J. Pharm. Pharmac., 1971, **23**, *Suppl.*, 136S–140S *Received January* 18, 1971

Inactivation of resistant *Pseudomonas aeruginosa* by antibacterial combinations

R. M. E. RICHARDS

Pharmaceutical Microbiology Group, Department of Pharmacy, Heriot-Watt University, Edinburgh, U.K.

P. aeruginosa resistant to preservative concentrations of benzalkonium chloride, phenylmercuric nitrate and chlorocresol in nutrient broth was inactivated by using phenylethanol-antibacterial combinations. EDTA-antibacterial combinations also showed increased activity using benzalkonium and chlorocresol. *P. aeruginosa* resistant to 0·25% chlorbutol was inactivated using phenylethanol–chlorbutol combinations at concentrations which were ineffective alone. Similarly *P. aeruginosa* having an increased resistance to chlorhexidine was inactivated by phenylethanol–chlorhexidine combinations at concentrations that were ineffective alone. Phenylethanol showed a greater general usefulness than EDTA at the concentrations tested.

Pseudomonas aeruginosa, a common contaminant of ophthalmic solutions, can become resistant to a wide range of chemical agents (Richards, 1967a, b). It has been shown, however (Brown & Richards, 1964b, 1965), that polysorbate 80 and disodium ethylenediaminetetra-acetate (EDTA) are both capable of affecting the resistance of logarithmic phase cells of *P. aeruginosa* to inactivation by chlorhexidine, polymyxin and benzalkonium. Apparently, both polysorbate 80 and EDTA exerted their effects by modifying the permeability properties of the *P. aeruginosa* cells. Although polysorbate 80 is not suitable for use in combination with most chemical agents, since it inactivates antibacterial action, polymixin is an exception (Brown & Richards, 1964b).

The American N.F.XII (1965) recommends benzalkonium chloride as the most reliable antibacterial agent for the preservation of ophthalmic solutions and states that "Resistant strains of *Pseudomonas aeruginosa* have been made sensitive to benzalkonium chloride by the inclusion of 0·01 to 0·1 per cent. of disodium ethylenediaminetetra-acetate . . .".

Richards (1967b) concluded that further evaluation of antibacterial agents suitable for using to preserve ophthalmic solutions was needed with special reference to the activity of combinations against resistant bacteria.

Phenylethanol has been shown to affect the resistance of *P. aeruginosa* to inactivation by phenylmercuric nitrate (PMN) (Richards, Suwanprakorn & others, 1969) —work extended to other antibacterials by Richards & McBride (1971). Phenylethanol was first recommended for use as a preservative for ophthalmic solutions by Brewer, Goldstein & McLaughlin (1953) following a report that it was active against Gram-negative organisms (Lilly & Brewer, 1953). Silver & Wendt (1967) showed that phenylethanol exerted its antibacterial effect by modifying the permeability properties of the bacterial cell.

The investigations now described compare the efficiency of phenylethanol–preservative combinations with that of EDTA-preservative combinations in killing resistant *P. aeruginosa* cells contaminating nutrient broth.

MATERIALS AND METHODS

P. aeruginosa strain NCTC 6750, *Escherichia coli* strain NCTC 8196 and *Staphylococcus aureus* strain NCTC 6751 were grown on Oxoid nutrient broth No. 2 for liquid cultures and Oxoid nutrient agar for solid cultures: incubation was at 37°. The EDTA, PMN, chlorbutol, *p*-chloro-*m*-cresol and 2-phenylethanol were all BDH laboratory reagents. Chlorhexidine acetate B.P.C. was from ICI and the benzalkonium chloride B.P. from Macarthy Ltd., Glasgow. Cell numbers were estimated by colony counts. The counting procedure and inactivating broth were described by Richards & others (1969), and the maintenance of stock cultures by Brown & Richards (1964a).

Evaluation of native resistance

A series of six replicates of four dilutions of each chemical was prepared in 10 ml volumes of nutrient broth. 0·1 ml overnight culture of each organism was used as the inocula to give a final concentration of approximately 6×10^6 cells/ml for *P. aeruginosa* and *E. coli* and 2×10^6 cells/ml for *S. aureus*. The resulting reaction mixtures in duplicate were incubated for 7 days. Cultures showing no growth were subcultured 0·5 ml into 10 ml inactivating broth and incubated for a further 3 days.

