Chlamydia trachomatis Persistence In Vitro: An Overview

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Chlamydiae growing in target mucosal human epithelial cells in vitro can transition from their normal developmental cycle progression, alternating between infectious but metabolically inactive elementary bodies to metabolically active but noninfectious reticulate bodies (RBs) and back to elementary bodies, into a state of persistence. Persistence in vitro is defined as viable but noncultivable chlamydiae involving morphologically enlarged, aberrant, and nondividing RBs. The condition is reversible, yielding infectious elementary bodies after removal of the inducers, including penicillin, interferon-γ, iron or nutrient starvation, concomitant herpes infection, or maturation of the host cell into its physiologically differentiated state. All aberrant RB phenotypes are not the same, owing to differing up- or down-regulated chlamydial gene sets and subsequent host responses. Although all persistence-inducing conditions exist in vivo, key questions include (1) whether or not aberrant chlamydial RBs occur in vivo during the alternating acute-silent chronic-acute chlamydial infection scenario that exists in infected patients and animals and (2) whether such aberrant RBs can contribute to prolonged, chronic inflammation, fibrosis, and scarring.

Genital strains of Chlamydia trachomatis serovariants (serovars) D–K target superficial mucosal epithelia of the urethra or the endocervix to initiate their infectious process. This process requires 2 morphologically distinct forms of the bacterium: the elementary body (EB) and the reticulate body (RB). The small (∼0.2–0.3 μm), dense, infectious EBs make contact with the epithelial cell surface and, after ligand-receptor interaction, are endocytosed (Figure 1A). Rapid modification of the EB-containing endocytic vesicle by the EBs allows escape of the EB endosome from the endocytic-lysosomal pathway and trafficking on cytoskeletal intermediate filaments to the nuclear hof/endoplasmic reticulum/Golgi activity center. After arrival at this destination, transformation of the essentially metabolically inactive EBs into the larger (∼0.8 μm), metabolically active RBs is triggered. The compacted EB DNA is relaxed; signals for DNA, RNA, and protein synthesis are activated; and RB cell division ensues (Figure 1B). The RB-containing endosome intercepts trans-Golgi vesicles for endosome membrane expansion to accommodate the increasing number of RB progeny. This now microscopically visible intracellular membrane-bound chlamydial microcolony is termed an “inclusion.” After several hours of continued logarithmic growth and expansion of the inclusion (Figure 1C), depletion of nutrients and adenosine triphosphate scavenged by the growing RBs from the infected host cell signal the maturation of the noninfectious RBs back into the infectious EBs (Figure 1D). EBs released from the extruded inclusion and/or lysis of the host cell can then infect neighboring epithelial cells to perpetuate the infectious process [1, 2].

This in vitro characterized developmental cycle is fundamental to all Chlamydia and varies only in timing (from 48 to 72 to 96 h, depending on the species) and in the number of inclusions per host cell (from one in a C. trachomatis–infected cell to several inclusions per Chlamydia pneumoniae–or Chlamydia psittaci–infected cell) (Figure 1E). Stages of this developmental cycle...
**DISCOVERY OF A C. TRACHOMATIS PERSISTENCE MODE IN VITRO**

Historically, *C. trachomatis* was associated with postgonococcal urethritis or cervicitis or nongonococcal urethritis or cervicitis. In the early 1960s and 1970s, patients with gonococcal urethritis or cervicitis were treated with penicillin, before *Neisseria gonorrhoeae* acquired penicillin resistance. However, ~3 months later, some of these patients would present with postgonococcal urethritis or cervicitis, and *C. trachomatis* was isolated from them [4, 5].

Matsumoto and Manire [6] exposed *C. psittaci* Cal-10–infected L929 cells in vitro to 200 μg/mL of penicillin 1 h after infection. EB to RB transformation proceeded, but the developmental cycle was apparently stalled, because no new infectious EB progeny could be recovered. Transmission electron photomicrographs revealed that the normal RBs continued to grow but could not undergo binary fission; thus, enlarged, morphologically aberrant RBs were visible. After the penicillin was removed and after a lag period of RB internal and external budding, the RBs returned to normal size, underwent cell division, and matured into infectious EBs. These morphological changes are illustrated in Figure 2, which shows *C. trachomatis*–infected endometrial (HEC-1B) cells exposed to 10 μg/mL of penicillin.

