Université de Montréal

Caractérisation des infections à *Chlamydia trachomatis* persistantes induites par l'action des antibiotiques

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Thèse présentée à la Faculté des études supérieures

en vue de l'obtention du grade de

Philosophiæ Doctor (Ph.D.)

en microbiologie et immunologie

Septembre, 2005

Université de Montréal
Faculté des études supérieures

Cette thèse intitulée :

« **Caractérisation des infections à *C. trachomatis* persistantes induites par l'action des antibiotiques** »

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RÉSUMÉ

Chlamydia trachomatis est la bactérie intracellulaire obligatoire de cellules épithéliales humaines responsables des maladies susceptibles de devenir chroniques. Lesdites maladies occasionnent les lésions tissulaires importantes. La cicatrisation caractéristique résultante conduit au dysfonctionnement tissulaire, d'où les séquelles permanentes engendrées: cécité, infertilité tubaire, grossesses ectopiques. Le développement de telles séquelles est imputé à la réponse immunitaire auto-pathologique chronique induite et nécessite que les individus soient plusieurs fois réinfectés ou que l'infection initiale souvent asymptomatique demeurent non résolue. Chez certains patients, malgré le traitement avec des antibiotiques appropriés, l'infection même aiguë, est demeurée non résolue. Le fait que *C. trachomatis* soit susceptible de développer une forme de latence, dite de persistance, permet de suggérer que cette forme puisse être impliquée dans le développement de maladies chroniques avec de réponses immunitaires délétères.

Ainsi, utilisant un modèle d'infection *in vitro*, des cellules cervicales épithéliales HeLa, l'efficacité de trois antibiotiques habituellement utilisées dans la thérapie des infections chlamydiennes a été testée. Il en résulte que la doxycycline, la tétracycline et l'érythromycine, bien qu'utilisées aux concentrations minimales bactéricides pendant 24 jours, ne résolvent pas l'infection à *C. trachomatis* serovar L2. L'infection semble résolue en apparence seulement: ni les inclusions typiques, ni les particules infectieuses ne sont détectées après 10 jours de traitement. Cependant le test de viabilité par RT-PCR, ciblant les trancripts d'ARNr 16S mature ou non mature, puis les ARNm du gène *Omp1* spécifique à *Chlamydia*, révèle la présence continue de la bactérie viable après 24 jours de traitement. À la différence de ce qui est observé dans les cellules HeLa, l'infection de cellules inflammatoires, les lignées de cellules monocytiques THP-1 et U-937, est résolue par le traitement à la doxycycline. Ceci suggère que l'action combinée de la doxycycline et des réactions de défense probablement plus intenses dans les cellules monocytiques (THP-1 et U-937) que dans les cellules épithéliales (HeLa), est requise pour résoudre l'infection chlamydienne. Ces résultats suggèrent que les cellules épithéliales pourraient constituer un réservoir de particules chlamydiennes persistantes, malgré l'antibiothérapie appropriée.

Les infections à *C. trachomatis* sont souvent accompagnées d'une inflammation chronique délétère. Comme les cytokines pro-inflammatoires sont requises dans la promotion de l'inflammation, les profils d'expression différentielle de certaines d'entre elles, l'IL-1β, IL-6, IL-8, IL-10, IL-12p70 et le TNF-α, selon que l'infection est productive ou persistante, ont été étudiés. Pour ce faire, 3 modèles d'infection *in vitro* ont été utilisés : les cellules HeLa, les cellules THP-1, les cellules HeLa et THP-1 en co-culture. Les différentes cytokines libérées dans le surnageant cellulaire ont été mesurées par le test de cytométrie en flux multiplexe. Les résultats montrent qu'en général toutes ces cytokines exhibent une expression accrue à la suite de l'infection chlamydienne, cependant avec le temps, cette synthèse accrue s'estompe. Quelques exceptions demeurent: l'IL-6 et l'IL-8 ont exhibé une expression accrue et continue dans le modèle de co-culture HeLa/THP-1 durant les 18 jours d'infection. Par contre, lorsque l'infection persistante est induite à la suite du traitement à la doxycycline, seulement l'IL-8 demeure exprimée de manière accrue et continue. Vu son expression accrue et soutenue durant l'infection persistante, l'IL-8 pourrait jouer un rôle, à déterminer, dans la pathogenèse des infections chlamydiennes chroniques.

MOTS-CLÉS:

Chlamydia trachomatis, Cytokines pro-inflammatoires, Doxycycline, Échec thérapeutique, Érythromycine, Infection chronique, Infection persistante, Inflammation chronique, Tétracycline.

SUMMARY

Chlamydia trachomatis is an obligate intracellular bacterium infecting human epithelial cells and leading to diseases that are liable to become chronic. The aforesaid diseases cause important tissue lesions. The resulting typical scar leads to tissue dysfunction that give rise to permanent sequelae such as preventable blindness, tubal infertility and ectopic pregnancy. Development of these sequelae is attributed to an induced chronic auto-pathological immune response and requires that individuals would be re-infected several times or that the asymptomatic initial infection remains unresolved. Some people with acute chlamydial infection remain infected despite treatment with suitable antibiotics. Since *C. trachomatis* is liable to acquire latency or persistence, we have suggested that this persistent form may play a part in the development of chronic diseases with deleterious immune responses.

