Ultrastructural Analysis of the Effects of Penicillin and Chlortetracycline on the Development of a Genital Tract Chlamydia

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The developmental cycle of a genital tract Chlamydia (MRC-1/G) growing in irradiated monkey kidney cell monolayers was analyzed by electron microscopy. Growth was essentially similar to that of C. psittaci and ocular isolates of C. trachomatis. Penicillin (50 units/ml) interfered with the growth cycle by producing greatly enlarged initial bodies which sometimes measured 3 μm instead of the normal 1 to 1.3 μm in diameter. In contrast to penicillin, chlortetracycline (10 μg/ml) did not grossly distort morphology of the initial body but had a definite inhibitory effect on the formation of elementary bodies depending upon the time postinfection the antibiotic was added. If added 24 hr or later postinfection, typical inclusions containing elementary bodies were formed. When administered 18 hr postinfection, the transition of initial bodies to elementary bodies was prevented, and the inclusion was much smaller when examined at 48 hr in comparison to a control without chlortetracycline. Addition of the drug at 6 or 12 hr postinfection resulted in very small inclusions which contained only one or two chlamydiae of an initial body type. It was not possible to detect chlamydiae when chlortetracycline was added immediately after adsorption (0 hr), and cells were examined by light and electron microscopy at 48 hr.

Members of the genus Chlamydia are obligate intracellular microorganisms which undergo a complex cycle of development. Entry of the mature infectious form, the elementary body, into a host cell initiates the cycle which begins with transformation of the elementary body into the more fragile initial body or reticulate body. Multiplication occurs and eventually elementary bodies are formed through a process of maturation and condensation. The ultrastructural aspects of this developmental cycle, as observed in C. psittaci and in isolates from lymphogranuloma venereum and trachoma (C. trachomatis), have been reported by several investigators (2-4, 15, 18). Higashi (11) noted no difference in the growth characteristics of C. psittaci and C. trachomatis as judged by electron microscopy.

More recently, C. trachomatis has been isolated frequently from the genital tract (6, 7, 10, 17) of men with nongonococcal urethritis and from the genital tract of women with cervicitis (5, 12, 16), although the etiological relationship between chlamydiae and these milder genital tract infections has not been completely established. For this study, strain MRC-1/G of C. trachomatis was chosen as a well-known genital tract isolate (13) to compare with the previously examined strains of different origins. The comparison included the effect of penicillin, which has been described for other isolates (3, 14, 19, 20-22). Chlortetracycline is used in the treatment of nongonococcal urethritis, and its effect on the developmental cycle of isolate MRC-1/G was investigated in detail in this study by electron microscopy.

MATERIALS AND METHODS

Organism. The genital tract isolate of C. trachomatis, designated as MRC-1/G (13), was used for infecting cell cultures. It was stored at -70°C as a 20% infected yolk sac suspension.

Tissue culture. A continuous line of monkey kidney cells (LLC-MK2) was carried on Eagle's minimum essential medium (MEM) containing 10% fetal calf serum, 1% MEM vitamin solution, and 2 mM L-glutamine. Stock cultures of the cells were subjected to
3,000 r of gamma or X irradiation because of the greater susceptibility to chlamydiae of irradiated cells, as described earlier (Dressler et al., Bacteriol. Proc., p. 117, 1965); the cells were then seeded into small roller bottles (roller vessel no. 7017, Bellco Glass Co., Inc., Vineland, N.J.) and maintained with the medium described above. Sufficient numbers of irradiated cells were used to provide a confluent monolayer in 4 or 5 days, after the surviving cells had increased in size.

**Infection procedure.** Maintenance medium was removed from monolayers of irradiated MK₂ cells; 0.5 ml of a 20% yolk sac suspension of MRC-1/G and 2.0 ml of MEM containing 20% horse serum, vitamins, l-glutamine, and 1% essential amino acids were added. The yolk sac suspension had been sonically treated for 5 min at 0 C immediately before use and had been maintained in an ice bath. Adsorption was for 2 hr at 2 rev/min in roller bottles held at 35 C. After adsorption, the inoculum was replaced with 20 ml of the same medium containing 50 μg of streptomycin per ml and 100 μg of vancomycin per ml. Incubation was at 35 C with a rotation rate of 0.5 rev/min.

