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Effects of New Quinolones on *Mycoplasma pneumoniae*-Infected Hamsters

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The efficacies of the new quinolones temafloxacin, ofloxacin, and ciprofloxacin were investigated against *Mycoplasma pneumoniae* in an experimental hamster pneumonia model. Hamsters were infected intratracheally with *M. pneumoniae* and sacrificed 18 h after the final medication, and their lungs were aseptically removed, homogenized, and cultured quantitatively. The efficacies of these drugs were determined by the CFU of *M. pneumoniae* in lungs. Temafloxacin and ofloxacin, but not ciprofloxacin, were active when the oral administration of 200 mg/kg of body weight per day (once per day) for 5 days was initiated 24 h after infection. Although no effect on the elimination of *M. pneumoniae* was observed after the administration of these drugs at 200 mg/kg/day at 5 days after infection, the continuous administration for 15 days of temafloxacin, but not ofloxacin or ciprofloxacin, significantly reduced viable *M. pneumoniae* in the lungs. These results suggest that temafloxacin and ofloxacin are effective in the acute phase of infection and, moreover, that temafloxacin is effective in the late stage of infection during which progressive lung alterations and continuous increases in mycoplasmal growth occurred. The peak levels of temafloxacin in sera and lungs after oral administration were similar to those of ofloxacin and higher than those of ciprofloxacin. The areas under the curve of temafloxacin in the lung tissue, however, were higher than those of ofloxacin and ciprofloxacin. On the basis of these results, temafloxacin and ofloxacin might be promising antimicrobial agents for the treatment of mycoplasmal infection.

Mycoplasma pneumoniae is the causative agent of upper respiratory tract infections and pneumonia in humans and the experimental pneumonia of hamsters (3, 5, 8, 10). Macrolide and tetracycline antibiotics have been widely used in chemotherapy against *M. pneumoniae* infections because of the susceptibility of the infectious agent to these antibiotics (1, 4, 13, 17, 23). At the same time, the reported incidences of mutants resistant to erythromycin from patients treated with or without the antibiotics have increased (14, 15).

Recently, the efficacy of new quinolones against various respiratory pathogens, including *M. pneumoniae*, has been demonstrated (4, 16, 22). We have also reported that temafloxacin, ofloxacin, and ciprofloxacin, among several new quinolones, possess more mycoplasmacidal activity against *M. pneumoniae* than macrolide and tetracycline antibiotics. In this report, we evaluated the in vivo potency of new quinolones, administered orally against *M. pneumoniae* pneumonia in hamsters, which exhibited the potent mycoplasmacidal activity in vitro described previously (1).

MATERIALS AND METHODS

***M. pneumoniae*.** *M. pneumoniae* 242, freshly isolated from the throat swab of a patient with mycoplasma pneumonia and passaged five to seven times in broth, was used as the challenge strain. Culture samples were kept at −80°C until used for inoculation. The MICs of temafloxacin, ofloxacin, and ciprofloxacin for the strain were 1.56, 3.12, and 3.12 μg/ml, and MBCs of these drugs were 12.5, 6.25, and 12.5 μg/ml, respectively, as described previously (1).

Hamsters. Young (female, 5- to 6-week-old, 60- to 80-g) healthy Syrian hamsters (SPF) were purchased from the Inoue Animal Center Co. Ltd. (Kumamoto, Japan).

Experimental *M. pneumoniae* model. Syrian hamsters were anesthetized subcutaneously with 0.3 ml of 2% sodium pentobarbital (Dainihon Pharmaceutical Co. Ltd., Osaka, Japan) and infected by the intratracheal peroral route as described by Barile et al. (3). Hamsters were mounted on a board slanted 30° from the vertical. The trachea of each hamster was cannulated with a blunt metal needle (23 gauge) by the oral route. The feel of the needle tip against the tracheal cartilage confirmed the location of the cannula. A 0.3-ml sample of the mycoplasma suspension (2×10^7 CFU) was delivered to each hamster via a syringe. The hamsters were maintained in a vertical position for 10 min.

