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Intracellular Activity of Tosufloxacin (T-3262) against *Salmonella enteritidis* and Ability To Penetrate into Tissue Culture Cells of Human Origin

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The intracellular antimicrobial activity of tosufloxacin was tested against *Salmonella enteritidis* C-32 by using human lung fibroid WI-38 cells and was compared with those of ofloxacin and norfloxacin. The intracellular antimicrobial activities of these drugs were evaluated by determining the numbers of viable organisms remaining within cells after treatment with various drug concentrations. At 0.2 and 0.78 μg/ml, tosufloxacin suppressed intracellular multiplication of *S. enteritidis* C-32 more effectively than ofloxacin and norfloxacin did. The ability of tosufloxacin to penetrate into WI-38 cells was also determined by the velocity gradient method. The ratio of the intracellular concentration to the extracellular concentration of tosufloxacin was 1.7- and 2.6-fold higher than those of ofloxacin and norfloxacin, respectively. The results indicate that the potent intracellular bactericidal activity of tosufloxacin may be due not only to its high in vitro activity but also to its ability to penetrate into cells at a high level.

It is known that intracellular parasites, such as members of the genera *Salmonella* (3), *Shigella* (19), *Legionella* (7, 15, 17), and *Staphylococcus* (13, 18), invade host cells and grow. Therefore, these pathogens evade the bactericidal potential of antimicrobial agents such as β-lactams that do not enter host cells (7, 8). The survivability of these pathogens in host cells makes drug therapy difficult.

Tosufloxacin, the *p*-toluenesulfonic acid salt of DL-7-(3-amino-1-pyrrolidinyl)-1-(2,4-difluorophenyl)-6-fluoro-1,4-dihydro-4-oxo-1,8-naphthyridine-3-carboxylic acid monohydrate, is a newly developed quinolone antimicrobial agent and has shown a broad spectrum of activity against gram-positive and gram-negative bacteria (4, 5). Tosufloxacin has potent in vitro antibacterial activity against intracellular parasites, including the genera *Salmonella* and *Shigella*. Tosufloxacin also has a high level of efficacy against intestinal infections caused by *Salmonella* species in clinical trials in Japan (G. Masuda, H. Sagara, R. Nakaya, T. Yasuda, and T. Noumi, Program Abstr. 28th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 251, 1988). In order to clarify in detail the reason for the good clinical response of tosufloxacin, the intracellular activity of tosufloxacin against *Salmonella* species and its ability to penetrate into normal human cells were examined by using tissue culture cells of the human lung fibroid cell line WI-38 and were compared with those of ofloxacin and norfloxacin.

MATERIALS AND METHODS

Bacterial strain and susceptibility test. *Salmonella enteritidis* C-32 was isolated from a patient with intestinal infection and was stored at −120°C until use. The susceptibility of this organism to drugs was ascertained by determining the MIC by the broth dilution method. Eagle growth medium (Nissui Seiyaku, Tokyo, Japan) containing 10% fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.) was used for the assay medium. The MIC was determined after incubation for 18 h at 37°C.

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Antibiotics. Tosufloxacin was synthesized at the Research Laboratory, Toyama Chemical Co., Ltd., Toyama, Japan. Ofloxacin and norfloxacin were extracted from commercially available tablets. All the drugs were dissolved in 50% dimethyl sulfoxide and diluted in distilled water, as appropriate.

Tissue culture cell. Human lung fibroid WI-38 cells were obtained from Dainippon Seiyaku Co., Ltd., Osaka, Japan, and were stored at −180°C until use. The WI-38 cells (ca. 10^4 cells per ml) were cultured at 37°C under 95% air–5% CO_2 in Eagle growth medium containing 10% fetal bovine serum and 6.25 μg of kanamycin (Nissui Seiyaku) per ml for 7 days. After the cells were harvested by centrifugation (500 × *g*, 5 min, 25°C), they were washed twice with Hanks balanced salt solution (HBSS; Nissui Seiyaku) and used for intracellular susceptibility tests and studies to test the ability of the antibiotics to penetrate into WI-38 cells. Cells that adhered to the culture bottle were detached with 0.025% trypsin–EDTA (Denka Seiken, Tokyo, Japan).