Studies of these events with use of transcriptomics and proteomics have confirmed that there is continued genome rep-
Penicillin-induced aberrant reticulate bodies (RBs) in Chlamydia trachomatis serovar E–infected human endometrial epithelial cells cultured in vitro. A, Standard inclusion containing RBs and elementary bodies (EBs) in a C. trachomatis–infected HEC-1B cell control at 24 h after infection, not exposed to penicillin. B and C, C. trachomatis–infected HEC-1B cells at 36 h after infection, previously exposed to penicillin G (20 μg/mL) at 1 h after infection. The RBs are swollen, abnormal, and nondividing (RB*). The altered metabolic disconnect between transcription and translation in abnormally enlarged, nondividing RBs and cell envelope component biosynthesis results in excessive shedding of RB outer envelope blebs into the inclusion lumen; these antigen-containing (eg, major outer membrane protein, lipopolysaccharide) blebbed vesicles somehow fuse with or cross the inclusion membrane and emerge into the cytoplasm of the infected host cell (arrowheads). D, C. trachomatis–infected HEC-1B cells preexposed to penicillin G (20 μg/mL) for 35 h, followed by removal of the antibiotic and continued cultivation of the infected cells for an additional 12 h. The appearance of miniature bodies is one morphological signal of recovering RBs and their reprogrammed attempt to undergo cell division to produce progeny. Bars represent 10 μm at ×2900 (A–C) or 2 μm at ×10,000 (D) magnification.

Concomitant with or soon thereafter, a flurry of additional experiments showed other experimental conditions that reproduced the viable but nondividing altered RB phenotype. The inducers included interferon (IFN)–γ, nutrient/amino acid starvation, iron deprivation, the host cell differentiation state, and herpes simplex virus (HSV) infection. In the final analysis, the findings by many different researchers using differing experimental conditions and different chlamydial species were the same as those described by Matsumoto and Manire [6]. Thus, it is now generally accepted by investigators in the field that there exists in vitro a state of chlamydial persistence, defined by “viable but noncultivable chlamydiae” involving “morphologically enlarged, nondividing, aberrant RBs,” which is “reversible,” yielding infectious EBs [9, pp 688–693].

Because there are 2 elegant and detailed reviews on chlamydial persistence by Beatty et al [9] and Hogan et al [10] that cover the extensive literature on the subject over a 40-year period, presented here is only a brief summary of chlamydial persistence and the aberrant RB phenotype in vitro, focusing on the key questions of the Chlamydia Immunology and Control Expert Advisory Meeting: what definitive evidence is needed to confirm whether C. trachomatis aberrant RB persistence can or does occur in vivo and, if it does, what might be the consequences? My apologies to colleagues for studies not acknowledged because of page limitations and the fact that the focus of this meeting was human C. trachomatis infection.
Chlamydial Persistence In Vitro