Pulmonary clearance studies. To evaluate the intrapulmonary killing of mycoplasmas, hamsters were treated by the oral administration of temafloxacin, ofloxacin, and ciprofloxacin at the various doses with a bent metal cannula at 1 and 5 days after infection. Controls receive identical treatment with isotonic saline. The thorax of each animal, which had been anesthetized with 0.3 ml of 2% sodium pentobarbital, was opened, and the blood was pooled in the axilar space and removed with a capillary pipette. The lungs were dissected free from the trachea and other structures and homogenized in 4 ml of mycoplasma broth medium (Difco Laboratories, Detroit, Mich.).

Viable mycoplasmas in the homogenates were quantitated by plating 0.1 ml of serial 10-fold dilutions on Hayflick's mycoplasma agar plates.

Histologic studies. In some experiments, the lungs of the hamster were fixed by in situ intratracheal instillation of 10% phosphate-buffered formalin under controlled pressure. Thin

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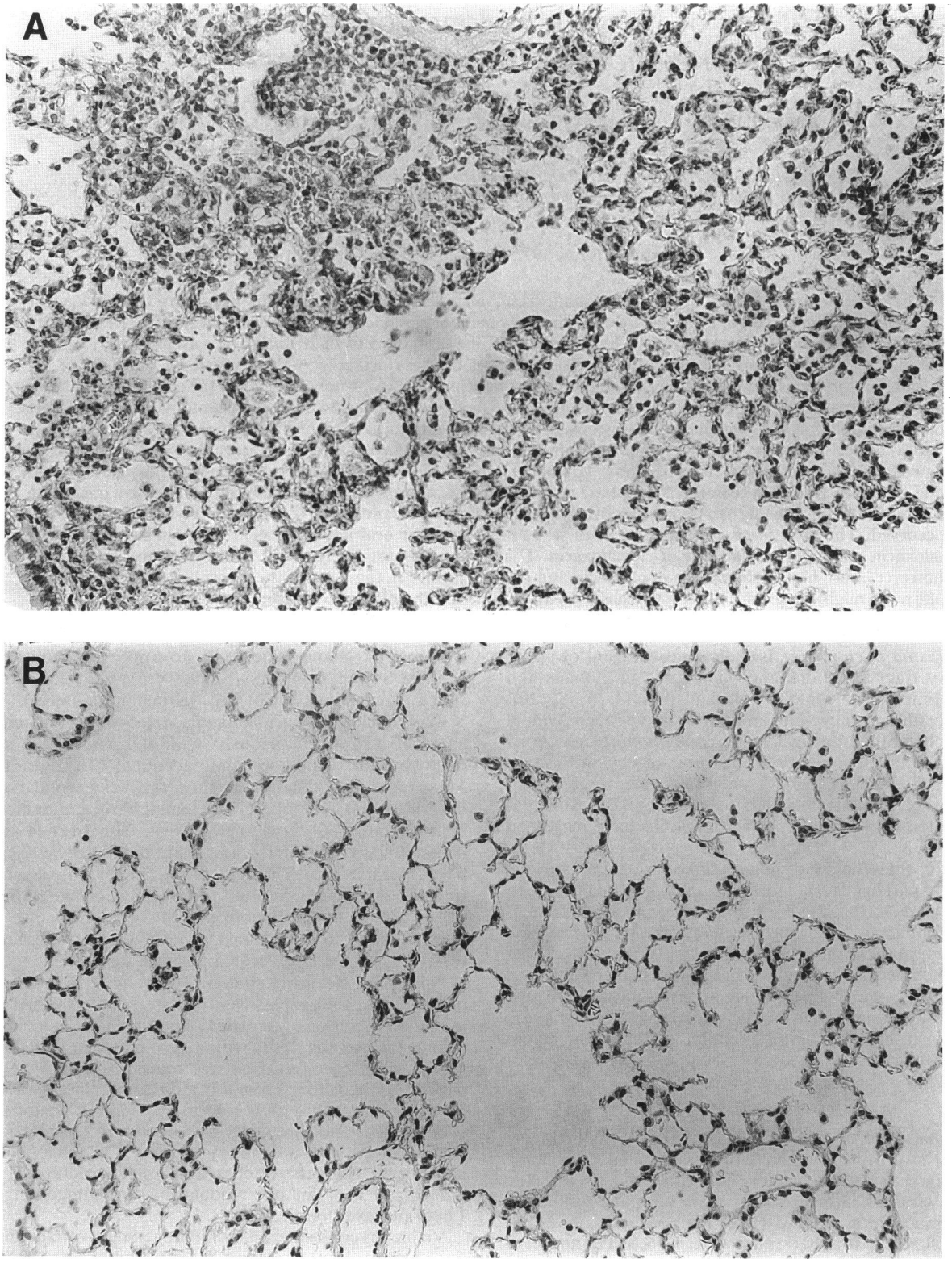

FIG. 1. Microscopic findings in hamster tissue on day 15 either after infection with *M. pneumoniae* (A) or with no *M. pneumoniae* infection (control) (B). Hematoxylin and eosin stain; magnification, ×100)

sections of lung (in paraffin) were stained with hematoxylin and eosin.

