In Vitro Assays of the Efficacy of Antimicrobial Agents in Controlling *Chlamydia trachomatis* Propagation

CYNTHIA K. LEE,¹ WILLIAM R. BOWIE,² AND E. RUSSELL ALEXANDER^{1*}

Departments of Epidemiology¹ and Medicine,² University of Washington, Seattle, Washington 98195

Received for publication 29 September 1977

The antimicrobial susceptibility of a low-laboratory-passage, slow-growing, genital *Chlamydia trachomatis* strain was studied by five different procedures with the use of McCoy cells pretreated with 5-iodo-2-deoxyuridine. The effects of antimicrobial agents when added to cultures on day 0 or day 2 after inoculation with *C. trachomatis* and the effects of washing and reincubating treated cultures in antimicrobial-free media were investigated. Tetracycline and erythromycin inhibited *C. trachomatis* growth at concentrations attainable in human serum, although their actions were reversible and significantly higher concentrations were needed to "cure" 48-h infected cultures. On a weight basis, spectinomycin was relatively ineffective in inhibiting *C. trachomatis* growth. The minimal inhibitory concentration of penicillin measured by our assay procedures was higher than that reported by other investigators. The five assay procedures used in this study were reproducible, and our results indicate that we can obtain more pertinent information about the efficacy of an antimicrobial agent in controlling *C. trachomatis* growth by using a combination of these assays than by simple minimal inhibitory concentration determinations, as had been previously described by other investigators. In addition, we failed to demonstrate changes in tetracycline susceptibility of *C. trachomatis* isolates from two patients who had received tetracycline therapy.

In vitro studies on the antimicrobial susceptibility of *Chlamydia trachomatis* in cell culture systems have been described (1-3, 8, 11). These studies often utilized fast-growing laboratory-adapted *C. trachomatis* strains, usually assayed inhibitory effects of drugs by the concurrent addition of drugs and *C. trachomatis*, and did not assay for the noninfectious reticulate body form of *C. trachomatis*.

In this study we examined the effects of tetracycline, erythromycin, spectinomycin, and penicillin on *C. trachomatis* in a 5-iodo-2-deoxyuridine (IUdR)-treated McCoy cell system. The bacteriostatic and bactericidal effects of these antimicrobial agents were measured by five different assay procedures. We attempted to parallel the in vivo situation by (i) using a non-lymphogranuloma venereum *C. trachomatis* strain that is slow growing, in low laboratory passage, and of a serotype common to United States isolates, (ii) treating already-infected cultures since, in vivo, antimicrobial agents must act on preestablished infections, and (iii) removing antimicrobial agents and reincubating cultures after treatment because antimicrobial agents are metabolically cleared in vivo and it is possible that organisms could survive in the

reticulate body form and develop to the infective stage after removal of the antimicrobial agents. In addition to the one test strain used in all experiments, we also tested the tetracycline susceptibility of post-tetracycline-treatment isolates from two individuals.

MATERIALS AND METHODS

Cells. McCoy cells (mouse heteroploid cells) (4) were carried in antibiotic-free medium for at least five weekly passages before being seeded into 1-dram (1.177-g) "shell" vials containing 12-mm glass cover slips at concentrations of 1.5×10^5 cells/vial; they were seeded in medium containing 10 μ g of IUdR per ml (14) and were incubated for 3 days before inoculation.

Medium. No antimicrobial agents other than the test drugs were incorporated in the medium, which contained Eagle minimal essential medium with 10% fetal calf serum, 7 mM sodium bicarbonate and 2 mM glutamine. Test vials of IUdR-treated cells were prepared in this medium supplemented with 5% glucose. Inoculated cells were maintained in medium supplemented with 5% glucose and 20 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid buffer at pH 7.5.

***C. trachomatis*.** The test strain used in all experiments was a urethral isolate from a patient with non-gonococcal urethritis (NGU), designated N24-1. The patient had not taken antimicrobial agents for at least

3 months prior to culture. The strain was serotyped as ED by the method of Wang et al. (13). The original specimen was collected in medium containing 50 µg of streptomycin per ml and 25 U of nystatin per ml, and the strain was isolated and passaged twice in medium containing 10 µg of gentamicin per ml and 25 U of nystatin per ml. For antimicrobial testing purposes, this strain was put through two serial passages in an antibiotic-free system. Portions were frozen in 0.2 M sucrose in 0.02 M phosphate buffer, pH 7.0 (2SP), at -70°C. A single lot of inocula was used for all experiments (passage four *C. trachomatis*); the inoculum was diluted to 5×10^4 inclusion-forming units/ml in 2SP immediately before use.

