Different Growth Rates of *Chlamydia trachomatis* Biovars Reflect Pathotype

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**Background.** Despite small genomic differences, *Chlamydia trachomatis* biovars exhibit diverse disease manifestations and different growth rates in vivo and in cell culture models.

**Methods.** Chlamydial inclusion-forming units were enumerated over time in HeLa cells, to evaluate the length of the developmental cycle for *C. trachomatis* strains A, B, C, and E/Bour (ocular strains) as well as D, EUW5/Cx, F, and L2 (genital strains). Prototype strains A, D, and L2 were selected for detailed analysis of reticulate body growth, division, and genomic replication. The impact that changing host cells and that coinfection with different strains has on growth was also assessed.

**Results.** The genital strains completed the developmental cycle in 36–44 h, whereas the ocular strains lagged behind considerably. Differences were the result of a longer lag phase (entry plus differentiation) and generation time for the ocular strains. A prototype ocular strain grew faster in conjunctival cells than in cervical cells. Coinfection with genital (D or L2) and ocular strains expedited recovery of the ocular strain.

**Conclusions.** Precise temporal evaluation of the chlamydial developmental cycle for selected genital and ocular *C. trachomatis* biovars provides a means for investigating genomic differences that define chlamydial pathotype.

*Chlamydia trachomatis* is a significant human pathogen; it is responsible for a spectrum of acute ocular and genital tract infections and for chronic diseases that lead to blindness and tubal-factor infertility, such as trachoma and salpingitis, respectively. *C. trachomatis* consists of 15 serovars (A, B, Ba, C–K, and L1–L3) according to major outer membrane protein (MOMP) antigenicity but are historically grouped into the trachoma biovar (serovars A–K) and the lymphogranuloma venereum (LGV) biovar (serovars L1–L3). The trachoma biovar is further divided into endemic trachoma strains (serovars A–C) and oculogenital strains (serovars D–K). Although endemic trachoma and oculogenital strains are capable of infecting epithelial cells of both the conjunctiva and genital tract, they are distinct in terms of organ-specific disease. The disease spectrum of endemic trachoma strains is limited to chronic conjunctival infection, whereas oculogenital strains cause a self-limited form of acute conjunctivitis and a variety of acute and chronic genital tract infections. Genome studies have provided evidence that, despite large differences in disease potential, the different *C. trachomatis* serovars share >99.6% genetic identity [1]. Therefore, differences in virulence, pathogenic profile, and disease syndrome must be attributable to a relatively small number of genetic differences. Modest genomic differences must also dictate growth distinctions among closely related *C. trachomatis* serovars.

All chlamydiae share a unique obligate intracellular developmental cycle consisting of attachment and endocytosis by the infectious elementary body (EB) followed by primary differentiation to the metabolically active reticulate body (RB) within a host cell cytoplasmic vesicle. The RB replicates by binary fission until redifferentiation to EB occurs [2]; this is followed by host cell lysis and EB release. The initial phase of attachment, endocytosis, and primary differentiation is equivalent to the conventional bacterial lag phase, in
which bacteria adapt to the environment before division occurs. The phase of RB replication before EB redifferentiation is equivalent to the bacterial log phase. Typically, Chlamydia organisms are quantified by an infectivity assay in which EB measurements are taken over a time course (one-step growth curve); this assay provides a good estimate of the overall time to completion of the entire developmental cycle but does not provide information on the lag and log phases. The temporal relationship of these events has been characterized qualitatively by ultrastructural analysis and transcriptional experiments [3] but only to a limited extent by quantitative measurements of pathogen growth. Thus, it is not precisely clear why some chlamydial strains have developmental cycles of 36–48 h, whereas others require 72–96 h to complete the intracellular growth cycle.