Detection of *M. pneumoniae* antibodies. Antibody titers in serum samples of *M. pneumoniae*-infected hamsters were measured by a metabolic inhibition test (MIT) (21) and an indirect hemagglutination (IHA) test as described previously (6). For the MIT, sera from hamsters (25 μl) were diluted in a twofold serial step in a microtiter plate (Nunc Co., Ltd., Copenhagen, Denmark). The broth suspension (25 μl) of *M. pneumoniae* FH (10^6 CFU/ml) was then added to each well except the one used as the medium control. Finally, each well received 150 μl of a freshly prepared mixture of 90% broth medium and 10% fresh human serum. The plates were sealed tightly and incubated at 37°C for 5 days. The highest serum dilution which prevented a color change of 50% (i.e., equivalent to approximately 0.25 pH unit) when compared with the same dilution of organisms grown without antiserum was recorded as the serum titer endpoint. The IHA test was performed by using Serodia Myco II (Fujirebio, Inc., Tokyo, Japan). A lyophilized preparation of gelatin particles sensitized with *M. pneumoniae* Mac antigen was added to twofold-diluted hamster serum in a microtiter plate. The plate was incubated at 15 to 25°C for 3 h, and the particle agglutinations were determined.

Pharmacokinetics in hamsters. The concentrations of temafloxacin, ofloxacin, and ciprofloxacin in the sera and lungs of female hamsters weighing 70 to 90 g were determined. Hamsters were fasted for 24 h before oral administration of the drugs. Blood and tissue samples were collected from groups of four hamsters at 0.125, 0.25, 0.5, 1, 2, 4, and 6 h after administration of the drugs. Blood was withdrawn from the femoral vein, kept at room temperature for 1 h, and centrifuged at 1,800 × *g* to obtain serum. Lungs were removed from exsanguinated hamsters, weighed and homogenized in 3 volumes of a phosphate buffer, and centrifuged at 1,800 × *g* for 20 min. The antibiotic concentrations in serum and lung samples were determined by a disk agar dilution bioassay procedure with *Bacillus subtilis* 6633 Kp used as the assay organism and heart infusion agar used as the growth medium for quinolones as described previously (9). Serum samples and standards were diluted in normal hamster serum, whereas tissue samples and standards were diluted in the phosphate buffer. For the pharmacokinetics in serum and lung samples, the areas under the curve (AUCs) were calculated by the log trapezoidal rule method from zeros to initiate time. The maximum drug concentrations (C_{max}) in serum and lung samples were graphically determined, and the time to C_{max} (T_{max}) was determined as the time to achieve the highest measured concentrations. The elimination half-life was calculated by dividing the natural logarithm of 2 by the elimination rate constant.

Antimicrobial agents. The following antimicrobial agents were used; temafloxacin (Tanabe, Osaka, Japan), ofloxacin (Daiichi, Tokyo, Japan), ciprofloxacin (Bayer, Osaka, Japan).

Statistical analysis. Means of CFU in each group of five hamsters were compared with those of the control group by Dunnett's multicomparison method (7).

RESULTS

Evaluation of the assay system. We first examined the development of the lung pathological findings, the colonization of the lung, and the rise of antibodies in the course of *M. pneumoniae* infection in hamsters. Histological studies of lung tissue from infected hamsters confirmed the presence of pneumonia. As shown in Fig. 1, the lungs were invaded by lymphocytes and polymorphonuclear cells, which thickened the alveolar walls by 14 days after infection. Perivascular infiltrated edema was also apparent. Interstitial and alveolar spaces were completely invaded by inflammatory cells, and bronchovascular areas were considerably distended with lymphoid patches. After infection with 7×10^6 CFU of *M. pneumoniae* per 0.2 ml per hamster, the colonies of *M. pneumoniae* in the lungs increased slowly, by ~1 $\log_{10}$ from 7 to 14 days. Thereafter, the numbers decreased gradually. Both MIT and IHA antibody titers increased 14 days after infection, and the mean antibody titers reached 76.1 and 46.0, respectively, 5 weeks after infection (Table 1). On the basis of our histological findings, antibody production indicates the establishment of a model of *M. pneumoniae* pneumonia in hamsters as described previously (3).

