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MINIREVIEW

Chlamydial Persistence: beyond the Biphasic Paradigm

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The chlamydiae are an evolutionarily distinct group of eubacteria sharing an obligate intracellular lifestyle and a unique developmental cycle that has been well characterized under favorable cell culture conditions. This cycle begins when infectious, metabolically inert elementary bodies (EB) attach to and stimulate uptake by the host cell. The internalized EB remains within a host-derived vacuole, termed an inclusion, and differentiates to a larger, metabolically active reticulate body (RB). The RB multiplies by binary fission, and after 8 to 12 rounds of multiplication, the RB differentiate to EB asynchronously (78). At 30 to 84 h postinfection (PI), depending primarily on the infecting species, EB progeny are released from the host cell to initiate another cycle (78, 122).

Shortly after Moulder (79) definitively reported the bacterial nature of chlamydiae in 1966, the genus *Chlamydia* was established (86) and divided into two species, *Chlamydia trachomatis* and *Chlamydia psittaci* (85). *Chlamydia pneumoniae* (42) and *Chlamydia pecorum* (34), formerly known as strains of *C. psittaci*, were designated as distinct species in 1989 and 1992, respectively. More recently, a new taxonomy was proposed that increases the number of species in the family *Chlamydiaceae* to nine and groups these species into two genera (31). However, this review will not use the emended taxonomy, since much debate continues on the issue (106).

Chlamydial species cause widespread infections in humans. *C. trachomatis* serovars D to K are considered the world's most common sexually transmitted bacterial pathogens (40) and, following vertical transmission through an infected birth canal, cause neonatal conjunctivitis (105) and pneumonia (12). Respiratory infection with *C. pneumoniae* occurs in almost everyone during his lifetime (120). *C. pneumoniae* is estimated to cause an average of 10% of community-acquired pneumonia cases and 5% of bronchitis and sinusitis cases (61). In addition, avian strains of *C. psittaci* have long been known to cause psittacosis in humans (11).

In addition to these acute chlamydial infections, chlamydiae are associated with a range of chronic diseases that are characterized by inflammation and scarring and result in significant damage to the host. The World Health Organization estimates that 146 million people have trachoma due to ocular infection by *C. trachomatis* serovars A to C and that 4.9 million of these are totally blind (121). Ascending infection by serovars D to K of the female upper genital tract, known as pelvic inflammatory disease, causes salpingitis, which in turn leads to fibrosis and scarring of the fallopian tubes, and eventual complications of ectopic pregnancy and tubal infertility (22). *C. trachomatis* originating from the genital tract is also associated with reactive arthritis, which develops in 1 to 3% of patients after genital chlamydial infection (123). *C. pneumoniae*, which can also disseminate from the site of the initial infection (74), has been associated with cardiovascular disease (62, 102) and late-onset Alzheimer's disease (4). In addition, unresolved respiratory *C. pneumoniae* infection may contribute to the pathogenesis of chronic inflammatory lung diseases, such as asthma (43) and chronic obstructive pulmonary disease (15). Similar *Chlamydia*-associated chronic diseases and their sequelae occur in many animals, for example, trachoma-like blindness (25) or infertility (71) in koalas and polyarthritis in sheep (117).

Recurrent chlamydial disease may result from either repeated infections or persistence of the organism after unresolved infections. Indeed, the high incidence of chlamydial infections and transient immunity typically observed after infection (90) present difficulties in differentiating between persistent infection and reinfection. Nonetheless, characterization of the in vitro persistent phase of chlamydiae and multiple lines of in vivo evidence suggest that chlamydiae persist in an altered form during chronic disease. This review will provide an update on chlamydial persistence, focusing on recent insights that have been obtained into the molecular basis of this important stage of chlamydial development.

CHLAMYDIAL PERSISTENCE IN VITRO

Chlamydial persistence has been described as a viable but noncultivable growth stage resulting in a long-term relationship with the infected host cell (9). Such relationships have been established in vitro, usually through deviations from conventional cell culture conditions for productive chlamydial development. The different in vitro persistence systems often share altered chlamydial growth characteristics, for example, a loss of infectivity and the development of relatively small inclusions containing fewer chlamydiae. In addition, these systems often produce common ultrastructural traits (Table 1).

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TABLE 1. Selected in vitro ultrastructural observations of atypical persistent chlamydiae

Induction	Infecting strain(s)	Specific conditions	Observations	Reference
Antibiotic exposure	*C. psittaci* MN/Cal-10	L cells, 200 U of penicillin/ml	0–12 h PI, normal EB-to-RB differentiation; 18–48 h PI, small vesicles pinched off enlarged RB; 20–48 h PI, membranes within enlarged RB; 28–48 h PI, flat membrane structures surrounding RB (subsequently released as membrane sheets); 36–48 h PI, multiple immature nucleoids within enlarged RB; after penicillin removal, budding and internal subdivision to normal RB	69
Amino acid deficiency	*C. trachomatis* serovar L2[a]	McCoy cells, 0–100% amino acid levels in Eagle's minimum essential medium, 1 μg of cycloheximide/ml	10% amino acids (48 h PI), RB-sized swollen intermediate forms or enlarged RB-like forms containing budding forms; 0% amino acids (48 h PI), enlarged inclusions containing few RB-like forms with multiple dense nucleoid centers and empty vesicle membranes, large irregular forms with a strand-like network and multiple cytoplasmic nucleoid-like masses, ring forms occupying most of inclusion	26
Iron deficiency	*C. trachomatis* serovar E	HEC-1B cells, 100 μM DAM, 0.5 μg of cycloheximide/ml	24 h PI, electron-dense material surrounding inclusions; 72 h PI, inclusions containing either numerous membrane blebs or normal-sized RB with dense and wavy outer membranes	95
IFN-γ exposure	*C. pneumoniae* A-03	HEp-2 cells, 25 U of IFN-γ/ml	48 h PI, small inclusions containing few chlamydiae, which were generally enlarged RB with sparse densitometric appearance	87
Monocyte infection	*C. trachomatis* serovar K	Primary human peripheral blood monocytes	5–7 days PI, vacuolization and electron-dense granules in chlamydiae; 10 days PI, inclusions dominated by aberrant oval RB	59
Phage infection	*C. psittaci* GPIC	HeLa cells, chlamydiae hyperinfected by ΦCPG1	18–32 h PI, small inclusions containing few chlamydiae, including maxi-RB 2–5 times the size of normal RB; 32–44 h PI, lysis of maxi-RB and inclusion membrane	49
Continuous infection	*C. trachomatis* genital isolate[b]	L cells, infections maintained for 6–7 continuous cycles	10–50% of host cells featured either (i) inclusions containing normal RB, (ii) inclusion-bound, 200-nm-diameter, RB protoplast-like bodies, or (iii) dense, 100-nm-diameter oval bodies bounded by single membranes	107
	C. pneumoniae TW-183 and CM-1	HEp-2 cells, infections maintained for 4 yr by periodic reseeding	48 h postsplit, 10% of inclusions were either altered (containing intrainclusional membranous material and a small proportion of aberrant RB 4–5 times the size of normal RB with homogeneous cytoplasm) or aberrant (smaller with no intrainclusional membranous material and dominated by aberrant but normal-sized RB)	63
Heat shock	*C. trachomatis* serovar L2	BGM cells, transferred to 42°C at 12 h PI	5 h after transfer, mosaic-like display of RB in some inclusions; 20 h after transfer, predominantly huge aberrant RB	53

[a] Similar development of abnormal forms was reported for infections (with or without cyclohexamide) with *C. trachomatis* serovar E or *C. psittaci* MN/Cal-10, 6BC, or GPIC.
[b] Moulder (78) proposed that the strain had been from the lymphogranuloma venereum biovar (serovars L1 to L3).