Ocular strains are considered to be slow growing, genital strains are considered to grow at an intermediate rate, and LGV strains are considered to be fast growing. A reasonable explanation for these observations is that growth differences reflect differences in the efficiency with which particular developmental steps are completed. These same factors may also reflect tissue tropism and differences in disease manifestation. Comparison of growth rates, therefore, may ultimately identify genetic differences that contribute to chlamydial tissue tropism and disease indications. An earlier attempt to study the chlamydial log phase used radiolabeled uridine and quantified total DNA in cycloheximide-treated host cells infected with C. psittaci [4]; the data suggested a 2-h generation time, which has been a generally accepted value. More-recent studies have used quantitative polymerase chain reaction (PCR) to measure levels of 16S rRNA [5, 6] or DNA [7] of C. trachomatis L2 and estimated the lag phase to be 6–8 h, with a generation time of 1.45–3.0 h. In each study, only 1 strain was evaluated, and, although basic growth-rate information was provided, details regarding times for cytokinesis and recovery of infectivity were lacking.

In the present study, we determined the length of the developmental cycle for representative examples of serovars A–F and L2 by use of one-step growth curves and then characterized the growth kinetics of representative C. trachomatis strains that exhibit rapid (L2), intermediate (D), and slow (A) growth characteristics in a human genital epithelial cell line (HeLa cells). We used the RB division rate in combination with a quantitative DNA assay and one-step growth curves to systematically evaluate chlamydial growth kinetics. We investigated the influence of host cell type by comparing the chlamydial growth characteristics observed in HeLa cells with those observed in diploid human conjunctival epithelial (HCjE) cells [8]. We also evaluated the effects that fast- and slow-growing chlamydial strains have on each other by comparing the growth patterns observed when cells were coinfecte.

to pathotype (genital tract infection vs. endemic trachoma). Differences were the result of a longer lag phase and slower generation time for the ocular (serovar A) strain. Slow growth was partially ablated in HCjE cells or by coinfection with either serovar D or L2. These results suggest that differences in growth rates may be a reflection of the efficiency at which developmental stage-specific host-pathogen interactions occur. These interactions likely determine tissue tropism and disease type.

**MATERIALS AND METHODS**

**Cell culture and growth of C. trachomatis.** HeLa 229 cells were used for propagation of all C. trachomatis strains used in the present study. HeLa cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Biowhittaker) supplemented with 10% heat-inactivated fetal bovine serum (HyClone), 10 μg/mL gentamicin, 2 mmol/L 1-glutamine, 100 mmol/L HEPES buffer, 1 mmol/L sodium pyruvate, and 55 μmol/L β-mercaptoethanol (Gibco). Cells were maintained at 37°C in 7% CO2 in cell culture flasks. C. trachomatis strains A/HAR-13, B/TW-5/OT, and C/TW-3/OT were propagated for 3 days, and strains D/UW-3/Cx, E/Bour (isolated from a patient with endemic trachoma [9]), E/UW-5/Cx (isolated from a patient with cervicitis [10]), F/UW6/Cx, and L2/434/Bu were propagated for 2 days, in HeLa cells pretreated with DEAE-dextran and 2 μg/mL cycloheximide (Sigma). C. trachomatis were collected from infected-cell sonicates by differential centrifugation and were partially purified by centrifugation through 30% renografin (Braaco Diagnostics). Stock titers were determined by infectivity assay in thawed samples that had been stored at −80°C. HCjE cells (provided by I. Gipson, Department of Ophthalmology, Harvard Medical School) were propagated and maintained as described elsewhere [8]. HCjE cells were switched to DMEM/F12 medium with high calcium (1 mol/L CaCl2) supplemented with 10% neonatal calf serum and 10 ng/mL epidermal growth factor, to promote stratification at confluence.

**RB growth curves.** RB growth curves were made by arresting cell division in the presence of penicillin added at specified times after infection and enumerating aberrant RBs within an inclusion by light microscopy 2–3 days later [11]. HeLa cells were grown on 8-well chamber slides and infected with a low MOI of C. trachomatis serovar A, D, or L2 (cells infected, 0.2%–0.5%), to avoid inclusion fusion. Penicillin (50 μg/mL) was added to quadruplicate samples at appropriate times after infection. Infected cells were fixed in methanol; stained with Giemsa at 36, 42, and 66 h after infection for serovars L2, D, and A, respectively; and examined by light microscopy (magnification, ×1000). The number of large, aberrant RBs in each inclusion was counted and averaged. The RBs in at least 200 inclusions were enumerated at each time point.

