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Susceptibility of *Legionella pneumophila* to Ofloxacin In Vitro and in Experimental *Legionella* Pneumonia in Guinea Pigs

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The antimicrobial activity of ofloxacin was tested against 15 standard strains and 37 clinical and environmental strains of *Legionella pneumophila* by agar dilution susceptibility studies with a new growth medium. The ofloxacin MICs were inoculum dependent and ranged from 0.03 to 0.125 μg/ml. The antibacterial activities of other agents tested relative to ofloxacin were rifampin > ofloxacin > josamycin > pipemidic acid. Ofloxacin, at concentrations equal to or greater than 0.05 μg/ml, inhibited the growth of *L. pneumophila* grown in human monocytes. The therapeutic efficacy of ofloxacin in experimental guinea pig *L. pneumophila* pneumonia was greater than that observed with erythromycin or josamycin therapy; it was less effective than was rifampin. Ofloxacin was very active against intracellular *L. pneumophila* in these experiments and should be studied in the therapy of human Legionnaires disease.

Ofloxacin (DL-8280) is a new synthetic antibacterial agent derived from benzoxazine. This agent has a broad antibacterial spectrum and strong antimicrobial activity against both gram-positive and gram-negative bacteria. It has excellent potency in vitro against *Haemophilus influenzae*, *Pseudomonas aeruginosa*, and obligate anaerobes including *Bacteroides fragilis*. Clinical trials have demonstrated efficacy in the treatment of respiratory infectious diseases (K. H. Spitzy and K. Karrer, Proc. 13th Int. Congr. Chemother., p. 125/1–125/83, 1983).

Legionella species, which are the causative organisms of Legionnaires disease, are susceptible to many chemotherapeutic agents which are ineffective clinically (4, 7). The main reason for this appears to be that only drugs which can penetrate phagocytic cells can attack *Legionella* spp. in their intracellular niche (9, 18). We therefore studied the activity of ofloxacin against *L. pneumophila* in an animal model of infection, as well as in infected monocytes.

MATERIALS AND METHODS

In vitro susceptibility testing. Agar dilution susceptibility testing was performed with buffered starch-yeast extract medium; its formula is shown in Table 1. This medium had previously been shown to cause less inactivation of erythromycin than did buffered charcoal-yeast extract alpha medium (20). The bacterial strains tested were 15 standard reference strains of *Legionella* spp. provided by the Centers for Disease Control, Atlanta, Ga., and Veterans Administration Wadsworth Medical Center, Los Angeles, Calif., and 37 nonstandard isolates of *L. pneumophila*. Of the 37 *L. pneumophila* isolates, 16 were clinical serogroup 1 strains isolate at the Veterans Administration Wadsworth Medical Center, 5 were clinical isolates from Japan (4 of serogroup 1 and 1 of serogroup 3), and 16 were environmental isolates from Japan (13 strains of serogroup 1, and 1 each of serogroups 3, 4, and 6). *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as the control strains.

Ofloxacin and rifampin were a gift of Daiichi Seiyaku Co., Ltd. Erythromycin was a donation from Abbott Laboratories, North Chicago, Ill.; josamycin was from Yamanouchi Pharmaceutical Inc.; pipemidic acid from Dainippon Pharmaceutical Inc. The concentration of each antibiotic agent tested was 64 to 0.03 μg/ml, in serial doubling dilutions.

