Effect of Osmolality on the Response of *Escherichia coli* to Mecillinam

DAVID GREENWOOD

Department of Microbiology, University of Nottingham, City Hospital, Nottingham, NG5 1PH, England

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The influence of osmolality on the effect of the novel β -lactam antibiotic, mecillinam, on *Escherichia coli* was examined in a turbidimetric system. Both sucrose and sodium chloride were able to protect *E. coli* from the effects of mecillinam during the stage of conversion to morphologically abnormal forms, and the additional presence of small amounts of magnesium sulfate protected the spherical forms from subsequent lysis. More phenotypically "resistant" survivors were recovered in osmotically protective media than in broth of low osmolality. Sucrose appeared a better protective agent than sodium chloride, but growth of the phenotypically resistant population was much slower in the sucrose-containing than in sodium chloride-containing medium.

Mecillinam, previously known by its manufacturer's code, FL 1060, is an anomalous β -lactam antibiotic whose behavior differs in several important respects from that of other penicillins and cephalosporins (4, 5, 10, 12). Among the unusual properties of mecillinam is its ability to convert susceptible gram-negative bacilli to spherical forms by a generalized effect on the cell wall (4, 6), whereas sufficient concentrations of most other β -lactam agents induce the formation of osmotically sensitive spheroplasts at discrete sites typically associated with incipient points of cell division (7, 9). It has been claimed that conversion of gram-negative bacilli to spherical forms by mecillinam is independent of sucrose concentration (10), implying that such forms are not osmotically sensitive, but our own studies (4, 8), using sodium chloride to vary the osmolality, have indicated that the formation and fate of spherical forms is very dependent on the osmotic environment. Recently, Tybring and Melchior (13) have suggested that NaCl, but not sucrose, is active in protecting the spherical forms and that the effect is related not to osmolality, but to specific conductivity of the medium.

The question of the fate of mecillinam-treated bacteria may be of some importance because, in contrast to the situation with other β -lactam agents, abnormal forms surviving the destructive effect of the drug are able to grow and divide in its presence and this may influence its therapeutic efficacy.

MATERIALS AND METHODS

A strain of *Escherichia coli* (laboratory code ECSA 2), originally isolated from infected urine,

was used throughout. *E. coli* ECSA 2 was chosen because in a previous study of the response of several *E. coli* strains to mecillinam (4) this strain showed an intermediate type of response to changes in the osmolality of the growth medium.

Mecillinam was a gift from Leo Laboratories Ltd. Suitable concentrations were freshly prepared as required in sterile distilled water.

Cultures were grown from small inocula in complete broth (7) containing the required amount of NaCl or sucrose, in the light path of a simple photometric device (14) connected to a potentiometric recorder. In this way the turbidity of the cultures could be continuously monitored. The cultures were mixed by using a magnetic stirrer. Mecillinam was added at a standard point in the logarithmic growth phase when the turbidity of the culture reached 12% of maximum.

Osmolality measurements were made by the cryostatic method using an advanced osmometer (Advanced Instruments Inc.).

Morphological observations were made on untreated wet preparations by interference contrast microscopy.

RESULTS

The osmolality of complete medium without supplements was found to be about 170 mOsm/kg; the addition of 10% (wt/vol) sucrose increased the osmolality to about 455 mOsm/kg; the addition of 1% (wt/vol) NaCl increased the osmolality to about 470 mOsm/kg.

Continuous turbidimetric monitoring was used in order to follow the kinetics of bacterial response to mecillinam. The relationship of turbidimetric changes to alterations in morphology and viability of *E. coli* has been discussed elsewhere (4).

Continuous turbidimetric records of *E. coli*

ECSA 2 exposed to mecillinam (10 $\mu\text{g/ml}$; 80 times the minimum inhibitory concentration) are shown in Fig. 1. In complete medium alone a small increase in opacity occurred after the addition of mecillinam over a period of about 90 min, corresponding to the period during which morphological changes develop (4), after which the opacity again declined. A fresh increase in opacity, shown by microscopy to be due to the growth of morphologically abnormal cells (4), occurred about 20 h after the addition of mecillinam. In the presence of 1% NaCl or 10% sucrose, the opacity of the culture continued to follow the normal growth curve for at least 90 min after the addition of mecillinam, after which a marked fall in opacity occurred. This fall in opacity occurred sooner and was more profound in the presence of NaCl than in the presence of sucrose. A fresh, progressive increase in opacity due to morphologically abnormal forms occurred as the lytic phase came to an end, but the increase was much slower in the presence of sucrose (Fig. 1C) than in the presence of NaCl (Fig. 1D).

