

Intracellular Activity of Zidovudine (3'-Azido-3'-Deoxythymidine, AZT) against *Salmonella typhimurium* in the Macrophage Cell Line J774-2

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Received 2 January 1992/Accepted 6 March 1992

The antibacterial effect of zidovudine (AZT) has been demonstrated both in vitro and in vivo with experimental models of gram-negative bacterial infections. It has been associated with the absence or low occurrence of nontyphoid *Salmonella* infections in AIDS patients treated with AZT. Using the macrophage cell line J774-2, we demonstrate the inhibition of intracellular growth of *Salmonella typhimurium* by AZT. This effect is obtained with one-half of the MIC (1 µg/ml) of AZT for *S. typhimurium*. Inhibition of intracellular growth is observed after 4 h of incubation and persists at 24 h. Maximal inhibition is shown at a concentration of 128 µg/ml, and no further effect is observed with higher concentrations. When the inhibitory effect of AZT is compared with that of pefloxacin or that of ceftriaxone at half their MICs (0.2 and 0.02 µg/ml, respectively), AZT and pefloxacin give better results than ceftriaxone. In this study, using an intracellular model, we show that AZT is able to inhibit the intracellular multiplication of *S. typhimurium* at a minimal effective concentration lower than the MIC, indicating its potential for antibacterial accumulation in the macrophages.

The antiviral and antibacterial activities of zidovudine (3'-azido-3'-deoxythymidine) (AZT) are derived from its analogous structure to thymidine, the 3'-hydroxy group being replaced by an azido group. The active form of AZT, triphosphorylated AZT, has 100-fold greater affinity for human immunodeficiency virus (HIV) reverse transcriptase than for cellular DNA polymerase. This selective activity is responsible for the inhibition of HIV DNA replication (7, 13).

AZT is also active against enterobacteria such as *Escherichia coli* and *Salmonella* and *Klebsiella* species, but it is not active against *Pseudomonas aeruginosa*, gram-positive cocci, and *Listeria* and *Mycobacteria* species (3, 8). As in HIV replication, incorporation of azidothymidylate into a growing DNA strand in the bacteria terminates DNA elongation, inhibiting DNA synthesis (3, 7, 8, 11, 13). AZT-resistant bacteria have been isolated from the stools of HIV patients receiving AZT (12). The mechanism of AZT resistance in enterobacteria is due to the loss of thymidine kinase activity (9). The absence of thymidine kinase activity is observed with *P. aeruginosa* and *Listeria* species that are naturally resistant to AZT. However, this resistance is not transferable (9, 10).

Salmonella are facultative intracellular pathogens responsible for gastroenteritis in humans. They are able to invade and multiply in phagocytic cells and to avoid being killed by the oxidative burst, lysosomal enzymes, or defensins present in macrophages and neutrophils (4, 5). *Salmonella* may persist in the host, often with debilitating results. After multiplying in the epithelial cells of the brush border and enterocytes, salmonella localize in the phagolysosomes of the macrophages of the Peyer's patches (6). Their elimination, as with other intracellular bacteria, depends upon cell-mediated immunity.

Decreased cellular immunity, observed in HIV patients by a reduction of CD4 cells, decreased production of gamma interferon, and impaired B-cell function, allows the intracellular development of these bacteria.

Several studies on the antibacterial activities of AZT have been made (3, 8, 11). In vitro determination of the MIC showed that salmonella are sensitive to AZT at concentrations of 0.03 to 2 µg/ml (3, 11, 14). However, the determination varies with the thymidine concentration of the medium. Kinetic studies have demonstrated that AZT is degraded during bacterial growth, with the maximal inhibitory effect of AZT occurring after 6 h of incubation (3, 11). AZT is completely inactive after 24 h of incubation, with the resulting bacterial count being equivalent to that of a control culture (3, 11). In vivo studies confirm this antibacterial activity, and AZT has been shown to protect animals against experimental gram-negative septicemia (8).

The discovery of the antibacterial activity of AZT can be related to results from epidemiological studies that show a decrease in the incidence of salmonellosis in HIV patients treated with AZT (1, 2, 11, 14). Salmonellosis, due mainly to *Salmonella typhimurium*, occurred more frequently in HIV patients than in the healthy population (1), with a high frequency of bacteremia 15 to 100 times the number of cases expected in the general population (16).

The fact that HIV patients are highly susceptible to opportunistic bacterial infections, the intramacrophage location of salmonella, and the antibacterial activity of AZT are reasons for studying intracellular growth inhibition of salmonella by AZT, and such studies will provide information on the decreased incidence of salmonellosis in HIV patients treated with AZT. The data presented here demonstrate that AZT is active against *S. typhimurium* in macrophages. This activity is comparable to those of two antibiotics currently used in the treatment of salmonellosis, pefloxacin (a fluoroquinolone) and ceftriaxone (a β-lactam).

