

MECHANISMS OF INTRACELLULAR CHLAMYDIAE SURVIVAL

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MEHANIZMI INTRACELULARNOG PREŽIVLJAVANJA HLAMIDIJA

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ABSTRACT

Chlamydiae are Gram-negative, non-motile, obligate intracellular, and spherically shaped bacteria with a diameter of 0.2-1.5 μm. Chlamydiae are present in several different morphological forms: the elementary body, the reticular body, and in the last several years, there has been the observation of a third form known as the persistent or atypical form. The intracellular localization of Chlamydia provides a unique replication cycle that occurs inside a membrane-surrounded vacuole in the host cell cytoplasm and is significantly different from the method of multiplication of other microorganisms. Chlamydiae are capable of manipulating different signalling pathways inside the infected cell, thus avoiding the host immune response. This ensures intracellular multiplication, survival, and long-term persistence of Chlamydiae. There are two basic means of achieving this persistence: inhibition of apoptosis and manipulation of NF-κB (nuclear factor kappa B)-mediated signals in the host.

Keywords: *Chlamydia, invasion, inclusion, intracellular survival, persistence*

SAŽETAK

Hlamidije su Gram-negativne, nepokretne, obligatno intracelularne bakterije, sferičnog oblika, prečnika od 0,2-1,5 μm. Pojavljuju se u više različitih morfoloških formi: elementarno telo, retikularno telo, a u poslednjih nekoliko godina uočava se postojanje i treće forme označene kao perzistentna ili atipična forma. Zahvaljujući intracelularnoj lokalizaciji, hlamidije imaju jedinstven replikativni ciklus koji se znatno razlikuje od ostalih načina umnožavanja mikroorganizama, a odvija se unutar vakuole oivičene membranom u citoplazmi ćelije domaćina. Hlamidije su unutar inficirane ćelije u stanju da manipulišu različitim signalnim putevima i da na taj način izbegavaju imunski odgovor domaćina, obezbeđujući sebi umnožavanje i dugotrajnu perzistenciju. Proučena su dva osnovna načina na koji ove bakterije to mogu: inhibicija apoptoze i manipulisane NF-κB (nuklearni faktor kapa V) posredovanim signalima.

Ključne reči: *Hlamidija, invazija, inkluzija, unutarćeljsko preživljavanje, perzistencija*



GENERAL CHARACTERISTICS AND MORPHOLOGY

Chlamydia trachomatis (CT) are strictly intracellular bacteria that target primarily cylindrical epithelial cells. These cells are found on the surfaces of the conjunctiva, urethra, endocervix, endometrium, and ovarian tube, thus explaining the localization of diseases caused by CT (1). Most Chlamydia infections remain undiagnosed because they are often asymptomatic and last for a long time. Consequently, this results in unrecognized and untreated infections that can be serious and difficult to treat (2). Bacteria within the *Chlamydiaceae* family are similar to viruses in their size and intracellular localization. On the other hand, they are considered bacteria due to some character-

istics, such as the existence of inner and outer membranes, simultaneous existence of both DNA and RNA, prokaryotic ribosomes, lipids and nucleic acids, sensitivity to many antibiotics, and capability of visualization with light microscopy (3). Chlamydiae are Gram-negative, non-motile, obligate intracellular bacteria, spherical in shape, with a diameter of 0.2-1.5 μm. The Chlamydia genome is characterized by double-helix DNA with an approximate size of 106 bp. All human serotypes of *Chlamydia trachomatis* have a common plasmid of 7.5 kbp (4), which is highly conserved and has a role in pathogenesis, thus it can be used for identification purposes (5). Chlamydiae are present in



two morphologically and functionally different forms. The metabolically inactive, infective form is called the basic or elementary body (EB), and the metabolically active, non-infectious form is called the gridded or reticular body (RB). Both represent evolutionary forms of chlamydiae adaptation to extracellular and intracellular living conditions (6). The latest research notes to existence of a third morphological form known as the persistent form.

