J. Pharm. Pharmac., 1972, **24**, 215–218 *Received November* 8, 1971

Relation between cation and lipid content of cell walls of *Pseudomonas aeruginosa, Proteus vulgaris* and *Klebsiella aerogenes* and their sensitivity to polymyxin B and other antibacterial agents

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Sensitivity to polymyxin correlated with amount of phospholipid in wall fractions of *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Klebsiella aerogenes* and to a lesser extent with wall cation. The affinity of polymyxin for phospholipid from *P. vulgaris* and *P. aeruginosa* was the same.

Several workers have correlated whole cell readily extractable lipid (REL) and resistance to antimicrobial agents (Hugo, 1967). Wall REL has been implicated in the resistance of *Pseudomonas aeruginosa* to such agents (Ivanov, Markov & others, 1964). Recently cell wall magnesium and wall REL, especially phospholipid, have been implicated in the sensitivity of *P. aeruginosa* to polymyxin (Brown & Melling, 1969; Brown & Watkins, 1970).

Proteus vulgaris is characteristically resistant to polymyxin and the main purpose of this work was to analyse the phospholipid and magnesium content of *P. vulgaris* walls.

In addition, the affinity of polymyxin for phospholipid from *P. vulgaris* was estimated. Polymyxin inactivation, enzymatic or otherwise, was also assessed.

Ca^{2+} has been reported as a major cation (with Mg^{2+}) in *P. aeruginosa* walls (Eagon, 1969). Furthermore, Ca^{2+} may replace Mg^{2+} for some functions of the wall associated with polymyxin sensitivity (Brown & Melling, 1969) and consequently wall calcium was also assayed in the present work. Comparison was also made with polymyxin-sensitive and resistant strains of *Klebsiella aerogenes*. In addition to polymyxin B, we tested sensitivity of these three species of organism to benzalkonium, chlorhexidine, carbenicillin, gentamycin, streptomycin and tetracycline.

METHODS

Bacterial strains. Pseudomonas aeruginosa strains NCTC 6750, BRL 2087 (Beecham Research Laboratories), NCIB 8625; *Klebsiella aerogenes* strains NCIB 418, NCIB 8017; *Proteus vulgaris* strains NCTC 8313, NCIB 67, NCIB 8066, NCIB 8261.

Preparation of cells and walls for analysis. Cultures were grown in Oxoid No. 1 nutrient broth in 8 litre volumes in 10 litre flasks aerated with the aid of magnetic stirrers. Whole cells and walls were prepared as before (Brown & Watkins, 1970).

Lipid and cation analysis. Readily extractable lipid (REL), including phospholipid, and cations were assayed as before (Brown & Watkins, 1970). Lanthanum 10 000

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ppm was used to eliminate phosphate suppression of calcium when this was assayed using a Unicam SP90 atomic absorption spectrophotometer.

Minimum Inhibitory Concentrations (MIC). MIC determinations were made using inocula of 10^6 bacteria in 5 ml nutrient broth and examined for growth after 1 and 5 days incubation at 37°.

Affinity of polymyxin B for P. vulgaris *phospholipid.* Phospholipid from whole cells of *P. vulgaris* NCTC 8313 and *P. aeruginosa* BRL 2087 was extracted (Brown & Watkins, 1970). Various emulsions were prepared containing from 0·1 to 2% phospholipid, 4% to 16% glycerol in nutrient broth or water. Several commercial preparations of lecithin were also used. Graded concentrations of polymyxin in water or broth were incubated with various emulsion formulations at 37° for 3 h to allow inactivation (Kohn, Gershenfeld & Barr, 1963). The mixtures were then made up to single strength nutrient broth and inoculated with 10^6 *P. aeruginosa* NCTC 6750 in a final volume of 5 ml. The cultures were incubated at 37° and examined after 1 and 5 days to give an apparent MIC. Comparison of the number and size of emulsion particles was made microscopically using phase contrast illumination.

Test for polymyxin B inactivation. Graded concentrations of polymyxin up to 10 000 units/ml in broth were inoculated in quintuplicate with 10^6 *P. vulgaris* in 5 ml. Growth occurred in every case and aliquots were sterilized by membrane filtration (Brown, Farwell & Rosenbluth, 1969), re-inoculated with polymyxin-sensitive *P. aeruginosa* and re-incubated at 37° to test for the presence of polymyxin. A thick suspension of *P. vulgaris* grown in the presence of polymyxin was disintegrated (Brown & Watkins, 1970) and a sterile supernatant containing intracellular material was obtained by dilution, centrifugation and filtration. Aliquots of the supernatant were incubated at 37° with graded concentrations of polymyxin in broth, to allow possible inactivation, and then inoculated with 10^6 polymyxin-sensitive *P. aeruginosa* in 5 ml to obtain an apparent MIC.

