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Ability of azlocillin and tobramycin in combination to delay or prevent resistance development in *Pseudomonas aeruginosa*

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The ability of combinations of azlocillin and tobramycin to prevent or delay resistance development in eight *Pseudomonas aeruginosa* isolates from cystic fibrosis (CF) patients was studied using chequerboard titration and in-vitro serial subculture. No isolate had developed resistance to tobramycin after 12 treatments with the antibiotic combination. Azlocillin resistance had not developed in four isolates after 16 exposures, and was delayed in the other four isolates for at least eight exposures. β-Lactamase production was responsible for azlocillin resistance in two isolates and occurred to a lesser extent in a third.

Introduction

The combination of azlocillin and tobramycin has been used successfully to treat *Pseudomonas aeruginosa* infections in cystic fibrosis (CF) patients.[1] A synergic interaction between the two antibiotics is one reason for using this combination.[2] A further indication for antibiotic combinations is to prevent emergence of resistance.[3] Synergic interactions are frequently assessed by chequerboard determinations, while serial subculture *in vitro* has been used to study resistance developed to antimicrobial agents.[4] In this study, we combined both procedures in order to examine the ability of an azlocillin/tobramycin combination to prevent or delay resistance development in CF isolates of *P. aeruginosa.*

Materials and methods

Antibiotics

Azlocillin and tobramycin were prepared in water from Securopen powder (Bayer plc, Newbury, UK) and Nebcin solution (Eli Lilly & Co. Ltd, Basingstoke, UK), respectively.

Pseudomonas aeruginosa isolates and MIC determinations

Eight isolates from the sputum of CF patients were identified as distinct strains by pyocin typing and serotyping, as described previously.[5] MICs were determined by broth microdilution in Iso-Sensitest broth (ISB; Oxoid, Basingstoke, UK) with a final inoculum of 10^5–10^6 cfu/mL, and incubation at 37°C for 24 h.

Development of resistance by in-vitro serial subculture

The procedure for studying resistance development consisted of subculturing and regrowing bacteria that survived in the presence of half of the MIC of the treatment antibiotic, and reassessing the MIC.[4] Controls in drug-free media were run in parallel.

Chequerboard broth microdilution method for serial subculture of antibiotic combinations

Eight doubling dilutions (based on initial MICs) of azlocillin and tobramycin in ISB were prepared. Each dilution (50 μL) of each antibiotic was placed in wells of a microtitre plate to give 64 drug combinations. The MICs of

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each antibiotic alone and in the combination (taken as the highest dilution of both antibiotics which failed to show visible growth) were determined after incubation at 37°C for 24 h.

A subculture (0.1 mL) was taken from the well containing one-half the MIC of both antibiotics, transferred to 9.9 mL of drug-free ISB and incubated at 37°C for 24 h. The MIC of the individual antibiotics was determined for this culture by the broth microdilution method. An additional sample of this culture was used to inoculate the next microtitre plate, and the process of treating with the combination of antibiotics, subculturing and re-growing was continued for at least eight treatments. Controls in drug-free media were included.

Measurement and characterization of β*-lactamases*

β-Lactamase levels were quantitatively determined based on the rate of degradation of nitrocefin (Glaxo research 87/312, Oxoid) and the p*I* of the enzymes characterized by isoelectric focusing, as described previously.[4]

Statistical treatment of results

Changes in MIC following serial subculture were tested for significance using the Wilcoxon Signed Rank Test.

Results

When isolates were exposed to the azlocillin/tobramycin combination, the MICs of neither azlocillin nor tobramycin had changed significantly ($P > 0.05$) after four subcultures, whereas the increase in MICs of the individual antibiotics was significant ($P < 0.05$) (Table I). On further serial subculturing, the MICs of azlocillin and tobramycin individually had risen significantly ($P < 0.05$) between the fourth and eighth subculture. For the tobramycin component of the combination, there was no significant change in MIC after eight subcultures. However, there was a significant increase in MIC of the azlocillin component after eight subcultures when compared with the start MICs for isolates. For three isolates (Nos 1–3), the MIC of the azlocillin component rose in a step-wise manner over the next four to six subcultures and increased between the fifteenth and sixteenth serial transfer of isolate No. 4 (Table II). For four isolates (Nos 5–8) there was little further change in azlocillin MIC up to 16 exposures to the antibiotic combination. Controls serially subcultured in drug-free media retained their original sensitivity.