OTHER INDUCERS OF C. TRACHOMATIS PERSISTENT ABERRANT RBs IN VITRO

IFN-γ and amino acid starvation. At the same time that the early penicillin studies were performed, other chlamydia researchers discovered the growth-inhibiting effects of immunological mediators on chlamydia-infected cells, the most notable being the cytokine IFN-γ [11, 12]. Clever studies by Byrne et al [13] revealed the cytokine effect to be on the host cell, which, in turn, affected chlamydial growth modulation. In human epithelial cells, IFN-γ induces indoleamine-2,3-dioxygenase, an enzyme that catalyzes the degradation of tryptophan, an essential amino acid for both eukaryotic cells and chlamydiae. However, addition of exogenous indole in the presence of IFN-γ results in a return to the normal chlamydial developmental cycle; this rescue event is evident for genital strains only [14]. Of interest, genital serovars possess functional tryptophan synthase (trpBA) genes to convert indole, secreted by local vaginal flora, into tryptophan, which allows these chlamydiae to escape killing by IFN-γ and establish persistent infection; ocular serovars have mutations in the Trp operon that inactivate the synthase enzyme, making them incapable of using endogenous indole to synthesize tryptophan. Subsequently, Beatty et al [15] revealed that exposure of C. trachomatis–infected cells to physiological concentrations of IFN-γ resulted in enlarged, aberrant RBs. Moreover, although these altered RBs were deficient in the cell envelope structural components, major outer membrane protein (MOMP), and lipopolysaccharide, they continued to synthesize and secrete heat shock protein 60 (HSP60), an immunodestructive antigen associated with the chronic inflammatory fallopian tube pathology that leads to pelvic inflammatory disease, ectopic pregnancy, and infertility [16]. The latter finding also raised the possibility that the persistent aberrant RB state was induced as a stress response to nutrient starvation.

Complete transcriptome analysis by Belland et al [2] of C. trachomatis serovar D growth in HeLa cells exposed to IFN-γ demonstrated the up-regulation of many genes involved in active metabolic processes in the aberrant RBs, including those involved in DNA repair and recombination, protein translation, and phospholipid utilization. Stress response gene up-regulation was also observed, as was down-regulation in the genes involved in proteolysis, peptide transportation, cell division, RB to EB redifferentiation, and compaction of EB DNA (histone genes). The authors’ interpretation was that the chlamydial response to IFN-γ–tryptophan nutrient limitation had evolved as a coordinated transcriptional response to control the transition between the classic EB-RB-EB active growth and the aberrant RB persistent growth states and that the latter represented an alternative lifestyle used by chlamydiae to avoid the host immune response. This premise has been challenged on the basis of in vitro experiments showing that cytotoxic T lymphocytes harvested from the spleens of mice previously immunized with C. trachomatis were able to recognize and kill L cells infected with aberrant RBs after exposure of the cells to IFN-γ or penicillin [17].

Because chlamydiae are obligate intracellular bacteria, an available supply of soluble nutrients from the host cell cytoplasm is essential for productive chlamydial growth. Therefore, with addition of the eukaryotic protein synthesis inhibitor cycloheximide to chlamydia-infected coverslip cultures, there is less competition by the host cell for nutrient pools, and chlamydial inclusions are considerably larger and easier to detect and count. Early studies by Moulder [18], who grew C. psittaci 6BC in fibroblasts in either nutrient-rich medium 199 or Eagle’s minimal essential medium, led to overt or covert “cryptic body” chronic infections, respectively. Extensive studies by Coles et al [19] examined the growth of C. trachomatis serovar L2 in McCoy cells in Eagle’s minimal essential medium containing 0%–100% amino acid levels. When the medium amino acid pools were reduced to 10%, chlamydial inclusions at 48 h after infection were filled with swollen and irregular budding RBs. A return to productive infection and maturation of RBs to EBs could be accomplished by addition of cysteine or L-isoleucine.

Iron deprivation. Virtually all prokaryotes have an absolute requirement for iron or related translational metals for survival; intracellular bacterial pathogens require iron for entry and replication in mammalian cells, and chlamydiae are no exception. Addition of the iron-chelating chemical desferal to the medium bathing C. trachomatis serovar E–infected polarized endometrial epithelial HEC-1B cells led to a distinctive form of persistent infection, with small inclusions containing enlarged, aberrant, nondividing RBs with loose, wavy outer envelope membranes. Reversal to productive infection and recovery of infectious EBs could be achieved with the removal of desferal and supplementation of the culture with iron-saturated transferrin [20]. Because C. trachomatis infections in the genital tract are most prevalent in young female individuals during their peak reproductive years [21], when iron concentrations fluctuate considerably from iron sufficient to iron deficient during the menstrual cycle, modulated in particular by estrogen, it is possible to envision chronic chlamydial infections in these women as a result of alternating aberrant RB persistent and productive infection episodes.