**Infection and preparation of cells for nucleic acid analysis and creation of one-step growth curves.** HeLa or HCjE cells...
Figure 1. One-step growth curves for Chlamydia trachomatis strains A, B, C, D, E/Bour, E/UW5/Cx, F, and L2. HeLa cells were infected at an MOI of 0.3 and then collected over a time course for determination of inclusion-forming units (IFUs). Ocular strains are represented by black symbols and lines, and genital strains are represented by white symbols and lines.

were plated onto 24-well plates at a density of $2 \times 10^5$ cells/well and incubated overnight. The cells were infected at an MOI of 0.3 in 0.2 mL of sucrose phosphate glutamate (SPG) and rocked at 37°C for 2 h. DMEM containing 2 μg/mL cycloheximide was added to HeLa cells, to compare strain-dependent growth differences. Stratification medium without cycloheximide (which is routinely used for HCjE cells) was added to HeLa and HCjE cells, to compare host cell–dependent growth differences for serovars A, D, and L2. The infected cells were incubated at 37°C in 7% CO₂, collected in triplicate by scraping at appropriate times after infection, suspended in SPG in microtubes with glass beads, vortexed, and frozen at −80°C. Thawed samples were serially diluted and plated onto HeLa cell monolayers for infectivity determinations.

Assessment of chlamydial and host DNA. Total nucleic acid was prepared from snap-frozen pellets of infected cells by use of the DNeasy Tissue Kit (Qiagen), in accordance with the manufacturer’s protocol. Chlamydial DNA was amplified by PCR with the oligonucleotides 5′-CATGCTGTGGGATTT-TGG-3′ and 5′-CTGGCTATATATTCTACCGTTCCTGTTT-3′ as specific primers and 5′-TATGCTTCTCCTTTATACGCA-3′ as a probe for a 78-bp region of the C. trachomatis groEL2 gene. PCR was performed with Taq DNA polymerase (TaqMan PCR Master Mix Kit; Applied Biosystems). The host cell β-actin gene was amplified by PCR, to normalize the data on the basis of on host cell genome copy numbers (TaqMan β-actin control reagent; Applied Biosystems). Probes were labeled with the FAM reporter fluorochrome and the TAMRA quencher. Amplification conditions were 2 min at 50°C, then 10 min at 95°C, and the 40 cycles of 15 s at 95°C and 60 s at 60°C; the ABI Prism 7000 Sequence Detection System was used.

Coinfection and assessment of infectivity. HeLa cells were infected with either serovar A alone (MOI, 3), serovars A and D (MOI, 3 [for both]), or serovars A and L2 (MOI, 3 [for both]) in 1.5 mL of SPG and incubated at 37°C in 7% CO₂ in the presence of cycloheximide. Some cell monolayers were fixed in methanol and incubated with monoclonal antibody (MAb) specific for the MOMP of serovar A (A20) for 1 h, then incubated with goat anti–mouse antibody coupled with Alexa Fluor 555 (Invitrogen) for 1 h. Cells were then washed several times with PBS-azide and incubated with murine MAb specific for the MOMP of serovar D, E, or L2 directly coupled with fluorescein isothiocyanate (Argene). Other infected-cell samples were collected by scraping at 25, 27, and 29 h after infection, and infectivity was assessed for serovar A.

RESULTS

Correlation between length of the developmental cycle and pathotype. The lengths of the developmental cycles for serovars A–F and L2 were established by documenting the time at which infectious EBs first appeared by use of one-step growth curves. Comparison of the tested serovars demonstrated a dichotomy in the lengths of the developmental cycles (figure 1). The genital strains (D, E/UW5/Cx, F, and L2) all initiated EB conversion by 20–24 h after infection and completed their developmental cycles by 30–48 h after infection; in contrast, the ocular strains (A, B, C, and E/Bour) did not initiate EB conversion until 30–36 h after infection and did not complete their developmental cycles until 48–68 h after infection. The data in figure 1 clearly illustrate how these 2 pathotypes fall into 2 distinct growth-rate groups.