**Penicillin.** Potassium penicillin G (50 units/ml) was added to the infected cell cultures after 48 hr, and cells were harvested for electron microscopy at 72 hr.

**Chloramphenicol.** Chloramphenicol hydrochloride (10 μg/ml) was added to infected cell cultures at 0, 6, 12, 18, 24, and 32 hr postinfection, and fixation for electron microscopy occurred at 48 hr.

**Electron microscopy.** Infected cell cultures were removed from the glass surfaces of the roller bottles by gentle scraping with a rubber policeman. Cells were concentrated by centrifugation (1,000 rev/min for 5 min) and washed three times in Hanks balanced salts solution before primary fixation with 5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.3) for 2 hr at 4 C. After glutaraldehyde fixation, the cells were washed three times in 0.1 M sodium cacodylate-0.2 m sucrose (pH 7.3) and postfixed in 2% OsO₄ in 0.1 M sodium cacodylate-0.2 M sucrose at 4 C for 1.5 hr. Cells were bathed in 0.5% aqueous uranyl acetate for 2 hr at room temperature, dehydrated in a graded ethanol series, passed through propylene oxide, and embedded in an Epon-Araldite mixture. Thin sections were poststained with uranyl acetate and lead citrate.

**Light microscopy.** As a correlation to electron microscopy, infected cells were stained by the Giemsa procedure and the Gimenez procedure (8) for light microscopic evaluation.

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**RESULTS AND DISCUSSION**

**Normal developmental cycle.** At an ultrastructural level, the development of the genital tract isolate of *C. trachomatis*, MRC-1/G (Fig. 1–6), was essentially similar to that of ocular isolates of *C. trachomatis* and also of *C. psittaci*.

At 12 hr postinfection (Fig. 1–2), the chlamydiae had transformed from the infectious form, the elementary body, into the initial body type. These initial bodies were contained in comparatively small inclusions which were surrounded by a membrane derived from the cell membrane of the host cell. The number of initial bodies per inclusion was small usually only four or five. The morphology of the initial bodies was heterogeneous even at this early stage of infection. Some were essentially spherical in shape, 1 μm in diameter, whereas others had maintained approximately the same diameter but had elongated to about 1.2 μm in length. Evidence for binary fission was seen among the elongated forms (Fig. 2).

The nature of the inclusion had changed by 18 hr postinfection in the sense that more chlamydiae of the initial body type were present, many cells gave evidence of active binary fission, and the inclusion itself was larger (Fig. 3–4). The chlamydiae were again not uniform in size. Most were spherical in shape with a diameter of about 0.8 μm whereas some were roughly two to three times larger. These larger forms are comparable to the "large bodies" or "giant bodies" which have been described by previous investigators (12, 15). The outer envelope of the initial body was seen as a loose-fitting wavy membrane and was reminiscent of the cell wall of gram-negative bacteria. The inner envelope, or cell membrane, was in close association with the cell cytoplasm.

The presence of several inclusions (Fig. 5) in a host cell at 24 hr postinfection provided one possible explanation for the large inclusions (Fig. 6) which were seen in the later stages of the developmental cycle. Coalescence of individual inclusions could result in the formation of one giant inclusion. The maturation of the initial body...
FIG. 5. Electron micrograph of a genital tract isolate of Chlamydia trachomatis strain MRC-I/G growing in irradiated MK₂ cells. The normal developmental cycle is shown. Fixation was at 24 hr postinfection. Several separate membrane-bound inclusions containing initial bodies are within the host cell; host cell nucleus (N) and mitochondria (M) are apparent. × 8,000.

FIG. 6. Electron micrograph of a genital tract isolate of Chlamydia trachomatis strain MRC-I/G growing in irradiated MK₂ cells. The normal developmental cycle is shown. Fixation was at 48 hr postinfection. Most of the cellular space is occupied by a large inclusion which contains elementary bodies (E) and transition forms (T) intermediate between the initial body type and the mature, infectious form—the elementary body. Ghost particles (G) and endospore-like particles (S) are also observed among the morphologically heterogeneous chlamydiae in the inclusion. × 20,000.
FIG. 7. Electron micrograph showing the effect of penicillin on the development of a genital tract isolate of Chlamydia trachomatis strain MRC-1/G growing in irradiated MK2 cells. Penicillin G (50 units/ml) caused a gross enlargement of the initial bodies (P). \( \times 39,000 \).