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Many studies have described enlarged, pleomorphic RB that are inhibited in binary fission and their differentiation to EB but nevertheless continue to accumulate chromosomes. These changes are generally reversible upon removal of the growth inhibitory factor. However, despite the general similarities, significant differences in growth and ultrastructural characteristics have also been reported among different systems or within a given system.

Antibiotic-induced persistence. Many early in vitro studies described abnormal chlamydial development after exposure to antibiotics. In general, agents that target bacterial protein or RNA synthesis can inhibit chlamydial differentiation either from EB to RB or from RB to EB, depending on when they are added to an in vitro infection, whereas those that target DNA or peptidoglycan synthesis specifically inhibit RB-to-EB differentiation (78). For example, 10 μg of erythromycin/ml, which reduces ribosome activity, inhibited *C. trachomatis* serovar A EB-to-RB differentiation in McCoy cells when added within 12 h PI (24). When the erythromycin was applied at later times PI, infections were established featuring enlarged RB that could not differentiate to EB (24). In contrast, early addition of 200 U of penicillin/ml, which targets peptidoglycan cross-linking, had no effect on *C. psittaci* MN/Cal-10 development in L cells up to 12 h PI, but from that time onwards, the RB became enlarged and progressively more aberrant (69) (Table 1). Upon transfer to penicillin-free medium, the productive developmental cycle resumed and normal RB were produced by budding from and endosporulation-like subdivision of enlarged RB (69).

More recently, exposure of *C. pneumoniae* AR-39 infections in HeLa cells to 50 μg of ampicillin/ml led to the development of aberrant, giant RB (122). Interestingly, when directly compared to *C. trachomatis* infections subjected to the same conditions, the persistent *C. pneumoniae* infections were extremely inefficient in reactivation after the removal of ampicillin (122). The explanation for this result is unclear at present, since observations of *C. pneumoniae* exposed to gamma interferon (IFN-γ) or deprived of tryptophan (72) (see below) argue against slow recovery from persistence as a distinguishing feature of this species. These examples and many other observations of persistence induced in vitro by various antibiotics (reviewed in reference 9) indicate that inadequate antimicrobial therapy may allow chlamydiae to persist in vivo.

Deficiency-induced persistence. In contrast to persistence induced by antibiotics, the depletion of essential nutrients from cell culture medium temporarily arrests the growth of both chlamydiae and their host cells until the missing nutrients are replaced (78). In the first ultrastructural characterization of deficiency-induced persistent chlamydiae, progressive depletion of all amino acids caused increasingly abnormal development of *C. trachomatis* serovar L2 in McCoy cells (Table 1), with partial recovery of particle size after reintroduction of amino acids (26). Induction of persistence in the same *Chlamydia*-host cell system by blood plasma concentrations of amino acids (45) suggested that amino acid levels could directly influence chlamydial development in vivo.

Minimum requirements for individual amino acids are likely to be even more important than total amino acid concentrations in determining the outcome of chlamydial infections in vivo. Allan and Pearce (2) reported that selected *C. trachomatis* serovars had differential requirements for specific amino acids in McCoy cell infections that correlated with their respective anatomical sites of origin. Of particular note, ocular (A to C) but not oculogenital (D to I) serovars required tryptophan for normal growth (2). However, a recent study of HeLa cell infections with reference strains representing all *C. trachomatis* serovars failed to reproduce this finding, since the growth of all serovars was inhibited in the absence of tryptophan (33). Instead, the tissue tropisms correlated with an indole-rescuable phenotype, since genital (D to K and L1 to L3) but not ocular (A to C and Ba) serovars growing in tryptophan-deficient medium were able to generate tryptophan from exogenous indole and therefore recover their infectivities (33). This finding was a crucial contribution towards establishing the in vivo relevance of IFN-γ-mediated persistence.

Depletion of nutrients other than amino acids from cell culture medium can also induce persistence. For example, *C. trachomatis* serovar L2 in McCoy cells became reversibly persistent in response to the removal of glucose from the cell culture medium, temporarily losing infectivity and showing abnormal morphology comparable to that seen during amino acid depletion (45). However, the major focus of recent research into deficiency-induced persistence has been iron deprivation (3, 46, 95). Exposure of *C. trachomatis* serovar E-infected polarized endometrial epithelial (HEC-1B) cells to 100 μM concentrations of the iron chelator deferoxamine mesylate (DAM) inhibited infectivity and caused significant morphological changes in the chlamydiae that were generally distinct from those observed in other persistence systems (95) (Table 1). The persistence of *C. pneumoniae* TW-183 in HEp-2 cells was similarly induced by exposure to 30 μM DAM and maintained for as long as 6 days, although not all of the morphological features described for the *C. trachomatis* cultures were observed (3). The addition of iron-saturated transferrin led to the recovery of infectivity and productive development for both species, thus supporting depleted host cell iron pools as the cause of the changes (3, 95). These studies support the hypothesis that fluctuating iron levels, for example under the influence of estradiol in endometrial tissue (57), may contribute to the outcome of chlamydial infections in vivo.

Cytokine-induced persistence. Exposure of in vitro chlamydial infections to cytokines, particularly IFN-γ, provides a system of indirect deficiency-induced persistence that could plausibly reflect in vivo events. In early studies, IFN-γ was identified as the active component in supernatant fluids from stimulated T cells that inhibited replication of *C. psittaci* 6BC in fibroblast (17) and macrophage (101) cultures that had been preexposed to the supernatant. Preexposure of epithelial cells for 24 h to high concentrations of IFN-γ inhibited inclusion formation by *C. trachomatis* serovar L2 (111), *C. psittaci* 6BC (18), and *C. pneumoniae* BAL-37 (118). However, lower IFN-γ levels only partially restricted chlamydial development (18, 111, 118).

