A Comparative Study of Characteristics of Current-Type and Conventional-Type Cationic Bactericides

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We have synthesized new polycationic bactericides, poly[oxyethylene(dimethyliminio)trimethylene(dimethyliminio)ethylene dichloride] (OXD) and poly(hexamethyleneguanidine phosphate) (HEP), in order to develop more active but less skin-irritative bactericides. The effects of these bactericides on *Pseudomonas aeruginosa*, *Escherichia coli*, *Serratia marcescens*, *Klebsiella pneumoniae*, methicillin resistant *Staphylococcus aureus* (MRSA) and the degree of their irritations on skin were compared with those of a widely used low molecular-weight cationic bactericide, benzalkonium chloride (BAC), and a polycationic bactericide, poly[2-hydroxyethylene(dimethyliminio)methylene chloride] (2HYC). The minimum bactericidal concentration (MBC) of OXD for 10 min contact incubation was 16 μg/ml against *P. aeruginosa*, *E. coli*, *S. marcescens* and *K. pneumoniae*, and >1000 μg/ml against MRSA. The MBC of HEP for 10 min contact incubation was 16 μg/ml against *P. aeruginosa*, 32 μg/ml against *E. coli* and *K. pneumoniae*, and 64 μg/ml against *S. marcescens* and MRSA. Itch, edema, erythema, heat, injury, desquamation and keratinization caused by skin irritation were examined in 21 subjects by patch tests. Only one subject treated with OXD experienced edema, and one subject with HEP experienced keratinization. However, BAC caused itch in 3 subjects, edema in 1, erythema in 10 and desquamation in 2, indicating that the incidence of skin irritation of BAC was higher than that of OXD or HEP. OXD and HEP had sterilization ability similar to BAC , however, they were less skin-irritative than BAC. This indicates that OXD and HEP can be used as safe bactericides.

Key words bactericide; polycationic biocide; benzalkonium chloride

Quaternary ammonium salts of low molecular-weight cationic bactericides have strong bactericidal effects and have been widely used as disinfectants and additives in ophthalmic solutions. The compounds are characterized by hydrophilic residues with a positive charge and hydrophobic alkyl chains in the molecules, and the length of the alkyl chains is an important factor that markedly affects bactericidal activity.[3—5)] A low molecular-weight cationic bactericide, benzalkonium chloride (BAC), is well-known and widely used. It has been demonstrated that BAC molecules with a chain length of C12—C14 exhibits strong bactericidal activity.[3,4)] Herein, we refer to the low molecular-weight cationic bactericide, BAC, as a conventional-type bactericide. To seek properties that are not observed in low molecular-weight cationic bactericides, the development of polycationic bactericides produced by the polymerization of organic cations has been actively performed. Polycationic bactericides are expected to have properties of strong bactericidal effects, low toxicity and low irritation, compared to the low molecular-weight cationic bactericides.[6—8)] For this purpose, poly[2-hydroxyethylene (dimethyliminio)methylene chloride] (2HYC) has recently been developed and partially characterized as a unique polycationic bactericide.

In recent years, we have synthesized new individual polycationic bactericides, poly[oxyethylene(dimethyliminio)-trimethylene(dimethyliminio)ethylene dichloride] (OXD) and poly(hexamethyleneguanidine phosphate) (HEP). These compounds are referred to as current-type bactericides in addition to 2HYC.

In this study, we compared the characteristics (the antibacterial activity against bacteria separated from clinical materials and the skin irritation) of OXD and HEP, with those of 2HYC and BAC.

MATERIALS AND METHODS

New Polycationic Bactericides and a Low Molecular-Weight Cationic Bactericide Figure 1 shows the chemical structure and molecular weight of poly[oxyethylene(dimethyliminio)trimethylene(dimethyliminio)ethylene dichloride] (OXD) and poly(hexamethyleneguanidine phosphate) (HEP), which were synthesized by the method reported elsewhere (submitted for publication) and poly[2-hydroxyethylene(dimethyliminio)methylene chloride] (2HYC) (Glokill PQ®, Rodia Inc.), which has been practically used.

As a low molecular-weight cation bactericide, benzalkonium chloride (BAC) (Osvan®, 10% (w/v), Nihon Pharmaceuticals Inc.) was used.

Bacterial Strains One strain each of *Pseudomonas aeruginosa*, *Escherichia coli*, *Serratia marcescens*, *Klebsiella pneumoniae*, methicillin resistant *Staphylococcus aureus* (MRSA), separated from clinical materials, was used.

