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Iron Increases Susceptibilities of *Pseudomonas aeruginosa* to Ofloxacin by Increasing the Permeability

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Iron increased the susceptibilities of clinical isolates of *Pseudomonas aeruginosa* to quinolones. In the presence of iron, increased susceptibilities to ofloxacin were observed in twenty-six out of thirty isolates and with no change in four isolates. In the case of norfloxacin, iron increased susceptibilities of twelve isolates but did not render any change in eighteen isolates. In the case of ciprofloxacin, iron decreased the MICs (Minimal Inhibitory Concentration) of twenty isolates, increased the MIC of one isolate, and did not change the MICs of nine isolates. To find out how iron increased susceptibility to ofloxacin, bacterial cells were grown in Muller Hinton (MH) media and succinate minimal media (SMM) to induce iron acquisition systems and the intracellular ofloxacin concentrations were assayed in the presence of iron. The addition of iron to the media decreased the MICs of cells whether they were grown in MH or SMM. Siderophores, carbonyl cyanide *m*-chlorophenylhydrazone (an inhibitor of proton motive force), and ouabain (an inhibitor of ATPase) did not decrease the effect of iron. Results suggested that the increase in the intracellular ofloxacin concentration by iron is accomplished not by decreasing the efflux but by increasing the ofloxacin permeability.

Key words: antimicrobial agent, efflux, iron, ofloxacin, permeability, *P. aeruginosa*

Pseudomonas aeruginosa is a common nosocomial pathogen with intrinsic resistance to many antibacterial agents and its resistance has been increasing. Quinolone resistance in *P. aeruginosa* results from changes in DNA gyrase and topoisomerase IV (5, 8, 9, 10, 11, 25, 26), and a reduced permeability and efflux mechanism (1, 4, 14, 18, 19, 20, 22, 28). One way to overcome quinolone resistance is increasing the intracellular quinolone concentration by increasing permeability or inhibiting the efflux system. During a study on the virulence mechanism of *P. aeruginosa*, we found that iron increased ofloxacin susceptibilities of most *P. aeruginosa*. Since iron is a virulence factor (2), this observation was rather surprising. There are several possible says for iron to influence MICs. An obvious possible way of increasing the intracellular ofloxacin concentration is by increasing the ofloxacin uptake or increasing the efflux. In this study, we found that ofloxacin MICs in *P. aeruginosa* decreased in the presence of iron by increasing the intracellular ofloxacin concentration.

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Materials and Methods

Bacterial strains

Thirty clinical isolates of *P. aeruginosa* from the Culture Collection of Antibiotics Resistant Microbes (CCARM) were used in this study. These strains were originally isolated from various sources at Asan Medical Center in Seoul. Bacterial cells were grown in Mueller-Hinton (MH) media (Difco Laboratories, Detroit, MI) or succinate minimal media (SMM).

MIC test

MICs were determined by the broth microdilution method of the National Committee for Clinical Laboratory Standards (17). To assay the effect of iron, 1 mM $FeCl_3 6H_2O$ was added to the medium. Ciprofloxacin, norfloxacin, and ofloxacin were purchased from Sigma Chemical Co. (St. Louis, MO).

Detection of siderophore production

To detect siderophore, bacterial cells were inoculated on Chrome Azurol S (CAS) agar plates (23). CAS and 2,3-dihydroxybenzoic acid (HDTMA) were purchased from Fluka Chemical Co. (Buchs, Switzerland), 1,4-piperazinediethanesulfonic acid (Pipes) from Sigma Chemical

Co., and casamino acids from Difco Laboratories Co.

Purification of pyoverdin and pyochelin

Pyoverdin and pyochelin were purified from strain No. 42 following the procedure described elsewhere (7).

Assay of intracellular ofloxacin concentrations

Intracellular ofloxacin concentrations were measured as described in a previous paper (12). Bacterial cells in log phase were concentrated to make A_{600} 10. The cells were added to the uptake medium containing 50 μg/ml quinolone and incubated. After 10 min, a portion of the cells were layered on 0.5 ml cold silicon oil in a 1.5 ml microcentrifuge tube and collected by centrifugation to remove quinolone outside of the cells. The lower part of the tube containing the cell pellet was cut with a tube cutter and the cell pellet was transferred into 1 ml distilled water. The cells were dispersed by vigrous shaking. The quinolone was extracted from cells by boiling. The quinolone concentration was measured in a flourescence spectrophotometer (Hitachi, Japan).