Persistent chlamydial infections are induced by exposing cultures to moderate IFN-γ levels, usually following infection. In this way, persistence was established for *C. trachomatis* serovar A in HeLa cells at IFN-γ levels as low as 0.2 ng (2.4 U)/ml (7) and for *C. pneumoniae* A-03 in HEp-2 cells at a level of 25 U/ml (87). Persistence of *C. trachomatis* serovar A was maintained for several weeks (10). Ultrastructurally, the IFN-γ-

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induced persistent chlamydiae were enlarged and aberrant (7, 87) (Table 1). In *C. trachomatis* serovar A, there was also evidence of budding and endopolygeny, the production of multiple progeny from a single enlarged form, during resumption of productive infection after removal of IFN-γ from the cultures (10). These morphological observations were consistent with those from other persistence induction systems (26, 69). However, a direct comparison of IFN-γ- and amino acid depletion-induced persistent *C. trachomatis* serovars E and L2 in HeLa cells revealed different growth characteristics between the two systems, since only IFN-γ-exposed cultures showed decreases in inclusion size and the number of infected cells (52).

The most important mechanism underlying the effects of IFN-γ on chlamydial growth in cultured human cells is tryptophan depletion through activation of the host tryptophan-degrading enzyme indoleamine 2,3-dioxygenase (IDO). Byrne et al. (18) showed that growth restriction of *C. psittaci* 6BC in human epithelial cells preexposed for 24 h to 20 ng (240 U) of IFN-γ/ml could be reversed by the addition of tryptophan following infection. IDO induction was later confirmed to be the major mechanism of IFN-γ-mediated persistence for *C. trachomatis* serovar A in HeLa cells (6) and *C. pneumoniae* A-03 in aortic smooth muscle cells (88). Recently, tryptophan depletion provided an important link between IFN-γ and differential tissue tropisms among *C. trachomatis* serovars. Caldwell and colleagues (20) showed that, in agreement with the previous study of direct tryptophan depletion (33) (see above), *C. trachomatis* serovar D, I, or L2 but not serovar A in HeLa cells displayed the indole-rescuable phenotype after exposure (24 h preexposure for serovar L2) to 5 ng (60 U) of IFN-γ/ml.

Interplay between the IFN-γ concentration and other factors may affect the outcome of a chlamydial infection in vivo. Such factors could include the susceptibility of the infecting strain to IFN-γ-mediated inhibition (76) and the activity of other cytokines such as tumor necrosis factor alpha (112, 118) and interleukin-1 (21) that may synergistically enhance the effects of IFN-γ on chlamydial growth. In the case of tryptophan depletion as the mechanism of inhibition, additional factors such as the availability of exogenous indole and ability of the infecting strain to use it (20, 33) and the IDO expression level of the host cell type (103) may also contribute. However, despite the evident importance of tryptophan catabolism, other mechanisms such as the inducible nitric oxide synthase effector pathway and iron deprivation could also be attributable to IFN-γ, thus adding to the potential complexity of the in vivo situation (50). In addition, the relative importance of these different mechanisms is likely to vary among host species. For example, inducible nitric oxide synthase induction seems to play a central role in IFN-γ-dependent inhibition of experimental chlamydial infection in mice, whereas IDO induction in response to chlamydial infection is yet to be observed in cultured murine cells (94).

Monocyte infections. In contrast to other persistence systems, chlamydiae become spontaneously persistent following infection of monocytes. Cell culture infections of freshly isolated human monocytes were morphologically characterized for *C. trachomatis* serovar K (59) and *C. pneumoniae* strain Kajaani 7 (1). In both studies, no normal RB (only aberrant RB) were observed at any time over the monocyte infection period and chlamydial mRNA continued to be detected, despite a loss of infectivity (1, 59). These observations suggested that the chlamydiae were surviving in a viable but culture-negative state. The addition of neither tryptophan nor antibodies against IFN-γ (nor antibodies against tumor necrosis factor alpha and IFN-α for *C. trachomatis*) (59) counteracted the chlamydial growth inhibition (1, 59). This finding, coupled with the unique morphological characteristics of this model (Table 1), led to the suggestion that a cytokine-independent mechanism is at least partially responsible for monocyte-induced persistence (59). The mechanism is also thought to be oxygen independent (100), but its precise nature remains obscure.

Phage infection of chlamydiae. Observations that lytic infection by naturally occurring bacteriophages can alter chlamydial development in vitro suggest yet another persistence induction mechanism. An early report of Chp1 infection of *C. psittaci* N352 described the formation of enlarged, distended RB (98). More recently, Hsia et al. (49) thoroughly characterized the ultrastructural effects of ΦCPG1 infection on *C. psittaci* GPIC. The maxi-RB observed had the basic characteristics of aberrant bodies seen in other cell culture models of persistence and were of a similar size to the distended RB induced by Chp1 (49) (Table 1).

If phage-induced chlamydial persistence represents an in vivo reality, it will only be relevant to certain chlamydial strains, since infecting phage was evident in neither the majority of complete genome sequences nor an additional analysis of six clinical *C. pneumoniae* isolates (55). However, the *C. pneumoniae* AR-39 genome sequence revealed that this strain is infected by a phage, ΦCpn1/ΦAR-39 (96), that is very similar to ΦCPG1 at the nucleotide level (5). Interestingly, a recent seroepidemiological study showed that seropositivity to ΦCpn1/ΦAR-39 Vp1 antigen correlated more strongly with the presence of aortic abdominal aneurysm than did seropositivity to *C. pneumoniae* AR-39 EB (55). One of the authors' conclusions was that phage-bearing strains may become persistent more readily in vivo, resulting in lower cultivability. There is the intriguing possibility that ΦCpn1/ΦAR-39 itself could be at least partially responsible for an increased tendency of *C. pneumoniae* AR-39 to become persistent. Studies are warranted to determine whether ΦCpn1/ΦAR-39 can affect *C. pneumoniae* AR-39 growth in a similar way to the induction of *C. psittaci* GPIC maxi-RB formation by ΦCPG1 and whether some maxi-RB and inclusions are indeed able to persist (escaping phage-induced lysis).

Continuous infections. In contrast to all other persistence systems, continuous cultures become spontaneously persistent when both chlamydiae and host cells are free to multiply in the absence of stresses (even spontaneous monocyte persistence must involve unidentified stresses on the chlamydiae). Continuous cultures are characterized by cycles consisting of several days of mostly inclusion-free host cell multiplication followed by rapid chlamydial multiplication leading to partial (84) or almost complete (65, 80) host cell destruction. These cycles continue indefinitely when the cultures are maintained by periodic washing and growth medium replacement.

Some continuous cultures seem to be established by a genetic block, as opposed to blocks caused by inhibitors or deficiencies, in the progression of a productive cell culture infection (65, 84). For example, an early report described continuous *C. psittaci* 6BC infections of Chang's human liver

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cells that showed large fluctuations in the percentage of inclusion-positive host cells among successive passages (84). Isolation of chlamydiae and host cells from selected passages revealed chlamydial variants that infected parent Chang's human liver cells with higher efficiency than wild-type *C. psittaci* and host cell variants that were more resistant to infection by the chlamydial variants (84). Thus, chlamydiae, their host cells, or both may undergo population shifts favoring genotypes that are more suited to a long-term persistent relationship.