Selection of resistant inocula of P. aeruginosa

The 7-day cultures growing in the presence of benzalkonium 0·02% (6×10^6)* and PMN 0·002% ($3{\cdot}8 \times 10^8$) were considered to be resistant cultures. Viable counts were made on the cultures and at the same time the cultures were used as sources of inocula for investigating the activity of preservative combinations.

The 7-day culture growing in the presence of 0·25% chlorbutol failed to grow in the presence of 0·5% chlorbutol and was recultured in the presence of 0·25% chlorbutol. This 7-day culture was counted ($3{\cdot}6 \times 10^7$) and used immediately as a source of inocula.

Cells showing an increased resistance to chlorhexidine were obtained using two methods. The first was to subculture in nutrient broth in the presence of increasing concentrations of chlorhexidine and incubate for 7 days. An inoculum of an overnight culture grew in the presence of chlorhexidine 0·001% and an inoculum from this grew in the presence of chlorhexidine 0·00143%. This culture was further subcultured in the presence of chlorhexidine 0·02% and the 7-day culture so obtained was counted ($1{\cdot}5 \times 10^8$) and used immediately as a source of inocula. The second method was to use 2 ml overnight culture to inoculate nutrient agar containing chlorhexidine 0·004% and incubate for 7 days. The surface culture was harvested, stored and used as a source of inocula as already described (Richards & others, 1969).

Cells growing in the presence of 0·05% chlorocresol were obtained by subculturing from a culture growing in the presence of 0·025% chlorocresol. The 7-day culture so obtained was also counted ($1{\cdot}4 \times 10^6$) and used immediately as a source of inocula in the next series of experiments.

Evaluation of antibacterial combinations

In this series of experiments each chemical was evaluated at two concentrations, both with EDTA and also with phenylethanol. [PMN was not tested with EDTA

* Figures in brackets indicate colony counts.

because PMN–EDTA combinations have no advantage over PMN alone (Brown, 1968).] A simultaneous test was made using a series of concentrations of the preservative, EDTA and phenylethanol as individual solutions in nutrient broth. The test procedure was the same as for "evaluation of native resistance" except that the sources of inocula were cultures having enhanced resistance to *P. aeruginosa*.

RESULTS AND DISCUSSION

Evaluation of native resistance

Chlorhexidine (0·0005–0·004%) showed growth at 0·001% with *P. aeruginosa* and at 0·0005% with all organisms.

PMN (0·00025–0·002%) showed growth at 0·00025% with *S. aureus* and at all concentrations with the other organisms.

Benzalkonium (0·005–0·02%) showed growth at all concentrations with *P. aeruginosa* and 0·001% with the other organisms.

Chlorbutol (0·05–0·5%) showed growth at 0·25% with all organisms.

Chlorocresol (0·01–0·10%) showed growth at 0·025% with *P. aeruginosa* and at 0·01% with the other organisms.

Therefore under the conditions of this experiment *P. aeruginosa* was resistant to concentrations of benzalkonium and PMN at concentrations recommended for the preservation of ophthalmic solutions. *E. coli* was likewise resistant to PMN. All three organisms showed a similar high resistance to chlorbutol and low resistance to chlorhexidine. *S. aureus* was sensitive to all the chemicals tested with the exception of chlorbutol.

Selection of resistant inocula of P. aeruginosa

The inocula obtained consisted of cells having either a native or cultivated resistance to the chemicals under test: colony counts/ml of *P. aeruginosa* have been given above.

Evaluation of antibacterial combinations

Table 1 shows chlorhexidine–phenylethanol combinations at all combinations of phenylethanol are more effective than either the chlorhexidine or phenylethanol alone. The chlorhexidine–EDTA combination showed no advantage over the chlorhexidine alone.

Benzalkonium 0·01% with EDTA 0·05% is more active than either benzalkonium or EDTA alone. However, no greater activity is shown with the benzalkonium–phenylethanol combinations than is shown by the phenylethanol alone. That phenylethanol alone at 0·4% is effective against the benzalkonium resistant cells indicates that there is no cross resistance between these two agents. The inactivation observed was apparently caused by the phenylethanol alone but Richards & McBride (1971), using a more sensitive technique, showed phenylethanol enhanced the action of benzalkonium against log phase *P. aeruginosa*.

PMN 0·001% with phenylethanol 0·4% and PMN 0·002% with phenylethanol 0·3% are more active against *P. aeruginosa* than either agent alone. These results agree with those of Richards & others (1969).

Chlorocresol 0·01% with phenylethanol 0·4%, and chlorocresol 0·05% with phenylethanol 0·3% are more active than either agent alone. Similarly, chlorocresol 0·01% with EDTA 0·05%, and chlorocresol 0·05% with EDTA 0·01% are more active than either agent alone.