Culture Media Nutrient broth (Kanto Kagaku, Inc.) was used for the pure culture of bacteria. Flat agar media were produced using purified agar (Kyokuto Pharmaceuticals, Inc.) and Soybean-Casein-Digest Agar with Lecithin and

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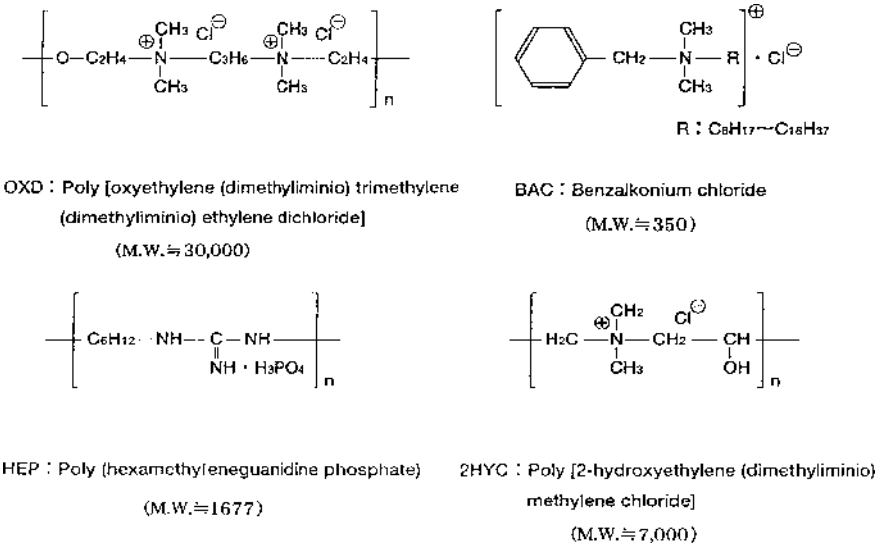

Fig. 1. Chemical Structures and Molecular Weight of Polycation-Type Bactericides and BAC

Polysorbate 80 (SCDLP) media "Daigo" (containing inactivation agent, Wako Pure Pharmaceuticals, Inc.) for measurement of the minimum bactericidal concentration (MBC).

Measurement of the MBC of the Polycation Bactericides and BAC against Bacteria The MBC was measured according to the Deutsche Gesellschaft fur Hygiene and Microbiogic (DGHH) method. Serial dilutions of the bactericides were made ranging from 1000 to 16 μg/ml with distilled water for injection. To 5 ml of each dilution, 0.05 ml of bacterial suspension, created by culturing at 37 °C for 1 d in nutrient broth adjusted to 10^{8-9} Colony-Forming Unit (CFU)/ml, was added and reacted for 3, 5, 10, 30, 60, and 120 min. After the reaction, one platinum loopful of the solution was inoculated on SCDLP media and cultured at room temperature for 72 h. MBC was judged by the presence of colonies. This measurement was performed twice.

Examination of Skin Irritation in Humans by Closed Patch tests Twenty-one healthy subjects, consisting of 13 males and 8 females, read a description of the purposes and methods of examination, possible changes in the skin and instructions, and orally gave their consent.

Adhesive tapes for patch tests (Torii Pharmaceuticals, Inc.), on which 0.2 ml of 0.2% w/v solution of each bactericide in distilled water had been dropped, were stuck on the bilateral medial upper limbs and in the sural region of the lower limbs, which had been cleansed using distilled water, and wiped. The patch tests were performed twice, and the second examination was performed one week after the first examination, when the effects of the first examination had disappeared. The adhesive tapes were removed 6 h after attachment, and changes in the skin, such as itching, edema, erythema, heat, injury, desquamation and keratinization were macroscopically examined, immediately and 24 h after the removal of the adhesive tapes.

RESULTS AND DISCUSSION

The number of hydrophilic positive charges and the length of hydrophobic alkyl chains in quaternary ammonium molecules are key factors upon the bactericidal activity. A polycationic bactericide, 2HYC, has been currently developed by increasing the molecular size. It has become apparent that a polycationic bactericide with many quaternary ammonium salts residues on the long chain-length has relatively strong bactericidal activity.[9,10] The conventional-type bactericide, BAC, which is widely used as a medical hand disinfectant, has a shortcoming of causing irritation to hands if used frequently, even though the contact period in each wash is short.[11,12] It may be pointed out that increasing the molecule size enhances the bactericidal activity and lowers the toxicity to the human body. Therefore, we have synthesized new polycationic compounds, referred to as the current-type bactericide, which is desired both to maintain bactericidal activity and lower irritation. The molecular weights of cationic bactericides used in a series of experiments were approximately 30000, 7000, 1700 and 350 for OXD, 2HYC, HEP and BAC, respectively. These compounds were investigated for bactericidal activity and skin irritation. Because of its strong bactericidal activity, BAC is used not only as a disinfectant but also as an additive to medical and non-medical products and cosmetics. It is also used in fields unrelated to medicine, as the in textile industry.