More recently, Kutlin et al. (64) established a continuous culture system in HEp-2 cells for *C. pneumoniae* CM-1 and TW-183. The authors suggested that population shifts analogous to those described for *C. psittaci* (84) and *C. trachomatis* biovar trachoma (65) occur in the *C. pneumoniae* system, although corresponding characterizations of isolated *C. pneumoniae* and host cells have not been reported. Ultrastructurally, continuous *C. pneumoniae* infections at 48 h postsplit largely resembled a productive infection, except for a subpopulation of atypical inclusions that were either partially or fully occupied by aberrantly enlarged RB resembling those described in other persistence systems (63) (Table 1). These infections also showed reduced sensitivity to azithromycin or ofloxacin at levels up to four times the MIC, as indicated by a significantly slower reduction of inclusion-forming units when compared over 6 days to primary infections similarly exposed from 0 h PI onwards (64). However, this property is not restricted to continuous cultures or even to established persistent cultures in general, since a similar effect was observed when established (48 h PI onwards) primary *C. trachomatis* serovar K infections of HEp-2 cells were exposed to ofloxacin at up to four times the MIC over 18 days (30). In that study, the antibiotic itself induced persistence, which in turn was considered to be responsible for the reduced antimicrobial susceptibility (30).

There is a special case of continuous culture that cannot be explained even by the genetic block hypothesis. Moulder et al. (80) reported continuous *C. psittaci* 6BC infections of L cells from which all isolated chlamydiae and host cells were found to be indistinguishable from their wild-type counterparts. In addition, nine clones taken from cultures with 25% inclusion-positive cells were initially inclusion free but subsequently all gave rise to persistently infected populations (80). To explain these observations, the authors proposed that some chlamydiae in a cryptic form survived each wipeout of host cells and that every host cell was always infected by either cryptic or productive chlamydiae (80). Moulder (78) has since raised the possibility that cryptic bodies may be related to single, inclusion-bound, dense oval bodies observed in *C. trachomatis*-L cell cultures (107) (Table 1) that behaved similarly to the *C. psittaci* 6BC-L cell cultures (80). However, the precise morphology of cryptic chlamydiae remains unknown.

Despite their relatively protected intracellular niche, chlamydiae are subject to a variety of insults, particularly in intact hosts with competent immune systems. Examples of naturally occurring insults include shortages of essential nutrients, which may be brought about by host-elaborated factors such as cytokines and hormones, and phage hyperinfection of certain chlamydial strains. In addition, antibiotics specifically target chlamydiae in multiple ways. Apart from these known factors, there are other mechanisms that remain incompletely explained, including those underlying monocyte-induced persistence and continuous cultures. The in vitro persistent state that chlamydiae enter after experiencing this broad range of hostile conditions may, at least in part, represent a general stress response that chlamydiae have evolved to ensure their survival under harsh conditions. This hypothesis is supported by the observation that heat shock of *C. trachomatis* serovar L2 in BGM cells at 42°C induced a morphological response that was similar to those often observed in other persistent cell culture systems (53) (Table 1). However, one potential inconsistency with this hypothesis is the occasional observation of aberrantly enlarged RB during productive cell culture infections (60, 122). Could such occurrences indicate localized areas of nutrient depletion in a cell culture? Further studies with more relevant systems, for example, polarized cells and cells other than epithelial cells, are required to further validate in vitro persistence in relation to in vivo events.

EVIDENCE FOR CHLAMYDIAL PERSISTENCE IN VIVO

The recognition that chlamydiae are likely to cause persistent infections in their hosts dates back to early descriptions of latent psittacosis in birds (73). Since these early studies of natural animal infections, the body of evidence that has accumulated for the existence of persistence in vivo has come mostly from experimental animal infections and clinical data from human disease. Various characteristics link these in vivo infections to the well-studied cell culture models of persistent chlamydial infection. Among the most convincing lines of evidence for persistence in vivo are observations of altered morphological forms in vivo, detection of chlamydial macromolecules in diseased hosts in the absence of cultivability, recurrences that occur when reinfection is unlikely, and clinical antibiotic resistance.

Electron microscopic visualization in diseased tissues of morphologically aberrant chlamydial forms resembling those observed in vitro (Table 1) indicates that such forms are unlikely to be mere laboratory artifacts. Nanagara et al. (81) showed that atypical, pleomorphic RB with poorly defined outer membranes dominated within infected fibroblasts and macrophages in synovial membrane samples from patients with *C. trachomatis*-associated reactive arthritis or Reiter's syndrome, despite antibiotic therapy. More recently, *C. pneumoniae* forms of a similar size to aberrant RB seen in vitro were observed within macrophages in aortic valve samples from patients with degenerative aortic valve stenosis (113). In addition, miniature *C. trachomatis* forms have been observed in total ejaculate and expressed prostatic secretion samples from patients with chronic chlamydial prostatitis (70) and in the oviducts of mice experimentally infected with *C. trachomatis* biovar mouse pneumonitis (92). These miniature forms may correspond to budding forms seen in vitro (10, 69). However, observations of ultrastructurally aberrant chlamydiae alone fall short of proving that chlamydiae persist in vivo, since the viability of these particles is uncertain.

The presence of viable but atypical chlamydiae in vivo is suggested by the detection of chlamydial macromolecules at diseased sites in the absence of cultivable organisms. Chlamydial DNA and antigen are often detected in tubal biopsy specimens from culture-negative women with postinfectious tubal infertility following antibiotic treatment (91). Even more convincingly, persistence of noncultivable chlamydiae in the va-

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gina, uterus, and oviduct of ewes experimentally infected with a naturally occurring strain of *C. psittaci* was documented for more than a year by the detection of DNA or antigen in these tissues (89). Although the detection of chlamydial DNA or antigen could reflect the presence of chlamydial cell debris remaining from resolved infections, PCR data showing that UV-inactivated *C. pneumoniae* organisms were cleared far more efficiently than live organisms from inoculated mice (74) argue against long-term persistence of chlamydial macromolecules. In addition, chlamydial RNA has been detected in the absence of cultivability in experimental trachoma of primates (48) as well as in synovial biopsy samples from patients with reactive arthritis or Reiter's syndrome (35, 93). Since RNA is highly labile, its detection in vivo strongly suggests the presence of viable organisms and correlates with similar data indicating viable but culture-negative chlamydiae in vitro (1, 30, 37, 59).

Experimental and clinical data provide evidence for reactivating persistent chlamydiae in vivo. Early reports described individuals who had experienced acute ocular *C. trachomatis* infection as children living in areas of trachoma endemicity and showed no more symptoms until they developed active trachoma decades after leaving those areas (119), indicating that the recurrences were more likely due to reactivations of persistent infection than to reinfections. Particularly convincing evidence of periodic reactivation of persistent infections comes from long-term studies that described individuals experiencing multiple recurrent infections with chlamydial isolates of the same genotype. In a study of women with genital *C. trachomatis* infection, Dean et al. (28) demonstrated recurrences of the original *ompA* genotype over 2 to 4.5 years, despite administration of accepted treatment regimens. Although this study was limited by the inability to control for reexposure to untreated partners, data from another study showed that 10% recurrence of genital *C. trachomatis* infection can occur even among subjects who report either abstinence or 100% condom use following treatment with azithromycin (56). Preliminary genotyping data from Dean et al. and Hammerschlag et al. (27, 44) suggested that *C. pneumoniae* can also persist for many years after initial respiratory infection. Animal infection models provide additional evidence for reactivation of persistent infections in vivo. In mice infected with either *C. trachomatis* (125) or *C. pneumoniae* (67), infections that had become asymptomatic reactivated to productive infections after treatment with cortisone. Since the reactivation occurred specifically after suppression of the immune system, these observations supported the hypothesis that immune factors such as IFN-γ play a significant role in persistence in vivo.