The increase in MIC of the azlocillin component of the combination that occurred after the eighth serial subculture was clearly related to an increase in β-lactamase activity and a change in p*I* of the enzyme for two of the three isolates (Nos 1 and 3) (Table II). In the case of the third isolate (No. 2), the β-lactamase activity increased, but to a lesser extent, and the p*I* did not change. Control levels of β-lactamase determined for isolate No. 3 after 8, 11 and 14 serial subcultures in ISB alone were 4.0, 3.3 and 2.4, respectively, and the corresponding p*I*s were 9.6, 9.7 and 9.7, which indicated that serial subculture *per se* did not have any effect on β-lactamase levels or p*I*s.

Discussion

When *P. aeruginosa* isolates were exposed to an azlocillin/tobramycin combination, no change in MIC of the tobra-

Table I. Influence on MIC (mg/L) of serial exposure of *P. aeruginosa* isolates to azlocillin or tobramycin alone and in combination

	MIC (mg/L) after serial exposure to									
	azlocillin alone			tobramycin alone			azlocillin/tobramycin in combination			
Isolate No.	0	4[a]	8[b]	0	4[a]	8[b]	0	4	8[c]	16
1	8	32	128	0.5	2	4	0.25/0.2	0.25/0.4	0.25/0.8	NT
2	16	256	512	0.5	4	16	0.5/0.2	0.2/0.2	2.0/0.2	NT
3	4	64	64	0.5	2	8	0.5/0.1	0.5/0.1	1.0/0.2	NT
4	2	16	64	0.5	4	8	0.25/0.1	0.25/0.4	0.5/0.2	4.0/0.1
5	8	64	128	0.5	4	8	1.0/0.2	2.0/0.2	2.0/0.2	4.0/0.1
6	16	512	512	1.0	4	8	0.12/0.8	0.12/0.8	1.0/0.4	2.0/0.2
7	16	16	32	0.5	2	4	2.0/0.1	2.0/0.2	2.0/0.4	1.0/0.4
8	4	64	64	0.5	4	2	0.5/0.4	2.0/0.2	2.0/0.2	1.0/0.2

Significant increase ($P < 0.05$, Wilcoxon Signed Rank Test) between MICs [a]at the start and after four serial subcultures, [b]between four and eight serial subcultures, and [c]between the start and after eight serial subcultures for the azlocillin component of combination.
NT, not tested.

Table II. Influence of serial exposure to sub-inhibitory concentrations of azlocillin and tobramycin in combination on MIC (mg/L) and β-lactamase activity (nU) of *P. aeruginosa* isolates

Isolate No.	Treatment No.	β-Lactamase activity (nU)[a]	p*I*	MIC (mg/L) in combination	
				azlocillin	tobramycin
1	0	4	9.2	0.25	0.2
	9	40	9.6	2	0.8
	10	90	8.1	16	0.8
	11	614	5.9	128	0.8
	12	40173	5.9	256	0.4
2	0	NT	NT	0.5	0.2
	9	5	8.1	4	0.4
	10	27	7.4	16	0.2
	11	36	7.4	16	0.4
	12	62	8.0	128	0.4
3	0	4	9.6	0.5	0.1
	10	3	9.6	2	0.4
	11	35	9.4	1	0.8
	12	312	8.1	8	0.4
	13	350	9.1	8	0.8
	14	756	5.9	16	0.8

[a]nU, nanomoles of nitrocefin hydrolysed/min/mg protein.
NT, not tested.

mycin component of the combination was observed for any isolate. This is in marked contrast to the development of resistance in all isolates to the individual agent. Absence of resistance to tobramycin during treatment of CF patients with aztreonam or piperacillin combined with tobramycin was reported previously,[6] and may be related to increased uptake.