Other strains tested were processed in the same way as N24-1. N40-2 was a passage five *C. trachomatis* serotype GF strain isolated from the urethra of an NGU patient who had not taken antimicrobial drugs for at least 3 months prior to culture. Rx109-1, Rx109-4, and Rx109-5 were isolates from the cervix of an NGU contact: Rx109-1 was a passage three *C. trachomatis* serotype GF (UW134) strain isolated prior to tetracycline treatment, Rx109-4 was a passage two *C. trachomatis* strain isolated 3 weeks after 5 weeks of tetracycline treatment, and Rx109-5 was a passage two *C. trachomatis* serotype GF (UW146) strain isolated 4 weeks after tetracycline treatment. E002-3 was a passage five *C. trachomatis* serotype ED strain isolated from the urethra of an epididymitis patient 5 weeks after 10 days of tetracycline treatment.

Antimicrobial agents. Tetracycline hydrochloride (intravenous, buffered with ascorbic acid) was from Pfizer Inc., erythromycin lactobionate (intravenous) was from Abbott Laboratories, and potassium penicillin G (for injection, USP) was from Eli Lilly & Co. Spectinomycin dihydrochloride pentahydrate (Trobicin susceptibility powder) was a gift from The Upjohn Co. All antimicrobial agents were supplied in

powder form and were reconstituted immediately before use.

Inoculation procedure. Three-day-old IUDR-treated McCoy cells were treated with 1 ml of 30 µg/ml diethylaminoethyl-dextran in 0.95% NaCl per vial for 30 min. Each vial was then inoculated with either 0.1 ml of the dilution of test strain that yielded 5×10^3 inclusions/cover slip or one vial of disrupted infected cells in cases where we were assaying for infectious *C. trachomatis*. These vials were centrifuged for 60 min at $2,380 \times g$ and 25°C. For examination, cover slips were fixed in situ and iodine stained by the methods of Gordon et al. (5).

Treatment of cultures at day 0 [MIC, MBC(0), W&R(0)] (refer to Table 1). After inoculation and centrifugation, vials were immediately overlaid with 1 ml of medium containing the proper dilution of test drug. These were incubated for 72 h at 37°C. Five vials per dilution were iodine stained, and the MIC was determined as that concentration which failed to yield iodine-staining inclusions in all five vials. At 72 h, another five vials per dilution were rinsed with GKN (0.1% glucose in a 0.85% NaCl and 0.4% KCl solution), freeze-thawed in 0.5 ml of 2SP per vial, and passaged by procedures already described. The MBC(0) was determined to be the lowest concentration that failed to yield infectious particles as assayed by passage. A third set of five vials per dilution was washed twice with GKN (30-min incubation at room temperature for each wash), overlaid with drug-free medium, and reincubated for 72 h. Direct cover slip examination was not routinely performed, but the cultures were passed into fresh monolayers after reincubation for assay of infectious particles. The lowest concentration that failed to yield infectious particles after wash and reincubation was termed W&R(0).

Treatment of cultures at day 2 [MBC(2), W&R(2)] (refer to Table 1). Forty-eight-hour in-

TABLE 1. Assay procedures for titration of *C. trachomatis* (CT) on McCoy cells pretreated with antimicrobial agents (Am)

Day	Antimicrobial assays					CT titration ^a
	MIC	MBC(0)	W & R(0)	MBC(2)	W & R(2)	
0	+CT +Am	+CT +Am	+CT +Am	+CT	+CT	+Am
1						
2				+Am	+Am	+CT
3	Direct examination for iodine-staining in- clusions	Removal of Am and passed ^b	Removal of Am			
4				Removal of Am and passed ^b	Removal of Am	
5						Direct examination for iodine-staining inclu- sions
6			Passed			
7					Passed	

^a CT titration = titration of *C. trachomatis* on McCoy cells pretreated with antimicrobial agents.

^b All passes examined for iodine-staining inclusions after 72 h of incubation.

fectected monolayers were treated with antimicrobial agents for 48 h, after which they were washed twice with GKN (30 min at room temperature for each wash). Five vials per dilution were passed into fresh monolayers of cells; the lowest concentration that failed to yield infectious *C. trachomatis* was termed MBC(2). Another five vials per dilution were washed, reincubated, and passaged as for W&R(0); the lowest concentration that failed to yield infectious *C. trachomatis* after this procedure was termed W&R(2).