Thus, to test the effectiveness of 3 antibiotics usually used in treatment of chlamydial infections, cervical epitheloid HeLa cells have been used as *in vitro* model of infection. result of this study shows that doxycycline, tetracycline and erythromycin are unable to resolve *C. trachomtis* serovar L2 infection, although they are used at the minimal bactericidal concentration for 24 days. Infection seems resolved outwardly: neither chlamydial inclusions, nor infectious particles are found 10 days after treatment. However, viability test by RT-PCR targeting unprocessed and processed 16S rRNA transcripts, and *Omp1* gene mRNA revealed that viable *C. trachomatis* continuously remained in culture, even after 24-days treatment. Unlike results found in HeLa cells, *C. trachomatis* serovar L2 infections in monocytic THP-1 and U-937 cells lines are resolved by treatment with doxycycline. This suggests that the combined action of doxycycline and defence reactions that are probably higher in inflammatory cells (THP-1 and U-937) than in epitheloid cells (HeLa) is required to resolve chlamydial infection. These results showed that epithelial cells could be a reservoir for persistent chlamydial particle despite suitable therapy.

Diseases due to *C. trachomatis* are often accompanied by deleterious chronic inflammation and cytokines can play a part in the promotion of this inflammation. Thus, IL-1β, Il-6, IL-8, IL-10, IL-12p70 and TNF-α expression profiles in chlamydial productive infection have been

compared with those in persistent infection. To do this, 3 *in vitro* infection models have been used: HeLa cells, THP-1 cells and co-cultured HeLa/THP-1 cells. Cytokines released in cell media have been measured using a cytometry bead array multiplexed assay. Results showed that all cytokines tested have increased expression following chlamydial infection. However, this increased expression falls with time. 2 exceptions are observed in the co-cultured HeLa/THP-1 model of infection: IL-6 and IL-8 exhibited continuous increased expression over 18 days of infection. On the other hand, when persistent infection was induced following treatment with doxycycline, only IL-8 remained continuously expressed with high intensity. This result suggests that IL-8 can play a role, to be determined, in the pathogenesis of chlamydial persistent infection.

KEY WORDS:

Chlamydia trachomatis, Chronic infection, Chronic inflammation, Doxycycline, Erythromycin, Persistent infection, Pro-inflammatory cytokines, Therapeutic failure, Tetracyclin.

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LISTE DES ABBRÉVIATIONS

ADCC:	Cytotoxycité dependent des anticorps
ADN ou DNA:	Acide désoxyribonucléique
APC:	"Antigen presenting cell"
ARN ou RNA:	Acide ribonucléique
ATCC:	"American Type Culture Collection"
ATP:	Adenosine triphosphate
CADD:	"*Chlamydia* protein associated with death domains"
CDC:	"Center for diseases control"
CHANCE :	**Ch**irurgie, **a**ntibiotique, **n**ettoyage facial, **e**nvironnement
CHO:	"Chineese hamster ovary"
CHUM:	Centre hospitalier de l'université de Montréal
CPAF:	"*Chlamydia* protease/proteasome-like activity factor"
CRP:	"Cysteine-rich protein"
Ctc:	"*Chlamydia* 2-component"
CTGF:	"Connective tissue growth factor"
DAG:	Diacylglycérol
DC:	"Dentritic cell"
DNase:	Désoxynucléase
dNTP:	Désoxynucléoside triphosphate
Dox:	Doxycycline
DTH:	"Delayed type hypersentivity"
DTNB:	"5,5'-dithiobis(2-nitrobenzoic acid)"
EB ou CE:	"Elemetary body" ou corps élémentaire
EBV:	Virus Epstein-Barr
Ery:	Érythromycine
GAG:	Glucosaminoglycane
GM-CSF:	"granulocyte macrophages- colony stimulating factor"
Hsp:	"Heat shock protein"
ICE:	Enzyme convertissant l'IL-1β

IDO:	Indoléamine dioxygénase
IFN-γ:	Interféron-γ
IFU ou UFI:	"Inclusion forming-units"
IL-:	Interleukin-
Inc:	Inclusion
LCR:	"Ligase chain reaction"
LPS:	Lipopolysaccharide
LVG:	lymphogranulome vénérienne
MOI:	Multiplicity of infection
MBC:	"Minimal bactericidal concentration"
MEC:	2,4-méthylérythritol 2,4-cyclodiphosphate
MEM/H:	Minimal essential medium avec les sels de Hank
MEP:	Méthylérythritol phosphate
MHC:	"Major Histocompability complex"
MIC:	"Minimal inhibitory concentration"
MOMP:	"Major outer membrane protein"
NF-κB :	"Nuclear factor-κB"
NK:	"natural killer cell"
NO:	"nitric oxide"
NOS :	"Nitric oxide synthase"
OMS :	Organisation mondiale de la santé
ORF:	"Open reading frame"
PBS:	"phosphate-buffered saline"
PDGF:	"Platelet derived growth factor"
PG:	Peptidoglycane
PID:	"Pelvic inflammatory diseases"
PIP_2:	Phosphatidylinositol 4,5 biphosphate
PKC:	Protein kinase C
Pmp or Pomp:	"Polymorphic outer membrane protein"
PrmC:	"Protein release factor methylation C"
RB ou CR:	"Reticulate body" ou corps reticulé

RNase:	Ribonucléase
rpm :	révolution par minute
RT-PCR:	"Reverse transcription-polymerase chain reaction"
SAFE:	"**S**urgery, **a**ntibiotic, **f**ace washing, **e**nvironmental improvement"
SEP:	Septum
SFB:	Serum foetal bovin
SOCS:	"Suppressor of cytokine signalling"
SPG:	"Sucrose-phosphate-glutamic acid buffer"
TACE:	"TNF converting enzyme"
Tarp:	"Translocated actin-recruting protein"
Tet:	Tétracycline
TGF-α	"Transforming growth factor-α"
Th:	"T helper"
TLR:	"Toll like receptor"
TNFR:	"Tumor necrosis factor receptor"
TNF-α:	"Tumor necrosis factor-α"
TTSS:	"Type III secretion system"
UDP:	Uridine diphosphate

DÉDICACE

À la mémoire de mon grand-père, da Ovourouga.