The influence of 0.1% magnesium sulfate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) on the response of *E. coli* ECSA 2 to mecillinam is shown in Fig. 2. Alone, it had little effect on the response curve, except that regrowth due to morphologically abnormal forms occurred somewhat sooner (16 h rather than 20 h after antibiotic addition). In medium containing 1% NaCl, the additional presence of MgSO_4 modified the rate of fall in opacity and allowed recovery to occur sooner, whereas in medium containing 10% sucrose, the additional presence of MgSO_4 totally abolished the lytic phase.

DISCUSSION

The death of gram-negative bacilli, after exposure to β -lactam antibiotics, is generally due to osmotic lysis (3, 7). However, two fractions of the bacterial population may survive exposure to β -lactam agents; morphologically normal

FIG. 1. Continuous opacity records of *E. coli* strain ECSA 2. (A) Normal growth curve; (B,C,D) mecillinam was added (arrow) to achieve a concentration of 10 $\mu\text{g/ml}$. Medium contained (A,B) no addition; (C) 10% sucrose; (D) 1% NaCl.

FIG. 2. Continuous opacity records of *E. coli* strain ECSA 2. (A) Normal growth curve; (B,C,D) mecillinam was added (arrow) to achieve a concentration of 10 $\mu\text{g/ml}$. Medium contained 0.1% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and (A,B) no further addition; (C) 10% sucrose; (D) 1% NaCl.

persisters (1, 2) and those cells having an intracellular osmolality below that of the environment, which survive as spheroplasts (3, 7). Mecillinam converts sensitive gram-negative bacilli to forms resembling spheroplasts, but, because it lacks the ability of other β -lactam agents to inhibit division, cells surviving exposure to mecillinam are (unlike conventional spheroplasts) able to grow and divide in a morphologically abnormal form (4).

The reliance of mecillinam on low environmental osmolality to achieve its lethal effect (4, 8) has been questioned by Tybring and Melchior (13) who relate protection to the specific conductivity of the medium. Results obtained in the present study, however, indicate that both sucrose and NaCl are active in protecting *E. coli* from the early effects of mecillinam, although lysis of part of the population subsequently occurred in the presence of both substances, when cell wall damage was sufficiently advanced (4); this delayed lysis occurred sooner and was more profound in the presence of NaCl. The additional presence of MgSO_4 , which has a stabilizing effect on cell wall-deficient *E. coli* (11), modified the lytic response in the presence of NaCl and abolished it in the presence of sucrose. The inferior protective ability of NaCl, which is also found in ampicillin-induced spheroplasts (3), is probably related to the flow of ions into the cell, whereas sucrose, a nonfermentable substrate for *E. coli*, is likely to be excluded.

Although sucrose appeared better able than NaCl to protect cells from the early effects of mecillinam, it was also found to allow growth of the surviving, phenotypically resistant fraction of the population at a much reduced rate compared with NaCl. This may explain the findings of Tybring and Melchior (13) that NaCl, but not sucrose, had a protective effect on me-

cillinam-treated gram-negative bacilli. In their experiments results were expressed as the minimal inhibitory concentration estimated in the presence of various osmotically active agents. Minimal inhibitory concentration tests read at a fixed point in time after overnight incubation may clearly be influenced by differences in growth rates of the surviving cells as a slow growing population may not achieve a visible turbidity by the time of inspection.

The question of whether the ready emergence of phenotypically resistant forms reduces the therapeutic efficacy of mecillinam has not yet been resolved. However, the markedly enhanced effect obtained when *E. coli* is exposed to mecillinam in conditions of relatively low osmolality suggests that it may be advisable to reduce the osmolality when practicable (for example, by increased fluid intake in urinary tract infection) in treatment with this antibiotic.

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