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Intracellular Activity of Azithromycin against Bacterial Enteric Pathogens

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Azithromycin, a new azalide antibiotic, is active in vitro against a variety of enteric bacterial pathogens. Since it is concentrated inside human neutrophils and other cells, it might be particularly useful in the treatment of infections caused by enteropathogens that invade host tissues. The intracellular activity of azithromycin against several enteric pathogens that had been phagocytosed by neutrophils was determined. Azithromycin was effective in reducing the intracellular viabilities of almost all strains tested, including representative strains of *Salmonella*, *Shigella*, and enteroinvasive, enteropathogenic, enterotoxigenic, and enterohemorrhagic *Escherichia coli*. Erythromycin was also effective in this model system, although azithromycin was generally more effective than erythromycin against strains of invasive enteric pathogens. Cefotaxime reduced intracellular bacterial viability to a lesser extent than either azithromycin or erythromycin. The presence of neutrophils did not significantly affect the activity of azithromycin in this system. Azithromycin may be a useful agent for the treatment of bacterial diarrhea, and clinical trials should be considered.

Infectious diarrhea is responsible for a high degree of morbidity and mortality worldwide (26, 27). While oral rehydration remains the primary therapy, treatment with antibiotics may be useful for patients with severe systemic illness (18), particularly illness caused by invasive bacterial pathogens (59), and may reduce morbidity, mortality, and the subsequent spread of the infectious agents.

Unfortunately, the progressive development of resistance to the commonly used antimicrobial agents has limited their effectiveness (8, 12, 44). In certain areas more than 80% of some species of *Shigella* and *Salmonella* are resistant to trimethoprim-sulfamethoxazole and to most or all of the other useful oral agents (11, 30, 37). While the fluoroquinolones have activity against a broad spectrum of bacterial enteropathogens, they are not yet approved for use in children and there have been some reports of resistance to these agents as well (1, 62). Oral broad-spectrum cephalosporins could also be effective, but more extensive use of these agents will likely select for organisms carrying TEM-like β-lactamases, as has occurred in the hospital environment (45). Erythromycin, an older macrolide, has been used to decontaminate the gastrointestinal tracts of patients undergoing chemotherapy (3) and to prevent traveler's diarrhea (4). While organisms from the family *Enterobacteriaceae* are typically resistant to erythromycin, with MICs ranging from 2 to 256 μg/ml, intraluminal levels in the intestine after oral administration are usually greater than these MICs (3). However, the use of erythromycin is associated with a high incidence of gastrointestinal side effects, and erythromycin would presumably not be effective in treating organisms which penetrate beyond the intestinal lumen. Thus, new therapies are needed for the treatment of bacterial diarrhea.

A variety of bacteria are commonly isolated from patients with infectious diarrhea (26). Some of these act, in part, by invading host tissues, including *Salmonella* spp. (33), *Shigella* spp. (61), enteroinvasive *Escherichia coli* (EIEC) (38), and enteropathogenic *E. coli* (EPEC) (19) and could potentially remain protected from the actions of extracellular antibiotics. Azithromycin, a new azalide antibiotic (52, 55), has good in vitro activity against a large number of bacterial enteric pathogens (25, 34, 35, 41). In addition, it is concentrated inside neutrophils (polymorphonuclear leukocytes [PMNs]) (22, 42, 49, 67) and other host cells (23, 39, 68) and may be delivered to sites of infection by PMNs (9, 21, 22, 56). Since invasive bacterial enteropathogens may be phagocytosed by PMNs and can potentially evade their antimicrobial systems, an antibiotic such as azithromycin that reaches high intracellular levels could be particularly useful for the treatment of these invasive infections.

We examined the intracellular activity of azithromycin against a variety of bacterial enteropathogens that had been phagocytosed by PMNs and compared its activity with those of erythromycin, another macrolide which reaches high levels inside PMNs (14, 29, 31, 54), and cefotaxime, a broad-spectrum cephalosporin which, like other β-lactam antibiotics, penetrates poorly into PMNs (28).