REMERCIEMENTS

Je désire tout d'abord exprimer ma gratitude à l'égard du Dr Madeleine Ravaorinoro, ma directrice de thèse qui a bien voulu diriger cette étude avec beaucoup de tact. Durant ces 4 années, Madeleine n'a pas fait que me guider, elle a aussi su susciter en moi différentes qualités ayant accrue mes capacités à mener à bien un projet de recherche. Je suis désormais dotée d'une rigueur et d'une curiosité scientifiques, mes connaissances et mes compétences sont maintenant plus accrues. Madeleine, je te suis énormément reconnaissante pour ta disponibilité et ton enseignement de qualité. À chaque fois que j'ai été confrontée à un problème, tu as toujours été présente pour répondre à mes questions et à mes inquiétudes. Merci!

Je tiens également à remercier le Programme Canadien des bourses de la Francophonie (PCBF) qui a assumé tous mes frais de subsistance et d'études doctorales. Je précise mes remerciements à Mr Yves Galipeau, Coordonnateur du PCBF, et à madame Colette Gagnon, conseillère pédagogique, qui en plus d'assurer convenablement leurs taches au sein du PCBF, ont su donner de leur temps pour m'écouter.

Mes remerciements s'adressent également à mes amis (es) ci-dessous à qui j'ai assailli de questions: Mohamadi kaouass, Mohamed Laakel (laboratoire du Dr Danuta Balicki), Sonia Deschênes et Hélène Brodeur (Laboratoire du Dr Pangala Bhat), Annie Chamberland et Geneviève Bélanger (laboratoire du Dr Cécile Tremblay) Jill Héneault (laboratoire du Dr Yves Raymond), Subadjini Selliah, Omar El Ared et Cathérine Carrier (laboratoire du Dr Madeleine Ravaoarinoro). Merci aussi à mon amie Sandra Akouamba (laboratoire du Dr Hugos Sudens) avec laquelle j'ai passé les nuits à discuter de mon examen pré-doctoral.

Je remercie les membres de mon comité de suivi et de ce présent jury, Dr Ali Ahmad, Dr Michel Desjardins, Dr Éric Frost et Dr Georges Szatmari, pour s'être intéressés à mon projet de recherche et d'avoir pris le temps de lire ma thèse.

Je remercie ma sœur Owanga Wilfried, ma fille Kounessoussa Carine et mon fils Epenit-Banas Wayne-P qui, ici à Montréal, ont vécu avec moi mes joies et mes peines.
Je profite de l'occasion pour renouveler mon affection pour mes deux frères, Ambroise Banas Germain et Jean-Pierre Kenké. Leurs encouragements constants ont été pour moi une source de force et d'énergie m'ayant permis d'affronter les difficultés quotidiennes et de mener jusqu'à la fin mes études.

Je remercie Mr Mondjo Epenit Mesmin de m'avoir encourager à entreprendre les études doctorales. Merci également à mon ami de coeur Mehdi Hermann Gaëtan Mvou-Kouma de m'avoir soutenu lors de la rédaction de cette thèse.
A ma mère, Véronique Kampalari, et à mon père, Camille Étougui, je réitère mon merci. Sans eux, je ne serais pas.

Je nomme les différents membres de ma famille ci-dessous pour le bien qu'ils ont pu m'apporter: Adjandji Marie-Pierrette, Atimbou Blanche, Ayaya Philomène, Benigui Monique, Boumas Marcel, Kassanga Josephine, Kayeghe Yvonne, Mme Kenké née Minko Judith, Lamboumi Élise, Mpiga Germaine, Ndjobolo Hortense, Ndjouba Rigobert, Ngabina Jacques, Ngouadzia Yvette, Obili Steeward, Olayi Sylvie, Ondjani Arlette, Ontoula Antoine, Otaly Émilie, Ontagha Bruno Bernard, Ntsiégué Claudine, Worogoyo Françoise, Yemayira Antoinette.

Merci à tous et à toutes!

PREMIÈRE PARTIE:
INTRODUCTION GÉNÉRALE ET REVUE DE LA LITTÉRATURE

INTRODUCTION GÉNÉRALE

Les infections causées par la bactérie intracellulaire obligatoire *Chlamydia trachomatis* sont en général asymptomatiques et peuvent être résolues spontanément, uniquement grâce à la réponse de défense générée (Morre et *al.*, 2002). Cependant, chez certaines personnes, les infections multiples et les infections initiales non résolues peuvent entraîner la réponse immunitaire spécifique et une inflammation chronique occasionnant les dommages tissulaires sévères (Joyner et *al.*, 2002). Les grossesses ectopiques, l'infertilité tubaire, la cécité curable sont quelques exemples de séquelles résultantes. Les mécanismes par lesquels les infections initiales aiguës deviennent chroniques sont peut-être multiples, mais sont encore indéterminés. Chez certains individus chroniquement atteints, *C. trachomatis* demeure difficile à cultiver alors que son ADN est révélé par PCR, suggérant la présence de l'organisme sous une forme non infectieuse (Grayston et Wang, 1975; Brunham *et al.,* 1985). De plus, malgré la thérapie avec les antibiotiques appropriés (doxycycline, tétracycline, érythromycine, azithromycine), dans certains cas, l'infection chlamydienne demeure toujours présente, quand les réinfections sont exclues par l'adoption de rapports sexuels protégés (Whittington et *al.*, 2001). Différents travaux réalisés *in vitro* ont permis de montrer qu'en présence de conditions non propices, *C. trachomatis* pouvait développer une forme latente ou persistante difficile à résoudre par les antibiotiques (Beatty et *al.*, 1994). Ces différents constats ont permis de poser l'hypothèse selon les antibiotiques habituellement utilisés dans la thérapie des infections à *C. trachomatis* pourraient induire la forme persistante à la place de résoudre l'infection.

