Synergism of Polymyxin and Sulfonamides in L-forms of *Staphylococcus aureus* and *Proteus mirabilis*

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Received for publication 24 August 1972

Earlier workers suggested that synergism of polymyxin and sulfonamides occurred because sulfonamides produced changes in the cell wall which permitted the more ready access of polymyxin. We tested this hypothesis by using L-forms, which lack cell wall. The demonstration that synergism of polymyxin and sulfonamides occurs in L-forms of *Proteus mirabilis* and *Staphylococcus aureus* suggested that the synergism is a result of some mechanism other than alterations of the cell wall.

Synergism of polymyxin and sulfonamides has been observed with *Proteus* species (3, 8, 10), *Serratia marcescens* (1), and some strains of *Pseudomonas aeruginosa* (9, 13). Polymyxin is considered to act on membrane phospholipids (6), and it has been suggested that sulfonamides alter the bacterial cell wall to increase transport of the polymyxin to the sensitive membrane (1, 7, 10). To examine the possible role of cell wall in the synergism, we studied cell wall-deficient L-forms of *P. mirabilis* and *Staphylococcus aureus*.

MATERIALS AND METHODS

A stable (nonreverting) L-form of *P. mirabilis* L 9 (ATCC 14168) and its parent bacterial form (ATCC 14273) were obtained from Ruth Wittler. *S. aureus* 61, coagulase positive, was isolated from a patient. The L-form of *S. aureus* 61 was methicillin produced and became stable (i.e., did not revert to the parent bacteria after removal of methicillin) after 116 transfers. It has since been transferred more than 40 times without reversion. Prior to their use in the following experiments *P. mirabilis* and *S. aureus* parent bacteria and L-forms were transferred five times in Trypticase soy broth (TSB; BBL) containing 0.5 M sucrose, 0.001% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 2% bovine serum albumin (BSA) and were stored at -15°C . Immediately before use, the organisms were passaged twice in the same medium. The same media were used for the L-forms and parent bacteria of both organisms in the following experiments.

To determine the minimal inhibiting concentration (MIC) for polymyxin and sulfadiazine, 10^6 organisms per ml (final concentration) were added to the TSB

medium described above, in doubling dilutions of polymyxin and sulfadiazine, and were incubated overnight (2). For minimal bactericidal concentrations (MBC) subcultures were made to Trypticase soy agar (BBL) containing 0.5 M sucrose, 0.001% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 2% BSA at 24 h.

Synergism was demonstrated by adding polymyxin in a concentration one-fourth the MBC (or approximately the highest antibiotic concentration tested when no MBC end point was reached). Because higher concentrations of sulfadiazine resulted in precipitation of the sulfadiazine in the reaction mixture, a sulfadiazine concentration of 1.6 mg/ml was used. Surviving organisms were assayed at 0, 1, 4, and 24 h after addition of antibiotics by culturing suitable dilutions of the cultures on the agar media described above.

RESULTS AND DISCUSSION

The MIC and MBC of polymyxin and sulfadiazine for L-forms and bacterial forms of *P.*

TABLE 1. MIC and MBC for bacterial forms and L-forms of *P. mirabilis* and *S. aureus* 61

Organism	Polymyxin ($\mu\text{g/ml}$)		Sulfadiazine (mg/ml)	
	MIC	MBC	MIC	MBC
<i>P. mirabilis</i>				
Bacteria	> 1,280	> 1,280	1.6	> 12.8
L-forms	5	10	12.8	12.8
<i>S. aureus</i>				
Bacteria	160	1,280	6.4	> 12.8
L-forms	0.6	20	0.2	12.8

mirabilis and *S. aureus* 61 are shown in Table 1. Studies of synergism between polymyxin and sulfadiazine in liquid media demonstrated that this reaction occurred with both bacterial forms and L-forms of these organisms (Fig. 1 and 2).

These results conflict with the suggestion that the sulfonamides alter cell wall metabolism to

increase permeability to polymyxin. The data show that synergism between polymyxin and sulfadiazine can occur in cell wall-deficient L-forms. Although it is difficult to be sure even with biochemical studies that there are no cell wall constituents present in L-forms, previous electron microscope studies by Weibull (15)

FIG. 1. Synergism of polymyxin and sulfadiazine for *S. aureus* 61 and its L-form. The concentrations of polymyxin and sulfadiazine used were 320 $\mu\text{g/ml}$ and 1.6 mg/ml for the bacteria and 5.0 $\mu\text{g/ml}$ and 1.6 mg/ml for the L-forms.

FIG. 2. Synergism of polymyxin and sulfadiazine for *P. mirabilis* and its L-form. The concentrations of polymyxin and sulfadiazine used were 640 $\mu\text{g/ml}$ and 1.6 mg/ml for the bacteria and 2.5 $\mu\text{g/ml}$ and 1.6 mg/ml for the L-forms.

FIG. 3. Electron micrograph of L-forms of *S. aureus* 61. $\times 13,400$.

showed only a single, triple-layered membrane in *P. mirabilis* L 9 L-forms, and no structured cell wall was visible in electron micrographs of our staphylococcal L-form (Fig. 3). In a biochemical study of the wall constituents, Weibull et al. (16) were unable to detect diaminopimelic acid in *P. mirabilis* L 9 L-forms.

A number of studies have shown that cell wall-deficient forms (L-forms and spheroplasts) of *P. morganii* and *P. vulgaris* (14), *P. mirabilis* (11, 12), and *S. aureus* (4), as well as L-forms of other bacteria (5), are usually more susceptible than the parent bacteria to all antibiotics except those acting on cell wall synthesis. Greater concentrations of polymyxin were therefore necessary with the parent bacterium than with the L-form to demonstrate synergism of polymyxin and sulfonamides. As a result of this, it was not possible to compare directly the amount of synergism observed with the L-forms and parent bacteria.

ACKNOWLEDGMENTS

We thank Charles Porter for technical help and are grateful to Tom Henshall and Morris Cohen for the electron micrograph of the staphylococcal L-form.

This investigation was supported by Public Health Service grant AI-02257 from the National Institute of Allergy and Infectious Diseases.

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