ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 1992, p. 2304–2309
0066-4804/92/102304-06$02.00/0

Vol. 36, No. 10

Azithromycin-Induced Block of Elementary Body Formation in *Chlamydia trachomatis*

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Received 10 March 1992/Accepted 20 July 1992

The mechanism of action of azithromycin on the murine strain of *Chlamydia trachomatis* grown in tissue culture epithelial cells is addressed. Azithromycin at a concentration of 100 ng/ml inhibits chlamydial growth in tissue culture, a value that agrees well with prior in vitro data from human strains of *C. trachomatis* grown in tissue culture. By morphological criteria, the block to chlamydial growth appears to occur early in its life cycle. Azithromycin is not directly toxic to chlamydial elementary bodies but does inhibit chlamydial protein synthesis in chlamydia-infected cells. This inhibition appears quite general in nature and is rapid. It is further shown that azithromycin does not directly inhibit mRNA synthesis. Azithromycin blocks chlamydial protein synthesis in host cell-free chlamydial reticulate bodies in a manner similar to its inhibition in infected cells, albeit at slightly higher concentrations. The inhibition of chlamydial protein synthesis following a brief exposure to azithromycin is more long lasting than that following a brief exposure to erythromycin.

Chlamydia trachomatis are gram-negative bacteria of major medical significance and intrinsic biological interest. Chlamydiae are the major cause of preventable infertility in well-developed countries and the primary cause of preventable blindness in underdeveloped countries (16). These organisms have developed a distinctive life cycle in which replication occurs only within the cells of their eukaryotic host—that is, they are obligate intracellular parasites (for a review, see reference 12). Outside of host cells, chlamydiae exist as sporelike forms (called elementary bodies or EBs). EBs are taken up by the host epithelial cells into membrane-bound vacuoles. Subsequently, they differentiate into more conventional bacterial forms (reticulate bodies or RBs) and then multiply by binary fission. The chlamydial vacuole escapes lysosomal destruction by the host cell. Following 24 to 48 h of multiplication, the RBs differentiate back into EBs and are released from the cell, thereby completing this dimorphic intracellular life cycle.

Azithromycin (AZI) is a novel azalide antibiotic with improved potency against gram-negative organisms. Clinical studies demonstrate that a single dose is sufficient to eradicate genital chlamydial and gonorrheal infections (7, 10, 18). Recent data suggest that synergism between AZI and phagocytic cells results in enhanced bacterial killing (19). Whether this putative interaction reflects simply the preferential accumulation of the antibiotic in macrophages and polymorphonuclear neutrophils (5) or whether the antibiotic affects other processes required for the survival of intracellular pathogens has not been investigated.

The exact mechanisms by which AZI inhibits chlamydial growth has not been rigorously tested. Like its close relative erythromycin (ERY), AZI may block chlamydial protein synthesis. In fact, studies suggest that AZI competes effectively for [^{14}C]erythromycin ribosome binding sites (14). Such a general inhibition of bacterial protein synthesis may be sufficient to inhibit chlamydial growth and replication. Alternatively or in addition, AZI might block a specific stage in the chlamydial intracellular life cycle, such as the differentiation of EBs to RBs or vice versa. Precedence for such a block and for the unexpected mechanism of action of a well-studied antibiotic is found in the action of the penicillin class of antibiotics on chlamydia. Treatment of chlamydia-infected cells with penicillin results in the accumulation of RBs, implying a block to EB differentiation (11). The exact mechanism by which penicillin inhibits the differentiation of RBs to EBs remains a mystery, as chlamydia lack detectable peptidoglycan (1, 4). Lastly, AZI might block the chlamydia-specified inhibition of phagolysosomal fusion that is critical to its intracellular survival. Very little is known about this process other than that it requires active chlamydial protein synthesis (for a review, see reference 12).

In this study, I have addressed directly the effect of AZI on chlamydia. I show that AZI does not inhibit chlamydial mRNA transcription but that it does inhibit protein synthesis, both in intracellular RBs as well as purified host-free RBs. Furthermore, as determined by light microscopy, AZI prevents the formation of chlamydial vacuoles, demonstrating that the block in chlamydial development occurs early in its life cycle.