Resistance to the azlocillin component of the combination was delayed considerably in four isolates, and could not be induced in another four isolates after 16 subcultures. In two of the isolates tested (Nos 1 and 3), levels of β-lactamase remained low and fairly constant for up to eight subcultures, and corresponded to a low MIC of azlocillin in the combination. In the subsequent three to four subcultures, the β-lactamase activity and the MIC of azlocillin gradually increased. The p*I* of the extracted enzymes changed also and may be due to increased production of existing enzymes or expression of aditional enzymes. The results suggest that resistance to the azlocillin component of the combination was due to selection of resistant mutants with β-lactamase activity and that this selection process was delayed by the presence of an additional antimicrobial agent even at subinhibitory concentrations. Resistance mechanisms in *P. aeruginosa* have recently been reviewed, and in the case of β-lactam antibiotics, the mechanism is usually mediated by chromosomal β-lactamases.[7] Stably resistant mutants containing Class I β-lactamases are present in most *P. aeruginosa* isolates,[8] and this is consistent with the low level of β-lactamase activity detected in the isolates before the serial subculture experiments. In the third isolate (No. 2), the increase in β-lactamase activity was of a lower order and there was no change in p*I* of the extracted enzymes.

The principle of combining two antibiotics to prevent emergence of resistance is well established, and clinical evidence for this happening in *P. aeruginosa* infections has been reviewed by Barriere.[9] This investigation describes a method to study resistance development to antibiotic combinations. Further investigation of the ability of antibiotic combinations to delay development of resistance in *P. aeruginosa* may be increasingly necessary as more antibiotics become ineffective when used as monotherapy.

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References

1. McLaughlin, F. J., Matthews, W. J., Strieder, D. J., Sullivan, B., Taneja, A., Murphy, P. *et al.* (1983). Clinical and bacteriological

responses to three antibiotic regimens for acute exacerbations of cystic fibrosis: ticarcillin–tobramycin, azlocillin–tobramycin, and azlocillin–placebo. *Journal of Infectious Diseases* **147**, 559–67.

2. Farrell, W., Wilks, M. & Drasar, F. A. (1979). Synergy between aminoglycosides and semi-synthetic penicillins against gentamicin-resistant Gram-negative rods. *Journal of Antimicrobial Chemotherapy* **5**, 23–9.

3. Moellering, R. C., Allsop, A., Bush, K., Quinn, J., Baquero, F. & Philipps, I. (1998). Antibiotic resistance: lessons for the future. *Clinical Infectious Diseases* **27**, *Suppl. 1*, S135–40.

4. Wu, Y. L., Scott, E. M., Li Wan Po, A. & Tariq, V. N. (1999). Development of resistance and cross-resistance in *Pseudomonas aeruginosa* exposed to subinhibitory antibiotic concentrations. *APMIS* **107**, in press.

5. Steen, H. J., Scott., E. M., Stevenson, M. I., Black, A. E., Redmond, A. O. B. & Collier, P. S. (1989). Clinical and pharmacokinetic aspects of ciprofloxacin in the treatment of acute exacerbations of *Pseudomonas* infection in cystic fibrosis patients. *Journal of Antimicrobial Chemotherapy* **24**, 787–95.

6. Giwercman, B., Lambert, P. A., Rosdahl, V. T., Shand, G. H. & Høiby, N. (1990). Rapid emergence of resistance in *Pseudomonas aeruginosa* in cystic fibrosis patients due to in-vivo selection of stable partially derepressed β-lactamase producing strains. *Journal of Antimicrobial Chemotherapy* **26**, 247–59.

7. Hancock, R. E. W. (1998). Resistance mechanisms in *Pseudomonas aeruginosa* and other nonfermentative Gram-negative bacteria. *Clinical Infectious Diseases* **27**, *Suppl. 1*, S93–9.

8. Eliopoulos, G. M. (1988). Induction of β-lactamases. *Journal of Antimicrobial Chemotherapy* **22**, *Suppl. A*, 37–44.

9. Barriere, S. L. (1992). Bacterial resistance to β-lactams, and its prevention with combination antimicrobial therapy. *Pharmacotherapy* **12**, 397–402.

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