Results

Changes of ofloxacin MIC by the addition of iron

When thirty randomly selected clinical isolates of *P. aeruginosa* were grown in MH containing 1 mM $FeCl_3$ (Fig. 1), twenty-six isolates showed two-to four-fold decrease in MIC to ofloxacin and four isolates showed no change. In the case of norfloxacin, iron increased susceptibilities of twelve isolates but did not render any change in eighteen isolates. In the case of ciprofloxacin, iron decreased the MICs of twenty isolates, increased the MIC of one isolate, and did not change the MICs of nine isolates. When cells were grown in the medium containing salicylate to suppress the outer membrane proteins, there was not much change in the MICs in each quinolone (Fig. 2).

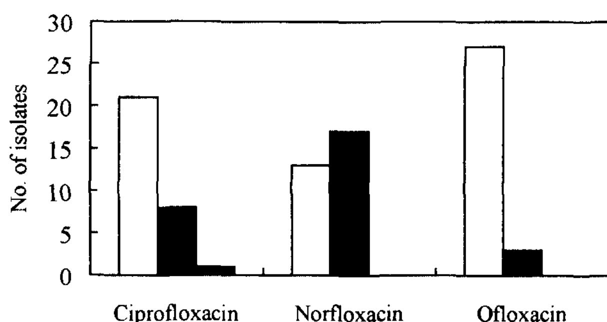

Fig. 1. The number of clinical isolates whose MIC were changed by the addition of iron. Cells were grown in MH and MICs were assayed in the absence/presence of iron. The number of isolates whose MICs changed in the presence of iron is shown as follows: ☐ the number of isolates whose MIC decreased; ■ the number of isolates whose MIC did not change; ▨ the number of isolates whose MIC increased.

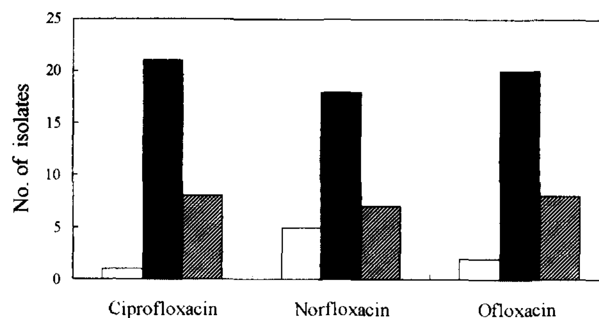

Fig. 2. The number of clinical isolates whose MIC were changed by the addition of iron. Cells were grown in MH containing salicylic acid and their MICs were assayed in the abscence/presence of iron. The number of isolates whose MICs changed in the presence of iron is shown as follows: ☐ the number of isolates whose MIC decreased; ■ the number of isolates whose MIC did not change; ▨ the number of isolates whose MIC increased.

Intracellular ofloxacin concentration in cells grown in MH and SMM

To find the relationship between iron and ofloxacin, bacterial cells were grown in SMM to induce the iron acquisition system. As shown Tables 1 and 2, the intracellular ofloxacin concentrations in cells grown in the presence of iron were much higher than those in cells grown in the absence of iron whether the medium was MH or SMM. Addition of pyoverdin or pyochelin to the cells did not affect the intracellular ofloxacin concentrations in cells grown in MH or SMM (data not shown).

Intracellular ofloxacin concentration in the presence of energy inhibitors

There are two possibilities for the increase in the intracellular ofloxacin concentration by iron : one is by decreasing the ofloxacin efflux and the other is by increasing the ofloxacin permeability. To differentiate between these two possibilities, CCCP, an inhibitor of the proton motive