The Chlamydia cell wall is similar to the cell walls of Gram-negative bacteria. It consists of inner and outer cytoplasmic membranes with penicillin-binding proteins but without a peptidoglycan layer in between (3). The LPS in Chlamydia can be located in the inclusion body inside the inclusion membrane, in the cytoplasm and surface of the host cell, or in the surrounding infected cells. LPS is important in the pathogenesis of chlamydial infections and in the exposure of infected cells to the host immune system (7). The major outer membrane protein (MOMP) represents a type-specific antigen that determines the chlamydial type and serotype and functions as a porin and an adhesin (8). The second major protein family is known as the polymorphic outer membrane protein (POMP) of as yet unknown biological function (7). Two cell wall proteins, OmcA and OmcB, are rich in cysteine and can interact with other proteins. Additionally, the OmcB protein participates in the adhesion process of chlamydia to a host cell in the early stages of interaction (9). The inclusion proteins, IncA, IncB, and IncC, have several functions, such as inclusion development, avoidance of lysosomal fusion, signalling of EB-RB-EB reorganization, etc. (10). Special spike-like structures on the surface of the chlamydiae elementary and reticular bodies have been observed by electron microscopy. Numerous studies note that these structures serve as channels between the host cell and parasites.

THE LIFE CYCLE OF CHLAMYDIAE

The infectious cycle starts when the EB establishes contact with the surface of the host cell. A large number of chlamydial proteins (i.e., MOMP, OmcB, PmpD, cysteine-rich proteins) function as adhesins. Adhesion occurs through glycosaminoglycans that act as “bridges” between receptors on the bacteria and receptors on the host cell (11). There are two described means of chlamydiae entry into the host cell: receptor-mediated endocytosis and microfilament-dependent phagocytosis. After infection, the EB becomes embedded inside a membrane connected to a vacuole called the inclusion. The EB differentiates into the metabolically active form called the RB that undergoes repeated cycles of binary fission and eventually secondary differentiation back to the EB form (7). Both EB and RB forms possess type III secretion systems (T3SS) that are envelope-spanning nano-machines conserved among diverse Gram-negative bacterial pathogens. T3SS translocate virulence effector proteins directly into host cells, where they subvert cellular

processes to promote bacterial entry, survival, and replication (12). Primary differentiation from the EB into the RB includes changes in the structure of the outer membrane, with the breaking of disulfide bonds between MOMP and other proteins of the outer membrane (13). The decrease in the number of disulfide bonds results in increased membrane permeability, easier transport of nutritive material, and increased metabolic activity, while simultaneously contributing to mechanic and osmotic sensitivity of the RT (14). Treatment of the EB with dithiothreitol results in a reduction of disulfide bonds, an increase in permeability and metabolic activity, and a decrease in osmolarity and infectivity (13). In contrast, the compact electronically dense nucleotide structure of the EB is preserved, which indicates that the mere reorganization of membrane structure is not enough to induce differentiation. DNA is loosely packed in the RB. In relation to this, changes in structural organization of nucleotides represent a crucial moment in the developmental cycle of Chlamydia. Decondensation of the chlamydial chromosome is the most important step in the activation of transcription and translation. The differentiation of the RB into the EB is followed by reincorporation of MOMP and other proteins of the outer membrane as the infectious cycle progresses (13). Lastly, the host cell lyses releasing EBs that infect surrounding cells (7).

THE PERSISTENT OR ALTERED DEVELOPMENTAL CYCLE of *C. trachomatis*

The term “persistent infection” represents the absence of visible development, suggesting the presence of Chlamydia in a form that is different from typical intracellular morphological forms (Table 1, Figure 1). This altered developmental cycle correlates with a decrease in metabolic activity, which limits growth and multiplication and postpones the differentiation into EB forms (13). Chlamydiae induce the secretion of interferon- γ (IFN γ), which completely inhibits bacterial development (7). Low concentrations of IFN γ induce the development of morphologically aberrant forms of chlamydia (13). The levels of chlamydial MOMP decrease with low concentrations of IFN γ resulting in the maintenance of a chronic infection and accumulation of high quantities of chlamydial heat shock protein 60 (HSP60) in infected host cells (15). The persistent forms of Chlamydia are not only morphologically atypical but also express different key chlamydial antigens. This is concurrent with a reduction in the synthesis of chlamydial MOMP and LPS and an increase in the synthesis of HSP60.