MATERIALS AND METHODS

Reagents. AZI was provided by Pfizer. It was dissolved in absolute ethanol at a concentration of 100 mg/ml. Other reagents were obtained from the following suppliers and used according to manufacturer's specifications: ERY, rifampin (RIF), and chloramphenicol (CAM), Sigma (St. Louis, Mo.); ^{32}P-containing radioisotopes, Amersham Corp. (Arlington Heights, Ill.); [^{35}S]methionine (Translabel), ICN (Irvine, Calif.); and protein molecular mass markers, Bethesda Research Laboratories (Bethesda, Md.).

Growth of chlamydia in culture. The Mouse Pneumonitis strain of *C. trachomatis*, a murine strain of chlamydia, was grown in HeLa cells as previously described (15). Monolayers of HeLa cells grown to approximately 70% confluence were infected with chlamydia at a multiplicity of infection of 1 to 5 PFU per cell in SPG (10 mM NaH_2PO_4 [pH 7.4], 5 mM glutamic acid, and 250 mM sucrose). Following adsorption for 1 to 2 h at room temperature, they were overlaid with Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (DMEM-5) and incubated at 37°C for 18 to 48 h as indicated. Cultures were examined by

light microscopy at 24 and 48 h to assess the number of chlamydial vacuoles and the extent of host cell lysis.

Protein gel electrophoresis. The HeLa cell monolayers were extracted at various times after infection with lysis buffer (10% glycerol, 50 mM TrisCl [pH 7.5], 150 mM NaCl, 0.2% Triton X-100). Samples were mixed with an equal volume of 2× Laemmli buffer (8) prior to electrophoresis on sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gels (8) and then fluorographed.

In vivo labelling of chlamydial proteins. Chlamydia-infected HeLa cells grown in 100 mM dishes for various times postinfection in the presence or absence of drug were then incubated for 30 min in methionine- and cysteine-free DMEM containing 100 μg of cycloheximide (CX) per ml to inhibit host (eukaryotic) cell protein synthesis and subsequently pulse-labelled with [^{35}S]methionine (100 μCi/ml) for 15 to 60 min. For some experiments, the drug was added 15 min prior to addition of [^{35}S]methionine to the cultures.

In vivo labelling of HeLa cell proteins. Uninfected HeLa cells were incubated with DMEM-5 containing AZI (1 μg/ml) for 18 h. The media were replaced with methionine- and cysteine-free DMEM for 30 min, and the cultures were then pulse-labelled with [^{35}S]methionine (100 μCi/ml) for 15 min. This experiment was carried out in the absence of CX.

Effect of pretreatment of EBs with AZI prior to infection of HeLa cells. EBs were preincubated with AZI (1 μg/ml) at room temperature in SPG for 15 min. They were pelleted and washed free of the drug-containing media, and this inoculum was used to infect HeLa cells. For the control experiment, EBs were treated in the same manner except that preincubation was with SPG alone. At 18 h postinfection (hpi), the infected cells were incubated in methionine- and cysteine-free DMEM containing CX for 30 min and then pulse-labelled with [^{35}S]methionine for 15 min.

Measurement of duration of drug effect in tissue culture. HeLa cells were infected with chlamydia for 12 or 21 h. AZI (1 μg/ml) or ERY (1 μg/ml) was added to the media, and the cultures were incubated for an additional 15 min at 37°C. The media were removed, and the cells were washed three times with DMEM-5. The cultures were then incubated for 0, 6, or 9 h in drug-free media. The media were replaced with methionine- and cysteine-free DMEM containing CX for 30 min followed by pulse-labelling with [^{35}S]methionine (100 μCi/ml) for 30 min.

Preparation of host-free RBs. Partially purified RBs were isolated from chlamydia-infected cells as described by Hatch (6) with the following modifications. The chlamydia-infected cells were removed from T-150 flasks with glass beads, pelleted by centrifugation at 5,000 × *g*, and homogenized with a Dounce homogenizer ca. 30 times in phosphate-buffered saline (a manipulation which breaks open the host cell but leaves the RBs metabolically intact [1a, 6]). Cellular debris was removed by centrifugation at low speed, and the supernatant was then spun at 18,000 × *g* to pellet the RBs. The RBs were resuspended in buffer (100 mM Tris-hydrochloride [pH 7.0], 50 mM KCl, 5 mM $MgCl_2$, 20 mM dithiothreitol, CX [10 mg/ml], 7.8 mM creatine phosphate, 1.5 mg of phosphocreatine kinase per ml, 19 unlabelled amino acids [each present at a final concentration of 50 μM], and 0.1 mM ATP). To measure the amount of inhibition of protein synthesis by AZI or ERY, the host-free RBs were incubated with various concentrations of the antibiotic for 15 min at 37°C and inhibition of protein synthesis was assessed by pulse-labelling with [^{35}S]methionine (200 μCi/ml) for 15 min at 37°C. The reaction was terminated, and the RBs were lysed by the addition of 2× Laemmli buffer (8). The samples were electrophoresed as described above.

