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Haemolytic action of *N*-alkylpolymethylenediamines

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Abstract—The haemolytic action of various *N*-alkyl derivatives (lauryl; $C_{12}H_{25}$-, myristyl; $C_{14}H_{29}$-, palmityl; $C_{16}H_{33}$-) of 1,3-diaminopropane, 1,4-diaminobutane, 1,5-diaminopentane, 1,6-diaminohexane, 1,7-diaminoheptane, 1,8-diaminooctane was examined using rabbit red blood cells. The activities of the various derivatives were compared with those of antiplaque agents commonly used as mouthwashes; cetylpyridinium chloride (CP) and chlorhexidine acetate (CH). The haemolytic activities of these agents were dependent on the length of the *N*-alkyl chain, whereas the number of methylene groups between the nitrogen atoms had little effect. The order of potency was CP, *N*-palmityl derivatives, *N*-myristyl derivatives > *N*-lauryl derivatives > CH which was similar to the order of the antiplaque effect evaluated in-vitro.

Some useful in-vitro activities of *N*-alkylpolymethylenediamines were shown in our previous studies on the prevention and therapy of dental caries, including antibacterial activity against pathogenic oral micro-organisms (Murata et al 1990a), inhibitory activity against glucosyltransferase (Miyamoto et al 1990) and antiplaque activity (Murata et al 1990b). However, surface-active agents such as these compounds interact with biological membranes (Neurath et al 1944). It has been reported that polymethylenediamine dihydrochlorides have weak haemolytic activity and that the hydrophobicity of these detergents plays an important role in their effect (Kondo & Tomizawa 1969). Recently, an in-vitro red blood cell assay was discussed as a simple method for estimating the irritation potential of detergents (Pape et al 1987; Vercoutre 1990).

In the present study, the haemolytic activity of eighteen *N*-alkyl derivatives of polymethylenediamines containing from three to eight methylene groups was measured and the relationship between their haemolytic activity and structure investigated. A comparison was also made with commercially available disinfectants, cetylpyridinium chloride (CP) and chlorhexidine acetate (CH), which are both used as mouthwashes for dental prophylaxis (Kidd & Joyston-Bechal 1987; Ciancio & Bourgault 1989).

Materials and methods

Materials. Eighteen *N*-alkyl derivatives (lauryl; $C_{12}H_{25}$-, myristyl; $C_{14}H_{29}$-, palmityl; $C_{16}H_{33}$-) of polymethylenediamines, 1,3-diaminopropane, 1,4-diaminobutane, 1,5-diaminopentane, 1,6-diaminohexane, 1,7-diaminoheptane, and 1,8-diaminooctane, were synthesized as the dihydrochloride salts in our laboratory (Murata et al 1990a). Other test compounds included the corresponding polymethylenediamine dihydrochlorides (Wako Pure Chemicals, Osaka), CH (Sigma Chemical Co., St Louis, MO), and CP (Wako Pure Chemicals, Osaka). All the chemicals were of reagent grade obtained commercially, and used without further purification.

Preparation of erythrocytes. The red-cell suspension used for the experiments was freshly prepared as follows. Heparinized blood from a male rabbit was centrifuged and the cells were washed three times with 0·9% NaCl (saline). Unless otherwise stated, the cells were then suspended in the same medium to give a 1·0% (v/v) suspension (1×10^8 cells mL^{-1}). The number of cells in the suspension was counted with a cell counter (Sysmex Microcell-counter Model CC-108, TOA Medical Electronics Co., Ltd, Kobe, Japan).

Determination of the percentage haemolysis. Determination of haemolytic activity was carried out as described previously (Kondo & Tomizawa 1969). Test solution (2 mL) was prepared with saline in a test tube, which was then capped and thermostated at 30 ± 0·1°C in a water bath. The same volume of cell suspension was pipetted and gently mixed. Saline, instead of the test solution, served as a blank. The reaction mixture was incubated for 15 min and then cooled with ice-cold water. The test solutions were centrifuged to remove the unhaemolysed cells (3000 rev min^{-1}, 5 min), and their supernatants were examined spectrophotometrically at 540 nm. The percentage haemolysis of each test solution was calculated relative to the value for complete haemolysis, which was determined in each experiment as follows. Two equal portions of red-cell suspension were prepared. Saline was added to one sample as a blank and the other was diluted with distilled water to cause complete haemolysis of erythrocytes; hypertonic sodium chloride solution was added to the final volume to obtain isotonicity. The absorbance was proportional to the red-cell volumes added and was unaffected by the presence of the tested drugs.