Table 1. Basic mechanisms of intracellular survival of chlamydiae

Mechanism	References
Persistence	13,15
Apoptosis inhibition	51,52,53,54,55,56,57,58
Modulation of NF- κ B signalling	59,60,61,62,63,64,65

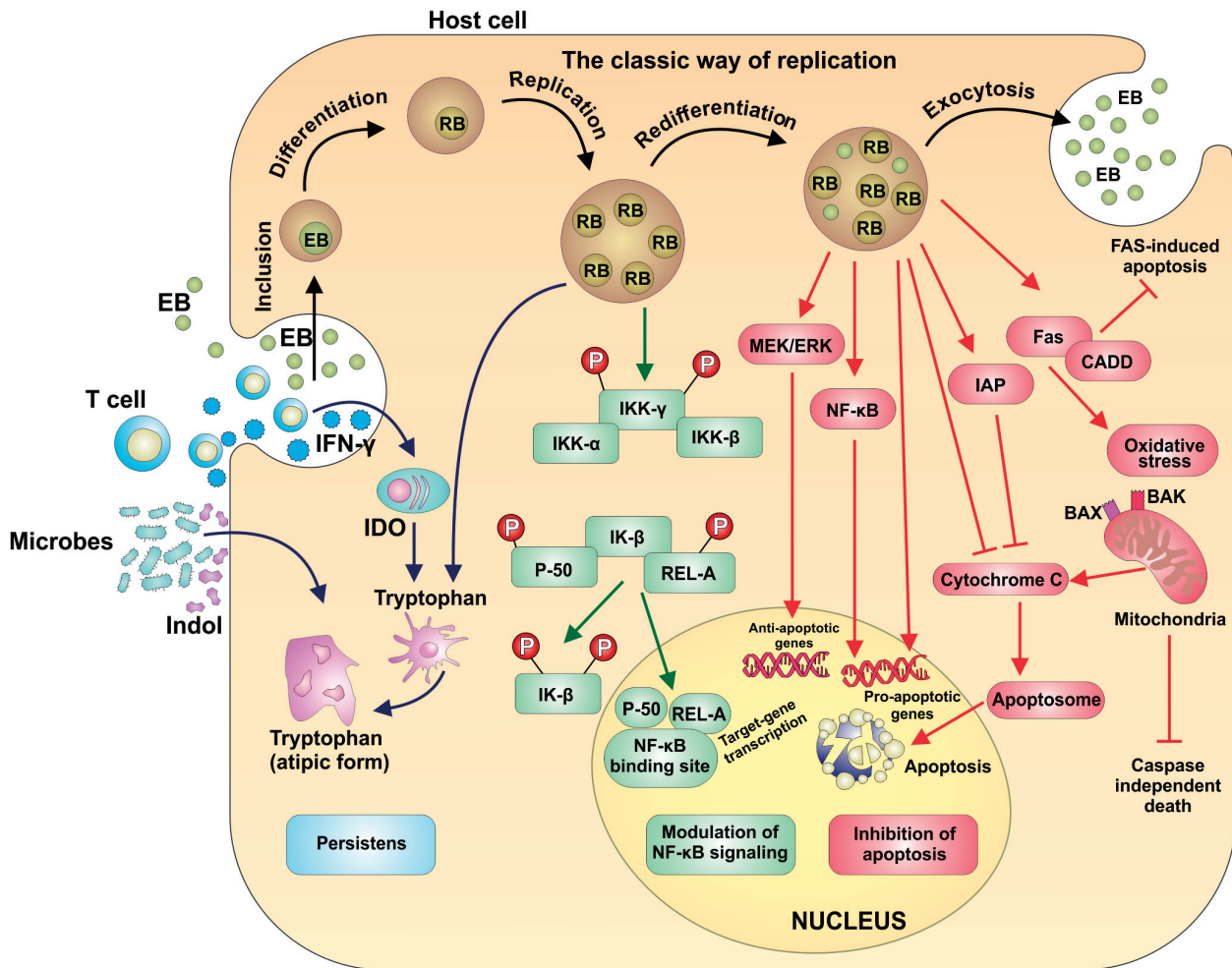


Figure 1. Developmental cycle and intracellular survival mechanisms of Chlamydia: black - classical replication pathway; blue - persistent developmental cycle; green - intracellular survival via modulation of NF-κB signalling; red - intracellular survival via inhibition of apoptosis.