Titration of *C. trachomatis* on McCoy cells pretreated with antimicrobial agents (refer to Table 1). IUdR-treated monolayers of McCoy cells were preincubated with antimicrobial agents for 48 h, and the monolayers were then washed by three different procedures before inoculation with *C. trachomatis*: (i) washed twice with GKN, 30-min incubation at room temperature for each wash; (ii) washed five times with GKN, last two washes with 30-min incubation at room temperature for each wash; (iii) washed twice with GKN, 30-min incubation at room temperature for each wash; after inoculation and 24-h incubation at 37°C, the medium was changed. *C. trachomatis* was titered on these monolayers.

RESULTS

Titration of *C. trachomatis* on McCoy cells pretreated with antimicrobial agents. Reduction of *C. trachomatis* inclusions was assayed on McCoy cells pretreated with tetracycline, erythromycin, or spectinomycin. The results presented in Table 2 show that, when the first wash procedure of two 30-min washes was used, cells pretreated with $\geq 8 \mu\text{g}$ of tetracycline per ml, $\leq 16 \mu\text{g}$ of erythromycin per ml, and $\geq 125 \mu\text{g}$ of spectinomycin per ml inhibited *C. tra-*

chomatis growth. Even at the lower concentrations where there was no reduction in inclusion-forming units, the inclusions were abnormal in appearance, probably as a result of the presence of residual amounts of the antimicrobial agents.

Antimicrobial assays. The results obtained with all five procedures used to assay antimicrobial effects on *C. trachomatis* are presented in Table 3. Each end point was determined by the absence of iodine-staining inclusions in five replicate vials. All tests were performed at least twice on separate occasions, and variations were expressed as a range. Tetracycline, erythromycin, and spectinomycin had reproducible end points, varying only in single twofold dilutions.

The MICs of penicillin were inconsistent, ranging from 16 to greater than 64 U/ml. The end point was neither clear-cut nor reproducible; beginning at 2 U/ml, only a few of the five replicate vials showed the presence of inclusion bodies. The MBC(0) results were also less reproducible for penicillin; the range brackets two twofold dilutions. By the remaining three procedures, no end points could be obtained at the concentrations of penicillin tested.

Susceptibility of other clinical isolates to tetracycline. Five other *C. trachomatis* isolates were tested for their susceptibility to tetracycline. None of the post-treatment isolates (Rx109-4, Rx109-5, and E002-3) differed from either the pretreatment isolates (Rx109-1 and N40-2) or the test strain (N24-1) in the MIC or MBC(0) of tetracycline required (0.125 $\mu\text{g}/\text{ml}$ in all cases).

TABLE 2. *C. trachomatis* titers on drug-treated McCoy cells^a

Antimicrobial drug	Wash procedures		
	(i) Two washes	(ii) Five washes	(iii) Three washes + 24-h medium change
Input (no drug)	1.92×10^{3b}		
Tetracycline			
4 $\mu\text{g}/\text{ml}$	1.50×10^3	6.18×10^2	ND ^c
8 $\mu\text{g}/\text{ml}$	~50	~75	ND
16 $\mu\text{g}/\text{ml}$	None	~50	ND
32 $\mu\text{g}/\text{ml}$	None	None	ND
Erythromycin			
16 $\mu\text{g}/\text{ml}$	~30	~50	ND
32 $\mu\text{g}/\text{ml}$	None	6	ND
64 $\mu\text{g}/\text{ml}$	None	None	ND
Spectinomycin			
62.5 $\mu\text{g}/\text{ml}$	2.86×10^3	1.04×10^3	1.35×10^3
125 $\mu\text{g}/\text{ml}$	$(5 \times 10^2)^d$	~50	~55
250 $\mu\text{g}/\text{ml}$	$(6)^d$	1.5	0.5
500 $\mu\text{g}/\text{ml}$	$(\text{None})^d$	0.5	0.5

^a No obvious cytotoxicity was noted by light microscope examination either before *C. trachomatis* inoculation (after antimicrobial treatment) or after incubation with *C. trachomatis*.

^b Iodine-staining inclusions per cover slip. All counts were based on the average of two cover slips.

^c Not done.

^d These results were from a separate experiment where the input was 4.3×10^3 inclusions/cover slip.