Table 1. *Effect of simple solutions and combinations against resistant* P. aeruginosa. All determinations made in duplicate. Minimum concentrations effecting sterility determined by observing growth or no growth after incubation for 7 days. All apparent no growths were subcultured into inactivating broth for a further 3 days.

Simple solutions		Inoculum for simple solutions and combinations		Combinations		
				Principal antibacterial ("P")	Adjuvant antibacterial	
Antibacterial concentration (%)	Minimum concentration (%) effecting sterility	7-day culture in broth plus antibacterial concentration (%)	Cells/ml in reaction mixtures	Concentration (%)	Minimum concentration % phenylethanol plus "P" effecting sterility	Minimum concentration % EDTA plus "P" effecting sterility
Chlorhexidine 0·001–0·01	0·005	Chlorhexidine 0·02	$1·5 \times 10^6$	Chlorhexidine 0·001	0·2	None
Phenylethanol 0·2–0·6	0·4			Chlorhexidine 0·01	0·2	0·005
EDTA 0·005–0·1	None					
Chlorhexidine 0·001–0·01	0·002	7-day culture on *Agar* plus chlorhexidine 0·04	6×10^6	Chlorhexidine 0·001	0·2	None
				Chlorhexidine 0·002	0·2	0·02
Benzalkonium 0·001–0·01	None			Benzalkonium 0·001	0·4	None
Phenylethanol 0·2–0·6	0·4	Benzalkonium 0·02	6×10^4	Benzalkonium 0·01	0·4	0·05
EDTA 0·005–0·1	None					
PMN 0·001–0·002	None			PMN 0·001	0·4	Not done
Phenylethanol 0·2–0·6	0·5	PMN 0·002	$3·8 \times 10^6$	PMN 0·002	0·3	Not done
Chlorocresol 0·01–0·1	0·1	Chlorocresol 0·05	$1·4 \times 10^4$	Chlorocresol 0·01	0·4	0·05
Phenylethanol 0·2–0·6	0·5			Chlorocresol 0·05	0·3	0·01
EDTA 0·005–0·1	None					
Chlorbutol 0·1–0·5	0·3	Chlorbutol 0·25	$3·6 \times 10^5$	Chlorbutol 0·1	0·5	None
Phenylethanol 0·2–0·6	None			Chlorbutol 0·5	0·2	0·005
EDTA 0·005–0·1	None					

Chlorbutol 0·1% with phenylethanol 0·5% shows an increased antibacterial effect over either chlorbutol or phenylethanol alone. Chlorbutol-EDTA combinations did not show increased activity over the activity of the separate agents at equivalent concentrations. Cells grown in the presence of 0·25% chlorbutol would not grow in the presence of 0·3% chlorbutol but were able to grow in the presence of 0·6% phenylethanol. This suggests that it is difficult to produce cells resistant to chlorbutol and also that cells having some measure of resistance to chlorbutol are likely to show cross resistance to phenylethanol. The inoculum to a final concentration of $3·6 \times 10^5$ *P. aeruginosa* was not the highest used, but nevertheless it was the only inoculum to produce growth in the presence of phenylethanol 0·6%. [EDTA 0·02% reversed this resistance to phenylethanol 0·2% (Richards, unpublished observation)].

In addition to affecting cell permeability properties, EDTA can also affect cell growth by removing Mg, an essential nutrient, from the growth medium. The work of Weiser & others (1968 & 1969) and Neu & Winshell (1970) investigating synergism with EDTA-antibiotic combinations was criticized by Brown (1971) for not taking this effect on the growth medium into consideration in the evaluation of their results. In this present work no concentration of EDTA in simple solution effected sterilization of the contaminated broth, although growth was not always evident until after sub-culture in the inactivating medium. This sub-culture procedure therefore eliminated no growths occurring solely as the result of the effect of EDTA on the medium. In the conditions pertaining in the reaction mixture, however, EDTA could be enhancing the activity of the antibacterial agents both by an effect on cell permeability and by making conditions less favourable for growth by affecting the medium.

The results obtained show that, except for the three combinations noted above, the phenylethanol-antibacterial agent and EDTA-antibacterial agent combinations,

at selected concentrations, are more effective than either agent individually in overcoming resistant *P. aeruginosa* cells contaminating nutrient broth.

The concentrations proposed for using in combination with other antibacterial agents, when there are no contraindications, are 0·4% for phenylethanol and 0·05% for EDTA.

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