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TABLE 1. Intracellular growth of *S. typhimurium* in macrophages at different MOIs

MOI	Growth (\log_{10} CFU/ml ^a) after:	
	2 h	24 h
1:1	3.12 \pm 0.05	10.29 \pm 0.05
1:10	4.51 \pm 0.03	10.43 \pm 0.03
1:50	5.41 \pm 0.06	11.80 \pm 0.04
1:100	5.80 \pm 0.07	12.18 \pm 0.47

^a Mean \pm standard deviation of total bacteria.

MATERIALS AND METHODS

Bacteria and growth conditions. *S. typhimurium* was isolated from the stool and blood culture of an HIV patient not receiving AZT. Bacteria were cultivated on Trypticase soy broth or agar at 37°C.

Cells. The macrophage cell line J774-2 (15) was kindly provided by G. Milon (Institut Pasteur, Paris, France). The cells were cultivated in 50- or 250-ml Falcon flasks in minimal essential medium (MEM) (BHK 21; GIBCO Laboratories, Cergy-Pontoise, France) supplemented with L-glutamine and 10% (vol/vol) fetal calf serum (FCS) at 37°C under 5% CO₂.

Infection of macrophages. Bacteria were grown overnight, diluted in Trypticase soy broth, and grown for 2 h to a concentration of 1×10^9 to 2×10^9 CFU/ml. The inoculum was evaluated by plating serial 10-fold dilutions on Trypticase soy agar.

Macrophages (1×10^5 to 2×10^5 cells per ml) were cultured in 24-well plates and incubated for 24 h, by which time the concentration had doubled.

Infection was done as described by Vladoianu et al. (17) but slightly modified. The cell monolayer was infected with *S. typhimurium* at cell-to-bacteria ratios of 1:1, 1:10, 1:50, 1:100 and incubated for 1 h at 37°C under 5% CO₂. The supernatant was then removed, and each well was washed three times with MEM without FCS. Extracellular bacteria were killed by incubation with 100 μ g of gentamicin per ml for 1 h at 37°C under 5% CO₂. Following this incubation, the supernatant was removed and the cells were washed once with MEM without FCS. The cells were then incubated at 37°C under 5% CO₂ in the culture medium. At 0, 2, 4, 6, 8, and 24 h of incubation, cells were lysed with cold distilled water and bacteria were counted by using 10-fold serial dilutions on Trypticase soy agar.

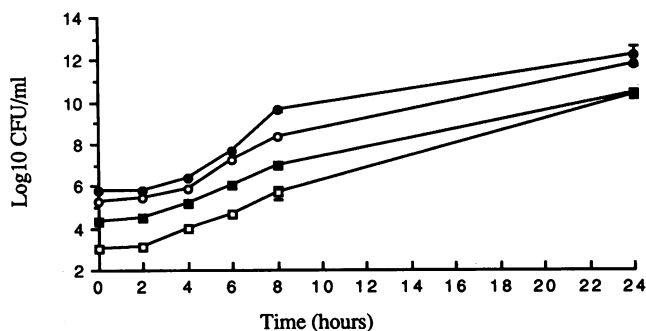


FIG. 1. Intracellular growth of *S. typhimurium* at MOIs of 1:1 (□), 1:10 (■), 1:50 (○), and 1:100 (●). Each point represents the mean of four values. The standard deviations of the mean are omitted, as they were always less than 0.10.

TABLE 2. Effects of different concentrations of AZT on intracellular growth of *S. typhimurium*^a

Concn of AZT (μ g/ml)	No. of expts	Growth (\log_{10} CFU/ml ^b)
0	6	12.03 \pm 0.24
1	8	5.22 \pm 0.07 ^c
4	10	3.94 \pm 0.4
128	2	3.21 \pm 0.03

^a MOI = 50.

^b Mean \pm standard deviation of total bacteria at 24 h with AZT.

^c $P < 0.001$ compared with that for control.

Antibiotics and the AZT experiment. AZT (purchased from Sigma, La Verpillière, France) was added to the medium at concentrations of 0.125 to 1,024 μ g/ml. Pefloxacin (Roger Bellon Laboratories, Paris, France) was used at a concentration of 0.2 μ g/ml, and ceftriaxone (Roche Laboratories, Zurich, Switzerland) was added at concentrations of 0.01 and 0.02 μ g/ml.