TABLE 1. Titers of MIT and IHA antibodies in *M. pneumoniae*-infected hamsters

Days after infection	Titer[a]	
	MIT	IHA
7	≤8	≤20
21	9.5	30.3
35	76.1	46.0

[a] Geometric mean titers of six hamsters in each test.

Pharmacokinetics profile in hamsters. After the oral administration of 50 mg of temafloxacin, ofloxacin, or ciprofloxacin per kg of body weight, the levels in serum and lung samples shown in Table 2 were reached. The peak levels of temafloxacin (11.82 μg/ml) and ofloxacin (25.5 μg/ml) in serum were higher than that of ciprofloxacin (1.34 μg/ml). The peak levels of temafloxacin (21.45 μg/ml) and ofloxacin (21.27 μg/ml) in lungs were also higher than that of ciprofloxacin (3.56 μg/ml). The AUCs of temafloxacin, ofloxacin, and ciprofloxacin in the serum samples were 23.0, 30.4, and 1.5 μg · h/ml, respectively. Those in lung samples were 55.3, 36.9, and 3.5 μg · h/ml, respectively. The half-lives of temafloxacin, ofloxacin, and ciprofloxacin in serum samples were 0.90, 0.82, and 1.07 h, respectively. The half-lives of these drugs in lung samples were 0.98, 0.97, and 0.62 h, respectively. These accumulated data suggest that temafloxacin and ofloxacin pharmacokinetics might be expected to have a therapeutic effect against *M. pneumoniae* infection.

In vivo assays. The effects of 5 days of oral administration of temafloxacin, ofloxacin, and ciprofloxacin after 24 h of infection were investigated. As shown in Fig. 2, the administration of 200 mg of temafloxacin or ofloxacin, but not

TABLE 2. Pharmacokinetics of temafloxacin, ofloxacin, and ciprofloxacin in hamsters after oral administration[a]

Tissue	Drug[b]	T_{max} (min)	C_{max}[c] (μg/ml)	$t_{1/2}$[d] (h)	AUC (μg · h/ml)
Serum	Temafloxacin	7.5	11.82 ± 1.91	0.90	23.0
	Ofloxacin	7.5	25.51 ± 8.22	0.82	30.4
	Ciprofloxacin	15.0	1.34 ± 0.30	1.07	1.5
Lung	Temafloxacin	30.0	21.45 ± 1.16	0.98	55.3
	Ofloxacin	7.5	21.27 ± 1.57	0.97	36.9
	Ciprofloxacin	7.5	3.56 ± 1.43	0.62	3.5

[a] Values are the means of four hamsters.
[b] Drugs were administered orally at doses of 50 mg/kg.
[c] Peak concentration (means ± standard deviations).
[d] Half-lives in serum and lung samples.

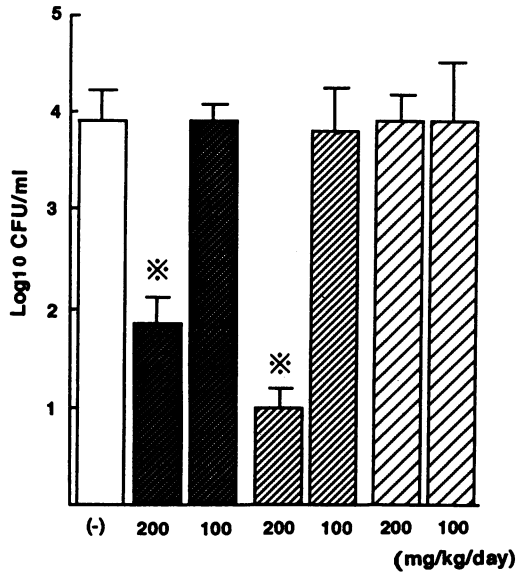

FIG. 2. Activity of temafloxacin, ofloxacin, and ciprofloxacin administered orally for 5 days against *M. pneumoniae* pneumonia in hamsters, with administration initiated after 24 h of infection. Symbols: □, control; ■, temafloxacin; ▨, ofloxacin; ▨, ciprofloxacin. Values are the means ± standard deviations of five hamsters. An asterisk indicates a significant difference between the drug administration and the control ($P < 0.05$).