MATERIALS AND METHODS

Special reagents. Azithromycin was obtained from Pfizer (Groton, Conn.) and was dissolved in 80% ethanol at 10 g/liter as a stock solution. Erythromycin, cefotaxime, and other reagents were obtained from Sigma (St. Louis, Mo.) unless indicated otherwise.

Bacterial strains. The strains of bacteria used in the study are listed in Table 1. All organisms, which were stored at −70°C, were from the culture collection of the Center for Infectious Diseases and were identified to the species level by routine biochemical tests. Identification of EIEC, EPEC, enterotoxigenic *E. coli* (ETEC), and enterohemorrhagic *E. coli* (EHEC) was performed by using previously described gene probes (10, 40, 47, 66). MICs (Table 1) were determined by dilution in Mueller-Hinton broth (Difco, Detroit, Mich.) at pH

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TABLE 1. Bacterial strains used in the study

Species or group	Strain	Reference or source	MIC (μg/ml)[a]		
			Azithromycin	Erythromycin	Cefotaxime
Salmonella typhi	S30	6	0.5	16	0.0625
Salmonella enteriditis	HD4052	6	1.0	16	0.125
Shigella dysenteriae	60R	53	1.0	8	0.0312
Shigella sonnei	CP171	53	1.0	32	0.125
EIEC	O167:H$^-$	16	0.25	16	0.0312
EIEC	D371-4	24	1.0	16	0.125
EPEC	O111	J. Mathewson	0.5	8	0.0312
EPEC	118A	65	0.5	16	0.5
ETEC	H19123	46	2.0	64	0.125
ETEC	20-005A	20	2.0	64	0.0625
EHEC	43-12	70	2.0	16	0.125
EHEC	E473	70	1.0	16	0.125
E. coli	ATCC 11775	ATCC[b]	0.5	16	0.0625

[a] MICs were determined by the tube dilution method in Mueller-Hinton broth (pH 7.4).
[b] ATCC, American Type Culture Collection.

7.4 (48). The MIC of ampicillin for *E. coli* ATCC 11775 was 2 μg/ml.

For analysis of the intracellular activities of the antibiotics, bacteria were streaked onto blood agar plates, inoculated into brain heart infusion broth, and incubated overnight at 37°C and were then diluted 1:100 in fresh brain heart infusion broth and grown to the mid-logarithmic phase for 3 to 4 h at 37°C with tumbling. Bacteria were washed twice and were resuspended to the required concentration in Hanks' balanced salt solution (HBSS).

Neutrophil isolation. PMNs were isolated from EDTA-anticoagulated venous blood by dextran sedimentation, Ficoll-Hypaque centrifugation, and hypotonic lysis of residual erythrocytes as described previously (58). Cells were suspended in HBSS at approximately 2×10^7 PMNs per ml. Cells were determined to be ≥95% neutrophils by Diff-Quick (Baxter Scientific Products, Miami, Fla.) staining, and viability was ≥96% as determined by trypan blue exclusion.

Intracellular activities of antibiotics. Approximately 10^8 bacteria were incubated with 10^7 PMNs and 10% autologous serum in HBSS containing 1 mM Ca^{2+} and 1 mM Mg^{2+} in a final volume of 400 μl and were tumbled for 30 min at 37°C to allow the bacteria to be phagocytosed. An assay of phagocytosis in which fluorescein isothiocyanate-labelled bacteria were used (5) revealed three to four intracellular organisms per PMN. PMNs, along with intracellular bacteria, were washed and resuspended in 400 μl of Dulbecco's modified Eagle's medium containing 10% heat-treated (56°C for 30 min) fetal calf serum (DMEM–10% FCS; pH 7.0) and were exposed to various concentrations of antibiotics at 37°C. Initially and at 1, 2, and 18 h, samples were withdrawn and PMNs were lysed in distilled water for 10 min. The bacteria were then serially diluted and bacterial viability was determined by the pour plate method by using brain heart infusion agar. After 18 h of incubation, 56% of the PMNs remained viable as assessed by trypan blue exclusion. For experiments lacking PMNs, an equivalent number of bacteria were suspended in HBSS–10% serum alone for 30 min, washed, resuspended in DMEM–10% FCS, and exposed to antibiotics as described above. Statistical significance was assessed by the use of Student's two-tailed *t* test.