Isolation of *S. typhimurium* resistant to AZT. As described by Lewin et al. (12), *S. typhimurium* was grown in Trypticase soy broth with either 10 or 100 μ g of AZT per ml for 18 h at 37°C with shaking. After this time, resistant bacteria were detected by plating dilutions of these cultures on Trypticase soy agar plates containing 20 μ g of AZT per ml.

MIC determinations. Determinations of the MICs were carried out as recommended by the French Committee of Antibiogram.

Expression of results. Each time point was measured in quadruplicate, and the results were expressed as the mean \log_{10} CFU \pm the standard deviation of *S. typhimurium* per ml. Student's *t* test to nonpaired data was used to determine the significant differences between each experiment.

RESULTS

Macrophage toxicity. Viability of the J774-2 cells assessed by the trypan blue dye exclusion test was always greater than 95%, with or without the concentrations of AZT used during experiments.

Intracellular multiplication of *S. typhimurium*. Four different cell-to-bacteria ratios were used (1:1, 1:10, 1:50, and 1:100). The results presented in Table 1 show that the mean number of CFU of *S. typhimurium* within macrophages was approximately two to three times greater 24 h postinfection than after 2 h. The growth curve is given in Fig. 1.

MICs of AZT, pefloxacin, and ceftriaxone in cell culture medium. The MIC of each drug was determined in MEM containing 10% FCS to establish activity in this enriched medium. The MICs of pefloxacin and ceftriaxone under these conditions were similar to those expected, 0.4 and ≤ 0.04 μ g/ml, respectively. The MIC of AZT for *S. typhimurium* was 2 μ g/ml, which was greater than the values given in previous reports (3, 11, 14).

The MICs of AZT obtained by culture of *S. typhimurium* in four different media (MEM with 10% FCS, Mueller-Hinton broth, M63 glucose broth, and Luria-Bertani broth) were 2, 0.8, 2, and 4 μ g/ml, respectively, showing that the MIC was influenced by the medium. Thereby the MIC obtained in cell culture medium was chosen for this study.

Multiplication of *S. typhimurium* in macrophages in the presence of AZT. AZT at concentrations of 0.125 to 1,024 μ g/ml was used to determine the minimal inhibitory extracellular concentration for the growth of *S. typhimurium*.

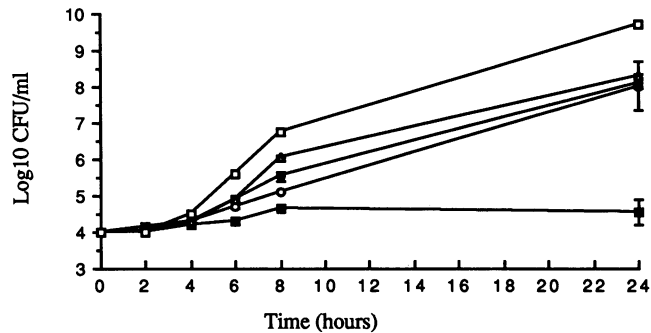


FIG. 2. Effects of different concentrations (in micrograms per milliliter) of AZT on the intracellular growth curves of *S. typhimurium* with an MOI of 1:50. An open box (\square) indicates the control bacterial culture without AZT. Concentrations of 1 $\mu\text{g/ml}$ (\blacksquare), 0.5 $\mu\text{g/ml}$ (\circ), 0.25 $\mu\text{g/ml}$ (\bullet), and 0.125 $\mu\text{g/ml}$ (Δ) were added to the medium. Each point represents the mean of four values \pm the standard deviations of the mean.

Twenty-four hours postinfection, the mean number of bacteria with a multiplicity of infection (MOI) of 1:50 (Table 2) was similar to that of intracellular bacteria following incubation with gentamicin in the presence of AZT and was significantly different from the control growth (Fig. 2). This effect was obtained with a minimal extracellular inhibitory concentration equivalent to half the MIC (1 $\mu\text{g/ml}$) obtained in cell-free medium. At lower concentrations of AZT (0.125 to 0.5 $\mu\text{g/ml}$), inhibition of growth was observed up to 6 h but bacterial regrowth commenced 8 h postinfection (Fig. 2). To explore the maximal inhibitory effect of AZT, higher concentrations, up to 1,024 $\mu\text{g/ml}$, were used. The highest inhibitory effect was at a concentration of 128 $\mu\text{g/ml}$ (Fig. 3), and no significant difference was observed with higher concentrations (data not shown). An important feature of this experiment was the inability of AZT to totally eradicate the bacteria by 24 h postinfection, which was mainly due to the selection of AZT-resistant mutants.