Le but de ce projet de recherche est d'associer les échecs thérapeutiques rapportés dans la littérature à la persistance chlamydienne et de déterminer les conséquences inflammatoires résultantes

Les objectifs sont alors les suivants :

1- Démontrer si le traitement des infections chlamydiennes *in vitro* par des antibiotiques habituellement utilisés dans la thérapie des infections chlamydiennes conduit à l'infection persistante à la place de la résoudre

2- Caractériser les réponses de cytokines pro-inflammatoires induites selon que l'infection est productive ou persistante, dans de modèles d'infection *in vitro*

Ce travail est subdivisé en 3 parties. La première partie est une revue de littérature décrivant la biologie chlamydienne, les pathologies occasionnées et les réponses immunitaires induites. La deuxième partie décrit: les effets de la doxycycline, la tétracycline et l'érythromycine sur l'infection chlamydiennes dans deux modèles de cultures *in vitro*; les cytokines inflammatoires induites selon que l'agent pathogène soit métaboliquement actif ou latent. Finalement, la troisième partie inclut la discussion générale pour une synthèse de l'étude.

CHAPITRE I: ARTICLE 1

CHLAMYDIA TRACHOMATIS: FROM ATTACHMENT TO INFECTION

Chlamydia trachomatis: from attachment to infection

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Submitted to

Journal of Medical Microbiology

1.1 SUMMARY

Chlamydia trachomatis is an obligate intracellular bacterium with a growth cycle that occurs in several stages. The infectious chlamydial particle, or elementary body (EB), enters a susceptible cell and remains in a vacuole without fusing to endocytic organelles. It then converts to a growth form, or reticulate body (RB), whose role is to multiply by binary fission. The generated RBs undergo maturation to EBs, which are released from the cell to initiate new rounds of infection in neighbouring cells. Most stages of its developmental cycle have been established, but corresponding molecular details need to be determined. In this review, we report and discuss recent knowledge in molecular biology on the growth cycle of *C. trachomatis* whose cycle complexity is already noted at the cell entry level, which involves enzymatic processes and requires several molecules or molecular domains as acceptors and receptors. It is suspected that Inc inclusion proteins can contribute to escape from phagolysosomal fusion, and specific isomerases and proteases can play a part in differentiation process. Finally, 3 transcription factors and a 2-component regulation system have been identified, but their role in transcription regulation of the chlamydial cycle remains to be elucidated.

Key words: *Chlamydia trachomatis* biology; elementary body; reticulate body; developmental cycle; inclusion.

1.2 RÉSUMÉ

Chlamydia trachomatis est une bactérie intracellulaire obligatoire dont le cycle de reproduction se produit en plusieurs étapes. La particule infectieuse chlamydienne, le corps élémentaire, pénètre dans la cellule-hôte et y demeure dans une vacuole ne fusionnant pas avec les organelles endocytiques. Il s'y différencie en forme de reproduction, le corps réticulé, dont le rôle est de se reproduire par fission binaire. Les corps réticulés engendrés se dédifférencieront en corps élémentaires qui seront expulsés de la cellule-hôte afin d'initier de nouveaux cycles d'infection. Si l'ensemble des étapes de ce cycle de reproduction est établi, il reste cependant à déterminer les détails moléculaires correspondants. Dans cette revue, nous relatons et discutons des dernières connaissances moléculaires de la biologie chlamydienne. La complexité du cycle s'observe déjà au niveau de la pénétration qui, en plus d'être un processus enzymatique, requiert plusieurs molécules ou domaines moléculaires accepteurs et récepteurs. On soupçonne que les protéines des inclusions Inc puissent contribuer à l'échappement à la fusion lysosomale, et les isomérases et protéases spécifiques à la différenciation. Finalement, trois facteurs de transcription et un système de régulation à deux composantes ont été identifiés, mais leur rôle dans la régulation transcriptionnelle du cycle chlamydien demeure à déterminer.

Mots-clés: Biologie chlamydienne, *Chlamydia trachomatis*, corps élémentaire; corps réticulé; cycle de développement; inclusion.

1.3 INTRODUCTION

Chlamydia trachomatis is a bacterium responsible for a broad spectrum of pathologies. Even though *C. trachomatis* is susceptible to many antibiotics [1-3], it remains the leading cause of sexually-transmitted diseases and the second leading cause of blindness worldwide [4, 5]. This Gram-negative prokaryote, belonging to the chlamydiaceae family, is described as an atypical obligate intracellular parasite with no peptidoglycan (PG) in wall. Although the eubacterial origin of *C. trachomatis* has been established, its precise phylum still remains to be determined. It is possible that the chlamydiaceae have a common ancestor with chloroplasts and cyanobacteria [6]. It is also suggested that *C. trachomatis* has undergone reductive evolution. According to this assumption, protochlamydia must have lost genes during its evolution as it adapted to different intracellular niches, which would explain why the *C. trachomatis* species is made up of many serovars, A to L3, differing in pathogenicity and tropism [7].

Several decades after its discovery, *C. trachomatis* still remains a mystery. Nevertheless, recent sequencing of the complete chlamydial genome has allowed us to learn a little more about this organism. *C. trachomatis* is a bacterium with one of the smallest genomes, roughly 1,042, 519 pb, that is, approximately 894 genes, of which 28% still remain to be assigned [8]. This genome sequence also reveals various chlamydial metabolic pathways, but several among them are interrupted, explaining why *C. trachomatis* has limited anabolic capacities, and, hence, its energy- and metabolite-dependence on the host. This dependence would partly justify strict chlamydial parasitism. However, despite chlamydial genome revelations, little is known about various aspects of chlamydial biology, specifically gene regulation and interactions with the host.