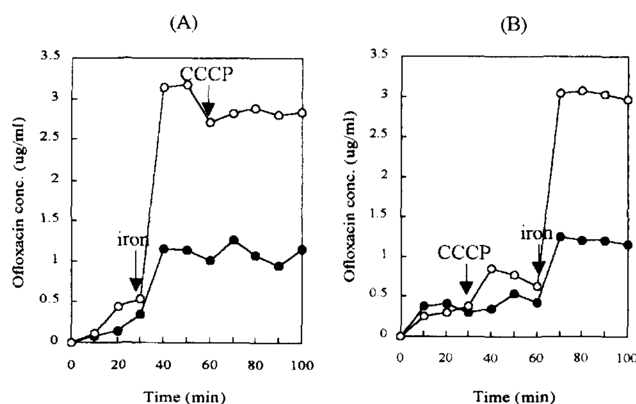

Fig. 3. Intracellular ofloxacin concentrations in the presence of CCCP and iron. CCCP and iron were added to cells at the times indicated with arrows. The intracellular ofloxacin concentrations were assayed at various times as described in Materials and Methods.

Table 1. Intracellular ofloxacin concentrations

Isolate No.	Relative intracellular concentration of ofloxacin[a] (%)				Isolate No.				
	-iron		+iron[b]			-iron		+iron	
	-CCCP[d]	+CCCP[e]	-CCCP	+CCCP		-CCCP	+CCCP	-CCCP	+CCCP
1	100	495	1222	1189	30	100	94	160	189
3	100	853	1241	2224	31	100	323	826	947
6	100	500	488	1088	39	100	238	258	450
8	100	221	234	562	42	100	290	327	463
9	100	332	282	445	44	100	162	189	456
11	100	160	153	232	49	100	110	155	303
13	100	198	245	375	55	100	127	149	210
14	100	144	172	155	64	100	390	377	750
15	100	60	256	297	67	100	889	5192	4261
16	100	362	269	552	68	100	191	211	339
18	100	182	160	379	77	100	197	109	200
21	100	105	169	155	79	100	67	143	263
25	100	318	515	641	83	100	118	38	217
26	100	1333	1144	1469	87	100	123	251	323
27	100	88	179	147	91	100	93	135	152

[a]ofloxacin (50 μg/ml) was added to the cells; [b]1 mM $FeCl_3$ was added to the cells; [c]CCCP (0.1 mM) was added to the cells; [d]intracellular ofloxacin concentrations in the absence of iron and CCCP were taken as 100%; [e]ofloxacin concentration in the presence of iron without CCCP.

Table 2. Intracellular ofloxacin concentrations in cells grown in the presence of salicylate.

Isolate No.	Relative intracellular concentration of ofloxacin[a] (%)				Isolate No.				
	-iron		+iron[b]			-iron		+iron	
	-CCCP[d]	+CCCP[e]	-CCCP	+CCCP		-CCCP	+CCCP	-CCCP	+CCCP
1	100	57	230	242	30	100	106	363	342
3	100	64	213	226	31	100	78	187	160
6	100	92	194	187	39	100	40	215	237
8	100	133	334	339	42	100	90	162	190
9	100	80	227	274	44	100	30	147	150
11	100	74	136	170	49	100	87	134	136
13	100	40	174	158	55	100	86	381	484
14	100	114	129	162	64	100	60	193	211
15	100	135	196	177	67	100	44	179	182
16	100	93	310	324	68	100	90	328	317
18	100	76	336	301	77	100	91	135	165
21	100	135	224	240	79	100	51	326	307
25	100	109	201	188	83	100	84	356	388
26	100	37	148	184	87	100	108	217	261
27	100	44	221	213	91	100	182	369	383

[a]ofloxacin (50 μg/ml) was added to the cells; [b]1 mM $FeCl_3$ was added to the cells; [c]CCCP (0.1 mM) was added to the cells; [d]intracellular ofloxacin concentrations in the absence of iron and CCCP were taken as 100%; [e]ofloxacin concentration in the presence of iron without CCCP.

force, was added to the cells to inhibit the efflux system. As shown in Tables 1 and 2, the intracellular ofloxacin concentration increased with the addition of CCCP but the increase was much smaller than the increase with the addition of iron. When iron was added to these cells treated with CCCP, it could still increase the intracellular ofloxacin concentration. It therefore is unlikely that the efflux system is involved. This idea is further strengthened with the observation that when cells were treated consecutively with iron and CCCP, the intracellular ofloxacin concentration increased markedly as soon as iron was added to the cells regardless of the presence of CCCP. The increase in the intracellular ofloxacin concentration by iron was observed in cells grown in MH as well as SMM.