Comparison of the abilities of AZT, pefloxacin, and ceftriaxone to inhibit intracellular growth of *S. typhimurium*. Knowing that the minimal inhibitory extracellular concentration of AZT for *S. typhimurium* corresponded to half the MIC of AZT for *S. typhimurium*, a comparison of each

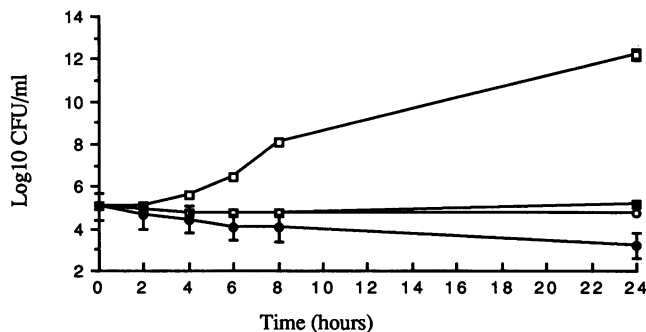


FIG. 3. Effects of different concentrations (in micrograms per milliliter) of AZT on the intracellular growth curves of *S. typhimurium* with an MOI of 1:50. An open box (\square) indicates the control bacterial culture without AZT. Concentrations of 1 $\mu\text{g/ml}$ (\blacksquare), 4 $\mu\text{g/ml}$ (\circ), and 128 $\mu\text{g/ml}$ (\bullet) were added to the medium. Each point represents the mean of four values \pm the standard deviations of the mean.

TABLE 3. Comparative effects of AZT, pefloxacin, and ceftriaxone on intracellular growth of *S. typhimurium*^a

Antimicrobial agent ^b (no. of expts)	Concn ^c ($\mu\text{g/ml}$)	Growth (log ₁₀ CFU/ml ^d)
AZT (4)	1	5.26 \pm 0.06 ^{d,e}
PEF (4)	0.2	5.55 \pm 0.03
CEF (4)	0.02	6.88 \pm 0.1
Control (4)		12.14 \pm 0.07

^a MOI = 20.

^b PEF, pefloxacin; CEF, ceftriaxone.

^c Antimicrobial agents were added at concentrations of half of the MICs for each drug determined in cell culture medium.

^d Mean \pm standard deviation of total bacteria at 24 h with antimicrobial agent.

^e $P < 0.001$ compared with that for ceftriaxone.

antibacterial product at concentrations equivalent to half of their MICs, as determined previously in MEM with 10% FCS (0.2 and 0.02 $\mu\text{g/ml}$, respectively, for pefloxacin and ceftriaxone), was performed. Intracellular inhibition of *S. typhimurium* grown in the presence of AZT (1 $\mu\text{g/ml}$) was as effective as that by pefloxacin for an MOI of 1:20 and higher than the intracellular inhibition induced by ceftriaxone (Table 3). Similar results were obtained at other MOIs (1:1, 1:10, and 1:50). For AZT and pefloxacin, the number of intracellular bacteria decreased slowly in the first 6 h postinfection. After 8 h of incubation, only partial bacterial regrowth occurred, but the 24-h bacterial count was still significantly different from that of the control culture (Fig. 4). In contrast with results with ceftriaxone, the number of intracellular bacteria increased slowly for 6 h and then remained stable. Ceftriaxone was also tested at a concentration of 0.01 $\mu\text{g/ml}$ (one-fourth its MIC), but the intracellular growth inhibition of *S. typhimurium* was even less effective (data not shown).

Intracellular growth of AZT-resistant salmonellae. Macrophages were infected with AZT-resistant *S. typhimurium* in the presence or the absence of a high concentration of AZT (128 $\mu\text{g/ml}$). At 24 h, the bacterial counts in both cases were equivalent to those of a control culture of AZT-sensitive *S. typhimurium* grown without AZT (data not shown), thereby showing that AZT has no synergistic effect on the intracel-

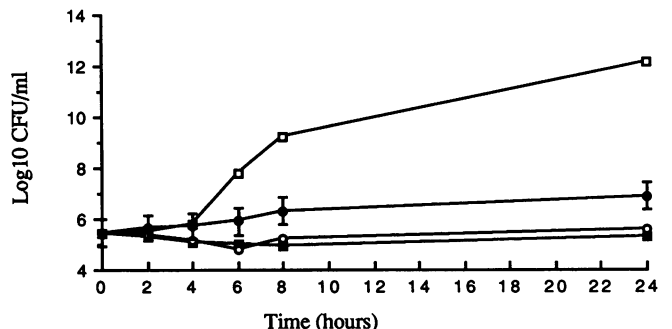


FIG. 4. Comparison of the intracellular activity of AZT with those of pefloxacin and ceftriaxone at an MOI of 1:20. A concentration of each drug corresponding to half of its MIC was added to the medium. An open box (\square) indicates the control bacterial culture without any drug. Concentrations of 1 μg of AZT per ml (\blacksquare), 0.2 μg of pefloxacin per ml (\circ), and 0.02 μg of ceftriaxone per ml (\bullet) were used. Each point represents the mean of four values \pm the standard deviations of the mean.

lular growth of AZT-resistant *S. typhimurium* in the presence of macrophages.