RESULTS

Intracellular activities of antibiotics against the neotype strain of *E. coli*. The activities of azithromycin and other antibiotics were first tested against *E. coli* ATCC 11775 (the neotype strain). *E. coli* ATCC 11775 organisms were phagocytosed by PMNs and exposed to azithromycin at 8 μg/ml (8× the MIC) or 0.125 μg/ml (1/8× the MIC), and viability was determined at intervals. As seen in Fig. 1A, exposure to azithromycin at 8× the MIC resulted in the progressive loss of viability of *E. coli* ATCC 11775, with a reduction in the log CFU per milliliter of 3.88 at 18 h. This pattern of microbicidal activity was not significantly different from that seen when organisms in suspension, without PMNs present, were exposed to an equivalent concentration of azithromycin (azithromycin alone at 8× the MIC). In this assay system, PMNs alone were ineffective in killing phagocytosed organisms, and viability rose by 2.56 log units after 18 h of incubation. Likewise, viability after exposure to azithromycin at 1/8× the MIC was similar to that after exposure to PMNs alone, with an increase in viability of 3.00 log units after 18 h.

Erythromycin, another macrolide antibiotic, had intracellular activity against *E. coli* ATCC 11775 similar to that of azithromycin (Fig. 1B). Erythromycin at 8× the MIC reduced viability by 3.2 log units at 18 h, while the numbers of bacteria increased by 2.6 log units during exposure to erythromycin at 1/8× the MIC. However, neither of the two β-lactams tested (ampicillin or cefotaxime) could kill intracellular *E. coli* ATCC 11775 even at concentrations equal to 8× the MIC for this organism, consistent with the poor penetration of β-lactam antibiotics into PMNs.

Intracellular activity of azithromycin against enteropathogens. A variety of enteropathogens, including strains of *Salmonella*, *Shigella*, EIEC, EPEC, ETEC, and EHEC, were phagocytosed by PMNs and were exposed to azithromycin at 8× or 1/8× the MIC. As seen in Fig. 2, azithromycin at 8× the MIC reduced bacterial viability for all strains except EHEC E473, whose viability increased slightly after 18 h. The loss of viability for all other strains ranged from 1.36 log units for *Salmonella enteriditis* HD4052 to 4.20 log units for EPEC 118A; 8 of the 12 strains had a ≥2-log-unit decline in viability (Table 2). As seen with *E. coli* ATCC 11775, azithromycin at 1/8× the MIC was ineffective in killing intracellular organisms. Except for ETEC H19123, whose viability declined slightly, all other organisms multiplied despite exposure to the antibiotic at this concentration.

Intracellular activity of erythromycin or cefotaxime against enteropathogens. Erythromycin at concentrations equal to 8× the MIC was effective in reducing viability for all strains tested

