**In vitro effects of azithromycin on Salmonella typhi: early inhibition by concentrations less than the MIC and reduction of MIC by alkaline pH and small inocula**

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To explain good clinical results of azithromycin in patients with typhoid fever, 10 strains of *Salmonella typhi* were grown in cation-adjusted Mueller–Hinton broth. MICs of azithromycin were 4–16 mg/L. At a sub-MIC of 2 mg/L, early inhibition of growth was shown at 2, 4 and 8 h of incubation, but at 24 and 48 h growth to turbidity occurred. At 4 mg/L, inhibition occurred up to 8 h, after which growth towards turbidity followed. Elongated curved bacilli formed in broth containing 4 mg/L after 24–48 h. Adjusting the pH of the broth with phosphate-citrate buffer to 7.5 and 8.0 caused reductions in MICs to 0.25–0.5 mg/L. Large inocula of 10⁹ cfu/mL resulted in median MICs four- to six-fold greater than with inocula of 10¹–10³ cfu/mL. An inoculum of 10 bacteria per mL in broth at pH 7.5 resulted in an MIC of 0.13 mg/L. Clinical benefits in patients may occur because of early inhibition by sub-MIC concentrations of azithromycin, and due to lower MICs at alkaline pH and lower MICs with small inocula that may correspond to the low-grade bacteraemia in typhoid fever.

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25381-34-21) was obtained from Pfizer, Inc. and dissolved in ethanol. Two-fold dilutions of azithromycin from 1 to 16 mg/L were prepared in broth. Tubes were incubated at 37°C, and at 2, 4, 8, 24 and 48 h of incubation, aliquots were removed, diluted and plated on to MacConkey agar (BBL, Cockeysville, MD, USA) for colony counts.

Buffered solutions of Mueller–Hinton broth were prepared using 0.2 M disodium phosphate (Sigma Chemical Company, St Louis, MO, USA) and 0.1 M citric acid (Fisher Scientific, Fair Lawn, NJ, USA) to obtain broth at pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5. Tris-buffered Mueller–Hinton broths were prepared by mixing 0.1 M solutions of Trizma hydrochloride and Trizma base (Sigma), and adjusting the pH after dissolving broth in buffered solutions. Tubes were inoculated with 10^5 bacteria per mL and MICs read at 24 h. Antibiotics (Sigma) were prepared by dissolving ciprofloxacin hydrochloride in water, chloramphenicol in ethanol and ampicillin sodium salt in water. In experiments to investigate inoculum effects, bacteria were grown for 4–6 h in trypticase soy broth at 37°C to reach turbidity estimated at 10^8 per mL. Serial 10-fold dilutions in cation-adjusted Mueller–Hinton broth were carried out for the inoculation of tubes containing azithromycin. Numbers of bacteria were determined from the 10^-4 dilution plated on MacConkey agar.

Results

Disc diffusion tests

Zones of inhibition around discs containing 15 μg azithromycin had indistinct boundaries, with growth occurring closer to the disc along linear streaks of heavier growth, which corresponded to the paths of the cotton swabs that streaked the inoculum, indicating that a heavier inoculum required a greater concentration of antibiotic to inhibit growth. When plates were allowed to incubate for 48 h, a ring of lighter growth occurred with a smaller diameter of inhibition than the outer zone of inhibition that had formed at 24 h. The rings indicated that bacteria were growing slowly at antibiotic concentrations that were just adequate to inhibit initial growth.

Growth curves

Determinations at 2, 4, 8, 24 and 48 h after inoculation showed that the sub-MIC of 2 mg/L produced inhibition at 2–8 h, leading to growth to turbidity at 24 h (Figure). In azithromycin 4 mg/L, growth was inhibited completely for up to 8 h of incubation, with a delay in growth to turbidity for some strains at 24 h, and for all at 48 h. For those strains that manifested turbidity at 24 h, the turbidity was less in the tubes with 4 mg/L than in control tubes without antibiotic. Likewise, at 48 h the tubes with 4 mg/L showed less turbidity than tubes with 0, 1 and 2 mg/L. Numbers of bacteria in these turbid tubes containing 4 mg/L were <10^7/mL and were c. 10- to 100-fold less than in control tubes without antibiotic. Tubes with 8 and 16 mg/L showed bacteriostasis at 2–8 h post-exposure and some killing of bacteria at 24 and 48 h, with reductions in cfu of 1–2 logs.