DISCUSSION

Many studies have tried to determine the ability of intracellular pathogens to survive the antimicrobial defenses of macrophages and replicate within them. *S. typhimurium*, after oral absorption and penetration of the epithelial barrier of the digestive tract, is located in the phagolysosomes of macrophages in the Peyer's patches. These bacteria are able to multiply intracellularly, and when cellular immunity is decreased, as with HIV patients, they can disseminate.

The discovery of a decreased incidence of salmonellosis due to minor *Salmonella* species in HIV patients treated with AZT shows the potential effect of this product in limiting the spread of these infections. With the antibacterial activity of AZT having been previously demonstrated (3, 8, 11), it was easy to correlate the absence of *Salmonella* infections and the use of this product in HIV patients.

To correlate these data, an in vitro cell model was necessary to quantify the intracellular activity of AZT in limiting the spread of *S. typhimurium* in HIV patients treated with this product. No previous studies on the potential intracellular effect of AZT have been made. Only one in vivo study with mice had shown the efficacy of AZT in limiting gram-negative septicemia (8). We chose to use the J774-2 cell line, which is a murine macrophagelike cell line. It possesses the same properties as monocytes and macrophages, phagocytosis and lysozyme and plasminogen secretion (15).

Having shown the intracellular growth of *S. typhimurium* at different MOIs (Fig. 1), we demonstrated the efficacy of AZT in inhibiting intracellular growth of *S. typhimurium* in macrophages. This activity was obtained at a concentration half that of the MIC and was the same as the intracellular activity of pefloxacin, which is known to have effective intracellular penetration. No major differences were observed when we increased the AZT concentration. A maximal effect was obtained with 128 $\mu\text{g/ml}$. However, even with a high concentration of AZT, the complete eradication of the bacteria was not obtained. We think that the ability of *Salmonella* species to acquire AZT resistance could be the principal problem. Kinetic studies in vitro have demonstrated that a maximal inhibitory effect was observed after 6 h and that regrowth occurred after this period of time (3, 11). Our results after 6 h were similar, but after 24 h the numbers were found to be the same as those after treatment with gentamicin. The fact that AZT was able to inhibit the intracellular growth of bacteria after 24 h compared with growth in vitro (3, 11) confirms the intracellular activity of AZT. Intracellular penetration of AZT at concentrations equivalent to concentrations in blood of HIV patients limited intracellular growth. The AZT present in the medium was still active against free bacteria liberated by cell lysis. It seems to us that a consumption of AZT occurred during in vitro culture which may have led to the start of new growth after 6 h. An AZT-resistant strain of *Salmonella* has been isolated from the blood of an HIV patient (14). The explanation given was the low dosage of AZT (≤ 400 mg per day) used for this patient. This result is consistent with the intracellular activity of AZT at concentrations lower than 1 $\mu\text{g/ml}$, showing an inhibitory effect only during the first 4 h with regrowth beginning at 6 h.

A comparison of the intracellular activity of AZT against salmonella with two antibiotics currently used in salmonellosis, pefloxacin and ceftriaxone, demonstrated that AZT

was as effective as both pefloxacin and ceftriaxone in limiting bacterial growth in macrophages after 24 h. The inhibitory effect after 6 h of AZT or pefloxacin was greater than that of ceftriaxone, mainly because of the ability of these two products to penetrate intracellularly but also because of the low concentration of ceftriaxone used in this study.

The action of AZT demonstrated in this report provides an in vitro model of its antibacterial activity. In addition, AZT shows an inhibitory activity similar to that of two antibiotics currently used in salmonellosis therapy. AZT is active at low concentrations comparable to the concentrations of AZT in the blood of HIV patients. The results presented in this study help to explain the decreased incidence of salmonellosis observed with HIV patients treated by AZT.

ACKNOWLEDGMENTS

We thank G. Milon, M. Popoff, and P. Rajagopalan-Leasseur for helpful discussions, J. MacDougall for help with revising the manuscript, and H. Sauvageon-Martre for providing AZT.

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