MECHANISMS OF INTRACELLULAR SURVIVAL

Different bacterial adhesins and ligands mediate the invasion of many cell lines, and their mode of “contribution” depends on the type of cell and type of Chlamydia involved in the process. Well defined adhesins are glycosaminoglycan (GAG), MOMP, OmcB, and PmpD (16). The adhesion of chlamydiae is a two-step process, which entails an initial reversible interaction between the EB and the target cell via heparan sulfate proteoglycans (HSPG). Following this initial interaction there is an irreversible adhesion step via a secondary receptor of high affinity (17). Alongside heparan sulfate, mannose-6-phosphate-receptors and oestrogen receptors are other surface receptors used by chlamydiae to enter the target cells (18). A chlamydial protein, known as Tarp, translocates during entry and facilitates the invasion and differentiation of EB to RB (19, 20), while Rab GTPs act as regulators that enable the formation of the inclusion harbouring bacteria (21).

Following adhesion, chlamydiae reorganize the host cell cytoskeleton via induction and activation of the Rho family of GTPs (22). Entry of Chlamydiae into non-phagocytic cells is mediated by small GTPase-dependent reorga-

nization of the actin cytoskeleton (17, 23-26). Activation of Rac1 results in the recruitment of the actin regulators WAVE2, Abi-1, and Arp2/3, which are necessary for *C. trachomatis*-induced actin reorganization (27). Both chlamydial and host proteins may function synergistically to promote invasion. After entering the cell, the EB is located inside a membrane-surrounded vacuole known as the inclusion. The newly formed inclusion moves along microtubes to the peri-Golgi space, thus preventing fusion with lysosomes (22). A pH>6 within the inclusions indicates that there has been no fusion with lysosomes (28). This complex set of interactions between the chlamydial inclusions and cell internal pathways is important in acquiring essential nutrients, such as amino-acids, lipids and iron, while at the same time limiting the capability of recognition by the host immune system.

After entry into the host cell, the newly formed chlamydial inclusion is transported alongside microtubes to the microtubule-organization centre on a dynein-dependent or dynein-independent fashion mediated by Src kinase (29, 30). Recent studies have shown that the inclusion membrane is not homogeneous and that micro domains are made of inclusion proteins (Inc), active Src kinases, and



cholesterol combined with centrosomes and dynein (31). The inclusion forms a dynein-dependent relation with the centrosome during the cell cycle. These findings are interesting in terms of a possible connection between chlamydiae and HPV-combined cervical cancer (32). Numerous studies have shown a close relation between chlamydial inclusions and the Golgi apparatus from which bacteria take exocytotic vesicles with sphingomyelin and cholesterol via a Brefeldin A-sensitive manner (33). Sphingomyelin is necessary for growth and stability of the inclusion membrane but not for the replication process of chlamydiae (34). On the other hand, ceramide transfer protein (CERT), a cytosolic protein that transports ceramide from the endoplasmic reticulum to the trans-Golgi region, is recruited from the inclusion membrane through an interaction with the inclusion membrane protein IncD and it is involved in acquiring sphingomyelin (35). Host cell glycerophospholipids, such as phosphatidylinositol and phosphatidylcholine, are also taken over in the process, which entails the activation of phospholipase A2 located in the cytosol (36). In addition to the Golgi apparatus, the inclusion interacts with other cellular organelles, such as the multivesicular bodies, which are also a significant source of sphingolipids and cholesterol, as well as lipid droplets that translocate into the inclusion lumen and serve as a source of neutral lipids (37-39). The entire set of chlamydial proteins show tropism towards lipid droplets, which are lipid storage organelles. Lipid droplets pass through the inclusion membrane and are in close connection with the chlamydia RB. These interactions facilitate nutrient acquisition necessary for replication of bacterial cells, as well as expansion and stability of the inclusion membrane (31). The metabolically inert EB undergoes morphological changes and is reorganized into the RB (23) while the disulfide bonds between the MOMP and other outer membrane proteins break apart. Chromatin is released from a condensed structure, and transcription becomes more intense. The RB becomes metabolically active and divides via binary division inside host cell endosomes. Following a growth and division period, the RB is once again reorganized into the EB. The differentiation of the RB into the EB is associated with the reincorporation of MOMP and other outer membrane proteins as the infectious cycle progresses. All intracellular pathogens must eventually exit the host cell (40, 41). *C. trachomatis* has evolved at least two, possibly three, distinct mechanisms of host-cell egress, lysis, and extrusion (42), in addition to a non-lytic exocytosis-like mechanism (43). The extrusion mechanism is believed to be dependent on actin polymerization, N-WASP, Rho GTPase, and myosin II as determined by the use of specific inhibitors for each of their activities (42). The recruitment of the actin coat to the inclusion prior to extrusion appears to be a sporadic and dynamic event relying on a combination of both bacterial and host factors (43).

