QUANTITATIVE CORRELATION OF UPTAKE WITH ANTIBIOTIC ACTIVITY OF POLYMYXIN B IN *SALMONELLA TYPHIMURIUM*

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1. Introduction

Polymyxins constitute an interesting group of antibiotics for the following reasons: (1) They exhibit a rather selective activity against gramnegative bacteria [1]. (2) Their mechanism of action has been suggested to be an electrostatic interaction of the cationic antibiotic molecules with anionic cell envelope and membrane phospholipids leading finally to a breakdown of the permeability barrier [2, 3].

Therefore, polymyxins should provide a suitable system to study antibiotic-membrane interactions *in vivo*.

Using biologically active mono-*N*-acetyl-^{14}C-derivatives of polymyxin B [4], the number of molecules absorbed at the minimal inhibitory concentration giving maximal inhibition was determined to be about 2×10^5 mol per cell. At 23°, this amount was bound within 30 sec. Saturation, however, afforded prolonged incubation at 37° (about 10^6 molecules/cell). The reported properties of this uptake suggest that polymyxin has to combine with a specific binding site, possibly cell envelope phospholipids, in order to exert its antibiotic activity.

2. Materials and methods

Salmonella typhimurium G 30, an UDP-galactose-4-epimerase lacking mutant, was grown at 37° into early logarithmic phase (5×10^8 viable cells/ml = 0.12 mg dry weight/ml) in a defined, phosphate-buffered medium with glycerol as carbon source [5]. This suspension was used without further manipulations. Mono-*N*-acetyl-^{14}C-derivatives of polymyxin B (60.5 μCi/μmole) were prepared as described [4]. For the binding studies, ^{14}C-polymyxin dissolved in 5 μl of 0.14 M NaCl was added to 0.1 ml of bacterial or envelope suspensions. Incubation was terminated by filtering samples within 2 sec through cellulose acetate filters (0.45 μm pore size; Sartorium, Göttingen). Cell-bound radioactivity was determined in a toluene-based scintillation liquid with 54% counting efficiency in a Beckman LS 100 spectrometer. The values are corrected for unspecifically filter-bound radioactivity. Incorporaton of ^{14}C-uracil into cold, 5% trichloroacetic acid-insoluble material was measured by incubating the polymyxin-treated, filtered bacteria together with the filters in 0.8 ml of medium for 30 min at 37° in the presence of 0.1 μCi uracil (61 μCi/μmole). Viable cells were enumerated on McConkey-agar (Difco). The inhibition of uracil incorporation is paralleled by inhibition of active transport or viability [5]. Cell envelopes were prepared by mechanical disintegration with glass beads, centrifugation at 30,000 *g* and washing with distilled water. Phospholipid phosphorus was determined after extraction with chloroform-methanol and oxidation with periodate [6]. Cells contained 0.081 μmoles lipid-bound phosphorus/mg dry weight. Polymyxin B sulfate was provided by Pfizer GmbH (Karlsruhe). To avoid adsorption of

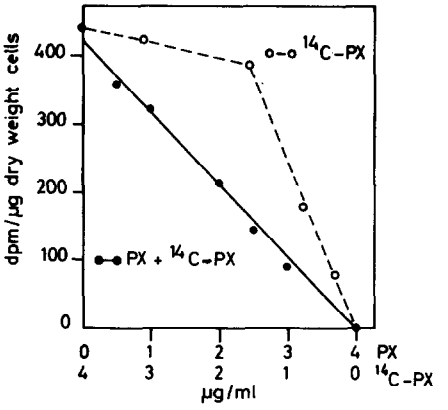

Fig. 1. Competition for NAc-polymyxin (^{14}C-PX) binding sites by not radioactive polymyxin B (PX). ^{14}C-PX and PX were mixed as indicated. Incubation was 3 min at 37°.

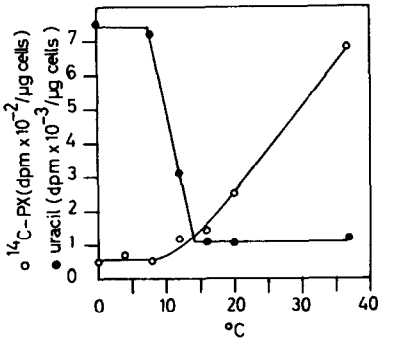

Fig. 2. Temperature dependence of uptake and antibiotic activity. Incubation was for 2 min with 4 µg NAc-polymyxin/ml. Uracil incorporation was determined after additional 30 min incubation of filtered bacteria at 37° without polymyxin.

polymyxin onto glass, all experiments were performed with polypropylene vials and pipettes. Abbreviation: NAc-polymyxin = mono-*N*-acetyl-^{14}C-polymyxin B.