Estimation of lag phase and generation time by penicillin treatment and enumeration of aberrant RB. Treatment of serovar A-, D-, and L2-infected cells with penicillin resulted in large, aberrant RBs that were visible by light microscopy. Penicillin treatment before initiation of division (6 h after infection) resulted in the formation of a single RB in >90% (186/200 inclusions) of infected cells examined. The proportions of inclusion-containing cells in the population were similar irre-
Figure 2. Growth-curve comparison. A, Comparison of growth curves for reticulate body (RB) division. HeLa cells infected at an MOI of 0.1 were treated with penicillin at different times after infection to interrupt RB division, allowing enumeration of aberrant RBs by light microscopy. Inserts show representative inclusions containing 8 RBs at 15, 18, and 24 h after infection for serovar L2, D, and A, respectively. Bars indicate 10 μm. The lag phase was 8.5 h for serovar L2, 10.5 h for serovar D, and 15 h for serovar A.

B, Comparison of growth curves for DNA replication of serovars A, D, and L2. HeLa cells infected at an MOI of 0.3 were collected over time and assessed for levels of chlamydial DNA. Data were normalized on the basis of the amount of host β-actin. The generation times for serovars L2 and D were nearly equal (2.2 and 2.4 h), whereas serovar A had a substantially slower growth rate (3.6 h).

C, Comparison of growth curves for E/Bour and E/UW5/Cx. The increase in genome copy number for E/Bour considerably lagged behind that for E/UW5/Cx, respectively. The relative increase in genome copy number closely paralleled the RB growth curve, indicating that cytokinesis was in balance with DNA replication (figure 2A and 2B). The genomic replication rate demonstrated that the generation time for serovars D and L2 were nearly equal at 2.2 and 2.4 h, whereas the generation time for serovar A was substantially longer (3.6 h). The exponential increase in genome copy number was linear until infectivity was confirmed (EB production), at which point it slowed substantially, indicating that conversion of RBs to EBs occurred relatively rapidly and, thus, limited further genomic replication. Comparison of genome copy numbers for E/UW5/Cx and E/Bour revealed a 2-h generation time for E/UW5/Cx but a significantly longer generation time for E/Bour (figure 2C). Thus, for these 2 serovar E strains, the genital strain exhibited growth characteristics similar to other genital strains, whereas the ocular strain exhibited growth characteristics similar to other ocular strains.

A similar pattern was observed in the one-step growth curve (figure 1).

Expiration of the growth rate of an ocular strain in conjunctival epithelial cells. The growth rates of serovars A, D, and L2 in genital tract cells (HeLa) and conjunctival epithelial cells (HCJE) under identical growth conditions were compared. One-step growth curves in HeLa cells showed the initial emergence of infectious EBs at 21 h for serovar L2, at 24 h for serovar D, and at 30 h for serovar A (figure 3). One-step growth curves in HCJE cells for serovars A, D, and L2 showed that serovar D grew slowly and relatively poorly, whereas serovar A grew more rapidly, in comparison to growth in HeLa cells (figure 3).

Expedited recovery of serovar A by coinfection with serovar D or L2. Coinfection resulted in single inclusions containing RBs and EBs for both infecting serovars (A and D or A and L2), indicating that these serovars can inhabit the same inclusion (figure 4A–C). Interestingly, coinfection with D or L2 resulted in the expedited recovery of infectious serovar A, compared with that of serovar A in the absence of faster strains (figure 4D).
DISCUSSION

Comparison of the lengths of developmental cycles demonstrated that *C. trachomatis* isolates could be grouped into fast and slow strains that corresponded to genital and trachoma pathotypes, respectively. Interestingly, the 2 serovar E strains, E/Bour (isolated from a patient with endemic trachoma [9]) and E/UW-5/Cx (isolated from a patient with cervicitis [10]), corresponded to their original clinical presentations. These findings confirm reports indicating that endemic trachoma strains grow slower than do genital strains. However, an extensive assessment of clinical strains is needed to determine whether this observation can be generalized.

