N., and Surolia, A. (1998) Kinetic and mechanism of the recognition of endotoxin by polymyxin B. *J. Am. Chem. Soc.*, **120**, 12428-12434.
- Vaara, M. (1992) Agents that increase the permeability of the outer membrane. *Microbiol. Rev.*, **56**, 395-411.