FIG. 8. Electron micrograph showing the effect of chlortetracycline on the development of a genital tract isolate of Chlamydia trachomatis strain MRC-1/G growing in irradiated MK2 cells. Chlortetracycline (10 \( \mu \)g/ml) was added at 32 hr postinfection, and cells were fixed for electron microscopy at 48 hr. Typical elementary bodies (E), with electron-dense centers and an outer envelope, are present. The inclusion is comparable in size to that shown in Fig. 6 and is indistinguishable from an infected control which was not exposed to chlortetracycline. Infected cells treated with chlortetracycline at 24 hr postinfection and fixed at 48 hr were similar to Fig. 6 and the 32-hr test (Fig. 8). \( \times 8,000 \).
into the smaller infectious elementary body (Fig. 6) by means of a morphological transformation signalled the termination of the developmental cycle. The ultrastructure of elementary bodies within the same inclusion was variable, with some appearing as uniformly electron-dense particles 0.3 μm in diameter and with only the outer envelope resolvable, whereas others had a central dense nucleoid surrounded by a more electron-transparent cytoplasm.

At 48 hr (Fig. 6), many typical elementary bodies were seen, but transition forms intermediate between initial bodies and elementary bodies and membrane-bound ghost particles were also present. The ghost particles may represent initial bodies which were defective and lysed during the developmental cycle. Those immediately adjacent to the boundary of the membrane-bound inclusion could possibly represent involutions of this host cell membrane, which, because of the plane of section, did not show continuity with the parent membrane. The presence of several endospore-like bodies surrounded by a common membrane (Fig. 6) may indicate that initial bodies can divide by a mechanism other than binary fission, or, like the ghost particles, they may represent degenerative forms which were not able to complete the developmental cycle.

Effect of penicillin. The genital tract isolate of C. trachomatis used in this study was influenced by penicillin in a manner similar to that described most recently by Matsumoto and Manire (14) for the meningopneumonitis strain of C. psittaci. Penicillin did not prevent the initial reorganization of the infecting elementary body into the initial body but instead inhibited binary fission by the initial body after 12 hr. As shown in Fig. 7, the growth of the MRC-1/G agent was not inhibited and abnormally large initial bodies were formed.

Effect of chlorotetracycline. The major point of interest in this study has been an electron microscopic evaluation of the effect of chlorotetracycline on the developmental cycle of a genital tract Chlamydia. Chlorotetracycline is used in the treatment of nongonococcal urethritis, a condition in which chlamydiae may have an etiological role. Antibiotic was added to cell cultures at selected intervals postinfection, and cells were fixed for electron microscopy at 48 hr postinfection. Chlorotetracycline-treated cell cultures were compared with controls, which were also infected with MRC-1/G but which did not receive antibiotic.

When the addition of chlorotetracycline was delayed until 24 or 32 hr postinfection and cells were fixed at 48 hr, there was no discernible effect of the antibiotic on the infection as observed by light or electron microscopy. In the 32-hr test (Fig. 8), the inclusions were large and contained numerous elementary bodies. When the drug was added at 24 hr, the inclusions were comparable in size to those of the control (Fig. 6) and the 32-hr test (Fig. 8) and contained elementary bodies. Ghost particles were no more frequent in the drug-treated preparations than they were in the control.

If chlorotetracycline was added at 18 hr postinfection, a definite difference could be noted in the inclusion and its contents by both electron and light microscopy. The electron microscopic appearance (Fig. 9) was quite different than the control (Fig. 6). The size of the inclusions was much smaller in the antibiotic-treated cells and no elementary bodies were present. The initial bodies in the inclusions were not visibly damaged, but the outer envelope of the chlamydial cells seemed to be in a loose association with the main body of the organism. Endospore-like bodies and ghost particles were present, but these may also be found in untreated controls (Fig. 3, 6) and they cannot be attributed to the action of the antibiotic. Light microscopic examination of Giemsa-stained smears of the antibiotic-treated cells revealed small inclusions containing few chlamydiae which were larger than elementary

Fig. 9. Electron micrograph showing the effect of chlorotetracycline added at 18 hr postinfection on the development of a genital tract isolate of Chlamydia trachomatis strain MRC-1/G growing in irradiated MKs cells. Cells were fixed for electron microscopy at 48 hr. The inclusion contains few chlamydiae and no elementary bodies are present. × 17,200.