The minimal bactericidal concentration of the compounds was determined as follows: The MBC of OXD and 2HYC against *P. aeruginosa* was 16 μg/ml for 3 min contact incubation, indicating that both have strong bactericidal activities. The MBC of HEP was 64 μg/ml for 3 and 5 min and 16 μg/ml for 10 min (Table 1). The MBC of BAC against *P. aeruginosa* was 16 μg/ml for 3 min and 16 μg/ml for 5 min, showing the same level of activity as OXD and 2HYC. The MBC of OXD against *E. coli*, *S. marcescens*, and *K. pneumoniae* was 32—16 μg/ml for 3—10 min incubation. The bactericidal activity of OXD against MRSA was >1000 μg/ml for 3—30 min, which was inferior to that against the other bacteria tested. OXD and 2HYC were less suitable as disinfectants for MRSA. The MBC of HEP against *E. coli* was 64 μg/ml for 3 and 5 min and 16 μg/ml for 30 min, while that against *S. marcescens* and *K. pneumoniae* was 125 μg/ml for 3 min, and 64 μg/ml for 5 min, indicating low bactericidal activity for a short time compared to OXD. How-

Table 1. MBC of Polycation-Type Bactericides and BAC against Various Bacteria (μg/ml)

Compound	Bacteria	Time of contact (min)					
		3	5	10	30	60	120
OXD[a]	*P. aeruginosa*	≦16	≦16	≦16	≦16	≦16	≦16
	E. coli	32	16	≦16	≦16	≦16	≦16
	S. marcescens	32	32	≦16	≦16	≦16	≦16
	K. pneumoniae	32	16	≦16	≦16	≦16	≦16
	MRSA	>1000	>1000	>1000	>1000	1000	125
HEP[b]	*P. aeruginosa*	64	64	≦16	≦16	≦16	≦16
	E. coli	64	64	32	≦16	≦16	≦16
	S. marcescens	125	64	64	16	≦16	≦16
	K. pneumoniae	125	64	32	16	≦16	≦16
	MRSA	250	64	64	32	32	32
2HYC[c]	*P. aeruginosa*	≦16	≦16	≦16	≦16	≦16	≦16
	E. coli	32	32	16	≦16	≦16	≦16
	S. marcescens	32	32	16	≦16	≦16	≦16
	K. pneumoniae	32	32	16	≦16	≦16	≦16
	MRSA	>1000	>1000	>1000	>1000	>1000	32
BAC[d]	*P. aeruginosa*	16	≦16	≦16	≦16	≦16	≦16
	E. coli	16	16	≦16	≦16	≦16	≦16
	S. marcescens	16	16	≦16	≦16	≦16	≦16
	K. pneumoniae	16	16	16	≦16	≦16	≦16
	MRSA	125	64	32	32	16	16

a) Poly[oxyethylene(dimethyliminio)trimethylene(dimethyliminio)ethylene dichloride]. *b*) Poly(hexamethyleneguanidine phosphate). c) Poly[2-hydroxyethylene(dimethyliminio)methylene chloride]. *d*) Benzalkonium chloride.

ever, HEP showed strong bactericidal activity against MRSA, 250 μg/ml for 3 min and 64 μg/ml for 5 and 10 min, showing stronger activity than OXD. 2HYC showed bactericidal activity equally as strong as OXD, with 32 μg/ml for 3 and 5 min, and 16 μg/ml for 10 min against *E. coli*, *S. marcescens*, and *K. pneumoniae*. For MRSA, it was >1000 μg/ml for 3—60 min contact incubation. The bactericidal activity of OXD and 2HYC was considered to be at a similar level, while that of HEP was lower than OXD and 2HYC for all bacterial species tested, except for the short-term effects on MRSA.