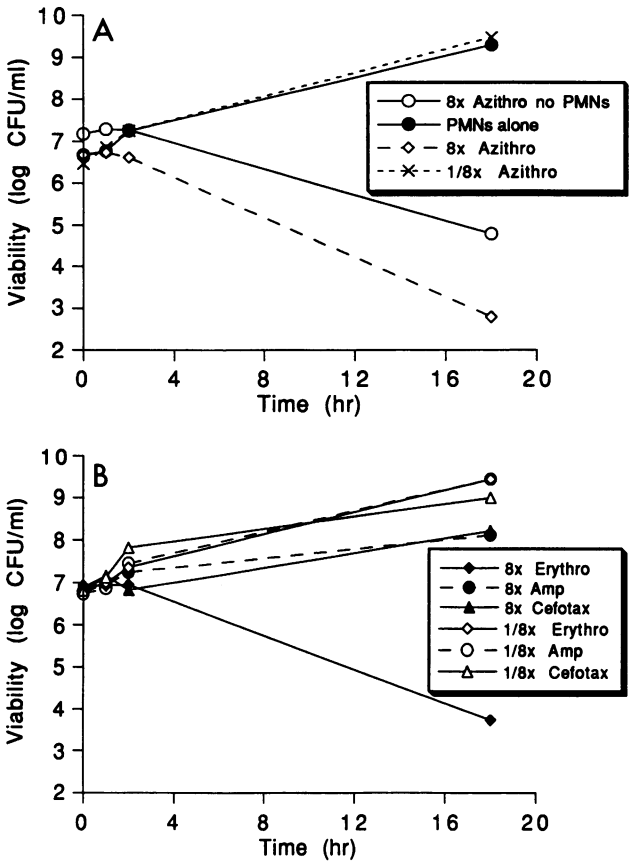

FIG. 1. Intracellular activity of antibiotics against *E. coli* ATCC 11775 (the neotype strain). *E. coli* ATCC 11775 was phagocytosed by PMNs and was exposed to azithromycin (A) or erythromycin, ampicillin, or cefotaxime (B). At the indicated times, samples were withdrawn and bacterial viability was determined. 8×, exposure to a concentration of antibiotic equal to 8 times the MIC; 1/8×, exposure to 1/8 times the MIC; Azithro, azithromycin; Erythro, erythromycin; Cefotax, cefotaxime; Amp, ampicillin. In the experiment with no PMNs and azithromycin at 8× the MIC, PMNs were omitted from the incubation; in the experiment with PMNs alone, azithromycin was omitted (n = 4 for azithromycin at 8× the MIC and no PMNs, azithromycin at 8× the MIC, and azithromycin at 1/8× the MIC; n = 10 for PMNs alone; n = 4 to 7 for erythromycin, ampicillin, and cefotaxime).

(Table 2); 7 of 12 strains had a ≥2-log-unit decline in viability. However, strains of *Salmonella* and *Shigella* were only slightly responsive to erythromycin, with declines in viability ranging from 0.79 to 1.25 log units. Azithromycin reduced viability significantly more than erythromycin for 5 of the 12 strains, while erythromycin was more active against 2 strains. To our surprise, cefotaxime, which does not penetrate well into PMNs and which was ineffective in killing intracellular *E. coli* ATCC 11775, reduced the viabilities of strains of EPEC, ETEC, EHEC, *Shigella*, and *Salmonella typhi*; however, only 2 of the 12 strains had a ≥2-log-unit decline in viability. Both strains of EIEC and the strain of *S. enteriditis* were able to grow intracellularly in the presence of 8× the MIC of cefotaxime. Azithromycin reduced viability significantly more than cefotaxime did for seven strains, while cefotaxime was better than azithromycin at reducing the viabilities of two strains.

Comparison of azithromycin combined with PMNs versus azithromycin alone. To determine the contribution of PMNs to the activity of azithromycin in the assay system described here, bacteria were also suspended in medium alone in the absence of PMNs and were exposed to 8× the MIC of azithromycin. As seen in Table 2, 7 of the 12 strains had a greater reduction in viability when PMNs were present in combination with azithromycin. The only statistically significant differences between killing in the presence of PMNs versus that in the absence of PMNs were seen with the two strains of EHEC; EHEC E473 grew unusually well in medium with azithromycin alone, while EHEC 43-12 was killed significantly more in medium with azithromycin alone. The growth of EHEC E473 in the presence of 8× the MIC of azithromycin may have been due to the number of organisms initially present in the assays (2.5×10^8 organisms per ml), in comparison with the much lower number used to determine MICs.