Host cell differentiation state. In the early 1980s, infection of human monocytes with C. psittaci resulted in inclusions containing enlarged abnormal RBs; only after the monocytes matured into macrophages did productive chlamydial growth of normal RBs and EBs resume [22]. Reproducible results of ultrastructurally abnormal RBs have also been reported by a group of investigators comparing C. trachomatis serovar K growth in human monocytes and synovial tissue samples from patients with chlamydial arthritis and with laryngeal Hep-2 cells.
as controls [23, 24]. All 11 gene groups, representing glycolysis, the pentose phosphate pathway, the tricarboxylic acid cycle, electron transportation, sigma factors, and DNA replication, were up-regulated during the active stages of growth; in aberrant RB phenotype infection, chromosome replication continued, but cytokinesis was severely down-regulated.

In 1985, Richmond [25] proposed that chlamydiae survive in a persistent aberrant RB phenotypic form in the undifferentiated epithelial cells at the base of endometrial glandular cells. The epithelial cells migrate up the glands, and differentiation into the productive form of infection is achieved as the epithelial cells reach and repopulate the mucosal surface. Partial support for this proposal has been provided by Guseva et al [26] with use of pig reproductive tissues cultured ex vivo. *Chlamydia suis* produces aberrant RB/persistent-type inclusions and few infectious chlamydiae within infected, primary swine glandular epithelial cells but follows a more normal developmental sequence in more differentiated swine luminal epithelial cells. Productive replication in luminal cells damages the epithelium and serves as a source of released infectious EBs, whereas aberrant RB phenotype forms in glandular tissue serve as a silent reservoir for continued infection.

Another example of this phenomenon is shown in Figure 3, in which transmission electron microscopy reveals the asynchronous inclusion development and aberrant RB in inclusions in *C. trachomatis*-infected glandular epithelial cells derived from ductal breast carcinoma cell line MCF-7. Host cell differentiation–dependent replication of obligate intracellular parasites is not without precedence; human papilloma virus is highly dependent on basal cell differentiation into keratinized squamous epithelial cells for productive replication, whereas persistent host infection is maintained by chronic viral episome infection of the basal cell layer. **HSV infection.** A somewhat surprising culture condition was recently reported to induce typical chlamydial persistence. Infection of HeLa cells 24 h after inoculation with *C. trachomatis* serovar E with HSV-2 resulted in swollen, aberrantly shaped, electron-translucent RBs 20 h later [27]. As reported elsewhere [15] with IFN-γ exposure, chlamydial MOMP was decreased, whereas HSP60–1 production was increased. However, new data suggest that coinfection of the same cell by chlamydiae and HSV-2 is not necessary to trigger a chlamydial persistent aberrant RB phenotype. Mere contact of chlamydiae-infected epithelial cells with ultraviolet-inactivated virions alone or with surface antigens on fixed cells infected singly with HSV infected cells activates a host cellular response resulting in chlamydial persistence, again with increased HSP60 production. Eventual degradation of the defective HSV virions by the host epithelial cell results in reversal of chlamydial persistence and a return of productive chlamydial infection [28]. Because there

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**Figure 3.** Asynchronous inclusion development and aberrant reticulate bodies (RBs) in *Chlamydia trachomatis* E–infected glandular epithelial cells derived from ductal breast carcinoma MCF-7 cells cultured in vitro. N, normal RBs and elementary bodies in a typical inclusion; P, morphologically enlarged, aberrant RBs in other inclusions in juxtaposed epithelial cells. Bar represents 2 μm at ×7000 magnification.
are considerable data on HSV-2 infection of endocervical epithelial cells, especially in Pap smears, there are a number of ways that C. trachomatis-infected cells could come in contact with the appropriate viral ligands in the absence of cellular coinfection in vivo. First, because HSV infection produces 50–200 defective viral particles per plaque forming unit, cellular coinfection with defective virions is probably more frequent than coinfection with replication-competent HSV. Second, viral glycoproteins released from infected cells could induce persistence by interacting with receptors on chlamydiae-infected epithelial cells. Third, contact between C. trachomatis–infected cells and viral glycoproteins on the surface of neighboring, HSV-infected cells or on infiltrating, monocytes or T cells abor-

tially infected with HSV could also produce this effect.