To examine whether the delayed growth of *S. typhi* at 24 and 48 h post-incubation was caused by decay of active antibiotic, tubes containing azithromycin were incubated at 37°C for 48 h before inoculation with bacteria. Six strains of *S. typhi* all had an MIC of 8 mg/L in freshly prepared solutions of azithromycin. Five of these strains showed MICs of 8 mg/L and one showed an MIC of 4 mg/L in the solutions of azithromycin that had been incubated previously for 48 h, indicating no detectable loss of antibiotic activity during 48 h of incubation.

To examine whether the delayed growth of *S. typhi* was caused by the growth of azithromycin-resistant mutants, MICs were performed on cultures of *S. typhi* that showed turbid growth after 48 h in the presence of 4 mg/L of azithromycin. Five strains with MICs of 4 or 8 mg/L had the same MIC after exposure to 4 mg/L, and one strain with an MIC of 4 mg/L had an MIC of 8 mg/L after exposure to azithromycin, indicating that antibiotic resistance had not developed.
Azithromycin and *Salmonella typhi*

**Bacterial morphology after exposure to azithromycin**

Gram-stained slides prepared from tubes incubated for 24 and 48 h with azithromycin at concentrations of 1, 2 and 4 mg/L showed elongated and curved bacilli. Cultures with azithromycin 4 mg/L incubated for 24 or 48 h showed the greatest proportions of elongated and curved bacilli.

**Effect of pH on the MIC of azithromycin**

Strains of *S. typhi* tested in Mueller–Hinton broth supplemented with phosphate-citrate buffers showed reductions in MICs with increases in pH. The median MIC decreased stepwise from 64 mg/L at pH 6 to 0.13 mg/L at pH 8.5. Tris-buffered Mueller–Hinton broth gave results similar to those of the phosphate-citrate buffers. Median MICs of azithromycin for four strains of *S. typhi* were 8 mg/L (pH 6.5), 2 mg/L (pH 7.0), 0.5 mg/L (pH 7.5) and 0.13 mg/L (pH 8.0). To investigate whether the increased osmolarity of the buffered broth solutions contributed to the greater antibacterial activity of azithromycin at higher pH, broths supplemented with sodium chloride solutions (0.15 and 0.3 M NaCl) were examined. MICs for two strains of *S. typhi* were 8 mg/L in standard Mueller–Hinton broth and ≥16 mg/L in the broths containing NaCl, indicating that the increased osmolarity resulted in increases in the MIC of azithromycin and could not explain the lower MICs obtained at alkaline pHs.

To examine whether a lower MIC at alkaline pH is a special property of azithromycin, MICs of ciprofloxacin, ampicillin and chloramphenicol for *S. typhi* were tested in buffered solutions of Mueller–Hinton broth. Decreases in MICs from pH 6 to pH 8 were 256-fold for azithromycin, 11-fold for ciprofloxacin, three-fold for ampicillin and 1.5-fold for chloramphenicol.

**Effects of divalent cations on the MIC of azithromycin**

To test the effects of the addition of calcium and magnesium to concentrations of 24 and 12 mg/L, respectively, as recommended by the NCCLS, Mueller–Hinton broth was used with and without the addition of cations. The results indicated that the addition of calcium and magnesium, either alone or in combination, caused four-fold increases in MIC in Mueller–Hinton broth and two- to four-fold increases in MIC in Tris-buffered Mueller–Hinton broth.

**Effect of inoculum size on MIC**

The testing of 10-fold increments in inoculum size from c. 10^5 to 10^9 per mL for six *S. typhi* strains showed a corresponding increase in MIC (Table). A four-fold increase in MIC was observed when the inoculum size was increased from 10^3 to 10^6 per mL in cation-adjusted Mueller–Hinton broth. To test whether an inoculum effect would affect MICs in broth at alkaline pH, inoculum sizes from 10^6 to 10 per mL were placed into buffered Mueller–Hinton broth at pH 7.5. An MIC as low as 0.13 mg/L was obtained with an inoculum of 10 organisms per mL (Table).

