Differences in Susceptibility to Quinolones of Outer Membrane Mutants of Salmonella typhimurium and Escherichia coli

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The mechanism of penetration of quinolones through the bacterial outer membrane was studied with lipopolysaccharide-deficient and porin-deficient mutants. The data indicated that the lipopolysaccharide layer might form a permeability barrier for hydrophobic quinolones such as nalidixic acid but not for hydrophilic quinolones such as norfloxacin and ciprofloxacin. The results also showed that quinolones with a low relative hydrophobicity appeared to permeate through OmpF porin, whereas quinolones with a high relative hydrophobicity appeared to permeate through both OmpF porin and phospholipid bilayers.

New quinoline derivatives which have broader and more potent antibacterial activity against gram-negative and gram-positive bacteria than old quinolones, such as nalidixic acid, have recently been developed. They also have greater antibacterial activity against nalidixic acid-resistant bacteria (5, 7, 9, 21, 24, 25).

We previously reported that the greater antibacterial activity of norfloxacin, one of these new quinolones, might be due to its greater ability to permeate bacterial cells and its potent inhibitory action on in vivo DNA synthesis (5).

The ability of drugs to pass through the bacterial outer membrane is a very important factor in their antibacterial activity and spectrum (2, 10, 13, 14, 22, 23, 26). There are many reports on the penetration into bacterial cells of various antimicrobial agents, especially beta-lactam antibiotics (4, 8, 15, 22, 23, 26), but the penetration mechanisms of quinolones have not been studied in detail.

We report here on the mechanisms of penetration of quinolones through the bacterial outer membrane with lipopolysaccharide (LPS)-deficient mutants of Salmonella typhimurium LT2 and porin-deficient mutants of Escherichia coli K-12. LPS-deficient mutants of S. typhimurium LT2 (19) were kindly provided by M. Inoue, Laboratory of Drug Resistance in Bacteria, Gunma University. Mutants lacking the OmpF or OmpC protein were isolated from E. coli CS109 with phages Tula and Tulf as described previously (17). The OmpF and OmpC proteins are parts of the receptors for phages Tula and Tulf, respectively. The absence of the OmpF or OmpC protein in the mutants thus isolated was confirmed by analysis of outer membrane proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (17). E. coli CS109 and the phages were kindly provided by T. Sawai, Chiba University, and S. Mizushima, Nagoya University, respectively.

Norfloxacin, pefloxacin, cinoxacin, miloxacin, oxolinic acid, rosoxacin, flumequine, and AM-833 (K. Hirai, M. Hosaka, Y. Oomori, S. Murayama, A. Ito, K. Takagi, T. Irikura, and S. Mitsuhashi, Program Abstr. 23rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 658, 1983) were synthesized at the Central Research Laboratories, Kyorin Pharmaceutical Co. Ltd. Other quinolones were obtained from the following sources: nalidixic acid and ofloxacin were from Daiichi Pharmaceutical Co. Ltd.; pipemidic acid, piromidic acid, and enoxacin were from Dainippon Pharmaceutical Co. Ltd.; and ciprofloxacin was from Bayer Yakuhin Co. Ltd. The partition coefficients of the quinolones were determined by the modified method of Nikaido (13). Solutions (10 μg/ml) of quinolones were made in 0.1 M phosphate buffer (pH 7.2). After shaking with an equal volume of n-octanol at 25°C for 48 h and centrifuging at 1,870 × g for phase separation, the concentrations of quinolones in the aqueous phase were determined with spectrophotometric assay by measuring the A372 for enoxacin, norfloxacin, and pefloxacin, the A286 for ciprofloxacin, the A364 for pipemidic acid, the A388 for cinoxacin, the A352 for AM-833 and piromidic acid, the A394 for ofloxacin, the A264 for miloxacin, the A300 for oxolinic acid, the A328 for nalidixic acid, the A328 for rosoxacin, and the A354 for flumequine. The partition coefficients were expressed as the ratio of the amount of compound in the n-octanol phase to that in the aqueous phase.

Susceptibility to quinolones was measured by the agar dilution method with Mueller-Hinton agar and an inoculum of 10⁶ CFU per spot as described previously (7). The data were expressed as MICs.