Oculogenital strains cause inclusion conjunctivitis and have the same tissue tropism as the endemic trachoma strains, despite differences in disease manifestation. Therefore, the difference in the progression of the developmental cycle is more likely to be reflective of disease type rather than tissue type. However, some reports have suggested that oculogenital strains obtained from neonates with inclusion conjunctivitis are slow growing [12], providing evidence that ocular isolates may be genetically distinct variants of infecting strains selected during perinatal transmission. Comparison of the lengths of the developmental cycles of paired isolates obtained from a mother’s genital tract and an infant’s conjunctiva may provide definitive evidence with respect to these claims.

Systematic analysis of RB division, genomic replication, and infectivity provide an accurate temporal and quantitative assessment of chlamydial growth kinetics, including lag and log phases and terminal differentiation (RB to EB conversion). Measurement of these parameters for different *C. trachomatis* pathotypes has helped to explain why some chlamydial strains complete their developmental cycle rapidly and others require substantially longer periods of time. The initial lag phase consists of attachment, endocytosis, and primary differentiation to initiate replication; this phase is represented by very small increase in DNA content and minimal evidence of cytokinesis during the first several hours. The log phase consists of binary fission of metabolically active RBs and is represented by logarithmic increases in DNA content, which proceed and parallel cytokinesis. The log phase is followed by the conversion of RBs to EBs, which is represented by detection of infectious EBs coinciding with the decrease in DNA replication.

The ability to quantitatively determine chlamydial growth kinetics has practical applications in understanding disease pathogenesis and in evaluating the effects that antimicrobials or immune mediators have on *C. trachomatis* infection. In the present study, comparison of the growth kinetics of the different serovars demonstrated obvious differences in the developmental life cycles of the genital and trachoma strains. Systematic comparison of the specific parameters of growth (RB, genome, and EB) for serovars A, D, and L2 showed that the differences were

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**Figure 3.** One-step growth curves for serovars A, D, and L2 in HCjE or HeLa cells. Cells grown in the same medium (without cycloheximide) were infected at an MOI of 0.3, and cell scrapings were collected over time for analysis of inclusion-forming units (IFUs). The data suggest expedited growth of an ocular strain (serovar A) in HCjE cells. Error bars represent 1 SD.
attributable to the length of the lag phase and to the generation
time (the log phase). Specifically, the lag phase was shortest for
serovar L2, longest for serovar A, and in between for serovar
D, with a difference of up to 6–8 h—indicating a difference in
the efficiency of attachment, endocytosis, and/or primary dif-
ferentiation. Several studies have shown that LGV strains are
more efficient at attachment and uptake than are ocular strains.
For example, ocular strains require pretreatment with DEAE-
dextran [13] or centrifugation [14] for efficient infection, whereas
LGV strains maintain high infectivity without treatment. On
attachment, C. trachomatis inject, via its type III secretion sys-
tem, transmembrane actin-recruiting protein (Tarp) into the
host cytoplasm [15]. Tarp is activated by tyrosine kinase–me-
diated phosphorylation, which results in actin recruitment to
induce endocytosis of chlamydial EB. Serovar L2 has 6 repeats
of the tyrosine-rich amino acid sequence, as opposed to 3 re-
peats for serovars A and D. This difference may, at least in
part, account for the more-efficient entry process and the
shorter lag phase observed for serovar L2. However, no clear
association between Tarp polymorphism and organ specificity
has been demonstrated by sequence analysis of 15 serovars [16].
Moreover, chlamydial uptake appears to occur relatively quickly
in all strains examined to date [17], suggesting that other factors
associated with primary differentiation might contribute to the
observed differences.

The generation times for serovars D and L2 were nearly
identical, whereas the generation time for serovar A was sub-
stantially slower. The generation time for bacteria is determined
by intrinsic and extrinsic (e.g., nutrients, pH, temperature, etc.)
factors. In the present study, optimal growth conditions (me-
dium enriched with cycloheximide) were applied to all strains,
to highlight intrinsic differences. For other bacteria, generation
time has been correlated with cell wall composition and altered
activities of penicillin-binding proteins [18], suggesting that the
efficiency of cytokinesis regulates the DNA-replication rate.
However, although the increase in the C. trachomatis genome
copy numbers correlated with RB division here, previous stud-
ies have shown that genome copy numbers increase after pen-
icillin treatment, suggesting that the rate of DNA replication
is independent of cytokinesis for Chlamydia organisms [11].
Therefore, intrinsic differences that affect DNA replication—
as well as transcription or translation—are candidates for fur-
ther investigation.