ciprofloxacin, was markedly effective in reducing the counts of viable *M. pneumoniae* in the lungs. These drugs however, were not effective when administered at 100 mg/kg/day. To determine the effects of these drugs on the reducing mycoplasmas which were colonized in the lung tissue, they were administered after 5 days of infection. The effects of 5 and 15 days of oral administration of these drugs on the counts of *M. pneumoniae* are shown in Fig. 3. The 5-day oral administration of temafloxacin, ofloxacin, or ciprofloxacin at 200 mg/kg/day was not effective, but the 15-day administration of temafloxacin, but not that of ofloxacin or ciprofloxacin, at 200 mg/kg/day significantly reduced the numbers of viable *M. pneumoniae*. The activities of temafloxacin were significantly superior to those of ofloxacin. Temafloxacin administered at 100 mg/kg twice per day was also effective in reducing the count of *M. pneumoniae* (Table 3).

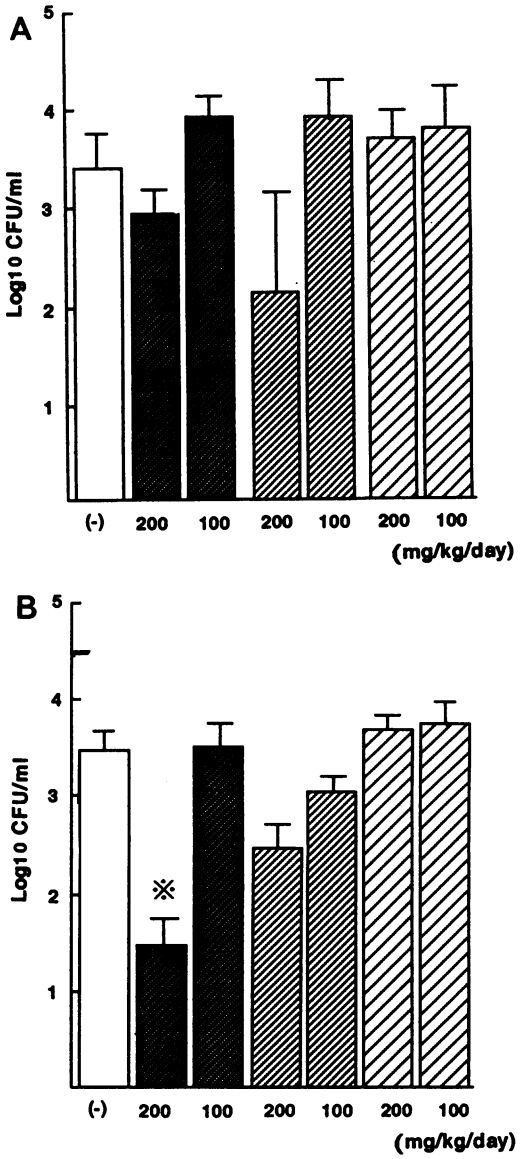

FIG. 3. Activity of temafloxacin, ofloxacin, and ciprofloxacin administered orally against *M. pneumoniae* pneumonia in hamsters for 5 (A) or 15 (B) days, with administration initiated after 5 days of infection. Symbols: □, control; ■, temafloxacin; ▨, ofloxacin; ▨, ciprofloxacin. Values are the means ± standard deviation of five hamsters. Asterisk indicates a significant difference between the drug administration and the control ($P < 0.05$).