RESULTS

Polymyxin B sensitivity. Table 1 shows that the polymyxin-resistant strains *P. vulgaris* (four) and *K. aerogenes* (one) all had relatively low wall-phospholipid contents. The calcium and magnesium content of *P. aeruginosa* wall fractions (each about 0·3%) compares with a cation content of *P. vulgaris* about 10-fold less, with one exception. The wall calcium of *P. vulgaris* NCTC 8313 is similar to that for *P. aeruginosa.* Both the polymyxin-sensitive and resistant *K. aerogenes* have low wall-cation contents. The cell calcium content of the *P. aeruginosa* strains was about 7-fold greater than the *P. vulgaris* strains, although all cultures were grown in the same complex medium.

Sensitivity to other antibacterial agents. There is a remarkable similarity in the spectrum of sensitivity of the two strains of *K. aerogenes* to the antibacterial agents, with the notable exception of polymyxin. The three bacterial species exhibit a relatively consistent between-species spectrum of sensitivity. The high streptomycin resistance of *P. aeruginosa* NCTC 6750 and BRL 2087 does not appear to relate with the limited chemical analyses.

Affinity of P. vulgaris *phospholipid for polymyxin B.* The concentration of glycerol had no significant effect upon apparent inactivation of polymyxin, and emulsions of phospholipid in water were more effective inactivators than in broth. There was about seven times more visible particles in broth emulsions than in water emulsions, indicating greater solubilization in the latter, hence greater availability of phospholipid for

inactivation of the polymyxin. The apparent MIC of polymyxin for *P. aeruginosa* after inactivation of polymyxin by 0·1% phospholipid emulsions in water containing 4% glycerol was (units/ml polymyxin) for *P. vulgaris* phospholipid, 100–200, for *P. aeruginosa* phospholipid, 100–200, for lecithin, 50–75, for control without phospholipid 5–10.

Test for polymyxin B inactivation. The re-inoculated supernatants gave similar apparent MIC's of polymyxin for *P. aeruginosa* as did controls not having grown *P. vulgaris.*

Table 1a. *Cation and lipid analyses of gram negative organisms.*

		K. aerogenes		*P. vulgaris*				*P. aeruginosa*		
Organism		NCIB 418	NCIB 8017	NCTC 8313	NCIB 67	NCIB 8066	NCIB 8261	NCTC 6750	BRL 2057	NCIB 8625
% Ca^{2+}	Cell	0·084	0·019	0·029	0·008	0·006	0·013	0·101	0·115	0·074
	Wall	0·024	0·057	0·29	0·03	0·045	0·026	0·187	0·28	0·198
% Mg^{2+}	Cell	0·191	0·13	0·20	0·41	0·15	0·21	0·214	0·266	0·262
	Wall	0·01	0·073	0·03	0·0052	0·013	0·009	0·304	0·279	0·303
% REL	Cell	14·2	25·2	8·4	8·8	16	9·5	13·5	11·3	10·7
	Wall	12·6	7·3	14·8	8·2	17·2	8·9	19·0	17	15·8
%EIF	Cell	1·5	3·6	2	5	2·3	1·5	0·1	1·0	<1
% PL	Cell	5·2	4·1	5·6	2·9	5·3	4·2	6·1	5·8	4·2
	Wall	3·5	<1	0·002	<0·5	<0·5	<0·5	2·9	3·0	3·1
% FN	Cell	7·5	17·5	0·8	0·9	8·4	3·8	7·3	4·5	6·5
	Wall	9·1	7·3	14·8	8·2	17·2	8·9	16·1	14·0	12·7

Table 1b. *Sensitivity to chemical antibacterial agents (minimum inhibitory concentration).*

	K. aerogenes		*P. vulgaris*				*P. aeruginosa*		
	NCIB 418	NCIB 8017	NCTC 8313	NCIB 67	NCIB 8066	NCIB 8261	NCTC 6750	BRL 2057	NCIB 8625
Polymyxin (units/ml)	0·5–1	2–5000	15–20 000	45–80 000	10–15 000	80–100 000	12–14	10–12	10–12
Benzalkonium (μg/ml)	1–10	10–25	1–10	10–25	1–10	10–25	50–75	100–250	50–75
Chlorhexidine (μg/ml)	2–5	2–5	10–25	5–10	2–5	5–10	5–10	5–10	2–5
Gentamycin (μg/ml)	1–2	0·5–1	1·5–2	2–5	2–5	2–5	0·5–0·75	0·6–0·8	0·2–0·4
Carbenicillin (μg/ml)	500–750	500–750	10–25	25–50	25–50	25–50	250–500	250–500	75–100
Tetracycline (μg/ml)	2–5	2–5	50–100	10–25	10–25	10–25	25–50	50–100	10–25
Streptomycin (μg/ml)	5–10	5–10	20–40	5–10	15–20	10–15	>20 000	>20 000	20–40

(REL), Readily extractable lipid (chloroform:methanol soluble).