TABLE 3. Antimicrobial susceptibility of *C. trachomatis* strain N24-1

Antimicrobial agent	MIC	MBC(0)	W & R(0)	MBC(2)	W & R(2)	Cytotoxicity ^a
Tetracycline, $\mu\text{g/ml}$	0.125	0.125		250	250	Cytotoxic at 250 $\mu\text{g/ml}$
Erythromycin, $\mu\text{g/ml}$	0.25 $\rightarrow$ 0.5	0.25 $\rightarrow$ 0.5	32	250	500	Cytotoxic at 250 $\mu\text{g/ml}$
Spectinomycin, $\mu\text{g/ml}$	125 $\rightarrow$ 250	125 $\rightarrow$ 250	125	2,000	250	No apparent cytotoxicity at these concentrations
Penicillin, U/ml	16 $\rightarrow$ >64	4 $\rightarrow$ 32	>64	>8,000	>8,000	No apparent cytotoxicity at these concentrations

^a Determined by light microscope examination of cell monolayer.

DISCUSSION

We have reported here a detailed study of the effects of four antimicrobial agents on *C. trachomatis* in cell culture. The in vitro MIC and MBC(0) of tetracycline and erythromycin were within the concentrations attainable in vivo in serum (10, 15). However, the inhibitory effects of these agents were reversible upon drug removal [W&R(0)]. Neither drug was very effective in "curing" already-infected cultures [MBC(2) and W&R(2)]. We conclude that these two drugs are mainly bacteriostatic and not bactericidal in their action against *C. trachomatis*. It appears that eradication of in vivo *C. trachomatis* infections with these drugs would have to be in conjunction with effective host-defense mechanisms.

Penicillin had been shown to inhibit *C. trachomatis* in vitro but to be ineffective in vivo (6). Previous investigators reported the in vitro MIC of penicillin to be 0.1 to 1 U/ml (2, 3, 8, 11), whereas we found the MIC to be 16 to >64 U/ml. This discrepancy may be due to our use of a slower-growing *C. trachomatis* strain which may be less susceptible to penicillin action on the cell walls of dividing cells. Another contributing factor may be the large number of replicas used in this study. Only some of the five replicate vials showed *C. trachomatis* inclusions at concentrations of 2 U/ml or greater. The reversibility of the inhibitory process [W&R(0)] was not tested beyond 64 U/ml. High titers of infectious particles were obtained from reincubated cultures at concentrations of penicillin that inhibited formation of infectious particles. Penicillin did not sterilize infected cultures at concentrations as high as 8,000 U/ml [MBC(2), W&R(2)]. There were no observable cytotoxic effects on the host cells. The titers were reduced and the iodine-staining inclusions were abnor-

mal in appearance after penicillin treatment: a few large bodies could be seen in each inclusion, and these were embedded in the dark-staining glycogen mass. These were undoubtedly the giant reticulate bodies described in transmission electron microscope studies on *C. trachomatis* (7) and on *C. psittaci* (9). After removal of penicillin and reincubation, the inclusions were normal again in appearance. This again corresponds with the transmission electron microscopy studies in which the giant reticulate bodies bud off and reorganize into normal-size reticulate and elementary bodies upon removal of penicillin.

The MIC of spectinomycin was relatively high, and the inhibitory effects were not reversible at this concentration. Our results indicate that the irreversibility may be due to factors other than the direct action of spectinomycin. As can be seen in Table 2, manipulation of wash procedures did not affect the *C. trachomatis* titers on spectinomycin-pretreated McCoy cells. This suggests either that the drug remained bound to the host cell or that the drug had irreversibly affected the host cell so that it could not support *C. trachomatis* propagation. In the treatment of preinfected cultures, at 2,000 $\mu\text{g/ml}$, no infectious particles were present after treatment. After wash and reincubation, the lowest concentration that prevented production of infectious particles decreased to 250 $\mu\text{g/ml}$. Even if there was toxicity to the host cell, infectious particles present after drug treatment at concentrations of less than 2,000 $\mu\text{g/ml}$ should still be viable after reincubation. We believe that these results imply that the intracellular spectinomycin could not be removed, and the 72-h reincubation period became an extra 72-h treatment period.

The main objective of this study was to examine the effects of antimicrobial agents on *C.*

trachomatis growth in vitro by procedures which parallel in vivo conditions. Our MIC and MBC(0) procedures were similar to those of previous studies (1, 8, 11, 12), but these two procedures may not adequately describe the efficacy of an antimicrobial agent against *C. trachomatis*. Our procedures included the comparison of the addition of antimicrobial agents at day 0 and day 2 after *C. trachomatis* inoculation and the removal of antimicrobial agents from the cultures after a treatment period to examine their effectiveness in eradicating noninfectious forms of *C. trachomatis*. Our results indicate that both of these variables significantly influence the "effective" concentration of an antimicrobial agent in controlling *C. trachomatis*. The five procedures described here were reproducible, and we believe that a combination of some or all of these procedures would better describe the efficacy of an antimicrobial agent in controlling *C. trachomatis* propagation. However, the validity of these in vitro assays is dependent on the concurrent examination of possible interfering factors such as host-cell toxicity and inefficiency in drug removal. We also advocate the use of *C. trachomatis* strains that are more typical of the majority of clinical isolates: non-lymphogranuloma venereum, slow growing, and in low laboratory passage.