DISCUSSION

In the present study, we demonstrated that azithromycin has good intracellular activity against a variety of bacterial enteric pathogens. Except for one strain of EHEC, azithromycin was effective in reducing intracellular viability for strains of *Salmonella* (including *S. typhi*), *Shigella*, and several different types of *E. coli*. Erythromycin, another macrolide antibiotic, was similarly efficacious against these pathogens in this in vitro model system. Although there was a large variability among organisms, in general, azithromycin lowered viability to a greater extent than erythromycin for strains of invasive organisms (strains of *Salmonella*, *Shigella*, EIEC, EPEC), while erythromycin was more effective than azithromycin for strains of noninvasive organisms (ETEC and EHEC).

Both azithromycin and erythromycin are concentrated inside PMNs (22, 42, 49, 67), consistent with their good intracellular activities in the present study. However, cefotaxime, which has been reported not to enter PMNs to an appreciable degree (28), was also active in the intracellular killing assay against 9 of the 12 strains of enteropathogens tested, in accord with other recent reports (60, 69). Although the reason for this is unclear, one explanation, at least for the present study, could be that the long incubation period used (18 h) allowed cefotaxime to enter the cells and kill intracellular organisms, since β-lactams actually can diffuse into the cytoplasm of phagocytes and kill organisms such as *Listeria* spp. in that cell compartment (63). Alternatively, although the majority of organisms were intracellular prior to incubation with antibiotics (data not shown), the death of almost half of the PMNs after 18 h could have produced a significant number of extracellular bacteria, and cefotaxime could have appeared to be effective by killing those extracellular organisms.

Azithromycin, as a weak base, is concentrated by PMNs primarily via passive diffusion and subsequent lysosomal trapping (22, 36), with intracellular:extracellular ratios ranging from 5 (42) to 300 (49, 67). Although typical levels in serum reach 0.4 μg/ml after administration of an oral dose of 500 mg (52), which would be just below the MICs for most of the organisms tested in the present study (range, 0.25 to 2.0 μg/ml), the concentration of azithromycin inside PMNs has been reported to reach 113 μg/ml after 5 days of oral administration (2). Nevertheless, in the present study, the extracellular concentrations of azithromycin equal to 1/8× the MIC for each organism were almost uniformly ineffective in killing intracellular enteropathogens. Although we did not measure the concentrations of azithromycin inside PMNs, the high intracellular:extracellular ratios described previously would suggest that intracellular concentrations in the present study should have been far higher than the MICs for each of the