Several studies have reported resistance of chlamydial isolates to antibiotics (reviewed in reference 99). Whether these data reflect direct resistance or phenotypic resistance manifested by altered chlamydial forms is unclear. Chlamydiae are capable of developing true genotypic resistance to antibiotics in vitro. For example, *C. trachomatis* serovar L2 mutants isolated from cell culture after several rounds of exposure to various fluoroquinolones consistently showed a point mutation in *gyrA* (encoding DNA gyrase subunit A), suggesting that DNA gyrase is the primary target of these antibiotics (29). However, a recent study described *C. trachomatis* isolates associated with treatment failure that were resistant to multiple drugs with diverse molecular targets (doxycycline, azithromycin, and ofloxacin at concentrations above 4 μg/ml) (115). This indicated the presence of a more global resistance mechanism such as the induction of a persistent phenotype that is refractory to multiple antibiotics, for example, through membrane alterations that affect drug intake. In some cases, the explanation for resistance could be more complex; certain genotypes could confer antibiotic resistance by encouraging development of the persistent phenotype. Such a scenario seems to occur in tetracycline-resistant porcine *C. trachomatis* strains, which produced large aberrant RB in response to the antibiotic at 2 μg/ml (66). Could the *gyrA* mutations that developed in cell culture in response to fluoroquinolone exposure (29) also favor the formation of a persistent phenotype, since alterations to DNA gyrase could inhibit RB-to-EB differentiation?

Taken together, the in vivo data suggest that chlamydiae persist in their hosts. However, these studies do not conclusively prove that the chlamydiae persist in an altered form. For example, detection of chlamydial macromolecules in the absence of cultivability could also reflect low-grade productive infections that are not detectable by the culture methods used. Similarly, although Beatty et al. (10) definitively proved that in vitro aberrant forms can give rise to productive infections, in vivo data indicating reactivation could also represent enhancement of an inapparent productive infection. A final line of evidence for altered forms in vivo that will be discussed below comes from studies that have demonstrated similarities in chlamydial gene or protein expression trends between persistent cell culture systems and tissue samples from sites of chronic disease.

MOLECULAR BASIS OF CHLAMYDIAL PERSISTENCE

Although our understanding of the molecular basis of chlamydial persistence is still at an early stage, the rate of progress in this area has significantly increased in recent years. This has been made possible primarily by the completion of several genome sequencing projects (54, 96, 97, 116) and by recent improvements in molecular methodology, in particular, quantitative PCR, microarrays, and proteomics. While persistence is a simple term, there are several systems for chlamydial persistence with a significant number of variables that make direct comparison of results difficult. Variables include the infecting chlamydial species and strain, the origin of host cells, the in vitro persistence induction, and times PI chosen for analysis. Despite this complexity, some common themes are emerging from the results of molecular studies undertaken to date (Table 2). The categories described below represent a current overview of a rapidly advancing field and are therefore likely to be modified as further data are generated, particularly from global microarray and proteomics studies.

MOMP and cHSP60. Until the recent availability of complete chlamydial genome sequences, most molecular studies of persistence focused on relative levels of the major outer membrane protein (MOMP) and chlamydial heat shock protein 60 (cHSP60). Using immunoelectron microscopy, Beatty et al. (8) found that exposure of *C. trachomatis* serovar A infections in HeLa cells to 0.5 ng (6 U) of IFN-γ/ml caused cHSP60 levels to increase slightly (1.4-fold) and MOMP levels to decrease twofold, after correcting for the decreased surface-to-volume ratio in enlarged persistent forms. Subsequent immunoblot analyses showed a decreased MOMP-to-cHSP60 ratio over

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TABLE 2. Postgenomic molecular studies of chlamydial persistence

Induction	Infecting strain(s)	Method of analysis	Gene or protein expression that was:				Reference
			Up-regulated	Down-regulated	Unchanged	Variable	
Antibiotic exposure	*C. trachomatis* serovar D	Microarray	*fabH*, *ftsK*, *himD*, *htrA*, *icc*, *mutS*, *pdhC*, *r16*, *tolB*, *yaeT*, *CT265*, *CT296*, *CT476*, *CT598*, *CT790*	*aroB*, *bpl*, *glgA*, *hctB*, *hemN*, *ltuB*, *sfhB*, *CT073*, *CT082*, *CT181*, *CT249*, *CT456*, *CT694*, *CT814*, *CT875*	*dnaA*, *ftsW*, *groEL-1*, *incA*, *nlpD*, *ompA*, *porB*, *pyk*		82
Amino acid deficiency	*C. trachomatis* serovars E and L2	Immunoblotting				60-kDa CRP, cHSP60, DnaK, LPS, Mip, MOMP, S1	52
Glucose deficiency	*C. trachomatis* serovar L2	sqRT-PCR		*glgA*, *glgP*, *gltT*, *omcB*, *ompA*, *pckA*, *ptsN*, *uhpC* MOMP[a]	*euo*		51
IFN-γ exposure	*C. trachomatis* serovars A, D, and L2	2-D gel	TrpA, TrpB				108, 109
	C. trachomatis serovars E and L2	Immunoblotting		Hc1, Hc2		60-kDa CRP, cHSP60, DnaK, LPS, Mip, MOMP, S1	52
	C. pneumoniae IOL-207	sqRT-PCR	*nlpD*, *ompA*, *porB*, *pyk*, *CPn0585*		*76kDa*, *groESL*, *omcB*, *pmpI*, *yaeT*, *CPn1046*		68
	C. pneumoniae TW-183	sqRT-PCR		*ftsK*, *ftsW*	*dnaA*, *minD*, *mutS*, *polA*		19
	C. pneumoniae A-03	2-D gel	AmiB, Amn, cHSP60, GlgP, GyrA, MOMP, Pgk, Pnp, RpoA, Rrf, YscN		DnaK, GatA, PepA, Pyk, ThrS, TufA, CPn0425, CPn0512, CPn0763, CPn0820		75
	C. pneumoniae CM-1	sqRT-PCR	*groEL-1*, *omcB*, *ompA*	*ftsK*, *lcrD*, *lcrE*, *lcrH-1*, *sycE*	*lcrH-2*, *yscC*, *yscJ*, *yscL*, *yscN*, *yscR*, *yscS*, *yscT*		114
	C. trachomatis serovars A and E	rtRT-PCR	*trpBA*, *trpR*[b]				20
	C. trachomatis serovars A and L2	rtRT-PCR, immunoblotting	*trpBA*, *trpR*, TrpA, TrpB[b]				124
	C. trachomatis serovar D	Microarray, rtRT-PCR[c]	*euo*, *fusA*, *pgsA-2*, *recA*, *trpB*, *CT228*	*ftsW*, *hctA*, *ihfA*, *ltuB*, *omcB*, *ompA*, *oppA-4*, *CT546*, *CT780*			13
Monocyte infection	*C. trachomatis* serovar K	sqRT-PCR		*ftsK*, *ftsW*, *ompA*	*dnaA*, *mutS*, *parB*, *polA*		38
				cydA, *gap*, *gnd*, *ompA*, *pgk*, *pyk*, *tal*	*cydB*, *fumC*, *mdhC*, *rpoD*, *rpoN*, *rpsD*		36
Continuous infection	*C. pneumoniae* TW-183	rtRT-PCR	*nlpD*, *ompA*, *pmpI*, *porB*, *CPn0483*		*ftsK*, *groESL*	*76kDa*, *omcB*, *pyk*, *yaeT*, *CPn0585*, *CPn1046*	47