Nucleic acid preparation and analysis. Total RNA was prepared from chlamydia-infected cells by a modification of the guanidinium thiocyanate method (9). Total RNA was harvested by collecting the cells from T-150 flasks by using glass beads and then a low-speed centrifugation to pellet the chlamydia-infected cells. The cell pellet was lysed with 4 M guanidinium thiocyanate and then phenol extracted and ethanol precipitated. The pelleted nucleic acids were treated with RNase-free DNase (Promega, Madison, Wis.), phenol extracted, and ethanol precipitated. Equal amounts of RNA were electrophoresed on 1% agarose gels containing formaldehyde (9). The gel was blotted to Hybond filters (Amersham) in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (9) and hybridized as described previously (2) to a DNA probe made by nick translation (9) of the KARP gene and then visualized by autoradiography.

RESULTS

Concentration of AZI that inhibits chlamydial growth and protein synthesis in tissue culture. HeLa cells were infected with chlamydia and incubated in the presence or absence of AZI for 48 h, and the number of inclusions was assessed by direct visualization under light microscopy. Parallel cultures were incubated for 18 h, at which time the amount of chlamydial de novo protein synthesis was assessed by labelling with [^{35}S]methionine for 1 h. Eukaryotic host cell protein synthesis was inhibited by the addition of CX 30 min prior to labelling proteins with [^{35}S]methionine (see Materials and Methods). Lysates from the cultures were electrophoresed in SDS-polyacrylamide gels, and the pattern and extent of inhibition of protein synthesis were evaluated.

At 30 ng of AZI per ml, the amount of chlamydia-induced host cell lysis observed was approximately 50% compared with that in the control culture containing no drug. At 100 ng of AZI per ml, absolutely no chlamydial inclusions or host cell lysis was observed (data not shown). For comparison, 10 ng of ERY per ml resulted in a 50% reduction of chlamydia-induced host cell lysis and 30 ng/ml completely inhibited the formation of chlamydial inclusions (data not shown).

Inhibition of chlamydial protein synthesis by AZI occurred at similar concentrations as did the inhibition of chlamydial growth. Figure 1A, lane 3 demonstrates that 30 ng of AZI per ml inhibited chlamydial protein synthesis compared with control cultures (lane 1). At 100 ng of AZI per ml, chlamydial protein synthesis was entirely inhibited (lane 4). All chlamydial proteins appeared equally sensitive to the inhibition. For comparison, the concentration at which ERY completely inhibited chlamydial protein synthesis was 30 ng/ml (Fig. 1A, lane 11). In control experiments, AZI had no effect on eukaryotic host cell protein synthesis (Fig. 2A). These results suggest that the MIC of AZI for 90% of chlamydia grown in cultured HeLa cells is 100 ng/ml and that AZI is about threefold less active than ERY, a finding that agrees well with previous estimates of chlamydial sensitivity to AZI (14, 17).

AZI is not directly toxic to EBs. The observed inhibition of chlamydial protein synthesis could have been the indirect result of toxicity to EBs. To assess this possibility further, EBs were treated with AZI (1 μg/ml) for 15 min. The EBs were pelleted to remove the antibiotic, diluted in SPG, and used to infect HeLa cells in tissue culture. At 18 hpi, the viability of the intracellular chlamydia was assessed by measuring de novo chlamydial protein synthesis by pulse-