DISCUSSION

In studies on the pathogenesis of *M. pneumoniae* infection, Syrian hamsters have proved to be a useful experimental model. Intratracheal inoculation of hamsters with *M. pneumoniae* results in sequences of pathological and immunological changes resembling those that occur in natural human disease (3, 5, 8). The purpose of the present study was to evaluate in vivo the antimycoplasmal activities of new quinolones by using hamsters infected intratracheally with *M. pneumoniae* by the method of Barile et al. (3), which was highly reproducible. There is little information on the preclinical prediction of antimycoplasmal activity of antimicrobial agents by using *M. pneumoniae*-infected models. On the basis of our previous report, we selected three new quinolones, temafloxacin, ofloxacin, and ciprofloxacin, which had low MICs and MBCs and were expected to be effective against mycoplasmal infection. Our results show that their efficacies appear to depend on the time after infection at which therapy is initiated. At an early stage (24 h after infection), temafloxacin and ofloxacin were active, but 120 h after infection, with increased mycoplasmal growth and inflammation progressing in the lung, ofloxacin was less active than in the case of the administration of the drug at the initial stage of infection. This evidence of the decreased efficacy of ofloxacin at advanced stages of disease has also been reported in antipneumococcal activity (2).

Little information was available for a profile of the pharmacokinetics of new quinolones in noninfected or infected hamsters. Pharmacokinetic data in uninfected hamsters showed that temafloxacin achieved higher concentrations in

TABLE 3. Effects of dose size and frequency of daily administration of temafloxacin and ofloxacin against *M. pneumoniae* infection

Drug	Dose (mg/kg)	No. of doses/day	Log_{10} CFU/ml ± SD[a]
Temafloxacin	200	1	2.0 ± 0.5*
	100	2	2.2 ± 0.3*
	100	1	4.8 ± 0.8
Ofloxacin	200	1	1.7 ± 0.5*
	100	2	3.0 ± 0.2
	100	1	4.0 ± 0.7
Control			3.6 ± 0.8

[a] Values are the means ± standard deviations for five hamsters. An asterisk indicates a significant difference between the drug administration and the control ($P < 0.05$).

serum and lung samples, longer half-lives in serum and lung samples, and a greater AUC than that of ofloxacin or ciprofloxacin. Moreover, in pneumococcus-infected mice, concentrations of temafloxacin in serum and lung samples were greater than those of uninfected mice, and despite the differences in the dose used, the serum AUCs are of the same order of magnitude, i.e., 23.0 μg · h/ml after an oral administration of 50 mg/kg in hamsters versus 23.4 μg · h/ml after a 300-mg administration in humans (2).

After a single oral dose of 50 mg of temafloxacin or ofloxacin per kg in hamsters, the concentrations exceeded the MIC and MBC for *M. pneumoniae* 242 for approximately 2.5 h in serum and 3.0 h in lungs. The concentrations of ciprofloxacin exceeded the MIC (3.12 μg/ml) for a short period but failed to exceed the MBC (6.25 μg/ml) in both serum and lung samples. Therefore, therapeutic concentrations of temafloxacin and ofloxacin persisted in the lungs of hamsters longer than the therapeutic concentration of ciprofloxacin did. It has been reported that temafloxacin and ofloxacin showed better absorption, greater AUC values, and higher peak levels and longer half-lives in serum and lung samples than ciprofloxacin did in mice (11), guinea pigs (20), and humans (12, 19), although the bioavailability of ciprofloxacin is slightly lower than that of temafloxacin or ofloxacin (18). Although the reason is not clear why ciprofloxacin absorption was low compared with that of temafloxacin or ofloxacin, cations, such as aluminum, magnesium, and multivitamin preparations containing zinc, have a very significant effect on the absorption of quinolones and, in the case of ciprofloxacin, dramatically reduce serum concentrations (19). Therefore, ciprofloxacin absorption was affected by cations in the gastrointestinal trace of hamsters more than that of temafloxacin or ofloxacin even after a 12-h fast. Although another possibility is that *C. difficile* infection might prevent drug absorption, we did not find diarrhea among the hamsters that were used for experiments within the course of quinolone therapy.

Niitu et al. reported the development of erythromycin resistance in *M. pneumoniae* that was accompanied by cross-resistance to other macrolide antibiotics in vitro; they also reported the isolation of *M. pneumoniae* that was resistant to erythromycin and other antibiotics after erythromycin therapy (14, 15). New quinolones might exhibit antimycoplasmal activity against macrolide- and tetracycline-resistant strains of *M. pneumoniae* because of their different mechanisms of action. Furthermore, new quinolones show excellent penetration into lung tissues and, in particular, bronchial secretions. The mycoplasmacidal activities and favorable pharmacokinetic profiles of temafloxacin and ofloxacin described above suggest that these antimicrobial agents might be useful in treating respiratory tract infections caused by *M. pneumoniae*.

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