C. trachomatis is characterized by a unique developmental cycle during which the extracellular stage alternates with the intracellular stage (Figure 1). The first stage is achieved by particles known as elementary bodies (EBs) that are of small size (0.3 μm of diameter), metabolically inactive and specialized in cell infection. To initiate the

developmental cycle, EBs must penetrate cells without specialized endocytosis. As the resulting phagosome does not fuse with the lysosome, the EBs can survive and differentiate into reticulate bodies (RBs) of 0.5-1.3 µm, which ensure growth and are less rigid, osmotically fragile and metabolically active. The RBs undergo multiple divisions by binary fission, leading to enlarged phagosomes called inclusions (Figure 2). At the end of growth, RBs are differentiated back into EBs for release from the cell to perpetuate the developmental cycle [9]. Reproductive cycle events are globally established, but the precise mechanisms of each cycle step remain to be determined. The objective of this paper is to update *C. trachomatis* biology, especially its structure and reproductive cycle.

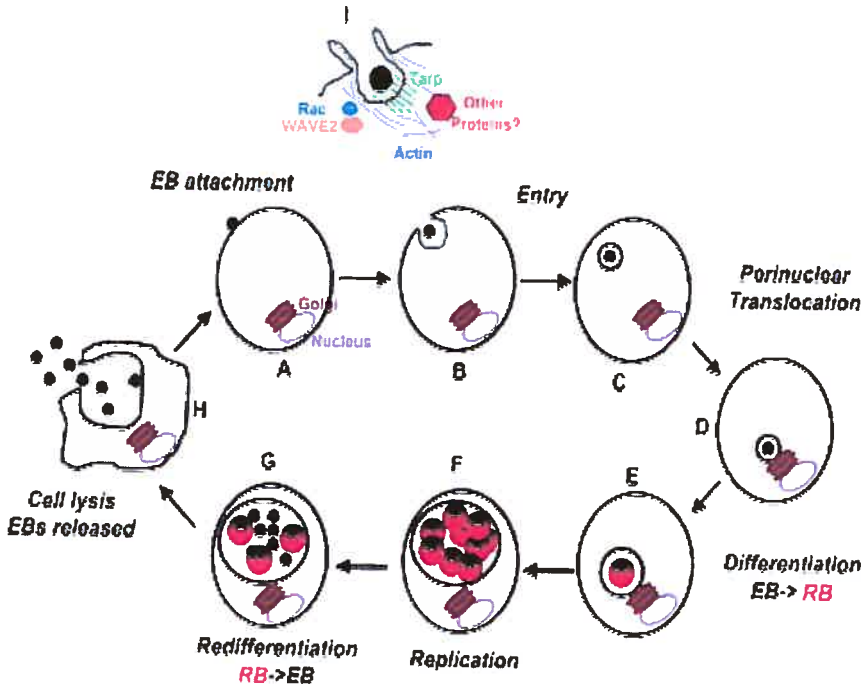

Figure 1. *Chlamydia trachomatis* life cycle. Interaction of elementary body with host cells plasma membrane lead to Rho GTPase member family Rac recruitment and the component of Arp2/3 complex, WAVE2. With phosphorylated recruited Tarp, these proteins allow actin recruitment at the site of attachment. Actin remodeling results in distinct microvillar reorganization throughout the host cell surface and the formation of pedestal-like structure and entry. EB, elementary body; RB, reticulated body; Tarp, translocated actin-recruting protein. **Adapted from J. Engel. 2004. Proc. Natl. Acad. Sci. U S A. 101, 9947-8 [10].**

1.4 EBs versus RBs

EBs are distinguished from RBs not only by their compacted nucleoid, but also by their rigid wall, conferring osmotic stability. As EBs are specialized in cell infection, it is important to study their wall ultrastructure to understand interactions that allow attachment and penetration. Despite the presence of all genes required for peptidoglycan (PG) synthesis in the chlamydial genome, the *C. trachomatis* wall does not have a fine PG layer that is specific for Gram-negative bacteria [8]. Instead of PG, there is a supramolecular structure made up of a trimeric major outer membrane protein (MOMP, 39.5 kDa), a large cystein-rich protein (CRP, 60/62 kDa) and a small CRP (12 kDa). This structure, within MOMP and CRPs, is highly disulfide cross-linked and confers EB wall rigidity [10]. In RBs, the wall possesses monomeric MOMP, but loses large and small CRPs; thus, RBs are osmotically fragile [12].

Several studies have attempted to clarify the ultrastructure of these parietal proteins. A topological model of the *C. trachomatis* MoPn MOMP has been built. According to this model, MOMP is made up of 5 constant domains separated by 4 variable antigenic domains. MOMP folds up 16 times so that its constant domains form transmembrane β-strands and short periplasmic loops, while its variable domains form long loops exposed at the cell surface. According to the authors, this model is in agreement with the porin function of MOMP [13, 14]. When MOMP is in the outer membrane, CRPs are at the inner surface of the outer membrane. A small CRP is proposed to be a periplasmic lipoprotein that, like Braun lipoprotein, connects the inner large CRP molecules to the outer membrane through disulfide bonds [15]. *C. trachomatis*, like other Gram-negative bacteria, has lipopolysaccharides (LPS) in its wall, but these LPS are truncated, terminating by 3-deoxy-D-mannose-octulosnic acid [16]. In addition to these wall compounds, examination of the completed chlamydial genome has led to prediction of the family of genes coding for polymorphic outer membrane proteins, called pomps or pmps, of 90-180 kDa. In *C. trachomatis* species, 9 genes have been identified (*pmpA* to *pmpI*), whereas *C. psittaci* has 6 (2 families: pomp90 and pomp98) and *C. pneumoniae*, 21 (pomp1 to pomp21). The corresponding coding proteins differ in their amino acid sequences, but are classified only according to their 2 polypeptide signatures (GGA (L,

V, I) and FXXN) which are repeated several times in an alternating fashion. Henderson and Lam [17] suggest that these proteins belong to the autotransporter family. Pmps B, F and H have so far been detected only in EBs, while Pmps C, D E, G and I have been found in both EBs and RBs. In contrast, PmpA has been detected only in RBs [18, 19]. Much work is needed to elucidate Pmps function.