3. Results

It was reported that the minimal inhibitory concentration was the same for polymyxin B and NAc-polymyxin [4]. The linearity between the NAc-polymyxin/polymyxin B ratio and uptake of NAc-polymyxin as shown by the competition experiment in fig. 1, justifies the use of these derivatives as a substitute for the natural polymycin. The unaltered antibiotic activity is probably due to the observation that polymyxins become preferentially *N*-substituted in the noncyclic part of the peptide chain [7] which is not necessary for activity [8]. That uptake of NAc-polymyxin is a requirement for activity is demonstrated in fig. 2. The absorption isotherms depicted in fig. 3 substantiate the uptake-activity relationship. Similar isotherms are obtained when the not cell-bound NAc-polymyxin is determined in the supernatant of cells after centrifugation. At bactericidal levels, binding is linear with cell mass. The time course of binding (fig. 4) reveals a multiphasic characteristic with intact cells but not with cell envelopes. This suggests differen-

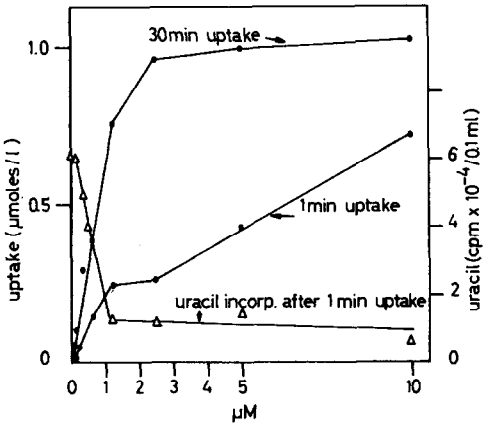

Fig. 3. Uptake isotherms of NAc-polymyxin compared to antibiotic action. Uracil incorporation was measured after 1 min polymyxin-treatment as indicated in fig. 2. Incubation temperature was 37°. Molarity is based on a calculated molecular weight of 1250 for NAc-polymyxin.

tially accessible binding sites in intact bacteria which is also evident from the absorption isotherms after short incubation. At 23 and 37°, the amount of NAc-polymyxin bound within 30 sec is sufficient to cause a 95% reduction of uracil incorporation and viability. At 23°, an average number of about 2 X 10^5 molecules NAc-polymyxin bound per cell is calculated for the 30 sec uptake.

The binding capacity of cell envelopes which is 1.7

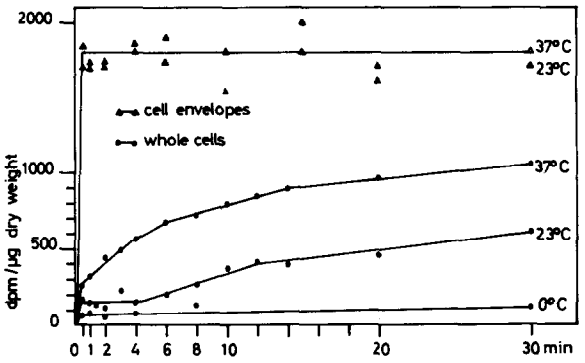

Fig. 4. Time course of uptake by cells and cell envelopes. NAc-polymyxin concentration was 3.1 µg/ml. Cell envelope concentration was 0.11 mg dry weight/ml.

times that of cells is reduced by 70% after extraction with chloroform–methanol (2:1). The cell-bound NAc-polymyxin is completely rèmoved by cellulose-thinlayer chromatography in *n*-butyl alcohol–pyridine–water (6:4:2) and to 85% by washing with 0.14 M NaCl, however, not by distilled water. At saturation levels of binding, the molar ration NAc-polymyxin/lipid phosphorus is 0.2 and 0.19 for intact cells and envelopes, respectively. Therefore, the bound NAc-polymyxin bearing 4 protonated γ-aminogroups of its diaminobutyric acid residues would suffice to neutralize most of the monovalent diester-linked phospholipid phosphorus.

4. Discussion

Though our results provide only indirect evidence for phospholipids as primary polymyxin receptors, they support experiments of Few [2] and Newton [3] which described interactions of polymyxin with phosphatidylethanolamine monolayers [2] and binding of fluorescent derivatives *in vivo* to the bacterial cell envelope as judged by direct microscopic observation [3]. The fact that polymyxin actually penetrates cephalin monolayers [2] might, in analogy to cytochrome *c*-phosphatidylethanolamine interactions [9], be interpreted as additional hydrophobic interaction of the D-phenylalanine-L-leucine-part of polymyxin B with the fatty acid residues of the phospholipids. This could mean that both electrostatic and hydrophobic forces as in the case of cytochrome *c* are involved. The characteristic temperature dependence of binding of NAc-polymyxin which was also seen with cell envelopes (Bader and Teuber, unpublished) could therefore reflect the known, fatty acid-dependent temperature transition point of phospholipids [10, 11]. With the described binding system, it should now be possible to test this hypothesis *in vivo* by using bacteria having known qualitative [11] or quantitative [12] variations in their cell envelope or membrane phospholipids.

Acknowledgement

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