Intracellular bactericidal activity of quinolones against *S. enteritidis* C-32. Freshly harvested WI-38 cells were cultured on a cover glass (22 by 22 mm) at 37°C under 95% air–5% CO_2 in Eagle growth medium containing kanamycin and 10%

TABLE 1. Number of *S. enteritidis* C-32 cells in human lung fibroid WI-38 cells in the presence of tosufloxacin, ofloxacin, and norfloxacin[a]

Drug	No. of *S. enteritidis* cells/WI-38 cell with the following drug concn (μg/ml)[b]:			MIC (μg/ml) for *S. enteritidis* C-32[c]
	0.05	0.2	0.78	
Tosufloxacin	7.2 ± 1.1	3.3 ± 0.6	2.4 ± 0.5	0.0125
Ofloxacin	8.2 ± 0.9	4.7 ± 0.6	4.3 ± 0.6	0.05
Norfloxacin	8.7 ± 1.1	8.2 ± 1.5	7.4 ± 1.3	0.05

[a] After 9 h of incubation, *S. enteritidis* C-32 multiplied to 10.2 ± 1.21 cells per WI-38 cell in the absence of quinolones.
[b] Values are means ± standard errors.
[c] MICs were determined by the broth dilution method by inoculating 10^6 bacterial cells per ml.