Results and discussion

Typical haemolysis curves for *N*-alkyl derivatives of pentamethylenediamine dihydrochloride are shown in Fig. 1. The haemolytic activity increased with increasing detergent concentration with

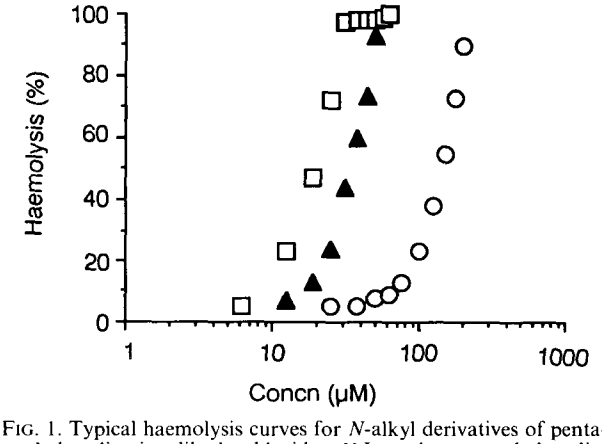

FIG. 1. Typical haemolysis curves for *N*-alkyl derivatives of pentamethylenediamine dihydrochlorides. *N*-Lauryl pentamethylenediamine dihydrochloride (○), *N*-myristyl pentamethylenediamine dihydrochloride (▲), *N*-palmityl pentamethylenediamine dihydrochloride (□).

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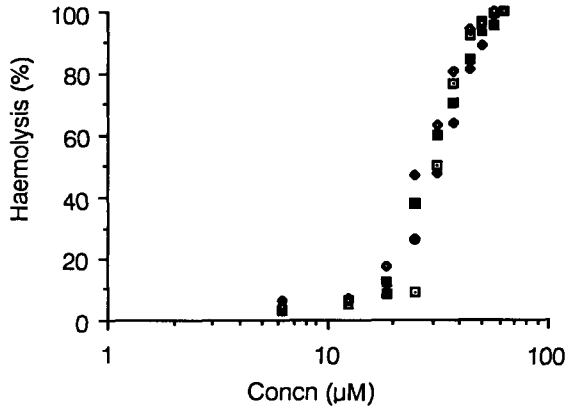

FIG. 2. Haemolysis curves for *N*-myristyl polymethylenediamine dihydrochloride vs drug concentration. 1,4-Diaminobutane (□), 1,7-diaminoheptane (■), 1,8-diaminooctane (◇).

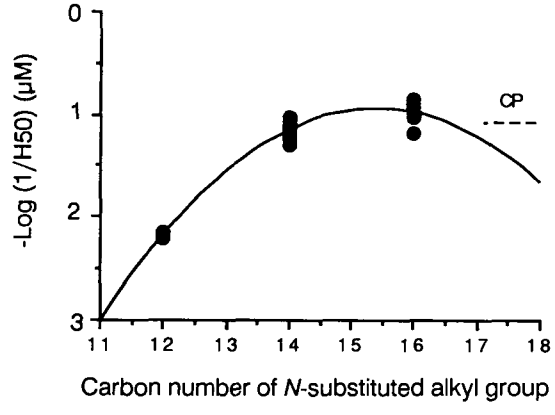

FIG. 3. The relationship between log (1/H50) of eighteen *N*-alkyl derivatives of polymethylenediamine dihydrochlorides and the carbon number of the *N*-substituted alkyl group. CP = cetylpyndinium chloride.

similar-shaped curves. Similar results were also obtained for the other homologues. The curves tended to shift toward a lower concentration with increasing *N*-alkyl chain length, but were little affected by the increasing number of methylene groups between the nitrogen atoms of the molecule (Fig. 2). The haemolytic potency of the detergents was compared in terms of H50 (μM), the concentration required to produce 50% haemolysis calculated from the linear portion of the curve, 30–70% haemolysis (Thron 1964), of each experiment. These values followed a straight line when plotted against cell concentration. Table 1 summarizes the H50 values of the test compounds. The relationship between the haemolytic potency, (log (1/H50)), and carbon number, CN, of the *N*-alkyl chain is shown in Fig. 3 (log $(1/H50) = -1{\cdot}06 \times 10^{-1}$ $CN^2 + 3{\cdot}28$ $CN - 26{\cdot}2$; $r = 0{\cdot}983$, $n = 18$). An increase of the *N*-alkyl chain length greatly affected haemolysis, whereas detergents with the same *N*-alkyl chain length showed similar potency despite an increase in the number of methylene groups between nitrogen atoms from 3 to 8. Under the same conditions, the corresponding polymethylenediamine dihydrochlorides produced little haemolysis, despite an increase of the contact concentration to about 2×10^3 μM. These results suggest that the hydrophobicity of the *N*-substituted group plays an important role in cell lysis. Kondo & Tomizawa (1969) described only a weak haemolytic activity of polymethylenediamine dihydrochlorides, with only dodeca- or decapolymethylenediamine causing haemolysis. Accordingly, they suggested the hydrophobic bond of the methylene groups was important for this activity. Unfortunately, the low solubility of *N*-substituted polymethylenediamine derivatives made it difficult to measure their haemolytic activities. Therefore, we were unable to investigate the effect on haemolysis of methylene groups between the nitrogen atoms under the present experimental conditions.