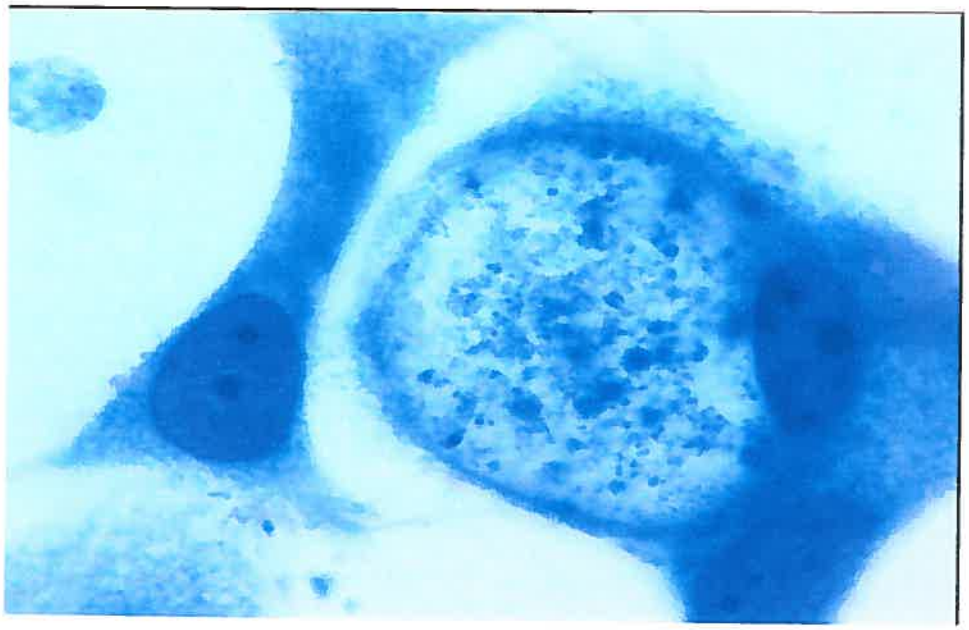

Figure 2. Optical micrography (100x) of *Chlamydia trachomatis* serovar L2 inclusion in HeLa cell. Infected HeLa cells were stained by the May-Grünwald-Giemsa method. Each point in inclusison is either elementary body, or reticulated body.

1.4 REPRODUCTIVE CYCLE

1.4.1 Attachment and penetration

Several chlamydial adhesins, such as MOMP, large CRP and 70 kDa heat shock protein (Hsp70), are considered to be ligands. However, only a cellular compound, heparan sulfated-like glucosaminoglycans (GAG), was studied as a cellular receptor during chlamydial entry. It is now known that these different compounds are not high affinity ligands or receptors. Possibly, a given serotype has several ways of penetrating, since inhibition of specific interactions between the cell and the EB surface confers only partial protection.

Carabeo and Hackstad [20] showed that attachment is a specific process that occurs in 2 steps. The initial step consists of electrostatic and reversible interactions that would allow intimate proximity of the EB and host cell. This proximity would promote the second step during which high-affinity ligand and receptor form specific and irreversible bonds. Actually, it is found that Chinese hamster ovary cells (CHO) deficient in GAG expression (mutant pgsA-745) remain resistant to infection by L2 serovar as well as MoPn serovar, whereas CHO expressing GAGs, but deficient in other receptors (D4.1-3 mutant) remain resistant to the L2 serovar, but not to MoPn serovar. This means that both serovars use GAGs as the primary receptor as well as 2 distinct but unknown secondary receptors.

The high affinity chlamydial ligand may not be exposed on the EB surface and must become accessible only after reduction of the cysteine-rich outer membrane protein lattice. This possibility was suggested as it was noted that EB penetration but not attachment was compromised by 5,5'-dithiobis(2-nitrobenzoic acid)(DTNB) [21]. DTNB is an impermeable reagent that covalently modifies surface sulfhydryls to prevent disulfide bond cleavage. Thus, penetration could be an enzymatic process requiring isomerases, enzymes taking part in reduction of the disulfide bonds. The importance of isomerases in chlamydial penetration has been proved with anti-cellular disulfide isomerase antibodies to reduce HEC-1B cell infection by *C. trachomatis* serovar E. This

55-kDa cellular isomerase was normally found to act in liaison with cellular Hsp70 and other additional proteins of 45, 48 and 90 kDa, activating estrogen receptors. Thus, Davis *et al.* [22] suggested that during the association of EB on the surface of endometrial epithelial cells, this isomerase would act as a universal trigger for reduction of the supramolecular cross-linked EB outer membrane complex, thereby exposing the high affinity MOMP or large CRP adhesin domains. Moreover, 2 chlamydial disulfide isomerases, whose existence was predicted by chlamydial genome analysis, could be activated for total dissolution of the disulfide bonds during this process.