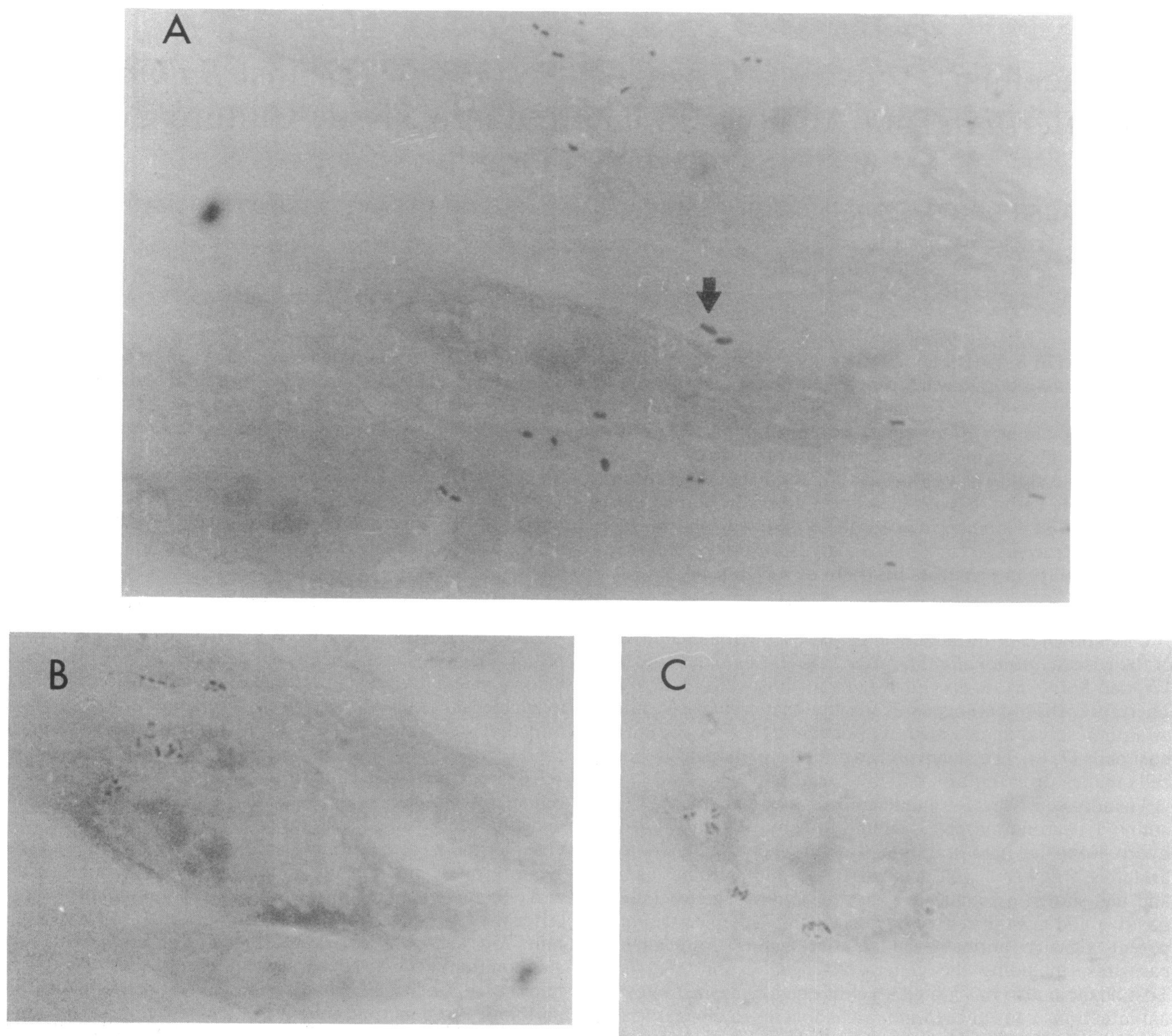

FIG. 1. Micrographs of a WI-38 cell incubated with *S. enteritidis* C-32. (A) Thirty minutes after the beginning of incubation; arrow indicates adherent bacteria; (B) adherent bacteria invading the WI-38 cell; (C) constantly multiplying, intracellular bacteria. Magnifications, ×1,000.

fetal bovine serum. After 5 days, growing cells were washed twice with HBSS. *S. enteritidis* C-32 (ca. 10^7 cells per ml) cells were allowed to attach to WI-38 cells and incubated at 37°C for 30 min under 5% CO_2 to achieve infections in antibiotic-free medium containing 10% fetal bovine serum. The cells were washed three times with HBSS, and then the medium was replaced with fresh medium containing various concentrations of quinolones and 6.25 μg of kanamycin per ml (kanamycin did not affect the multiplication of intracellular organisms). The cells were incubated for an additional 6 h and then stained with 10% Giemsa solution.

The number of intracellular bacteria per WI-38 cell was calculated by microscopic observation (×1,000) of 100 infected cells.

Ability of quinolones to penetrate into cells. The ability of the drugs to penetrate into cultured cells was determined by a modification of the velocity gradient technique of Koga (12). The freshly harvested cells from the culture dish were suspended in 10 ml of HBSS to give a concentration of 10^6 cells per ml. Antibiotic solutions were added to the WI-38 cell suspension at a final concentration of 5 μg/ml. Then, the suspension was mixed and shaken at 37°C in room air. After shaking for 30 min, the cells were rapidly separated from the extracellular solution by the velocity gradient technique. The suspension was centrifuged (8,000 × *g*, 5 min, 4°C) through a water-impermeable barrier of silicone oil (KF-96; Shinetsu Chemical). The water layer was used for determining the extracellular drug concentration. The separated cells were suspended in 0.5 ml of HBSS and were disrupted by boiling them for 10 min. Then, the disrupted cells were centrifuged (8,000 × *g*, 5 min, 4°C). The resulting supernatant was used for determining the intracellular drug concentrations. Drug concentrations were measured by high-performance liquid chromatography. The ability of these drugs