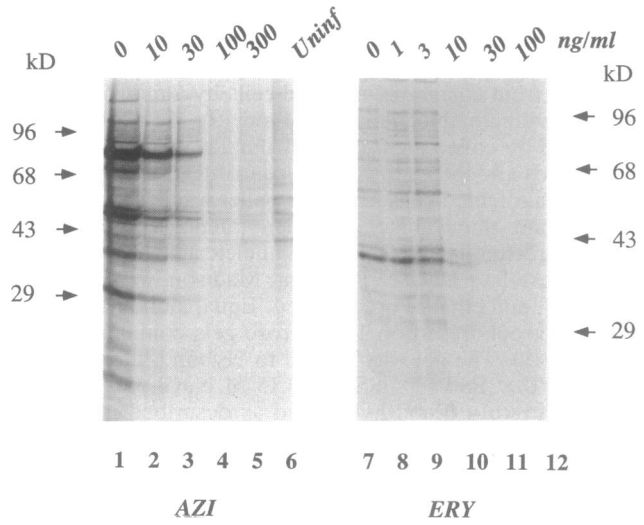

FIG. 1. Inhibition of chlamydial protein synthesis by AZI and ERY. HeLa cells were infected with chlamydia for 18 h in media containing drug at the indicated concentrations and then pulse-labelled with [^{35}S]methionine for 15 min. Lane 1, no drug; lane 2, 10 ng of AZI per ml; lane 3, 30 ng of AZI per ml; lane 4, 100 ng of AZI per ml; lane 5, 300 ng of AZI per ml; lane 6, uninfected HeLa cells; lane 7, no drug; lane 8, 1 ng of ERY per ml; lane 9, 3 ng of ERY per ml; lane 10, 10 ng of ERY per ml; lane 11, 30 ng of ERY per ml; lane 12, 100 ng of ERY per ml.

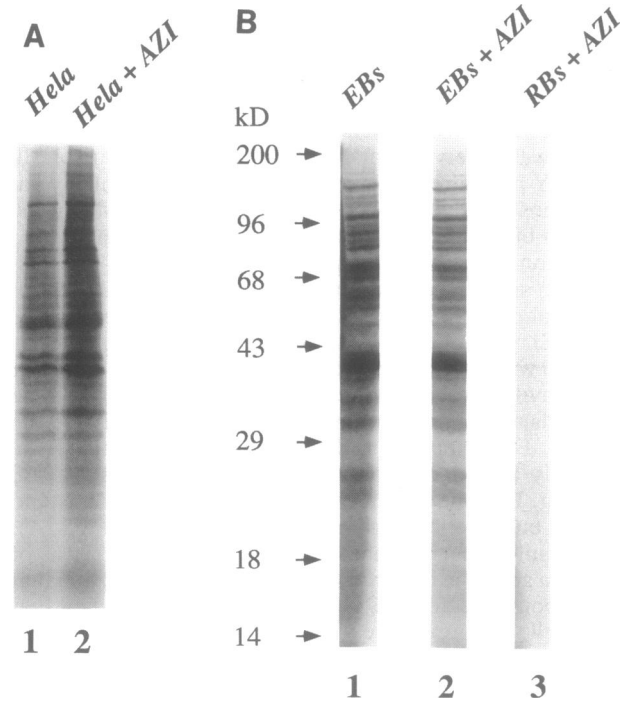

FIG. 2. (A) AZI does not inhibit HeLa cell protein synthesis. Lane 1, uninfected HeLa cells; lane 2, uninfected HeLa cells treated with AZI for 18 h. (B) AZI is not toxic to EBs. Lane 1, EBs not exposed to AZI prior to infecting HeLa cells for 18 h; lane 2, EBs exposed to AZI (1 μg/ml) for 15 min and then washed prior to infecting HeLa cells for 18 h; lane 3, HeLa cells infected with chlamydia for 18 h and incubated with AZI for 15 min.

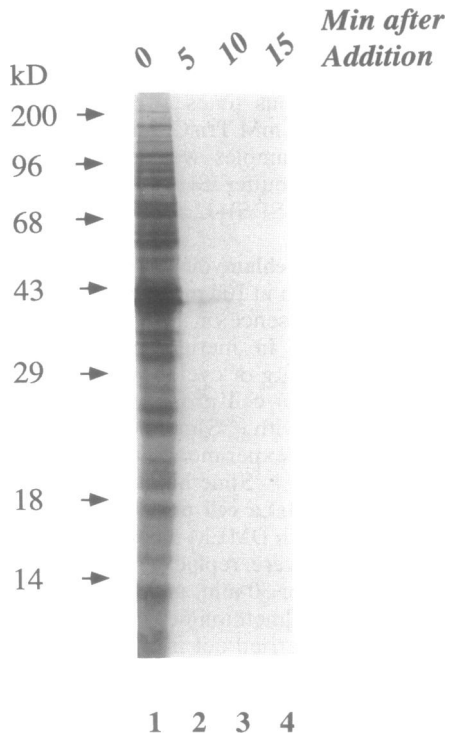

FIG. 3. Time course of inhibition of protein synthesis by AZI. HeLa cells were infected with chlamydia for 18 h. AZI (1 μg/ml) was added, and then pulse-labelling was done for 5 min with [^{35}S]methionine at 0 min (lane 1), 5 min (lane 2), 10 min (lane 3), or 15 min (lane 4) after the addition of drug.