We tested the antibacterial activity of quinolones against LPS-deficient (rfa) mutants of S. typhimurium LT2 (19) (Table 1). The susceptibility of the bacterial strains to five quinolones, including norfloxacin, enoxacin, and ciprofloxacin, was not affected by alterations in LPS structure. In contrast, the susceptibility of LPS-deficient mutants (rfaG, rfaF, and rfaE) to other quinolones was increased. The data indicated that the higher the hydrophobicity of the quinolones, the greater was the increase in antibacterial activity against rfa mutants. Differences in the susceptibility of LPS-deficient mutants and the parent strain to hydrophobic compounds that had partition coefficients higher than 2.0, such as nalidixic acid, enoxacin, and flumequine, were significant. However, new quinolones, such as norfloxacin, ciprofloxacin, enoxacin, ofloxacin, and AM-833, were hydrophilic, with a partition coefficient lower than 1.0, and their efficacy was little affected by alterations in LPS structure. There was a good correlation between the hydrophobicity of the quinolones and their relative efficacy against LPS-deficient mutants. These results indicated that a mutational alteration of the LPS structure of the outer membrane

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significantly affected the permeability of the bacterial membrane to hydrophobic quinolones like nalidixic acid but not to the new quinolones that were hydrophilic, with a partition coefficient lower than 1.0.

In previous work (5), we reported that EDTA treatment enhanced the inhibitory activity of nalidixic acid but not that of norfloxacin on DNA synthesis in *E. coli* KL-16 and in a *nalB* mutant. It is known that EDTA treatment causes the release of LPS or LPS-protein complexes and disrupts the outer membrane barrier (10, 23). Our previous and present results suggested that the outer membrane might act as a permeability barrier for hydrophobic quinolones but not hydrophilic quinolones, such as the new quinolones, and that this barrier function could be diminished by EDTA treatment or by a mutational alteration in LPS structure.

Hrebenda et al. (6) recently demonstrated the possibility that nalidixic acid might penetrate directly through the phospholipid bilayer. Our demonstration of an increased susceptibility of LPS-deficient mutants, as compared with a wild-type strain, to quinolones of increasing hydrophobicity, also indicated that quinolones with a high relative hydrophobicity might be able to penetrate through phospholipid bilayers. This difference in permeability among quinolones seemed to be one of the reasons that new quinolones did not show any cross-resistance to certain nalidixic acid-resistant strains, including the *nalB* mutant of *E. coli* K-12 (5).

Small hydrophilic molecules are believed to diffuse through porin pores that are formed by outer membrane proteins such as OmpF and OmpC in *E. coli* K-12 (4, 8, 15). Mutants lacking the porin proteins were resistant to compounds that could pass through those porin pores (4, 8). To determine whether quinolones diffuse through these porin pores, we compared the susceptibility of OmpF- and OmpC-deficient mutants with that of the wild-type strain (CS109).

When compared with the wild-type strain, the mutant lacking OmpF was less susceptible to all the quinolones, but the OmpC-deficient mutant was equally susceptible to all these compounds (Table 2). Differences in the susceptibility of the OmpF-deficient mutant and the wild-type strain to quinolones depended neither on their hydrophobicity nor on their ionic type. It is possible that the quinolones penetrated the outer membrane of *E. coli* K-12 through the OmpF porin.

| Compound       | Hydrophobicitya | Mol wt | Ionic type | Efficacy ratiob |
|----------------|-----------------|--------|------------|-----------------|
|                |                 |        |            | SL3770 (smooth)c | SL3749 (raf/d) | SL3750 (raf/c) | SL3769 (raf/cG) | SL3789 (raf/eD) | SL1102 (raf/eD) |
| Enoxacin       | 0.007           | 320.3  | Amphoteric | 1 (0.20)        | 1 1 1 1 1       |
| Norfloxacin    | 0.01            | 319.3  | Amphoteric | 1 (0.10)        | 1 1 1 1 1       |
| Ciprofloxacin  | 0.02            | 331.3  | Amphoteric | 1 (0.03)        | 1 1 1 1 1       |
| Pipemidic acid | 0.03            | 303.3  | Amphoteric | 1 (0.13)        | 1 1 1 1 1       |
| Cinoxacin      | 0.03            | 262.2  | Acidic     | 1 (0.10)        | 0.5 1 0.5 0.5 0.5 |
| AM-833         | 0.08            | 369.3  | Amphoteric | 1 (0.10)        | 0.5 1 0.5 0.5 0.5 |
| Ofloxacin      | 0.33            | 360.4  | Amphoteric | 1 (0.09)        | 1 1 0.5 0.5 0.5 |
| Miloxacin      | 1.12            | 263.2  | Acidic     | 1 (0.13)        | 1 1 0.25 0.25 0.13 |
| Pefloxacin     | 1.32            | 333.4  | Amphoteric | 1 (0.12)        | 1 1 0.13 0.13 0.06 |
| Oxolinic acid  | 2.23            | 261.2  | Acidic     | 1 (0.07)        | 0.5 1 0.13 0.13 0.06 |
| Nalidixic acid | 3.92            | 232.2  | Acidic     | 1 (0.07)        | 0.5 1 0.13 0.13 0.06 |
| Rosoxacin      | 10.74           | 312.3  | Acidic     | 1 (0.12)        | 0.5 1 0.13 0.13 0.06 |
| Piromidic acid | 11.7            | 288.3  | Acidic     | 1 (0.25)        | 1 1 0.13 0.13 0.06 |
| Flumequine     | 13.0            | 261.3  | Acidic     | 1 (0.07)        | 0.5 1 0.13 0.13 0.06 |