**ARE ALL CHLAMYDIA ABERRANT RBs THE SAME?**

Extensive comparative genomic, transcriptomic, and proteomic analyses have been applied recently to C. trachomatis [29], C. pneumoniae [30], and C. psittaci [31] growing under normal versus aberrant RB persistent conditions induced by penicillin, IFN-γ, or iron deprivation. The bottom line is that, although the phenotypic criteria remain similar for morphologically enlarged, aberrant, nondividing RBs, which are viable but non-cultivable, emerging transcriptional profiles show significant differences in up- or down-regulated gene profiles between the different persistence models. Data on this complex subject are reviewed in Hogan et al [10]. In addition, Beatty et al [14] found that IFN-γ–induced aberrant C. trachomatis RBs reduced production of MOMP and increased production of HSP60, whereas Nicholson and Stephens [7] found no change in the ompA/groEL gene transcripts in penicillin–induced aberrant C. trachomatis RBs. Conversely, Mathews et al [32] detected up-regulation of the ompA gene encoding MOMP in IFN-γ–in-
duced aberrant C. pneumoniae RBs. Furthermore, Gerard et al [33] reported that HSP60–1/groEL was expressed predomi-
nantly during acute phase growth of C. trachomatis serovar K and that the HSP60-copy 2/CT604 gene transcript/protein was increased in iron-induced persistent cultures. The latter finding was confirmed by LaRue et al [34] in iron-deprived cultures of C. trachomatis serovar E aberrant RBs. Thus, the current consensus is that the transcriptional response of the chlamydiae differs according to the persistence-inducing stimuli and, fur-
thermore, that each of these probably generates a different host cell response.

The rationale for such comparisons is to find genes that are consistently specific for the aberrant RB phenotype in chla-
mydial infection in vitro, to extend these studies successfully to patient tissues. The ultimate goal is to determine whether or not the aberrant RB phenotype exists in chlamydiae-infected tissues in vivo and, if possible, to determine the inducing mech-
anism(s). Leading candidate gene sets for in vivo investigations may be up-regulation of the early gene _evo_ with down-regu-
lation of the _hcTB_ gene. The latter encodes the Hc2 histone protein that modulates or compacts chlamydial DNA for RB-
to-EB redifferentiation and production of infectious EBs.

**EXISTENCE AND CONSEQUENCES OF THE CHLAMYDIA ABERRANT RB PHENOTYPE IN Vivo**

Historically, latent chronic chlamydial infections in birds and animals were well known to early chlamydia investigators. In a few cases, presumably resolved chlamydial infections could be reactivated by immunosuppressive agents, such as cyclo-
phosphomide. Clinical conditions that could be ascribed to persistent in vivo infections with very low levels or even non-
existent EB production could include (1) asymptomatic ure-
thritis in male individuals and cervicitis in female individuals; (2) silent pelvic inflammatory disease in female individuals; (3) reactivation or reisolation with the same genotype after weeks or months, despite denials of reexposure, in culture-positive individuals treated with antibiotics with apparent resolution of infection; and (4) negative culture results for individuals with strong serological titers and epidemiological associations. Additional data are available in the articles by Beatty et al [9] and Hogan et al [10].

It should be noted that use of the term “persistence” to describe the in vitro phenomenon has created great confusion in the literature, because the same term is used to describe clinically silent, chronic infection in humans and animals. In the context of in vivo chlamydial infection, “persistence” does not necessarily connote the aberrant RB phenotype identified by in vitro studies, as described above. In fact, little is known about the biological mechanisms used by chlamydiae to persist in vivo. To avoid confusion in this article, the term “aberrant RB phenotype” rather than “persistence” has been used to refer to the phenomenon defined in vitro.