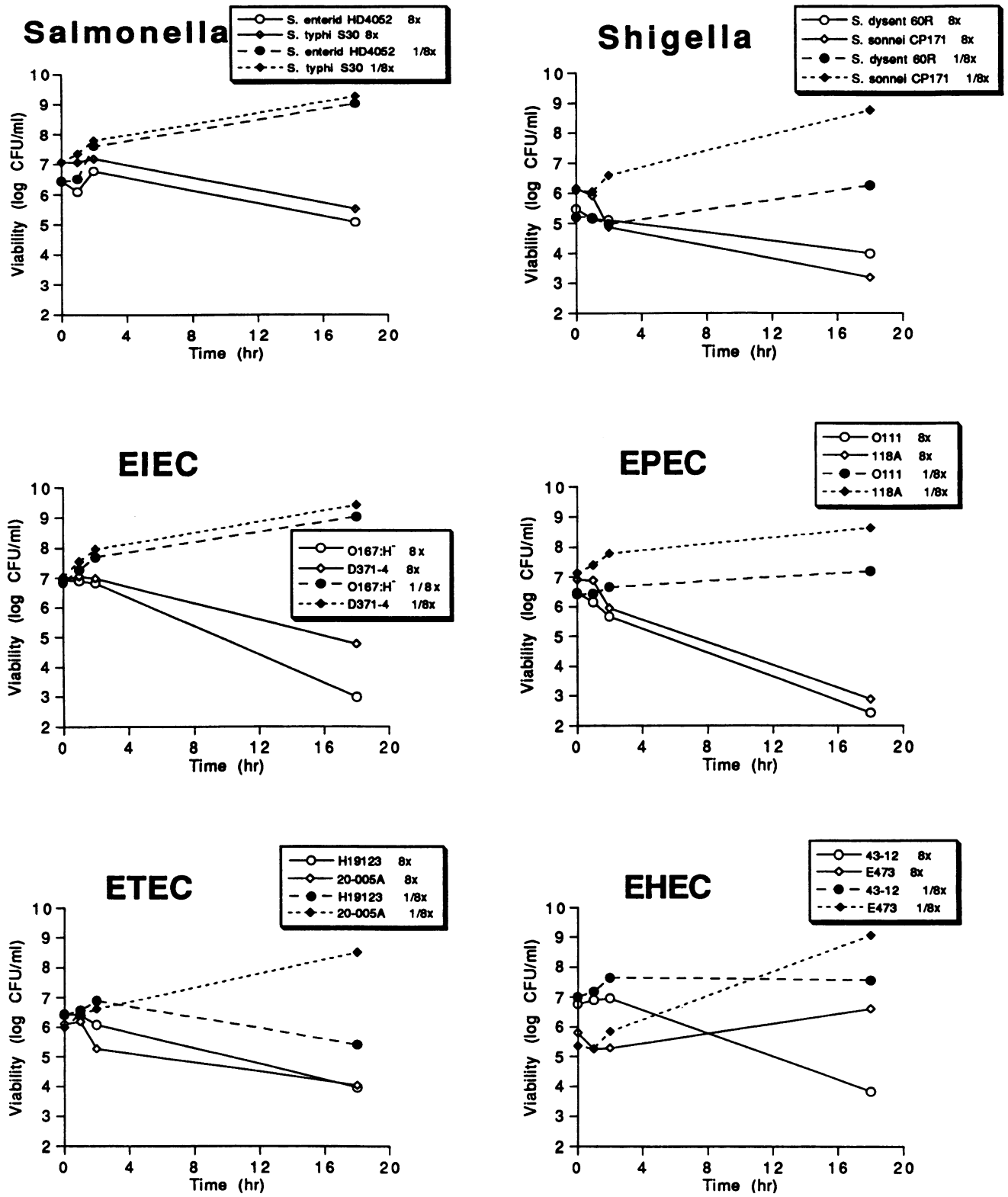

FIG. 2. Intracellular activity of azithromycin against enteric pathogens. Bacteria were phagocytosed by PMNs and were exposed to azithromycin (see legend to Fig. 1). 8×, exposure to a concentration of azithromycin equal to 8 times the MIC; 1/8×, exposure to 1/8 times the MIC (n = 4 to 7). S. enterid, *S. enteritidis*; S. dysent, *Shigella dysenteriae*.

TABLE 2. Change in intracellular bacterial viability after 18 h of exposure to azithromycin, erythromycin, or cefotaxime[a]