**Discussion**

High intracellular concentrations of azithromycin, which can be c. 100 times greater than serum concentrations, can explain its clinical efficacy, but the extracellular bacteraemia in typhoid fever probably contributes to symptoms in patients and needs to be controlled by effective antibiotic treatment. Our observation that at the physiological extracellular pH of 7.5, the MIC of azithromycin was <1 mg/L suggests that antimicrobial benefits may occur in the extracellular milieu, as well as intracellularly. Furthermore, the inoculum effect observed in our experiments, such that 10^3 bacteria per mL were inhibited by c. four-fold less antibiotic than the standard inoculum of 10^5 bacteria per mL, suggests that *in vivo* benefits could occur in typhoid fever, in which low-grade baceraemia with <10^3 bacteria per mL of blood occurs.

**Table.** Inoculum effect of *S. typhi* on the MIC of azithromycin

<table>
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<tr>
<th><em>S. typhi</em> inoculum size (cfu/mL)</th>
<th>Median MIC (range) (mg/L)</th>
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<tr>
<td></td>
<td>cation-adjusted Mueller–Hinton broth, pH 7.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>24 (16–32)</td>
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<tr>
<td>10&lt;sup&gt;5&lt;/sup&gt;</td>
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<td>4 (2–8)</td>
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<tr>
<td>10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>4 (1–8)</td>
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<sup>a</sup>Actual inoculum sizes from plate counts for 10<sup>4</sup> dilution were from 1.2 × 10<sup>5</sup> to 4.0 × 10<sup>5</sup> with a geometric mean of 2.1 × 10<sup>5</sup> per mL for cation-adjusted Mueller–Hinton broth, and from 5.4 × 10<sup>4</sup> to 1.8 × 10<sup>5</sup> with a geometric mean of 9.5 × 10<sup>4</sup> per mL for Mueller–Hinton broth in phosphate-citrate buffer.

<sup>b</sup>Median (range) of MICs of azithromycin for six strains tested in three experiments.
The earlier observation that azithromycin in agar diffusion susceptibility tests resulted in indistinct zones of inhibition around discs, with trailing of light growth toward discs, and the finding of transitional light-growth tubes between clear and turbid tubes in two-fold series of dilution for MIC determinations suggested that azithromycin, at sub-MIC concentrations, would show partial inhibition of bacterial growth. Growth curves demonstrated early inhibition of growth with 2 mg/L at 2, 4 and 8 h post-exposure. At the transitional MIC of 4 mg/L, growth was inhibited at 8 h, with turbidity developing at 24 h for some strains and for all strains at 48 h. Delayed growth to turbidity in the presence of 4 mg/L of antibiotic was not caused by the emergence of resistance or by loss of antibiotic activity during incubation, and was associated with development of elongated bacilli. These elongated forms suggest that azithromycin at sub-MIC concentrations might prevent septation in the later stages of cell division. The elongated bacterial forms explain the lower colony counts obtained in turbid broths containing 4 mg/L than in broth without antibiotic at 24 and 48 h of incubation because the elongated bacteria would produce fewer colonies relative to bacterial mass while still contributing to the turbidity of the broth. Assuming that the elongated bacteria remain viable, each would produce one colony when subcultured to solid media.

The mechanism of the effect of pH is likely to be greater transport of azithromycin into bacterial cells or greater access to ribosomal attachment sites at alkaline pH. The pH effect appeared not to occur by direct action on the bacterium because little or no effect on the MIC was observed with chloramphenicol and ampicillin. With ciprofloxacin, there was a decrease in MIC at alkaline pH, but the effect was much less than with azithromycin. Calcium and magnesium ions may play a role in the pH effect because the latter was partially abrogated in the presence of increased concentrations of divalent cations.

An inoculum effect of macrolide antibiotics against staphylococci and streptococci has also been reported. This may explain the range of MICs of 4–16 mg/L for S. typhi reported by Metchock and the difficulty we reported in reading zones of inhibition in disc diffusion, as well as the streaks of heavier inoculum extending closer to the discs that occurred in these experiments.

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References

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