ACKNOWLEDGMENTS

This research was supported by Public Health Service program project AI-12192 from the National Institute of Allergy and Infectious Diseases and by a fellowship from the Medical Research Council of Canada (W.R.B.).

LITERATURE CITED

1. Alexander, E. R., P. Skahen, and K. K. Holmes. 1977. Antibiotic susceptibility of *Chlamydia trachomatis* in cell culture, p. 223-226. In D. Hobson and K. K. Holmes (ed.), Nongonococcal urethritis and related infections. American Society for Microbiology, Washington, D.C.
2. Barker, L. F. 1969. Determination of antibiotic susceptibility of rickettsiae and chlamydiae in BS-C-1 cell cultures, p. 425-428. Antimicrob. Agents Chemother. 1968.
3. Bernkopf, H., P. Mashiah, and Y. Becker. 1962. Susceptibility of a trachoma agent grown in FL cell cultures to antibiotics and a sulfa drug. Ann. N.Y. Acad. Sci. 98:61-67.
4. Gordon, F. B., H. R. Dressler, A. L. Quan, W. T. McQuilkin, and J. I. Thomas. 1972. Effect of ionizing irradiation on susceptibility of McCoy cell cultures to *Chlamydia trachomatis*. Appl. Microbiol. 23:123-129.
5. Gordon, F. B., I. A. Harper, A. L. Quan, J. D. Treharne, R. St. C. Dwyer, and J. A. Garland. 1969. Detection of Chlamydia in certain infections in man. I. Laboratory procedure: comparison of yolk sac and cell culture for detection and isolation. J. Infect. Dis. 120:451-462.
6. Hobson, D., E. Rees, F. W. A. Johnson, and I. A. Tait. 1977. Effect of penicillin on *Chlamydia trachomatis* in vitro and in vivo, p. 227-229. In D. Hobson and K. K. Holmes (ed.), Nongonococcal urethritis and related infections. American Society for Microbiology, Washington, D.C.
7. Kramer, M. J., and F. B. Gordon. 1971. ultrastructural analysis of the effects of penicillin and chlortetracycline on the development of a genital tract *Chlamydia*. Infect. Immun. 3:333-341.
8. Kuo, C.-C., S.-P. Wang, and J. T. Grayston. 1977. Antimicrobial activity of several antibiotics and a sulfonamide against *Chlamydia trachomatis* organisms in cell culture. Antimicrob. Agents Chemother. 12:80-83.
9. Matsumoto, A., and G. P. Manire. 1970. Electron microscopic observations on the effects of penicillin on the morphology of *Chlamydia psittaci*. J. Bacteriol. 101:278-285.
10. Ory, E. M. 1974. The tetracyclines, p. 95-104. In B. M. Kagan (ed.), Antimicrobial therapy, 2nd ed. W. B. Saunders Co., Philadelphia.
11. Ridgway, G. L., J. M. Owen, and J. D. Oriel. 1976. A method for testing the antibiotic susceptibility of *Chlamydia trachomatis* in a cell culture system. J. Antimicrob. Chemother. 2:71-76.
12. Treharne, J. D., J. Day, C. K. Yeo, B. R. Jones, and S. Squires. 1977. Susceptibility of chlamydiae to chemotherapeutic agents, p. 214-222. In D. Hobson and K. K. Holmes (ed.), Nongonococcal urethritis and related infections. American Society for Microbiology, Washington, D.C.
13. Wang, S.-P., C.-C. Kuo, and J. T. Grayston. 1973. A simplified method for immunological typing of trachoma-inclusion conjunctivitis-lymphogranuloma venereum organisms. Infect. Immun. 7:356-360.
14. Wentworth, B. B., E. R. Alexander. 1974. Isolation of *Chlamydia trachomatis* by use of 5-iodo-2-deoxyuridine-treated cells. Appl. Microbiol. 27:912-916.
15. Woodsell, S. H., J. T. Wilson. 1974. Erythromycin and troleandomycin, p. 71-82. In B. M. Kagan (ed.), Antimicrobial therapy, 2nd ed. W. B. Saunders Co., Philadelphia.