(EIF), Ether insoluble fraction of REL. (See Brown & Watkins, 1970).

(FN), Free fatty acids and neutral lipids.

(PL), Phospholipid.

DISCUSSION

The affinity of polymyxin for *P. vulgaris* phospholipid was not significantly different from that for *P. aeruginosa* phospholipid, This result, together with the absence of any evidence for polymyxin inactivation is compatible with the polymyxin sensitivity of *Proteus mirabilis* spheroplasts (Teuber, 1969) and liposomes (Sud & Feingold, 1970).

There is a striking relation between polymyxin sensitivity and lack of wall-phospholipid and, to a lesser extent, with lack of wall-cation for all organisms studied. This work relates with other work on polymyxin-sensitive and resistant strains of *P. aeruginosa* (Brown & Melling, 1969; Brown & Watkins, 1970).

Sud & Feingold (1970) found that the lipid composition of polymyxin-sensitive and -resistant strains of *P. mirabilis* were similar and it was proposed that lipid composition was not important in polymyxin resistance: the presence of a wall factor responsible for polymyxin resistance was postulated. These workers did not distinguish wall lipid from membrane lipid and their findings are not incompatible with our results. Comparison of lipid of a variety of antibiotic-sensitive and -resistant Gram negative bacteria showed that there was a higher concentration of unsaturated acids and a lower concentration of cyclopropane acids in the lipid from resistant strains (Dunnick & O'Leary, 1970). These latter workers also did not distinguish wall lipid from membrane lipid.

Our results support the hypothesis (Brown & Watkins, 1970) that polymyxin is remarkable in requiring the presence of wall phospholipid for significant antimicrobial activity. Wall REL may also be involved in a non-specific way in excluding chemical antimicrobial agents. The role of Mg^{2+} and Ca^{2+} may be related to that of the phospholipid (Brown & Melling, 1969).

Brown & Watkins (1970) analysed the walls of both a polymyxin-sensitive and resistant strain of *P. aeruginosa*. They found that the percentage of wall phosphorus extracted with the REL from resistant walls was much greater than for sensitive walls, even though the total phosphorus content of the resistant walls was lower. Thus, for resistant walls there was a much smaller phosphorus content at sites not associated with readily extractable lipid, possibly implying a low lipopolysaccharide content.

A recent review on the mechanism of the general resistance of *P. aeruginosa* to chemical agents has suggested an important role for the wall with special reference to cation and lipid (Brown, 1971). The results reported here support the hypothesis that the amount and kind of cation and lipid in the wall plays a key role in determining the bacterial spectrum of drug sensitivity by an exclusion mechanism.

Acknowledgements

We gratefully thank the Medical Research Council for supporting this work. The gift of *P. aeruginosa* BRL 2087 from Beecham Research Laboratories is gratefully acknowledged. We are grateful to Dr. T. D. Martin of the Royal Berkshire Hospital for helpful discussions.

REFERENCES

Brown, M. R. W. (1971). *Inhibition and destruction of Pseudomonas aeruginosa* p. 358. In: *Inhibition and Destruction of the Microbial Cell.* Editor: Hugo, W. B., London: Academic Press.

Brown, M. R. W., Farwell, J. A. & Rosenbluth, S. A. (1969). *Analyt. Biochem.*, **27**, 484–491.

Brown, M. R. W. & Melling, J. (1969). *J. gen. Microbiol.*, **59**, 263–274.

Brown, M. R. W. & Watkins, W. M. (1970). *Nature, Lond.*, **227**, 1360–1361.

Dunnick, J. K. & O'Leary, W. M. (1970). *J. Bact.*, **101**, 892–900.

Eagon, R. G. (1969). *Can. J. Microbiol.*, **15**, 235.

Hugo, W. B. (1967). *J. appl. Bact.*, **30**, 17–50.

Ivanov, V., Markov, K. I., Golovinskii, E. & Kharisanova, T. (1964). *Z. Naturf.*, **196**, 604–606.

Kohn, S. R., Gershenfeld, L. & Barr, M. (1963). *J. pharm. Sci.*, **52**, 967–974.

Sud, I. J. & Feingold, D. S. (1970). *J. Bact.*, **104**, 289–294.

Teuber, M. (1969). *Ibid.*, **98**, 347–350.