Table 1. Concentrations of compounds required to cause 50% haemolysis (H50) of rabbit blood. Concentrations were calculated as the final concentration of the reaction mixture obtained from 3–6 experiments at 30 ± 0·1 °C and a final red cell concentration of 5×10^7 cells mL^{-1}.

Compound	H50 (μM ± s.d.)
Lauryl	
1,3-diaminopropane	142 ± 10·6
1,4-diaminobutane	157 ± 7·6
1,5-diaminopentane	153 ± 11·4
1,6-diaminohexane	153 ± 7·9
1,7-diaminoheptane	152 ± 6·8
1,8-diaminooctane	151 ± 8·2
Myristyl	
1,3-diaminopropane	14·8 ± 3·1
1,4-diaminobutane	12·5 ± 2·2
1,5-diaminopentane	14·2 ± 1·6
1,6-diaminohexane	18·9 ± 1·8
1,7-diaminoheptane	11·5 ± 3·2
1,8-diaminooctane	14·6 ± 2·9
Palmityl	
1,3-diaminopropane	9·7 ± 2·2
1,4-diaminobutane	9·2 ± 1·2
1,5-diaminopentane	7·5 ± 0·5
1,6-diaminohexane	10·6 ± 3·1
1,7-diaminoheptane	10·6 ± 1·1
1,8-diaminooctane	10·0 ± 0·5
Chlorhexidine	11·9 ± 2·0
Cetylpyridinium chloride	211 ± 21·6

The *N*-myristyl ($C_{14}H_{29}$-) and *N*-palmityl ($C_{16}H_{33}$-) derivatives which have good antiplaque activity (Murata et al 1990b), showed relatively high haemolytic activity. This tendency was similar to antiplaque activity evaluated in-vitro using glass tubes as a model of tooth enamel (Murata et al 1990b). However, the potencies were of similar order to the activity of CP, which is used commercially as a mouthwash (Fig. 3). On the other hand, the haemolytic potency of CH, which is generally used as a mouthrinse in dental care, was weak (H50 2×10^2 μM), although this was predictable from our results in-vitro (Murata et al 1990b). CH is classified as a cationic surface-active agent (Heard & Ashworth 1968) and its plaque control effect has been evaluated in-vivo (Kidd & Joyston-Bechal 1987). A large deviation against available effects of CH, in-vivo has also been found, based on measurements of enamel surface energies (Perdok et al 1989). Thus the antiplaque effect of chlorhexidine may be different from that of cationic detergents.

The haemolytic potencies of the *N*-alkylpolymethylenediamines tested here were similar or lower than that of CP, which is used as a mouthwash. These detergents, especially the *N*-myristyl and *N*-palmityl derivatives, showed useful activities in-vitro, i.e. antibacterial activity against pathogenic oral microorganisms, inhibitory activity against glucosyltransferase and antiplaque activity (Murata et al 1990a, b; Miyamoto et al 1990). Therefore, it should be possible to use these derivatives in the oral cavity for the prevention or therapy of dental caries.

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References

Ciancio, S. G., Bourgault, P. C. (1989) Prophylaxis pastes, dentifrices, and mouthwashes. In: Clinical Pharmacology for Dental Professionals. 3rd edn, Year Book Medical Publishers, Inc., Chicago, pp 222–236

Heard, D. D., Ashworth, R. W. (1968) The colloidal properties of chlorhexidine and its interaction with some macromolecules. J. Pharm. Pharmacol. 20: 505–512

Kidd, E. A. M., Joyston-Bechal, S. (1987) Essentials of Dental Caries: the Disease and its Management. Wright, Bristol, pp 120–142

Kondo, T., Tomizawa, M. (1969) Mechanism of haemolysis by cationic surface-active agents. J. Pharm. Sci. 58: 1378–1381

Miyamoto, E., Murata, Y., Kawashima, S., Ueda, M. (1990) Inhibitory activity of *N*-alkylpolymethylenediamines against glucosyl-transferase from *Streptococcus sobrinus* OMZ176. J. Pharm. Sci. Tech. Japan 50: 62–64

Murata, Y., Ueda, M., Miyamoto, E. (1990a) Antimicrobial activity of *N*-alkylpolymethylenediamines against dental plaque bacteria. Caries Res. 24: 33–35