[a] MOMP was only down-regulated in serovar A.
[b] Expression of these genes and proteins was normalized in genital serovars but not in serovar A after the addition of indole.
[c] Only genes that were confirmed by rtRT-PCR to be differentially expressed have been included.

10-day *C. trachomatis* serovar K infections in either HEp-2 cell cultures exposed to 0.5 μg of ciprofloxacin/ml (30) or unexposed monocyte cultures (37). In addition, semiquantitative reverse transcriptase PCR (sqRT-PCR) analyses demonstrated selectively down-regulated transcription of the *ompA* gene in *C. trachomatis* serovar K-infected monocyte cultures (36–38). Finally, two-dimensional (2-D) protein gel electrophoresis data showed that exposure to 100 U of IFN-γ/ml caused down-regulated MOMP expression in *C. trachomatis* serovar A (108) but not in serovars D and L2 (108, 109) in HeLa cells. Since the

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latter two serovars are among the most resistant to IFN-γ (76), those cultures may have required preexposure at the concentration used to become persistent. Indeed, preexposure of HeLa cells to 50 U of IFN-γ/ml was sufficient to induce persistence of *C. trachomatis* serovar D and down-regulation of *ompA*, as assessed by microarray and real-time RT-PCR (rtRT-PCR) (13). Gérard et al. (39) recently used rtRT-PCR to quantitate the relative transcript levels of the three *groEL* genes (encoding cHSP60 homologues) in *C. trachomatis* serovar K-infected monocytes over 7 days. Interestingly, transcription of *groEL-2* was markedly higher than that of *groEL-1* and *groEL-3* in monocyte cultures, a trend not seen in HEp-2 cell control infections (39). However, the data also seemed to suggest that *groEL-1* and *groEL-3* expression was strongly down-regulated in monocyte persistence relative to the controls. In addition, many other recent studies, mostly involving *C. pneumoniae*, have reported data contrary to a decreased *ompA*/MOMP-to-*groEL*/cHSP60 ratio, indicating that this may not be as universal a marker of persistence as was once thought (47, 52, 68, 75, 82, 114) (Table 2).

In general, the in vivo data support the decreased *ompA*/MOMP-to-*groEL*/cHSP60 ratio described in many cell culture studies of persistent *C. trachomatis*. Immunoelectron microscopic (81) and sqRT-PCR (35, 36, 38) analyses of synovial tissue samples from patients with *C. trachomatis*-associated chronic arthritis demonstrated diminished levels of MOMP and *ompA* transcripts, respectively. In apparent contrast, recent data from similar samples indicate down-regulated expression of all three *groEL* genes, including the virtual absence of *groEL-3* transcripts (39). Nonetheless, many studies have reported enhanced production of cHSP60-specific antibodies in various chronic chlamydial infections (reviewed in reference 58).

Whether an altered MOMP-to-cHSP60 ratio has any significance to chlamydial pathogenesis beyond serving as a marker of persistence in vivo is a subject of much debate. Beatty et al. (7) proposed that reduced levels of MOMP, an immunoprotective antigen, could enable chlamydiae to avoid the development of protective immunity. At the same time, according to the traditional immunological paradigm of chlamydial pathogenesis, steady or increased cHSP60 levels would promote immunopathology through delayed-type hypersensitivity or cross-reactivity with HSP60 from either the human host or other pathogens. Another potential explanation for the relative abundance of cHSP60 during *C. trachomatis* persistence is its function as a stress response chaperone (77), which is likely to be of particular importance under the conditions that induce persistence. In support of this view, the production of five proteins thought to be major heat shock proteins, including cHSP60, was strongly increased as assessed by one-dimensional gel electrophoresis after heat shock of *C. trachomatis* (53).

Cell division and chromosome replication and partitioning. Recent sqRT-PCR investigations have provided molecular insights into the common observation that persistent chlamydiae are inhibited in cell division and yet continue to accumulate chromosomes (Table 1). These studies analyzed expression of genes encoding products predicted to function in DNA replication (*polA*, *dnaA*, and *mutS*), chromosome partitioning (*parB* and *minD*), and cell division (*ftsK* and *ftsW*) in various persistence systems; all genes studied were strongly expressed during productive infections of HEp-2 cells used as controls (19, 38). In *C. trachomatis* serovar K-infected monocytes, *polA*, *dnaA*, *mutS*, and *parB* transcripts were produced over the whole 7-day infection period, whereas *ftsK* and *ftsW* were not detected after 1 day PI (38). Expression profiles in synovial tissue samples from patients with *C. trachomatis*-associated reactive arthritis reflected the in vitro results; *polA*, *dnaA*, *mutS*, and *parB* were weakly detected but neither *ftsK* nor *ftsW* was detected (38). In *C. pneumoniae* TW-183-infected HEp-2 cell cultures exposed to 0.5 ng (6 U) of IFN-γ/ml, *polA*, *dnaA*, *mutS*, and *minD* expression was steady, whereas that of *ftsK* and *ftsW* was absent (19). When the *C. pneumoniae* infections were exposed to an IFN-γ concentration (0.15 ng [1.8 U]/ml) that caused no significant morphological alterations to the chlamydiae compared to unexposed controls, *ftsK* and *ftsW* expression was present but significantly attenuated (19). Taken together, these studies demonstrated that down-regulated *ftsK* and *ftsW* expression occurs in different in vitro persistence models and in vivo and that these genetic alterations can precede evidence of morphological alterations during the establishment of persistence.