After subculturing on buffered charcoal-yeast extract agar, all the strains tested were incubated with shaking in buffered starch-yeast extract broth at 35°C for 18 h. Final inocula of both 10^4 and 10^6 CFU, prepared by dilution of buffered starch-yeast extract broth, were applied to agar by a replication spot device. Plates were incubated at 35°C for 48 h. The MIC was defined as the lowest concentration of

TABLE 1. Composition buffered starch-yeast extract agar medium[a]

System	material	Amt (g)	Source
A	Basal medium		
	Yeast extract	10.0	Difco Laboratories, Detroit, Mich.
	N-(2-Acetamide)-2-aminoethyl sulfonic acid	10.0	Aldrich Chemical Co., Inc., Milwaukee, Wis.
	Potassium hydroxide	2.5	Sigma Chemical Co., St. Louis, Mo.
	Sodium L-glutamate	5.0	
	Soluble starch	15.0	Difco
	Agar	15.0	Difco
	Purified water	980 ml	
B	L-Cysteine hydrochloride	0.4	ICN Pharmaceuticals Inc., Cleveland, Ohio
	Purified water	10 ml	
C	Ferric PP_i	0.25	Mallinckrodt, Inc., St. Louis, Mo.
	Purified water	10 ml	

[a] System A was autoclaved at 121°C for 15 min and cooled to 55°C; filter-sterilized systems B and C were added to A, and the mixture was adjusted to pH 6.90 ± 0.05 with 1 N KOH.

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TABLE 2. Susceptibility of standard reference strains of *Legionella* spp. to ofloxacin and other antibiotic agents

Bacterial strain (serogroup)	Inoculum (CFU/spot)	MIC (μg/ml)				
		Ofloxacin	Pipemidic acid	Erythromycin	Josamycin	Rifampin
L. pneumophila						
ATCC 33152 (1, Ph. 1)	10^6	0.0625	4.0	0.25	0.5	≤0.0313
	10^4	≤0.0313	2.0	0.125	0.25	≤0.0313
ATCC 33153 (1, Kx. 1)	10^6	≤0.0313	2.0	0.125	0.25	≤0.0313
	10^4	≤0.0313	2.0	0.125	0.25	≤0.0313
ATCC 33154 (2)	10^6	0.0625	4.0	0.25	0.5	≤0.0313
	10^4	≤0.0313	2.0	0.125	0.25	≤0.0313
ATCC 33155 (3)	10^6	0.0625	2.0	0.125	0.25	≤0.0313
	10^4	≤0.0313	0.5	0.125	0.25	≤0.0313
ATCC 33156 (4)	10^6	0.0625	2.0	0.125	0.5	≤0.0313
	10^4	≤0.0313	0.5	0.125	0.25	≤0.0313
ATCC 33216 (5)	10^6	0.0625	2.0	0.25	0.5	≤0.0313
	10^4	≤0.0313	1.0	0.125	0.5	≤0.0313
ATCC 33215 (6)	10^6	0.0625	4.0	0.5	0.5	≤0.0313
	10^4	≤0.0313	2.0	0.25	0.5	≤0.0313
L. micdadei						
ATCC 33218 (1)	10^6	≤0.0313	0.5	0.125	0.5	≤0.0313
	10^4	≤0.0313	0.5	≤0.0313	≤0.0313	≤0.0313
L. bozemanii						
ATCC 33217 (1)	10^6	0.0625	2.0	0.5	0.25	≤0.0313
	10^4	≤0.0313	1.0	0.125	0.125	≤0.0313
L. dumoffii						
ATCC 33279 (1)	10^6	0.0625	2.0	0.25	0.5	≤0.0313
	10^4	≤0.0313	1.0	0.125	0.25	≤0.0313
L. longbeachae						
ATCC 33462 (1)	10^6	≤0.0313	2.0	0.25	0.25	≤0.0313
	10^4	≤0.0313	0.5	≤0.0313	0.0625	≤0.0313
ATCC 33483 (2)	10^6	≤0.0313	2.0	0.125	0.25	≤0.0313
	10^4	≤0.0313	0.5	≤0.0313	≤0.0313	≤0.0313
L. gormanii						
ATCC 33279 (1)	10^6	≤0.0313	2.0	0.125	0.0625	≤0.0313
	10^4	≤0.0313	0.5	≤0.0313	≤0.0313	≤0.0313
L. jordanis						
ATCC 33623 (1)	10^6	≤0.0313	0.5	0.5	0.5	≤0.0313
	10^4	≤0.0313	1.0	≤0.0313	≤0.0313	≤0.0313
L. wadsworthii						
ATCC 33877 (1)	10^6	0.0625	2.0	0.25	0.5	≤0.0313
	10^4	≤0.0313	2.0	0.0625	0.125	≤0.0313
E. coli						
ATCC 25922	10^6	0.125 (0.0625[a])	4.0 (4.0)	64< (64<)	64< (64<)	16.0 (8.0)
	10^4	0.125 (0.0625)	2.0 (2.0)	64< (64)	64< (64<)	8.0 (8.0)
S. aureus						
ATCC 25923	10^6	0.5 (0.5)	64 (64)	0.25 (0.125)	1.0 (0.5)	≤0.0313 (≤0.0313)
	10^4	0.25 (0.25)	64 (32)	0.125 (0.125)	0.25 (0.25)	≤0.0313 (≤0.0313)