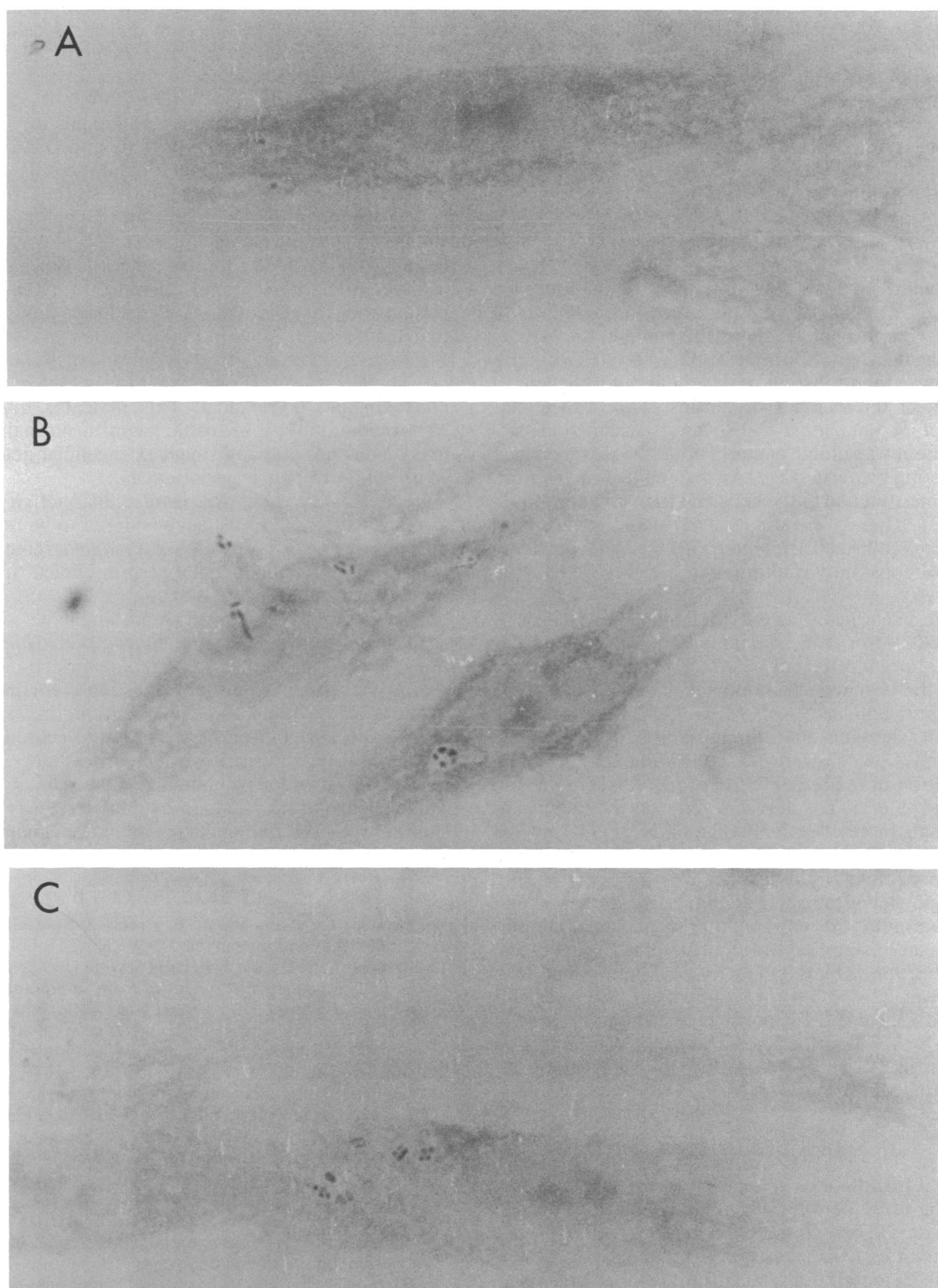

FIG. 2. Micrographs of infected WI-38 cells after incubation with tosufloxacin (A), ofloxacin (B), and norfloxacin (C). The reduced numbers of bacteria were observed in all quinolone-treated (0.78 μg/ml) WI-38 cells compared with those observed in the nontreated cells shown in Fig. 1C. Magnifications, ×1,000.

to penetrate into cells was expressed by the ratio of the intracellular drug concentration to the extracellular drug concentration. The volume of WI-38 cells was calculated from the average diameter of 100 round cells, which were prepared from adherent cells on the culture bottle by treating the bottle with adherent cells with 0.025% trypsin–EDTA. The calculated volume of WI-38 cells was $(3.88 \pm 0.21) \times 10^{-7}$ μl.

Antibiotic assay. The high-performance liquid chromatographic assay was performed with a high-performance liquid chromatograph (model LC-6; Shimadzu). Samples were run on a column (250 by 4 mm) of Nucleosil C-18 at room

temperature and at a flow rate of 2.0 ml/min. The mobile phase consisted of 200 to 250 ml of CH_3CN (for tosufloxacin, 250 ml; for ofloxacin, 200 ml; and for norfloxacin, 220 ml), 60 ml of 1 M sodium citrate dibasic, and 100 ml of 10% methansulfonic acid–10% triethylamine solution in 1,000 ml of H_2O. The eluate was monitored at 320 nm. Quantitative standards were run for each drug, and a standard curve was determined by using the total area under the peak of interest, as determined by electronic integration.

RESULTS

MIC determination. The MICs of the three quinolones for *S. enteritidis* C-32, which were determined in Eagle growth medium, are given in Table 1. By this technique, the MIC of tosufloxacin against *S. enteritidis* C-32 was 0.0125 μg/ml, which was lower than those of ofloxacin and norfloxacin.