labelling with [^{35}S]methionine for 30 min. Figure 2B demonstrates that de novo protein synthesis in chlamydia was not inhibited if the inoculating EBs had been exposed previously to AZI (lanes 1 and 2). In contrast, when chlamydia were treated with AZI for 15 min at 18 hpi (at which time they are predominantly intracellular RBs), complete inhibition of de novo protein synthesis was observed (lane 3). Thus, AZI does not inhibit chlamydial protein synthesis indirectly via a toxic effect upon metabolically inactive EB.

Time course of inhibition of chlamydial protein synthesis by AZI. To measure how rapidly AZI inhibited chlamydial protein synthesis in tissue culture, HeLa cells were infected with chlamydia for 18 h. The cells were pulse-labelled with [^{35}S]methionine for 5 min at various times after the addition of AZI (1 μg/ml), and extracts were electrophoresed on SDS-polyacrylamide gels to assess the pattern and amount of protein synthesis. Figure 3, lane 2, demonstrates that within 5 min after the addition or AZI, chlamydial protein synthesis was completely inhibited. Thus, the onset of inhibition of protein synthesis by AZI was quite rapid, and the inhibition of chlamydial protein synthesis affected the synthesis of all proteins equally well.

The inhibition of chlamydial protein synthesis by AZI is a direct effect. These results demonstrate that AZI inhibits chlamydial protein synthesis, but they do not address the issue as to whether this effect is direct or indirect. Specifically, AZI could inhibit chlamydial protein synthesis indirectly by inhibiting chlamydial RNA synthesis or by promoting phagolysosmal fusion, resulting in rapid death of the intracellular RBs. Although the observation that AZI inhibited de novo chlamydial protein synthesis within 5 min after

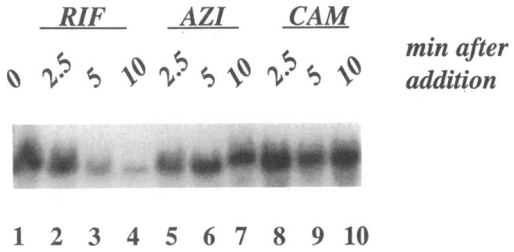

FIG. 4. AZI does not inhibit mRNA transcription. HeLa cells were infected with chlamydia for 20 h, and RIF (100 μg/ml, lanes 2 to 4), AZI (1 μg/ml, lanes 5 to 7), and CAM (100 μg/ml, lanes 8 to 10) were added. At 0 min (lane 1), 2.5 min (lanes 2, 5, and 8), 5 min (lanes 3, 6, and 9), or 10 min (lanes 4, 7, and 10) after addition of the drug, total RNA was electrophoresed, Northern blotted, and hybridized to a DNA probe made by nick translation of the KARP gene.

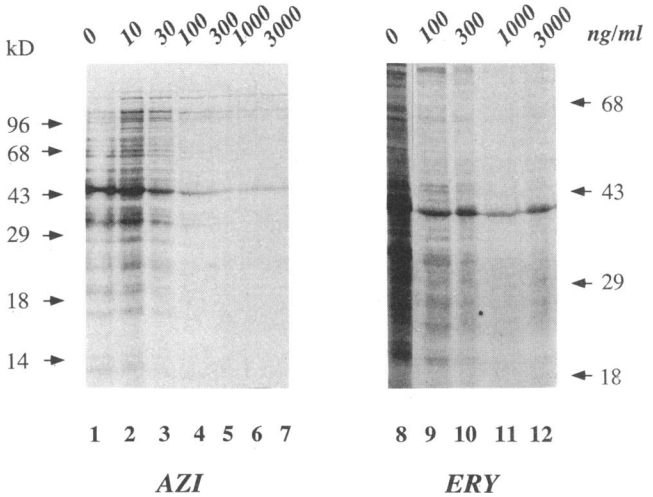

FIG. 5. Inhibitory concentration of AZI on protein synthesis in host-free RBs. Host-free RBs prepared from HeLa cells infected with chlamydia for 18 h were incubated in RB buffer containing no drug (lanes 1 and 8), AZI (lane 2, 10 ng/ml; lane 3, 30 ng/ml; lane 4, 100 ng/ml; lane 5, 300 ng/ml; lane 6, 1,000 ng/ml; lane 7, 3,000 ng/ml) or ERY (lane 9, 100 ng/ml; lane 10, 300 ng/ml; lane 11, 1,000 ng/ml; lane 12, 3,000 ng/ml) for 15 min at 37°C and then pulse-labelled with [^{35}S]methionine.