Fig. 10. Electron micrograph showing the effect of chlorotetracycline added at 12 hr postinfection on the development of a genital tract isolate of Chlamydia trachomatis strain MRC-1/G growing in irradiated MKs cells. Cells were fixed for electron microscopy at 48 hr. Two very small inclusions are adjacent to the host cell nucleus (N). No elementary bodies are present. Ghost particles or spurious membranes are present in the upper inclusion. × 24,000.

Fig. 11. Electron micrograph showing the effect of chlorotetracycline added at 6 hr postinfection on the development of a genital tract isolate of Chlamydia trachomatis strain MRC-1/G growing in irradiated MKs cells. Cells were fixed for electron microscopy at 48 hr. Several inclusions containing only a single chlamydial are present. Ghost particles (G) are seen and some inclusions contain chlamydiae which appear to be damaged (D). Division of the organism was inhibited. A portion of the cell nucleus (N) and mitochondria (M) are seen at the bottom of the figure. × 20,000.
bodies and stained blue, whereas the elementary bodies that were present in the control cells stained red.

There were some similarities between the inclusions of cells administered antibiotic 18 hr postinfection (Fig. 9) with fixation at 48 hr and control cells which were infected and fixed at 18 hr postinfection (Fig. 3 and 4). The size of the inclusions was approximately the same, and the number of chlamydiae per inclusion was roughly equal. In contrast to the control cells, the antibiotic-treated cells (Fig. 9) contained chlamydiae of an initial body type in which the cytoplasm was appreciably electron-transparent in certain areas. Untreated control cells at 18 hr postinfection contained initial bodies with cytoplasms uniformly granular and moderately electron-dense (Fig. 3 and 4).

Observation of the preparations at 48 hr when chlortetracycline had been added at 12 hr postinfection (Fig. 10) revealed a drastic reduction in the size of the inclusions. The number of initial bodies per inclusion was less than that observed in an untreated control fixed at 12 hr postinfection. Only rarely did an inclusion contain more than one initial body in the antibiotic-treated cultures. Elementary bodies were never seen, but ghost particles were present.

When chlortetracycline was added at 6 hr postinfection (Fig. 11), inclusions, observed at 48 hr, generally contained only a single chlamydial cell. In Fig. 11, several individual membrane-bound inclusions are shown, presumably resulting from an initial infection by several individual elementary bodies. Large ghost particles possessing a granular matrix were present, and some chlamydiae within the inclusions showed obvious signs of degeneration. The limiting membranes of the chlamydial cell did not appear to be completely intact in some cases; the cytoplasm of some appeared plasmolytic, whereas still other inclusions contained only disorganized electron-dense material which was probably the remnants of chlamydial cells. It was not possible to detect infected MK6 cells either by light or by electron microscopy when chlortetracycline was added at 0 hr.

On the basis of observations of infected yolk sacs and light microscopy (9), the action of chlortetracycline on chlamydiae was interpreted as being primarily bacteriostatic. The results of this study have confirmed that conclusion and have shown, within a single developmental cycle, that a bacteriostatic effect is demonstrable when chlortetracycline is added up to 18 hr after infection but not at 24 hr or later. Addition of chlortetracycline during the very early stages of infection (0 to 6 hr) appeared to result in the destruct-
tion or removal of chlamydiae by 48 hr. Chlamydiae were obviously damaged when the drug was added at 6 hr (Fig. 11), i.e., at 42 hr before fixation, in marked contrast to their appearance (Fig. 10) when drug was added at 12 hr (36 hr before fixation). The absence of detectable infected cells when the antibiotic was added at the end of the absorption period (0 hr) probably cannot be entirely explained by the low probability of seeing only single elementary bodies that did not begin the developmental cycle.

The destruction or damage to chlamydiae at early stages may result from the synergistic action of a bacteriostatic antibiotic and lysozymes. During the very early stages of infection, when protein synthesis and growth are beginning, the chlamydial cell may be especially susceptible to the action of drugs which inhibit protein synthesis. Alexander (1) demonstrated that infectious C. psittaci (meningopneumonitis strain) agent was not formed in L cells which had been treated with chloramphenicol or chlortetracycline during the first 20 hr after infection, whereas puromycin had to be added during the first 10 hr postinfection to produce a similar effect.

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LITERATURE CITED