[a] Numbers in parentheses are MICs obtained with Mueller-Hinton medium (pH 6.9).

antibiotic that inhibited development of visible growth on agar.

Effect of ofloxacin on *L. pneumophila* grown in human monocytes. Blood samples were collected from healthy adult volunteers, and the monocytes were fractionated by the Ficoll-Conray specific gradient method (2). *L. pneumophila* serogroup 1 (Philadelphia 1, ATCC 33152) was added to RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd., Japan) containing 15% fetal calf serum for a final concentration of 5×10^3 to 5×10^5 CFU/ml of *L. pneumophila*; monocytes were then added for a final concentration of 2.0×10^6/ml. The mixture was rotated for 1 h at 37°C and was then transferred to a 37°C, 5.0% CO_2 incubator. After 24 h, ofloxacin was added in five different concentrations ranging

from 0.005 to 5.0 μg/ml. The concentration of *L. pneumophila* in the broth was determined by viable plate counts on buffered charcoal-yeast extract agar at intervals of 24 h until day 4. A bacteria-monocyte suspension without added ofloxacin was used as a growth control, as was *L. pneumophila* inoculated in RPMI 1640 medium without monocytes.

Concentrations of ofloxacin in guinea pig serum and tissue. Ofloxacin was given orally in a dose of 20 mg/kg to each of 13 male Hartley guinea pigs weighing 280 to 320 g. Animals were sacrificed in groups of three by administration of CO_2 gas at 0.5, 1, 2, 4, and 6 h after administration of ofloxacin. Ofloxacin concentrations in serum, lungs, liver, kidneys, and spleen were measured by the paper disk (8.0 mm in diameter) method with *E. coli* Kp as the indicator organism (Spitzy and Karrer, 13th Int. Congr. Chemother.). Phosphate buffer solution (0.1 M, pH 7.0) was used as the diluent.

Experimental pneumonia in guinea pigs. Animals were infected according to the method of Pennington and Ehrie (17). *L. pneumophila*, serogroup 1 (80-045), which was isolated from the first case in Japan, was used as the challenge strain (19). The MIC of ofloxacin for the organism was 0.0625 μg/ml with 10^6 CFU per spot and 0.0313 μg/ml with 10^4 CFU per spot as the inoculum size. The guinea pigs were anesthetized with an intraperitoneal injection of a mixture of 5.0 mg of xylaxine sulfate (Celactal, Bayer AG) per ml and 100 mg of ketamine sulfate (Ketalar, Sankyo Pharmaceutical Inc.) per ml. Lidocaine (0.5%) was used as a local anesthetic. The trachea of each animal was then exposed, and 0.5 ml of bacterial suspension was infused into the trachea with a syringe equipped with a 26-gauge needle.