Localization of intracellular organisms. The intracellular localization of *S. enteritidis* C-32 was ascertained microscopically. The intracellular bacteria could be distinguished from the adhering bacteria (Fig. 1A). After 3 h of incubation, these organisms attached to the cells and then invaded WI-38 cells (Fig. 1B). There were 4.8 ± 0.62 viable intracellular organisms per WI-38 cell. *S. enteritidis* C-32 invaded the WI-38 cell and constantly multiplied in the WI-38 cell (Fig. 1C).

Intracellular bactericidal activity of quinolones against *S. enteritidis* C-32. After 9 h of incubation, intracellular *S. enteritidis* C-32 organisms multiplied to 10.2 ± 1.21 cells per WI-38 cell in the absence of quinolones. Tosufloxacin effectively killed intracellular *S. enteritidis* C-32 cells in WI-38 cells (Fig. 2), showing that the numbers of the viable organisms per WI-38 cell were 3.3 ± 0.57 and 2.4 ± 0.45 6 h after the addition of 0.2 and 0.78 μg of tosufloxacin per ml, respectively (Table 1). Tosufloxacin reduced the number of viable organisms more effectively than ofloxacin (4.7 ± 0.63 and 4.3 ± 0.54, respectively) or norfloxacin (8.2 ± 1.50 and 7.4 ± 1.30, respectively) did (Table 1).

Ability of quinolones to penetrate into cells. The intracellular concentration to the extracellular concentration ratio (C/E ratio; mean ± standard error) of the three quinolones, which was determined by the velocity gradient method with WI-38 cells, was 22.4 ± 1.4 for tosufloxacin, 8.6 ± 2.4 for ofloxacin, and 13.2 ± 1.2 for norfloxacin. Quinolones were well taken up by WI-38 cells. The intracellular concentration of quinolones in WI-38 cells was much higher than the extracellular concentration.

DISCUSSION

The ability of antibiotics to enter cells is an important factor affecting drug therapy for infections caused by intracellular parasites. There have been many reports on the ability of antibiotics to penetrate into cells and the bactericidal activities of antibiotics against intracellular organisms (1, 2, 6–8, 10, 11, 20). Easmon et al. (3) investigated ciprofloxacin therapy against systemic salmonella infections in mice and showed that the efficacy of ciprofloxacin therapy reflected both the in vitro activity of ciprofloxacin against *Salmonella typhimurium* and its good penetration into phagocytes (2). Havlichek et al. (9) have demonstrated that β-lactam antibiotics such as cefoxitin and thienamycin do not inhibit intraphagocytic *Legionella pneumophila* multiplication, despite their extracellular activities. On the other hand, quinolones showed good activity against cell-associated *L. pneumophila*. From the results of these reports with phagocytes, the intracellular activities of antibiotics are thought to depend not only on their extracellular activities but also on their ability to enter the cells.

However, there have been few reports dealing with the intracellular bactericidal activity of antibiotics and their ability to penetrate into cells of human origin other than phagocytes (14, 19). Recently, Une and Osada (19) investigated the intracellular bactericidal activity of ofloxacin against *Shigella* species in cultured epithelial cells without measuring the intracellular level of ofloxacin. Therefore, we evaluated both the intracellular bactericidal activity of tosufloxacin and its ability to penetrate into normal human cells in order to clarify in detail the reason for the good clinical response of tosufloxacin. The results of this study showed that tosufloxacin inhibits multiplication of *S. enteritidis* in WI-38 cells more effectively than ofloxacin and norfloxacin do. The C/E ratio of tosufloxacin was higher than those of ofloxacin and norfloxacin. This indicates that the potent bactericidal activity of tosufloxacin in normal human cells may be due not only to its high extracellular activity but also to its ability to penetrate into cells.

In conclusion, the potent intracellular activity of tosufloxacin against *S. enteritidis* is considered to be reflected by its high efficacy against intestinal *Salmonella* infections (salmonella is an intracellular pathogen) in clinical trials in Japan (Masuda et al., 28th ICAAC).

This indicates that tosufloxacin might be a useful agent against *Legionella*, *Staphylococcus*, and *Chlamydia* infections, as well as against *S. enteritidis* infections.

However, many important questions remain unanswered with regard to the intracellular antimicrobial activities of drugs and their ability to penetrate into cells. For example, quinolones need a much higher intracellular concentration than MICs in order to eliminate intracellular *S. enteritidis* C-32. Further work needs to be done to clarify the relationship between the intracellular and extracellular activities of drugs.

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