Although many researchers strongly suspect that the insidious nature of some chlamydial infections and the chronic in-
flammatory consequences of genital reproductive disease se-
queae are attributable, in part, to phenotypically aberrant chlamydiae, the collective body of data is still circumstantial and the topic is controversial [35]. Barriers to research in this subject are that infections due to the aberrant RB phenotype are not detectable by culture; detection of the presence of chla-
mydial DNA and/or antigen in diseased tissue samples from patients may result from delayed nucleic acid clearance after successful eradication of viable organisms; ultrastructural evi-
dence of abnormal pleomorphic RBs in macrophages from chlamydiae-infected patient tissue samples is suggestive but, in isolation, not proof; and high titers of antibodies to the Chla-
mydia-immunodestructive HSP60 antigen in a group of indi-
vials at high risk of infection is not necessarily synonymous with current chronic infection.

How to detect the existence of aberrant RB phenotype infections in vivo was one of the key questions posed to the workshop participants and will be a challenge to clinicians, investigators using experimental animal models, and researchers involved in translational studies. One possibility might be to collect only chlamydiae-positive endocervical and endometrial biopsy specimens, survey tissue specimens for morphologically aberrant or normal-appearing inclusions, and undertake extensive, painstaking analyses of chlamydial inclusions by such methods as electron microscopy, confocal microscopy, and molecular studies of isolated inclusions. All such data could be placed in the context of clinical findings and data collected from patients, such as demographic characteristics, sexual risk factors, past sexually transmitted infections, and antibiotic treatment history. Noninvasive approaches, such as the use of cytobrushes for collecting organisms and cells from the genital tract (as pioneered by the Quayle laboratory) to track changes in the T cell repertoire during chlamydial infection, might also provide valuable information on chlamydial growth characteristics and evidence of in vivo persistence [36]. However, it should be remembered that chlamydia-specific stimulation of adaptive immune response effectors can be indicative of either viable organisms or the presence of chlamydial antigen.

All of the aforementioned inducers of the chlamydial aberrant RB phenotype in vitro are likely to function in vivo at physiological concentrations. Penicillin, amoxicillin, and ampicillin, all 3 of which can induce the chlamydial aberrant RB phenotype in vitro (J. Kintner, D. Lajoie, A. Mabe, J. Whittimore, R.V. Schoborg, unpublished data), are widely used in the United States, with >34 million prescriptions dispensed in 2006 [37]. In fact, 2 reports claim that penicillin-induced aberrant RBs are more refractory to killing via azithromycin [38, 39] because of their slowed metabolism. These observations support the case for the aberrant RB phenotype as one of the mechanisms, if not the most important one, used by chlamydiae to persist in vivo.

Finally, another key question related to the potential consequences of the chlamydia aberrant RB phenotype in vivo is the nature of the immune response triggered by aberrant RB–infected versus acutely infected epithelial cells. Can the aberrant RB chlamydiae state (ie, swollen, nondividing, but viable RBs) actually result in a protracted, chronic infection that leads to immune-mediated damaging sequelae? At the research bench, experiments can be designed to ask whether different chemokine signals are generated in acutely and aberrant RB–infected endocervical and endometrial cells exposed in vitro to penicillin versus IFN-γ versus iron deprivation and whether the signals are antiinflammatory (interleukin-11) or proinflammatory (interleukin-8). Actually, aberrant RBs induced by penicillin may be the better model in this case, because both IFN-γ and iron deprivation have a definite effect on epithelial cells that may confound the data. Our fundamental understanding of the immunological consequences of acute and damaging chlamydial infection has been generated in experimental animal models, and these contributions have been invaluable [40–42]. In this supplement, Darville and Hiltke [43] review the pathogenic immune responses to acute chronic infection, and Rank and Whittum-Hudson [44] review the development of protective immunity to chlamydia infection in animal models and the underlying effector immune mechanisms.

In summary, the Chlamydia Immunology and Control Expert Advisory Meeting, sponsored by the Centers for Disease Control and Prevention, provided an appropriate venue for an in–depth look at how far we have come in understanding chlamydial infection, with an emphasis on genital C. trachomatis infection and, importantly, where we need to focus our immediate future research directions. The timing is right for a shift to more translational studies on tissue samples from patients with chlamydiae infection. Although the complexities and limitations of human studies are not trivial, well-designed prospective protocols and analyses will yield valuable information. Support for such efforts is necessary.

References