To determine the 50% lethal dose (LD_{50}), six different doses of bacterial suspension ranging from 1.9×10^2 to 1.9×10^8 CFU/0.5 ml were inoculated into groups of five guinea pigs. The bacterial suspension used in all animal studies was frozen at −80°C in tubes containing 1.9×10^9 CFU/ml. A new tube was used for each experiment. Growth from the plate was suspended in 0.01 M phosphate-buffered saline (pH 7.2) to match the turbidity of a McFarland no. 5 barium sulfate standard. The actual cell count was done at the time of challenge. The LD_{50} was calculated with the Bahrens-Karber method (13). For the treatment study, each animal was given 10 times the LD_{50} in a 0.5-ml volume. Antibiotic treatment was started 24 h after the challenge. The drugs were mixed with 0.25% sodium carboxymethyl cellulose (Kishida Chemical Co., Ltd., Japan) to solubilize them; they were administered in 1.0-ml volumes through an orogastric tube twice a day for 7 days. The doses used daily were as follows (in milligrams per kilogram): ofloxacin, 10 and 50; rifampin, 7.5 and 37.5; and erythromycin and josamycin 20 and 100 each. The lower dose was the same as a usual human dose based on weight, while the higher dose was five times greater. The therapeutic efficacy of each antibacterial agent was evaluated on the basis of survival rates followed for 10 days after bacterial challenge.

The therapy trial in guinea pigs was repeated to confirm the original results. Twenty, rather than five, animals per treatment group were studied. The observation period was also prolonged to 14 days.

TABLE 3. Antimicrobial activities against 37 strains of *L. pneumophila*

Drug	Inoculum (CFU/ml)	MIC_{50}[a] (μg/ml)	MIC_{90}[a] (μg/ml)	Range
Ofloxacin	10^4	≤0.03	≤0.03	≤0.03
	10^6	0.0625	0.0625	≤0.03–0.125
Rifampin	10^4	≤0.03	≤0.03	≤0.03
	10^6	≤0.03	≤0.03	≤0.03
Erythromycin	10^4	0.125	0.25	≤0.03–0.25
	10^6	0.25	0.5	0.0625–0.5
Josamycin	10^4	0.25	0.25	0.0625–0.5
	10^6	0.5	1.0	0.125–1.0
Pipemidic acid	10^4	2.0	2.0	1.0–4.0
	10^6	2.0	4.0	1.0–8.0

[a] Concentration required to inhibit 50 and 90% of strains, respectively.