More recent cell culture data have been inconclusive regarding cell division and DNA replication gene expression during persistence. In our own laboratory (R. J. Hogan, D. A. Good, S. A. Mathews, S. Mukhopadhyay, J. T. Summersgill, and P. Timms, unpublished data), rtRT-PCR analysis of *C. pneumoniae* A-03-infected HEp-2 cells exposed to 50 U of IFN-γ/ml revealed strong down-regulation of *ftsK* but insignificant differential expression of *ftsW*. Microarray studies of persistent *C. trachomatis* serovar D in HeLa cell cultures have also provided mixed data; exposure of such infections to 100 U of penicillin/ml was associated with up-regulated *ftsK* and unchanged *ftsW* expression (82), whereas exposure to IFN-γ lead to unchanged *ftsK* and down-regulated *ftsW* expression (13). These discrepancies are not surprising, since the precise functions of chlamydial FtsK and FtsW are unlikely to be directly related. FtsW is a predicted septum-peptidoglycan biosynthetic protein involved in cell wall formation, whereas FtsK is required for chromosome segregation (116). Similarly, DNA replication gene expression profiles were varied in the microarray study of IFN-γ-exposed *C. trachomatis*, with some genes up-regulated (*dnaB*, *topA*, and *xerC*) and others down-regulated (*dnaA-2*, *dnlJ*, and *ihfA*) (13).

Energy metabolism. Using a similar experimental design to that of their previous study (38) (see above), Gérard and colleagues (36) studied genes encoding enzymes predicted to be involved in energy metabolism in both cultured *C. trachomatis*-infected monocytes and synovial tissue samples from patients with *C. trachomatis*-associated reactive arthritis. Since genes encoding enzymes belonging to glycolysis (*pyk*, *gap*, and *pgk*) and the pentose phosphate pathway (*gnd* and *tal*) were found to be selectively down-regulated in vitro and in vivo relative to genes encoding enzymes in the tricarboxylic acid cycle (*mdhC* and *fumC*), the authors concluded that persistence may be characterized by a shift from a partial to a full reliance on the host cell for ATP (36). The microarray expression data for genes encoding tricarboxylic acid cycle enzymes in IFN-γ-induced persistence of *C. trachomatis* were mixed. Genes encoding 2-oxoglutarate dehydrogenase (*sucA*, *sucB-1*, and *sucB-2*)

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and succinate thiokinase (*sucC* and *sucD*) were down-regulated, whereas genes encoding other enzymes in the cycle were either up-regulated (*fumC* and *sdhB*) or unchanged (*mdhC*, *sdhA*, and *sdhC*). There was little evidence of down-regulation in glycolysis or the pentose phosphate pathway (13). Also in apparent contrast to the results of Gérard et al. (36), 2-D gel analysis of *C. pneumoniae* A-03 exposed to 50 U of IFN-γ/ml showed up-regulated Pgk and unchanged Pyk levels (75).

Tryptophan metabolism. Tryptophan metabolism in relation to persistence has recently become an area of intensive research. Using 2-D gel analysis, Shaw and colleagues (108, 109) showed that exposure of *C. trachomatis* serovars A, D, and L2 to 100 U of IFN-γ/ml caused significant tryptophan-reversible up-regulation of the α and β chains of chlamydial tryptophan synthase. Induction of this enzyme is a plausible mechanism by which chlamydiae would counteract tryptophan deficiency induced by exposure to IFN-γ. However, another important finding was the presence of frameshift mutations in the gene encoding the α chain, *trpA*, for serovars A and C and the resultant synthesis of a truncated α chain by these serovars (109).

More recent studies (20, 33, 124) have developed the initial observations of Shaw and colleagues (108, 109) into a paradigm that links the tissue tropisms of *C. trachomatis* serovars to their relative abilities to synthesize tryptophan. Fehlner-Gardiner et al. (33) cloned and sequenced a section of the plasticity zone, a highly variable region of the chlamydial genome that contains the *trpBA* operon encoding both chains of tryptophan synthase, from 15 reference strains representing all human-infecting serovars of *C. trachomatis*. With the exception of serovar B, which was missing the *trpBA* operon, these authors concluded that ocular (A to C and Ba) but not genital (D to K and L1 to L3) serovars have the *trpA* mutation (33). Further investigation with several experimental approaches revealed that this mutation was responsible for the inability of ocular serovars to synthesize tryptophan from indole (33) (see above). Caldwell et al. (20) continued the sequencing approach to confirm that this paradigm also applies to clinical isolates of *C. trachomatis* and used rtRT-PCR to analyze *trpBA* expression for selected ocular (A) and genital (E) serovars in infections of HeLa cells. While both serovars strongly up-regulated their *trpBA* expression in response to 5 ng (60 U) of IFN-γ/ml, only serovar E *trpBA* expression returned to original levels following the addition of 100 μM indole, providing further support for the paradigm that ocular serovars are not indole rescuable (20). The authors concluded that the evolution of genital strains to utilize indole for tryptophan synthesis represents an immunoavoidance strategy, since these serovars are more likely to survive under IFN-γ pressure in vivo (20).

C. pneumoniae does not contain a *trpBA* operon in its genome (54) and may therefore have an alternative strategy to counteract the effects of IDO activity, which this species no doubt encounters in vivo (103). A recent study revealed that respiratory strains of *C. pneumoniae* possess multiple copies of the *tyrP* gene, encoding a tyrosine-tryptophan permease, while vascular strains encode only one copy (41). Since the presence of extra *tyrP* copies correlated with increased mRNA levels and higher uptake of the substrate tyrosine in respiratory strains, the authors hypothesized that a reduced capacity for amino acid transport may contribute to a greater tendency of vascular strains to become persistent in vivo (41).

CPAF. Another immunoavoidance strategy that has been identified in both *C. trachomatis* (126) and *C. pneumoniae* (32) is the secretion of a chlamydial protease-like activity factor (CPAF) into the host cell cytoplasm that cleaves eukaryotic transcription factors required for both major histocompatibility complex class I and II antigen expression. Recent rtRT-PCR (R. J. Hogan, S. A. Mathews, S. Mukhopadhyay, J. T. Summersgill, and P. Timms, unpublished data), 2-D gel (110), and immunoblot (46) analyses of IFN-γ-exposed chlamydial infections, in addition to immunoblotting of iron-depleted *C. pneumoniae* cultures (46), revealed little or no differential expression of CPAF between persistent and productive infections. These data indicated that CPAF production is important in both persistent and normal infections. Interestingly, Heuer et al. (46) demonstrated inhibited CPAF translocation to the host (HEp-2) cell cytoplasm during both IFN-γ- and iron deficiency-induced persistence of *C. pneumoniae* CWL-029. These authors reasoned that if CPAF is translocated by the same mechanism as other chlamydial proteins, and if this mechanism is inhibited during persistence, then the resultant general decrease in chlamydial antigen processing and presentation would reduce the requirement for CPAF activity during this phase (46). The absence of CPAF protease activity on host cell proteins could also reduce the availability of readily transportable amino acids (including tryptophan) to chlamydiae and therefore contribute to the maintenance of persistence.