Cells of the innate immune system express receptors important for the recognition of microorganisms. Some of these receptors are located on the cell surface, while oth-

ers are located in the cytoplasm or endoplasmic reticulum. As with most bacteria, chlamydia infections are detected by host pattern recognition receptors (PRRs) that recognize chlamydial LPS via Toll-like receptor 4 (TLR4) (44-47) and Hsp60 via TLR2 and TLR4 (48). Signals from TLRs, which are specific for different bacterial antigens (49), enable the production of cytokines and enzymes involved in a variety of antimicrobial functions. Chlamydiae are capable of manipulating these signalling pathways and prevent the initiation of the innate immune response (50). There are two primary ways of achieving prevention: inhibition of apoptosis and manipulation of NF- κ B-mediated signals (Table 1).

The effects of Chlamydiae on the apoptotic signalling programme are complex (Figure 1). Chlamydiae inhibit apoptosis primarily via the inhibition of mitochondrial cytochrome C releasing, thus preventing early death of the host cell (51, 52). The Bcl-2 family of proteins regulates the release of mitochondrial cytochrome C. Chlamydiae induce the degradation of BH3-only Bcl-2 family proteins. (52). However, the cleavage of BH3-only proteins in cell lines engineered to express active recombinant CPAF occurs with different kinetics from canonical substrates and is prevented by the proteasome-specific inhibitor MG-132. This suggests that degradation of BH3-only proteins occurs via a proteasome-dependent mechanism indirectly influenced by CPAF (53). Although its anti-apoptotic role is unclear, CPAF is considered a central immune regulatory protein. Other potential anti-apoptotic mechanisms include the stabilization of inhibitor of apoptosis (IAP) proteins (54) and the sequestration of pro-apoptotic phosphorylated BAD and protein kinase C δ (PKC δ) at the chlamydial inclusion (55, 56). The increased expression of the anti-apoptotic protein Mcl-1 in infected cells has also been linked to the activation of Raf/MEK/ERK (57), a signalling cascade that affects inflammatory responses (58).

The interference with NF- κ B signalling is crucial in the modulation of the host immunity by chlamydial (59, 60). The NF- κ B subunits RelA (p65) and p50 form a heterodimer complex that translocates into the nucleus and acts as a transcription activator (Figure 1). During chlamydial infection, proteolysis of RelA occurs with the participation of *Chlamydia trachomatis* Tsp-like protease (Ct441), thus blocking translocation of NF- κ B (60, 61). Chlamydia may also block NF- κ B activation by regulating ubiquitin-mediated protein degradation. Nuclear translocation depends on the degradation of the inhibitor I κ B α via ubiquitin-mediated proteolysis during the canonical NF- κ B activation pathway (62). Ectopically expressed ChlaDub1 binds to I κ B α and inhibits its ubiquitination. This in turn suppresses degradation of I κ B α and subsequent activation of NF- κ B (63). Although CPAF is an extensively characterized protease with numerous potential substrates relevant to innate immunity (64), a recent report suggests that several proteins are targeted by CPAF, including the NF- κ B p65/RelA subunit, RFX5, Bim, and Puma (discussed below), all of which may not be bona fide CPAF substrates in vivo settings (65).



CONCLUSIONS

Taken together, there is still much to learn from using a combination of structural, cellular, and molecular approaches to study the critical early interactions between *C. trachomatis* and host cells. Although the developmental cycle of chlamydiae is well studied, the signals that start the conversion of EB into RB and vice versa are still unknown. The biology and means of intracellular survival of chlamydiae are still not completely understood, thus there is a need for all methods of research to understand how these intracellular bacteria survive extremely well within an infected cell. The exact mechanism of control and regulation of chlamydial intracellular development is still unknown and therefore, should be a focus of present and future studies.

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