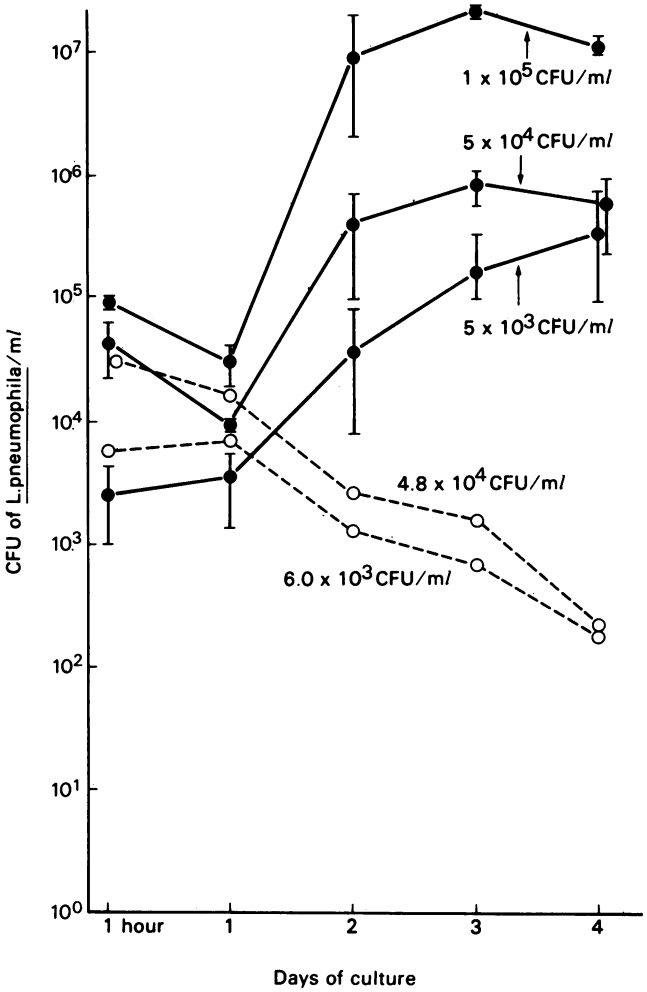

FIG. 1. Growth of *L. pneumophila* in RPMI 1640 (○) and in human monocytes (●). Vertical bars, standard deviation.

RESULTS

Antimicrobial activity in vitro. The MIC of ofloxacin for 15 standard reference strains of *Legionella* spp., *E. coli* ATCC 25922, and *S. aureus* ATCC 25923 are shown in Table 2. Rifampin had the greatest activity, with MICs lower than 0.0313 μg/ml for all strains of *Legionella* at both inoculum sizes. Ofloxacin was the next most active drug, with mean MICs lower than 0.0313 μg/ml for the small inoculum and 0.0625 μg/ml for the large inoculum. Pipemidic acid, which is a synthetic antibacterial agent of the same origin as

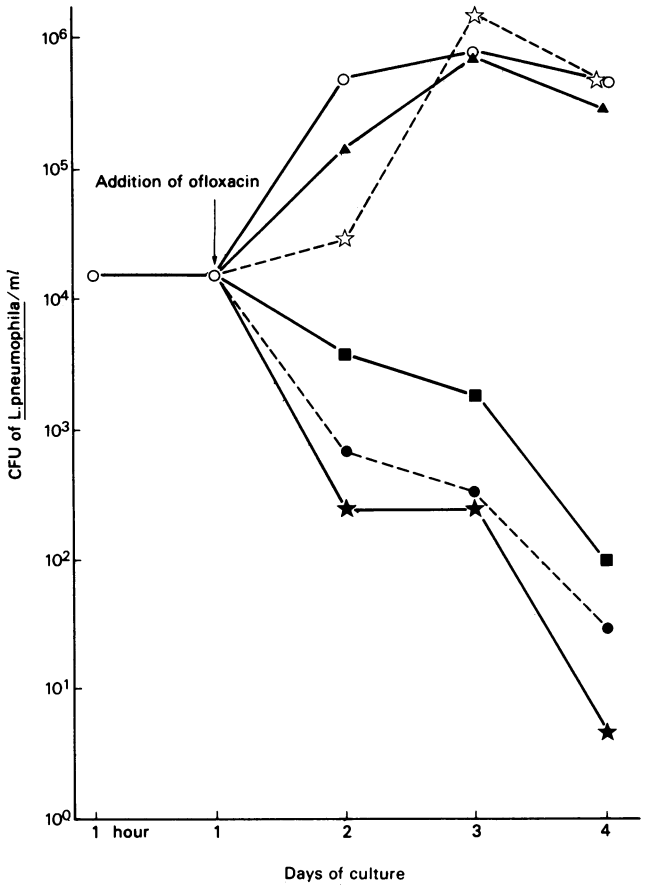

FIG. 2. Effects of ofloxacin against *L. pneumophila* in its proliferation within human monocytes. Symbols: ○, control; ★, ofloxacin, 5 μg/ml; ●, 0.5 μg/ml; ■, 0.05 μg/ml; ▲, 0.005 μg/ml; ☆, 0.0005 μg/ml.

ofloxacin, showed extremely weak activity, with mean MIC of 1.0 and 2.0 μg/ml for small and large inocula, respectively. The MIC of ofloxacin were two to four times lower than those of erythromycin and two to three times lower than those of josamycin.