Discussion

Although iron is abundant in nature, the extremely low solubility of Fe^{3+} at pH 7 presents most organisms with

the problem of obtaining sufficient iron from their environment (2). This is the reason why iron is a frequent limiting factor for *P. aeruginosa*, which prefers an aerobic metabolism that requires iron-containing respiratory enzymes. It was a rather intriguing observation that iron could increase the intracellular ofloxacin concentration making cells susceptible to antibiotics. *P. aeruginosa* has an intrinsic resistance against many antibiotics including quinolones because of low permeability and efflux pump. If iron increased the ofloxacin uptake, it could be accomplished by increasing the permeability or decreasing the efflux. Ofloxacin is imported through the outer membrane protein (13, 18) and effluxed via special efflux systems (19, 20). Cells grown in the presence of salicylic acid express reduced amounts of outer membrane proteins including ofloxacin import proteins (3, 21, 24). When iron was added to these cells, it did not render any change in ofloxacin MIC suggesting that iron increased ofloxacin permeability through outer membrane proteins which are the gates for ofloxacin (18).

Another possible route for ofloxacin permeability might be through the iron acquisition system (6, 7, 8)-the production and efflux of siderophores and uptake of the siderophore-iron complexes. It could be expected based on this hypothesis that, SMM-grown cells with more receptors for iron-siderophore complex would import more ofloxacin resulting in a reduced ofloxacin uptake. By contrast, the addition of siderophores did not affect the intracellular ofloxacin concentration. However, it was interesting that cells grown in SMM showed higher intracellular ofloxacin concentration than cells grown in MH. It is suggested that there are other possible routes for ofloxacin uptake besides porins. We are currently working to find out whether or not the iron and ofloxacin can form a complex and be imported through receptors overexpressed in SMM.

Another possibility for iron to increase the intracellular ofloxacin concentration is by inhibiting the efflux system. In *P. aeruginosa,* three homologous systems, MexAB-OprM, MexCD-OprJ, and MexEF-OprN containing resistance-nodulation-division (RND) transporters have been reported (15, 20, 28). These pumps consist of a cytoplasmic membrane component of the RND family that functions as a proton antiport exporter (i.e., MexB), an outer membrane component that forms channels (i.e., OprM), and a membrane fusion protein that links MexB and OprM. Substrates for MexA-MexB-OprM include the compounds as structurally diverse as β-lactamase inhibitors, quinolones, tetracyclin, trimethoprim, chloramphenicol, marcrolides, and novobiocin (28). Some RND transporters also pump out an extremely wide range of substrates, including practically all lipophilic and amphilic antibiotics, chemotherapeutic agents, metabolic inhibitors such as cerulenin, dyes, detergents and solvents (20). After CCCP was added to the cells to block the bacterial export systems energized by the proton motive force, iron was still capabale of increasing the intracellular ofloxacin concentration in all clinical isolates. Moreover, it was also noted that the addition of iron increased the intracellular ofloxacin concentration as soon as it was added to the cells. This showed that iron increased the intracellular ofloxacin concentration not by decreasing the efflux operated by the intrinsic ofloxacin efflux system. It is still possibile that ofloxacin is effluxed via other efflux systems which are energized by ATPase, such as the siderophore efflux system for iron uptake or TonB (16, 27). However, we observed that ouabain (an ATPase inhibitor) did not increase the intracellular ofloxacin concentration (data not shown). It was concluded that the increase in the intracellular ofloxacin concentrations by iron was due to the increase in the uptake rather than the decrease in the efflux activity.

Results from this study can be summarized as follows: 1) the intracellular ofloxacin concentration in cells with small amounts of porins was not affected by iron; 2) the intracellular ofloxacin concentration was affected by iron to a greater extent in cells with a large number of iron acquisition systems; 3) the increase by iron was not energy-dependent; and 4) siderophore is not involved in the ofloxacin permeability. All these results suggested that iron increases the ofloxacin uptake through the outer membrane protein(s) and energy is not required for this process. MICs of many resistant strains decreased more than four-fold in the presence of iron (data not shown). Based on these results, we suggest considering iron supplements to solve some antibiotic resistant problems.

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