antibiotic addition makes the second possibility less likely, both the effect of AZI on chlamydial RNA synthesis and the effect of AZI on protein synthesis in host cell-free RBs were further investigated.

(i) Inhibition of RNA synthesis. To determine whether AZI directly affects RNA synthesis, the half-life of a specific chlamydial mRNA in the presence of AZI, CAM, or RIF (a known inhibitor of prokaryotic RNA synthesis) was measured by Northern (RNA) blot analysis. The concentrations of CAM and RIF used in this experiment have been used previously for measurement of the effect of inhibitors of RNA and protein synthesis on mRNA synthesis in bacteria (3). AZI, CAM, RIF, or no drug was added to chlamydia-infected HeLa cells at 18 hpi. At 0, 2.5, 5.0, and 10 min after the addition of drug, total RNA was prepared, Northern blotted, and hybridized to a DNA probe made from a representative cloned chlamydial gene, the KARP gene. This gene encodes a protein made late during chlamydial infection, and its mRNA is one of the few chlamydial mRNAs easily detectable on Northern blots (13). Figure 4 demonstrates that while RIF, a known direct inhibitor of bacterial transcription, decreased the steady-state levels of the chlamydial KARP mRNA, treatment with AZI or CAM had no such effect. Thus, AZI does not inhibit KARP mRNA synthesis and probably has no direct effect on the chlamydial RNA metabolism.

(ii) AZI inhibits chlamydial protein synthesis in host cell-free RBs. To test whether the direct effect of AZI might have been to affect the fate of chlamydia in the endocytic pathway rather than to directly inhibit chlamydial protein synthesis, the effect of AZI on chlamydial protein synthesis in host cell-free RBs (which are no longer enclosed in endosomes) was investigated. Figure 5 demonstrates that AZI does inhibit de novo chlamydial protein synthesis in host cell-free RBs following a 15-min exposure to the antibiotic. On the basis of several experiments, the drug concentration at which de novo chlamydial protein synthesis was entirely abolished was 300 to 1,000 ng/ml. In parallel experiments, the concentration at which ERY entirely inhibited de novo chlamydial protein synthesis was 1,000 to 3,000 ng/ml.

The duration of action of AZI compared with that of ERY in chlamydia-infected cells. To compare the duration of action of AZI compared with that of ERY in chlamydia grown in epithelial cells, the rate at which the inhibition of chlamydial protein synthesis decayed following removal of the drug was measured. HeLa cells were infected with chlamydia and then exposed to AZI (1 μg/ml) or ERY (1 μg/ml) for 15 min. The cultures were washed free of drug, and chlamydial protein synthesis was measured by pulse-labelling with [^{35}S]methionine for 30 min at various times after removal of the antibiotic. Two hours after removal of AZI or ERY, chlamydial protein synthesis was inhibited (data not shown). Figure 6 demonstrates that 6 h following removal of AZI, chlamydial protein synthesis was still inhibited (lane 5). In contrast, chlamydial protein synthesis in the cultures treated 6 h previously with ERY was no longer inhibited (lane 6). However, by 9 h after removal of AZI, chlamydial protein synthesis resumed (lane 9). These experiments suggest that the inhibition of chlamydial protein synthesis by AZI persists longer after a 15-min exposure to the drug than does the inhibition by ERY, despite the fact that the potency of ERY is threefold greater. These observations are consistent with the clinical studies that demonstrate that AZI requires less frequent dosing than ERY to adequately treat most human chlamydial infections of the genital tract.