MIC of five antibacterial agents against the 37 nonstandard strains of *L. pneumophila* are shown in Table 3. The results obtained were similar to those found with the standard reference strains.

Antimicrobial activity of ofloxacin against *L. pneumophila* grown in human monocytes. The survival curves of *L. pneumophila* in RPMI 1640 medium and *L. pneumophila* multiplication in human monocytes are shown in Fig. 1. *L. pneumophila* did not grow in the RPMI 1640 medium alone and gradually died. On the other hand, the bacterial cells in the RPMI 1640 medium with human monocytes tended to decrease slightly at day 1 but increased by day 2. Results of adding ofloxacin to the bacteria-monocyte suspensions are shown in Fig. 2. Ofloxacin reduced bacterial growth rates in the monocyte suspension at almost the same concentration (0.05 μg/ml) as the MIC found in vitro for the same strain (0.0625 μg/ml).

Treatment of experimental pneumonia in guinea pigs. Tissue and serum concentrations of ofloxacin of guinea pigs are shown in Fig. 3. The LD_{50} was determined to be 1.5×10^5 CFU; thus, the challenge dose used in the treatment study was 1.5×10^6, given in 0.5 ml.

The efficacies of the antimicrobial agents given at the lower dosage tested were as follows. All animals in the control group died on day 4 or 5. The survival rates up to day 10 after the challenge were 100% in groups treated with rifampin, 80% in those treated with ofloxacin, 60% in those treated with erythromycin, and 0% in the josamycin-treated group. The results of high-dosage therapy differed in that all animals survived in the rifampin-, ofloxacin-, and erythromycin-treated groups, whereas no josamycin-treated animal survived. The results of the repeat experiments with groups of 20 guinea pigs are shown in Fig. 4. Almost the same therapeutic efficacies were observed.

DISCUSSION

Since *L. pneumophila* produces beta-lactamase, some of the beta-lactam antibiotics have little or no antibacterial activity (5, 15, 16). Beta-lactam agents of the second or third generation are stable to beta-lactamase and show high activity against *L. pneumophila* in vitro. However, they are ineffective clinically for the treatment of Legionnaires disease. Also, they are not effective in the treatment of experimental pneumonia due to *L. pneumophila* (6, 7, 14, 21). The major reason for this is probably the intraphagocytic location of *L. pneumophila*; beta-lactam antibiotics cannot penetrate into these cells (11, 18). Erythromycin and rifampin are currently used as therapeutic agents for Legionnaires disease and have shown excellent efficacy in many cases (6, 14). They are also effective in the treatment of experimental pneumonia (3, 7, 8).

These two antibiotics are concentrated by the liver, and if the patients have highly advanced hepatic dysfunction, the agents cannot be used for treatment. Also, if patients are allergic to these drugs or if they develop side effects, no other drug with established clinical efficacy is available. Clinical studies of chloramphenicol, tetracyclines (minocycline and doxycycline), or sulfamethoxazole-trimethoprim have not been done.

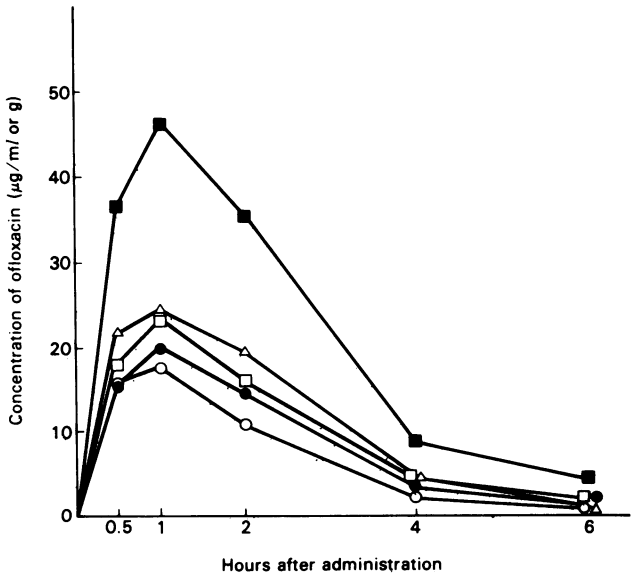

FIG. 3. Serum and tissue concentration (mean of three animals) of ofloxacin in guinea pigs (20 mg/kg, administered orally). Symbols: ○, serum; ●, lung; □, liver; ■, kidney; △, spleen.