The high local concentrations of the active residues, which are linked together by covalent bonds to form a polymer in the polycationic molecules, are considered to be responsible for their high bactericidal activity.[8,13]

On the other hand, the larger size of the high molecular-weight bactericides results in a lower permeability through the bacterial membrane, because they cannot reach their target sites if they are inside the cell. However, the cationic bactericides have their target sites on the cell surface. Therefore, the increase in molecular size due to polymerization does not lead to a reduction in bactericidal activity, but rather makes high bactericidal activity possible.[10,14,15] Ikeda *et al*. demonstrated the molecular dependence of antibacterial activity against *Staphylococcus aureus* using poly(vinylbenzyl ammonium chloride) polycationic biocides.[7,10] Similar molecular dependence was observed in our data, that is, adsorption of the polycations onto the negatively charged bacterial cell surface was enhanced with the increasing charge density of the polycations. On the other hand, cross-linked poly(*N*-benzyl-4-vinylpyridinium bromide) was slightly dependent on the molecular weight and showed strong antibacterial activity against gram-positive bacteria, whereas it was less active against gram-negative bacteria.[15] The polycationic compounds synthesized in this study had different bactericidal activities against gram-positive and gram-negative bacteria. It is yet to be elucidated completely how the correlation between the structural and biochemical properties of the surface of the bacteria and the molecular size of the compounds gives rise to differences in their bactericidal activities.

The harmonization of antibacterial activity and healthy use is important for pharmaceutical products.[16] To examine the degree of skin irritation caused by the compounds, patch tests were conducted with respect to the following 7 items: itching, edema, erythema, heat, injury, desquamation and keratinization (Table 2). Only OXD and HEP caused edema in the upper limbs in 1 subject (4.8%) and keratinization in the lower limbs in 1 subject (4.8%), respectively. None of the irritations were recorded with 2HYC. On the other hand, BAC caused itching in the upper limbs in 2 subjects (9.5%), itching, in the lower limbs in 1 subject (4.8%), edema in the lower limbs in 1 subject (4.8%), erythema in the upper limbs in 8 subjects (38.1%), erythema in the lower limbs in 2 subjects (9.5%), and desquamation in the upper limbs in 2 subjects (9.5%).

BAC has strong bactericidal effects and has been widely used as a disinfectant for hands, medical equipment, and also for the disinfection of environments. However, its repeated use often causes severe irritation to the skin, especially to hands, and elimination of such side effects has long been a difficult problem.[11,12]

In the present study, we tested not only antibacterial activity but also the degree of skin irritation of newly synthesized polycationic bactericides. Polycations were examined for their effects on the skin at the same concentration as is generally used for BAC (0.2% w/v). The results indicate that the new compounds have strong antibacterial activity against gram-negative bacteria and less irritation on the skin than BAC, and are unlikely to cause skin roughness after heavy use.

From the above results, we have concluded that the newly

Table 2. Skin Irritation of Polycation-Type Bactericides

		Control		OXD[a]		HEP[b]		2HYC[c]		BAC[d]	
		Number of occurrences	Rate (%)	Number of occurrences	Rate (%)	Number of occurrences	Rate (%)	Number of occurrences	Rate (%)	Number of occurrences	Rate (%)
Itch	Upper limb	0	0	0	0	0	0	0	0	2	9.5
	Lower limb	0	0	0	0	0	0	0	0	1	4.8
Edema	Upper limb	0	0	1	4.8	0	0	0	0	0	0
	Lower limb	0	0	0	0	0	0	0	0	1	4.8
Erythema	Upper limb	0	0	0	0	0	0	0	0	8	38.1
	Lower limb	0	0	0	0	0	0	0	0	2	9.5
Heat	Upper limb	0	0	0	0	0	0	0	0	0	0
	Lower limb	0	0	0	0	0	0	0	0	0	0
Injury	Upper limb	0	0	0	0	0	0	0	0	0	0
	Lower limb	0	0	0	0	0	0	0	0	0	0
Desquamation	Upper limb	0	0	0	0	0	0	0	0	2	9.5
	Lower limb	0	0	0	0	0	0	0	0	0	0
Keratinization	Upper limb	0	0	0	0	0	0	0	0	0	0
	Lower limb	0	0	0	0	1	4.8	0	0	0	0

a) Poly[oxyethylene(dimethyliminio)trimethylene(dimethyliminio)ethylene dichloride]. *b*) Poly(hexamethyleneguanidine phosphate). *c*) Poly[2-hydroxyethylene(dimethyliminio)methylene chloride]. *d*) Benzalkonium chloride.

developed polycations, which do not have immediate bactericidal effects on MRSA but are less irritative than BAC, are suitable as additives to pharmaceuticals and for the sterilization of swimming pools which require low-irritating disinfectants but not immediate bactericidal effects. In addition, we have considered the possibility of attaining high bactericidal activity while maintaining the minimum irritation by the combining the polycations and low molecular-weight cations at low concentrations.

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