The detailed mechanisms that account for the growth dif-
fferences among pathotypes remain largely unknown. A recent
report describes a slow-growing, nonfusogenic variant strain of

Figure 4. HeLa cells coinfected with serovars A and D (A), serovars A and L2 (B), and serovar A alone (C). Cells were fixed and stained 40 h after
infection. Serovar A was stained with specific monoclonal antibody (MAb; A20) and Alexa Fluor 555, and serovars D and L2 were stained with specific
MAb directly coupled with fluorescein isothiocyanate. Nuclei were stained with Hoechst dye. D, Recovery of infectious serovar A reticulate bodies at 25,
27, and 29 h after infection. Diamonds represent infection with serovar A alone (MOI, 3); squares represent infection with serovars A and L2 (MOI, 3 [for
both]); and triangles represent infection with serovars A and D (MOI, 3 [for both]). Error bars represent 1 SD. IFUs, inclusion-forming units.
C. trachomatis that is associated with milder clinical disease [19]. This finding highlights the possibility that differences in growth rates are a reflection of biological function and patho-

mological differences. Comparison of the genomes of C. trachomatis serovars A and D reveal a relatively small net change of <2000 bp (out of >1 million bp), resulting in only a few candidate gene differences that might account for the differences in tissue tropism (ocular vs. genital) and disease manifestations among C. trachomatis pathotypes [16]. There are 8 predicted open reading frame interruptions in the serovar A genome, compared with the serovar D genome. Of these, tryptophan synthase and the chlamydial cytotoxin genes are functionally conserved in genital strains and have been implicated as immune-evasion mechanisms against the interferon-γ-mediated antichlamydial effectors indoleamine deoxygenase and p47GTPases, respectively [20, 21].

Other interrupted genes, such as the arginine/ornithine antiporter (arcD) [22], may impact 1-arginine acquisition, which in turn could affect generation time; however, its role in chlamydial growth is unknown. Polymorphic membrane protein genes (pmpE, pmpF, and pmpH) are known to have a large number of single-nucleotide polymorphisms that correspond to pathobio-
types and also may play a role in tissue-specific host response and antigen recognition [16, 23]. However, given that the dif-
f erences in growth rates we observed occurred after cyclohexi-
mide treatment, they are more likely to reflect differences in the intrinsic growth potential of the chlamydial strains investigated rather than effects of host immune responses. Other epigenomic changes may also be important in distinguishing pathotypes. These more-subtle differences include alterations in transcriptional patterns of commonly held genes, and further investigation is warranted.

Different growth conditions were found to influence the overall rate of growth as well. For example, the more-rapid initiation of RB-to-EB conversion for an ocular strain in conjunctival cells provides a hint that chlamydial growth may, at least in part, depend on host cell factors. Coinfection of cells with fast (serovars D or L2) and slow (serovar A) strains expedited the recovery of infectious EB for the slow-growing strain. This result demonstrates a gain of function for serovar A that may be attributable to alteration of the environment by a gene function that is active in the faster-growing serovar D or L2 isolate but not in the serovar A isolate. Our study shows that differences in the length of the developmental cycle between genital and ocular strains may be lessened in conditions that favor the ocular strains. This suggests that the rate of chlamydial growth is correlated with tropic cell type. Alteration of growth rates by targeting a specific step in the developmental cycle may be sought as a marker for pathotypic profiling. For example, interfering with the unique entry process of genital strains would be expected to prolong the lag phase for genital strains but not for trachoma strains. Examination of chlamydial clinical isolates by functional genomics may also reveal growth variants with differing potential to cause disease resulting from small genomic differences. These sorts of studies will be useful in refining our understanding of chlamydial pathogenesis at the molecular level.

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