DISCUSSION

In this paper, the mechanism of action of AZI on the murine strain of *C. trachomatis* grown in tissue culture epithelial cells is addressed. The concentration at which AZI completely inhibits chlamydial growth and protein synthesis in cultured epithelial cells is shown to be approximately 100 ng/ml, a value that agrees well with prior in vitro data from human strains of *C. trachomatis* grown in tissue culture. By morphological criteria, the block to chlamydial growth appears to occur early in its life cycle, as no chlamydial vacuoles are observed under light microscopy in AZI-treated cultures. This finding contrasts the effect of the penicillin class of antibiotics on chlamydial growth; these drugs block

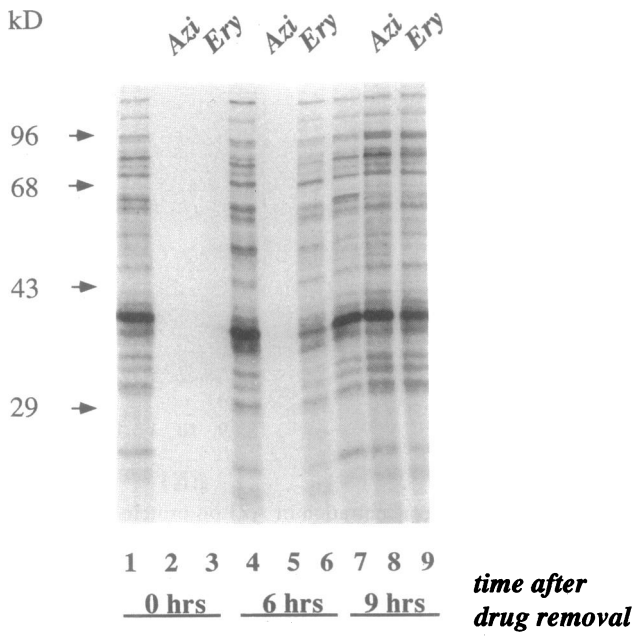

FIG. 6. Duration of action of AZI and ERY. HeLa cells were infected with chlamydia for 12 h (lanes 7 to 9) or 21 h (lanes 1 to 6). AZI (1 μg/ml) or ERY (1 μg/ml) was added to the media, and the cultures were incubated for an additional 15 min at 37°C. The cells were washed and then incubated for 0 h (lanes 1 to 3), 6 h (lanes 4 to 6), or 9 h (lanes 7 to 9) without drug and then pulse-labelled with [^{35}S]methionine.

the chlamydial life cycle at the RB-to-EB transition (11) and result in large chlamydia-laden vacuoles arrested at the RB stage. AZI is not directly toxic to EBs; EBs can be washed free of the drug after a brief exposure, and their subsequent infection proceeds normally. AZI does inhibit chlamydial protein synthesis. This inhibition appears quite general in nature; there is no selective inhibition of a particular subclass of chlamydial proteins. Moreover, the onset of the inhibition of protein synthesis is quite rapid and can be seen as early as 5 min after exposure to the drug.

That the inhibition of chlamydial protein synthesis by AZI appears to be a direct effect on translation is based on two criteria. First, AZI does not inhibit mRNA synthesis of chlamydial mRNAs. In the presence of AZI, the steady-state levels of the KARP gene mRNA do not decrease as assessed by Northern blot analysis, whereas the steady-state level of KARP mRNA does decrease in the presence of RIF. In fact, the RIF experiment allows the half-life of the KARP mRNA to be estimated to be on the order of 1 to 2 min. Second, AZI blocks chlamydial protein synthesis in host-free RBs in a manner indistinguishable from its inhibition in infected cells, although it requires a slightly higher concentration of drug. The onset of this inhibition is rapid and can be seen at least as early as 15 min after addition of the drug (earlier times were not tested). Since these host-free RBs are no longer in a vacuole, it is unlikely that the inhibition of chlamydial protein synthesis by AZI occurs primarily by overriding the chlamydial block to lysosomal fusion. It was surprising that the concentration of AZI or ERY needed to inhibit protein synthesis in host-free RBs was actually greater than the concentration needed to inhibit chlamydial protein synthesis intracellularly. Interestingly, the inhibition of chlamydial protein synthesis following a brief exposure to AZI was more long lasting than that following a brief exposure to ERY. Taken together, these findings may reflect the selective accumulation of AZI in tissue culture epithelial cells; notably, AZI has been previously found to accumulate in macrophages and neutrophils (19). These observations may explain the apparent success of single-dose therapy in clinical trials of AZI for the eradication of genital chlamydial infections (7, 10, 18).

ACKNOWLEDGMENTS

This work was supported by a grant from Pfizer.
I thank Don Ganem for helpful discussions.

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