EBs entry does not occur randomly, but in defined areas of plasma membranes called lipid rafts, characterized by high cholesterol and glycosphingolipid content. These lipid microdomains are enriched in molecules involved in signal transduction events. Coalescence of lipid rafts may trigger EB internalization. Disturbance of lipid rafts in the plasma membrane inhibits *C. trachomatis* serovar L2 entry into cells [23-25]. Although most studies argue for the involvement of cholesterol in *Chlamydia* entry, there is nevertheless one that questions this possibility [26].

Upon irreversible *C. trachomatis* EB attachment to the host cell, various events trigger EB internalization. In addition to lipid raft coalescence, actin remodelling is required to facilitate infection. Carabeo *et al.* [27] noted that the presence of cytochalasin D, an inhibitor of actin formation, prevented cell infection. Microscopy has revealed actin recruitment at the attachment site of *C. trachomatis* serovar L2 with HeLa cells. Accumulated actin would lead to the formation of a pedestal-like structure carrying EB and, finally, the formation of microvillar extensions, which would coat the EB. In this entry process, the Rho GTPase family of proteins, which controls actin filament assembly and organization, plays an important role. Rac, but not Rho or Cdc42, is specifically activated after *C. trachomatis* serovar L2 and D attachment. Clostridial toxin B, which is a known enzymatic inhibitor of Rac, Rho and Cdc42, significantly reduces *C. trachomatis* invasion of HeLa cells [28, 29] (Figures 1 and 3).

The *Chlamydia* signal required to trigger a cascade of events for entry remains unknown. A recent study has shown that immediately upon attachment, EB secreted, by a type III system, a protein named translocated actin-recruiting protein (Tarp) of about 103 kDa, encoded by chlamydial ORF ct456. This protein is tyrosine-phosphorylated at the cytoplsamic face of plasma membrane at the site of EB attachment. Tarp appears to initiate or participate in signalling events that regulate actin recruitment which, ultimately, leads to EB internalization [30, 31] (Figures 1 and 3). Despite this knowledge, much remains to be done to clarify the *Chlamydia* entry process.

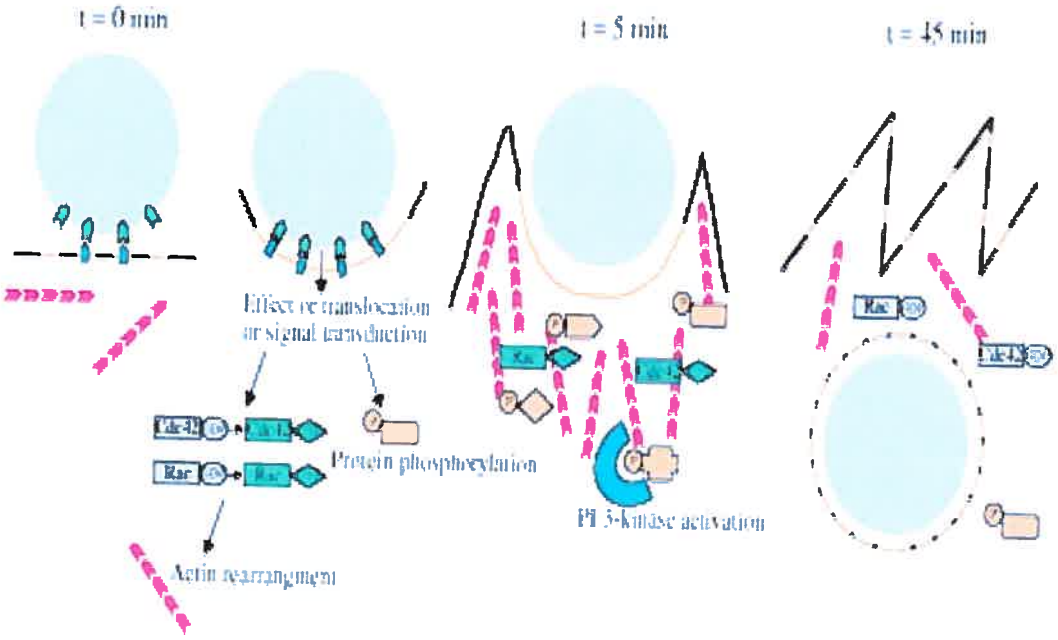

Figure 3. An example of early events during *Chlamydia caviae* entry. Interaction of bacterium (grey circle) with the host plasma membrane induces the clustering of cholesterol rich membrane domain (orange line). This interaction initiates intracellular signalling that is also mediated by activated recruited small GTPase Cdc42 and Rac. Phospohorylated proteins (orange shapes) accumulate at the site of entry where they interact with PI 3 kinase (blue) and participate in the signalling cascade. The activation of these different proteins is followed by actin recruitement and depolarization and bacterium entry. **Reproduced from A. Subtil *et al.* 2004. J. cell. Sci. 117, 3923-3933 [29].**

1.4.2 Chlamydial inclusion and lysosomal fusion escape

After penetration, the EB finds itself in the cell, internalized by a phagosomal or endosomal membrane. To survive in the cell, it must avoid being lysed by lysosomal enzymes contained in endophytic vesicles. Whereas many bacteria, such as *Coxiella burnetii*, survive in cells by resisting lytic enzymes, *C. trachomatis* EB must limit its contact with these enzymes by preventing phagolysosomal fusion. Actually, early endosomal markers such as Rab5 and EE1, are not found in the inclusion membrane. Markers of late endosomes and lysosomes, including LAMP1, LAMP2 and the hydrolase cathepsin, are also excluded. The proton pump ATPase is equally not found, explaining why the lumen of the inclusion is not acidified, and an inhibitor of vacuole acidification, such as bafilomycin A1, does not affect *C. trachomatis* replication. Only the early endosome marker transferrin is seen near, but not in the inclusion [32, 33].