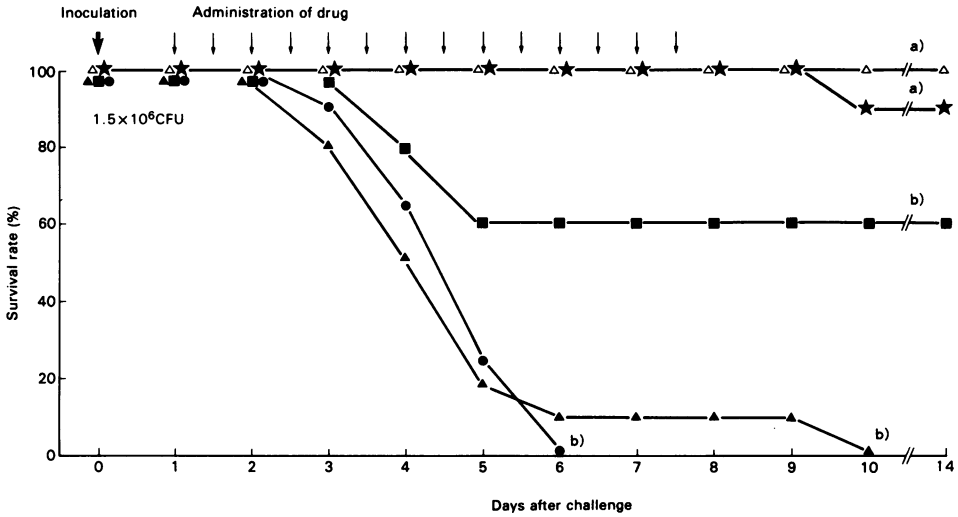

FIG. 4. Therapeutic effect of antibacterial agents (low doses) against experimental *Legionella* pneumonia. A preliminary study with fewer animals gave almost identical results. Symbols: △, ofloxacin, 10 mg/kg per day; ★, rifampin, 7.5 mg/kg per day; ■, erythromycin, 20 mg/kg per day; ▲, josamycin, 20 mg/kg per day; ●, control. Each group contained 20 animals. a, No significant difference; b, significantly different ($P < 0.01$) from ofloxacin.

Ofloxacin, in a concentration lower than the in vitro MIC, had high activity against *L. pneumophila* in human monocytes. This finding suggests that the drug would penetrate into human monocytes in high concentrations and would be effective in treating humans. Horwitz, Bacheson, and their colleagues (1, 10) have reported on the growth of *L. pneumophila* in human monocytes and on the effects of adding erythromycin and rifampin to the bacteria-monocyte suspension; they found that both of these drugs inhibited intracellular bacterial growth. Our study showed similar results with ofloxacin.

With regard to the measurement of ofloxacin concentrations in serum and tissue, guinea pigs were killed with CO_2 gas after the development of pulmonary edema; this may have altered ofloxacin concentrations in lung tissue or blood. Also, we used phosphate buffer as a diluent rather than the relevant tissue suspensions or serum. This method has some problems, as reported by Kaplan et al. (12).

Previous studies have been reported on other animal models of *Legionella* infection, with either intraperitoneal or aerosol inoculation. The former does not produce significant pneumonia, and the latter is very expensive to perform. Our method was simple to use and mimicked the results found in human disease. This method could possibly be applied to many other types of experimental bacterial pneumonia.

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