Organism	Change in viability (log CFU/ml) after exposure to:			
	Azithromycin	Erythromycin	Cefotaxime	Azithromycin without PMNs
S. enteriditis HD4052	−1.36 ± 0.63	−0.79 ± 0.21	1.26 ± 0.32[b]	−0.71 ± 2.00
S. typhi S30	−1.55 ± 0.21	−1.23 ± 0.15[b]	−2.62 ± 0.96	−1.82 ± 0.35
S. dysenteriae 60R	−1.49 ± 0.37	−1.04 ± 0.20[b]	−3.57 ± 1.11[b]	−1.15 ± 0.21
S. sonnei CP171	−2.98 ± 0.43	−1.25 ± 0.62[b]	−1.84 ± 0.42[b]	−2.38 ± 1.40
EIEC O167:H$^-$	−3.97 ± 0.88	−1.72 ± 0.50[b]	1.73 ± 0.61[b]	−3.97 ± 0.48
EIEC D371-4	−2.18 ± 1.40	−2.35 ± 0.96	1.81 ± 0.43[b]	−1.07 ± 0.22
EPEC O111	−4.05 ± 1.06	−3.04 ± 0.26	−0.42 ± 0.75[b]	−4.71 ± 0.52
EPEC 118A	−4.20 ± 0.52	−2.06 ± 0.32[b]	−1.78 ± 0.46[b]	−4.06 ± 0.30
ETEC H19123	−2.49 ± 1.39	−3.58 ± 0.44	−1.55 ± 0.56	−3.41 ± 0.28
ETEC 20-005A	−2.08 ± 1.53	−4.29 ± 0.27[b]	−1.50 ± 0.48	−1.93 ± 0.62
EHEC 43-12	−2.94 ± 0.36	−3.40 ± 0.32	−1.78 ± 0.73[b]	−3.97 ± 0.31[b]
EHEC E473	0.80 ± 1.53	−2.59 ± 0.30[b]	−1.21 ± 0.93[b]	3.65 ± 0.35[b]

[a] Bacteria were phagocytosed by PMNs or were suspended in 10% serum alone for the column azithromycin without PMNs and were exposed to 8× the MIC of the antibiotic for 18 h. Data are expressed as log (CFU/ml at 18 h) − log (CFU/ml at 0 h) (mean ± standard deviation; n = 4 to 7 for experiments with PMNs, n = 2 to 4 for experiments without PMNs).

[b] $P < 0.05$ versus azithromycin by Student's two tailed t test.

organisms tested. This disparity between the intracellular concentration obtained and the intracellular killing activity has been demonstrated previously for azithromycin by some investigators (43, 50) but not by others (42, 51, 67). Azithromycin uptake by host cells may be dependent on extracellular pH (36), and the pH of 7.0 in DMEM–10% FCS may have limited intracellular uptake (42). pH and divalent cation concentrations may also affect azithromycin activity in vitro (7, 57). The pH of activated PMN phagolysosomes containing engulfed microorganisms drops to 5.0 to 6.0 (15, 32), and the activity of azithromycin at this pH is markedly diminished. Alternatively, the potential presence of a significant number of extracellular organisms after 18 h could have contributed to the ineffectiveness of 1/8× the MIC of azithromycin.

Although a variety of interactions have been reported between antibiotics and PMNs with regard to their antimicrobial effects (64), the presence of PMNs in the experimental system described here did not appear to affect the overall antimicrobial activity of azithromycin. Similar findings have been demonstrated by others (42), although this may be dependent on both the species of bacteria and the host cell used (17, 42). One potential explanation for this in the present study is the poor antimicrobial activity of PMNs alone in our in vitro system over the 18-h incubation period.

In summary, azithromycin has good intracellular activity against a variety of common bacterial enteric pathogens. This intracellular activity, combined with the relatively low MICs of this agent for enteropathogens, makes azithromycin an attractive option for the treatment of diarrhea caused by invasive pathogens. Preliminary evidence has suggested a beneficial effect in the treatment of clinical infections caused by *S. typhi* (13). The pediatric population may particularly benefit from this antibiotic, since enteric pathogens have become increasingly resistant to the usual antimicrobial agents and the fluoroquinolones are not usually recommended for use in this age group. It is noteworthy that the development of high-level erythromycin resistance may be associated with concomitant resistance to azithromycin, as has been demonstrated in some *Campylobacter* spp. (62). Fortunately, this resistance does not appear to be widespread at present (25), although it will remain a potentially serious problem in the future. Azithromycin may become an important addition to our antimicrobial armamentarium for the treatment of bacterial diarrhea, and clinical trials should be considered.

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