Late genes. Down-regulated expression during persistence of genes and proteins that are specifically expressed late in the productive developmental cycle is a common observation most likely reflecting the inhibited RB-to-EB differentiation that characterizes persistence. A well-known example is the 60-kDa cysteine-rich protein (CRP), which is abundant in EB envelopes. The 60-kDa CRP was either markedly diminished or completely absent in early studies of *C. trachomatis* strains exposed to β-lactam antibiotics (23, 104), IFN-γ (7, 10), or heat shock (53). Another study reported the absence of the Hc-1 and Hc-2 DNA-binding proteins (involved in chromosomal condensation) in IFN-γ-exposed *C. trachomatis*, although interestingly, the 60-kDa CRP was not down-regulated (52) (Table 2). Similarly, both persistence microarray studies carried out to date reported down-regulated *hctB* (encoding Hc-2) expression in *C. trachomatis*, whereas *omcB* (encoding the 60-kDa CRP) down-regulation was associated with exposure to IFN-γ (13) but not to penicillin (82). As was the case for *ompA*/MOMP, many other studies have also failed to confirm *omcB*/60-kDa CRP down-regulation in persistent cultures (47, 52, 68, 114). Therefore, *hctB*/Hc-2 down-regulation currently appears to be a more reliable marker of persistence. Nonetheless, the penicillin microarray study (82) strongly supported the late gene shut-down hypothesis, since 14 of the 15 most down-regulated genes in that study were subsequently confirmed to be late genes (14, 83) (Table 2). Finally, this hypothesis was used to explain selective down-regulation of late-stage-specific type III secretion genes in an sqRT-PCR study of *C. pneumoniae* CM-1-infected HEp-2 cell cultures exposed to 40 ng (480 U) of IFN-γ/ml (114).

Microarray analysis: new persistence gene categories identified. The recent microarray study of IFN-γ-exposed *C. trachomatis* by Belland et al. (13) provided the first published transcriptome-wide data set for chlamydiae in a persistence

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system. While many of the differentially expressed genes fall into categories discussed above, other groups were identified that have not previously been considered in the context of chlamydial persistence. Many *rl* and *rs* genes (encoding ribosomal proteins) were markedly up-regulated, and none were significantly down-regulated (13). Early genes that were up-regulated in persistence included *euo* and the *incD-G* operon, although another early gene (*oppA-4*) was reported to be down-regulated (13). The *euo* result was consistent with data from *C. trachomatis* serovar L2-infected HeLa cells cultured in the absence of glucose (51), conditions known to induce persistence in this species (45). In that study, transcripts from *euo* but not from eight other genes of interest were detectable by sqRT-PCR (51) (Table 2). Other novel observations from the microarray data included up-regulation of three members of a gene family encoding phospholipase D-like enzymes and down-regulation of four *clp* and three *opp* genes, encoding proteins involved in proteolysis and peptide transport, respectively (13).

Belland et al. (13) used rtRT-PCR to clarify the magnitude of relative changes for 15 genes (Table 2). For example, three genes, *CT228*, *euo*, and *trpB*, with up-regulated microarray changes of a similar magnitude (approximately 6-fold) showed rtRT-PCR changes of 12.6-, 28.7-, and 458-fold, respectively (13). The dramatic variation in these figures emphasizes the importance of quantitative PCR in verifying microarray data, and perhaps this should include data for genes that show no significant relative change by microarray analysis.

Regulatory mechanisms in persistence. The gene expression data described in the above categories imply that many persistence gene profiles are transcriptionally regulated. Based on their microarray data, Belland et al. (13) proposed the existence of a chlamydial persistence stimulon that is more complex than a general stress response. The strongly up-regulated *euo* expression observed during persistence in that study may provide the first insight into the molecular mechanisms of regulation, since these authors proposed that Euo may contribute towards silencing late gene expression in persistence (13). The precise mechanisms of regulation for other differentially expressed gene categories remain unknown. Another unanswered question is to what degree a persistence stimulon may vary among persistence systems resulting from different induction mechanisms.

In addition, translational control may contribute to some observed profiles, at least in IFN-γ-mediated persistence. Beatty and colleagues (6) proposed that direct control at the translational level may explain their observations of steady cHSP60 levels and reduced membrane antigen levels in IFN-γ-mediated persistence. This proposal is based on gene sequence data revealing that both MOMP and the 60-kDa CRP, but not cHSP60, contain significant numbers of tryptophan residues and hence require adequate tryptophan levels for their synthesis (6). Brown and Rockey (16) also suggested this mechanism to potentially explain their observation that expression of an unidentified antigen (termed SEP) that localizes to the septum of dividing chlamydiae was severely attenuated in IFN-γ-exposed *C. trachomatis* serovar L2 but not in IFN-γ-exposed *C. psittaci* GPIC and not after exposure of either strain to ampicillin. If SEP is a peptidoglycan, the inhibition may have been due to a biosynthetic enzyme containing more tryptophan residues in *C. trachomatis* serovar L2 than are contained in *C. psittaci* GPIC (16).

CONCLUDING REMARKS

While the exact molecular mechanism by which chlamydiae enter and exit the persistent phase is not yet understood, there is little doubt that this stage plays an important role in chlamydial development. The well-accepted biphasic paradigm of chlamydial development warrants the addition of a persistent phase that represents a critical survival mechanism of this well-adapted intracellular pathogen. Down-regulated expression of the major structural protein MOMP was one of the first markers of persistence but has not been confirmed in all subsequent studies on persistence. On closer examination of these data, most *C. trachomatis* studies reported down-regulation of *ompA*/MOMP, whereas the *C. pneumoniae* studies observed up-regulation of *ompA*/MOMP. Perhaps this could be explained by different roles played by MOMP in the two species. In *C. pneumoniae*, MOMP is apparently less important structurally, whereas its role as a porin might be more critical.

Another example of a species-specific persistence marker relates to the elegant work of Caldwell et al. and Fehlner-Gardiner et al. (20, 33), who clearly showed the key role of a functional tryptophan synthase in the development of *C. trachomatis* experiencing tryptophan depletion. This is also an example of an expression pattern that relates to the key metabolic limitation in a particular system of persistence. Such patterns are presumably not a direct effect of persistence but rather the chlamydiae trying to compensate for limitations in other parts of the same or related pathways.

While most persistence patterns appear complex at this stage of our understanding of chlamydial cell biology, it is hoped they can be unraveled by the multiple molecular approaches that are currently being pursued, for example RT-PCR, proteomics, and microarrays. Since these methods complement each other, their combined use will be critical for answering biological questions about persistence. The establishment of gene and protein expression patterns representing persistence in vitro will allow the development of persistence assays to be applied to in vivo samples, resulting in the correlation of model systems to clinical reality.

An important question that remains is whether the persistence phenotype is simply a survival response by chlamydiae to a range of unfavorable environmental conditions, with the subsequent complex gene expression profiles being the uncontrolled outcome. Alternatively, have chlamydiae evolved over millions of years to deliberately enter the persistent state to maximize evasion of host cell immune mechanisms and increase growth and survival opportunities in different host niches? Indeed, can we further document the overall prevalence of this persistent state in vivo? Have different ecotypes of *Chlamydia* (serovars and strains) evolved subtly different ways of responding to environmental cues so as to enter this quiescent phase to enhance survival or transmission? As we continue to better understand the molecular and cellular mechanisms of chlamydiae, we will also begin to understand the role of persistence in this uniquely adapted obligate intracellular pathogen.

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