a Given as the partition coefficient in n-octanol-0.1 M phosphate buffer (pH 7.2).
b Given as the ratio of the MIC for the rough mutant to that for the smooth mutant.
c Numbers in parentheses are MICs (micrograms per milliliter).

| Compound       | MIC (μg/ml) for: |
|----------------|---------------|
|                | CS109 (wild type) | KE11 (OmpC') | KE7 (OmpF') |
| Enoxacin       | 0.10          | 0.10         | 0.39        |
| Norfloxacin    | 0.05          | 0.05         | 0.20        |
| Ciprofloxacin  | 0.013         | 0.013        | 0.05        |
| Pipemidic acid | 1.56          | 1.56         | 6.25        |
| Cinoxacin      | 1.56          | 1.56         | 3.13        |
| Rosoxacin      | 0.05          | 0.05         | 0.20        |
| Miloxacin      | 0.20          | 0.20         | 0.78        |
| Pefloxacin     | 0.05          | 0.05         | 0.20        |
| Oxolinic acid  | 0.20          | 0.20         | 0.78        |
| Nalidixic acid | 3.13          | 3.13         | 12.5        |
| Flumequine     | 12.5          | 12.5         | 25          |

Differences in the susceptibility of the OmpF-deficient mutant and the wild-type strain to quinolones depended neither on their hydrophobicity nor on their ionic type.
acin was calculated by subtracting the adsorbed norfloxacin at zero time from the total norfloxacin eluted. The uptake of norfloxacin by the OmpF-deficient strain was about twofold lower than that of the wild-type strain, but the OmpC-deficient strain had a norfloxacin uptake rate almost equal to that of CS109 (Fig. 1). In the case of nalidixic acid, uptake by the OmpF-deficient strain was also lower than that by the wild-type and OmpC-deficient strains (data not shown).

This study with porin-deficient mutants indicated that all quinolones might penetrate, at least in part, through the porins formed by OmpF, and that alterations in outer membrane proteins might be associated with resistance to quinolones. It has been reported that nalidixic acid resistant-mutants (nalB and nalD) have altered permeability for nalidixic acid (1, 6, 16). However, the precise mechanisms of these transport mutants have not been defined. Alterations in outer membrane proteins, possibly porins, have previously been demonstrated to be associated with quinolone resistance in Klebsiella, Enterobacter, and Serratia spp. (3, 20).

In this study, we found a correlation between the decrease in susceptibility to quinolones and reductions in the amounts of the OmpF porin of E. coli. However, the MICs of the quinolones for the OmpF-deficient mutant increased by factors of only two to four. These results may be interpreted to mean that quinolones penetrate the outer membrane through OmpF porin pores very efficiently, like aminoglycoside antibiotics (12), or through another specific site(s). As mentioned above, one of the other possible pathways for hydrophobic quinolones such as nalidixic acid is the phospholipid bilayer, but hydrophobic quinolones such as norfloxacin and ciprofloxacin seemed to be unable to permeate this bilayer. Additional studies will be required to determine the precise mechanisms of penetration of new quinolones through the outer membranes of gram-negative bacteria.

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ERRATUM

Differences in Susceptibility to Quinolones of Outer Membrane Mutants of
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Volume 29, no. 3, p. 535, column 2. Lines 11 to 17 should read: "... spectrophotometric assay by measuring the $A_{264}$ for
enoxacin and pipemidic acid, the $A_{272}$ for norfloxacin, pefloxacin, ciprofloxacin, and rosoxacin, the $A_{278}$ for cinoxacin, the $A_{282}$
for AM-833 and piromidic acid, the $A_{288}$ for ofloxacin, the $A_{260}$ for miloxacin, the $A_{280}$ for oxolinic acid, the $A_{258}$ for nalidixic
acid, and the $A_{248}$ for flumequine. The partition coefficients were...".