Murata, Y., Miyamoto, E., Ueda, M. (1990b) Antiplaque activity of some antimicrobial agents using simple in vitro method. Ibid. 24: 113–116

Neurath, H., Greenstein, J. P., Putnam, F. W., Erickson, J. O. (1944) The chemistry of protein denaturation. Chem. Rev. 34: 157–265

Pape, W. J. W., Pfannenbecker, U., Hoppe, U. (1987) Validation of the red blood cell test system as in vitro assay for the rapid screening of irritation potentials of surfactants. Mol. Toxicol. 1: 525–536

Perdok, J. F., van der Mei, H. C., Genet, M. J., Rouxhet, P. G., Busscher, H. J. (1989) Elemental surface concentration ratios and surface free energies of human enamel after application of chlorhexidine and adsorption of salivary constituents. Caries Res. 23: 297–302

Thron, C. D. (1964) Haemolysis by holothurin A, digitonin, and quillaia saponin: estimates of the required cellular lysin uptakes and free lysin concentrations. J. Pharmacol. 145: 194–202

Vercoutre, M. P. (1990) The importance of being mildest. Comun. Jorn. Com. Esp. Deter. 21: 159–166

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The use of albumin microspheres in the treatment of carrageenan-induced inflammation in the rat

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Abstract—Free hydrocortisone, hydrocortisone incorporated into microspheres and empty microspheres have been administered orally to rats with carrageenan-induced hindpaw inflammation. Hydrocortisone administered in particles was effective at a lower dose than free steroid in reducing inflammation. Inflammatory exudates were able to release steroid from the microspheres by proteolytic degradation.

Targeting drugs employed in the treatment of inflammatory disease to local sites, e.g. inflamed joints in rheumatoid arthritis, would result in a reduction in the amount of drug necessary to control the disease, with possible additional benefits in decreasing or even eliminating adverse side-effects. Recent work (Alpar et al 1989b) has shown that colloidal particles introduced into the circulation can concentrate in inflammatory exudates. Earlier reports (Volkheimer & Schultz 1968a, b) gave evidence for the transfer of small microparticles from the gastrointestinal tract to the circulation, a process called persorption. In order to test the possibility of a passive targeting system where orally administered particles would reach inflammatory exudates and tissues, we recently (Alpar et al 1989a, b) administered latex UV fluorescent microspheres (1–3 μm diam.) to rats with carrageenan-induced inflammatory air-pouches. We found that particles were rapidly transferred from the gastrointestinal tract to the circulation and that about 0·2% of the particles administered were detected in the exudate and tissues of the air-pouches. The transfer of orally administered latex particles from the gastrointestinal tract to various organs and to the circulation has recently been demonstrated (Jani et al 1990). In this communication we report on the use of orally administered biodegradable microspheres, prepared from bovine serum albumin and hydrocortisone, in the treatment of carrageenan-induced inflammation in the rat.

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Materials and methods

Preparation of plain microspheres. The method employed was based on that of Gallo et al (1984) except that olive oil was substituted for cotton-seed oil. A water in oil emulsion (w/o) was prepared from a mixture of bovine serum albumin (BSA) (250 mg dissolved in 0·5 mL of water) and olive oil (30 mL). The mixture was homogenized for 1 min at 14000 rev min^{-1} in an Omi homogenizer. The emulsion was then added at 40 drops min^{-1} to 100 mL of rapidly stirred olive oil at 125°C. This denatured the albumin which remained in suspension as microspheres. The temperature of the oil bath was maintained with stirring at 125°C for 10 min and then allowed to cool to room temperature (22°C). The olive oil was diluted with ether (60 mL) and the microspheres obtained by centrifugation at 3000 rev min^{-1} for 10 min. The particles were washed with ether and freeze-dried.

Preparation of microspheres labelled with fluorescein. The particles were prepared as described above but were suspended in a saturated solution of fluorescein isothiocyanate (FITC) in 1% sodium bicarbonate solution for 1 h. Excess FITC was removed by washing with water and the particles were centrifuged and dried by freeze-drying.

Preparation of microspheres loaded with steroid. Two methods were employed. Initially, plain particles, prepared as described above, were allowed to soak for 1 h in a saturated solution of hydrocortisone in methanol. After centrifugation, and washing with water to remove excess steroid, the particles were freeze-dried. However, higher and more uniform loading was obtained by employing a modification of the procedure for preparing microspheres previously described. In this modification the composition of the original emulsion was altered by adding 100 mg of hydrocortisone sodium phosphate and 5 mg of alkaline phosphatase to the original mixture which was pre-cooled to 4°C