Several hypotheses have been offered to explain how *Chlamydia* escapes lysosomal fusion. Heinzen *et al.* [34] demonstrated that after formation, chlamydial inclusions underwent retrograde transport, moving then to the exocytic pathway of the Golgi apparatus where they fused with exocytic vesicles containing sphingomyelin (Figure 4). This interaction allows chlamydial inclusions to be hidden in the exocytic network, which would explain how they escape from phagolysosomal fusion [35]. However, recent studies showing that even the initial phagosome does not contain any early endosomal markers raised the possibility that chlamydial components transform the inclusion membrane so that it is not recognized as being destined to fuse with the lysosome [34, 36]. This attractive possibility was reinforced by Taraska *et al.* [37] who reported that if an inclusion membrane seems derived from the plasma membrane, it does not necessarily contain host proteins, but could be rather decorated with various chlamydial Inc proteins [38].

Initially discovered in *C. psittaci* [39], Inc proteins as a group of proteins differ in their sequence, but resemble each other by their unique bilobed hydrophobic domain of 50 to

80 amino acids. In *C. trachomatis*, even if 40 open reading frames (ORFs) coding for these special proteins are present in the genome, only some of them have been identified (IncA to IncG). IncA, IncF and IncG are exposed at the cytoplasmic side of the inclusion [40]. The various functions of these proteins are not known. It is only clear that IncA is involved in homotypic fusion between *C. trachomatis* inclusions [41]. The inclusion membrane would be covered with these proteins, each playing a unique function, conferring multiple properties. One of their possible functions would be, notably, participation in avoidance of phagolysosomal fusion. Other functions must be considered, such as participation in nutrient acquisition and in EB differentiation into RBs and of RBs into EBs. Given the importance of inclusion for the establishment of conditions favourable to chlamydial growth and in its direct interactions with the host cytosol, the identification of various Inc proteins as well as their functions will provide much more information on chlamydial biology.

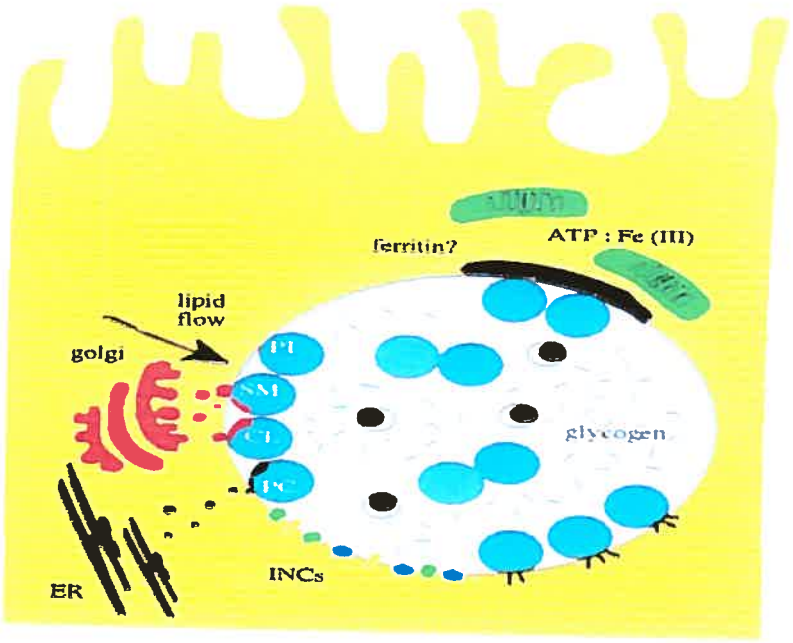

Figure 4. Chlamydial growth inside a membrane-bound inclusion. The nascent inclusion intercepts epithelial exotic vesicles carrying sphingomyelin (SM), phosphatidylinositol (PI), phosphatidylcholine (PC) and cholesterol (CL) for inclusion membrane expansion. ER, endoplasmic reticulum; Inc, inclusion proteins. **Reproduced from P. Wyrick. 2000. Cell. Microbiol. 2, 275-282 [9].**

1.4.3 Differentiation and de-differentiation: EB ↔ RB

Two hours after penetration, EBs must differentiate into RBs. Differentiation can be defined as all events taking place after internalisation, leading to breakdown of the supramolecular structure responsible for wall rigidity, and to nucleoid decondensation. The result is an increased membrane permeability with cytoplasmic decondensation and chromatin dispersion. These changes ultimately elicit metabolic activation. As EBs derive from the de-differentiation of RBs after multiple binary fissions at the reproductive cycle end, various authors have attempted to find late proteins expressed at the time of dedifferentiation of RBs into EBs, to understand the distinctive characteristics of EBs. Some of them succeeded in cloning and sequencing genes whose expression in *Escherichia coli* led to DNA compaction. It was suggested that *Chlamydia* histone H1-like protein, Hc1, could be involved in the compaction of chlamydial DNA, and that its expression during the reproductive cycle could trigger RB de-differentiation into EB [42]. Pedersen *et al.* [43] showed that the C-terminal domain of Hc1 is able to bind to DNA and RNA in limited proteolysis, SouthWestern blotting and gel retardation assays, whereas the N-terminal extremity is a dimerization domain. This could explain how Hc1 at high molarity allowed DNA and RNA compaction, as revealed by microscopic observations [44]. In addition, previous studies also disclosed that Hc1 binding to DNA strongly repressed its transcription and translation *in vitro* [45]. These different facts indicated that late Hc1 expression could bring some change of DNA topology, allowing differential late protein expression. High production of Hc1 and other proteins would likely cause, finally, the total compaction of DNA and RNA, inhibiting transcription and replication. These events would coincide with the de-differentiation of RBs into EBs.

The dissociation or degradation of proteins compacting chromatin, such as Hc1, is necessary at the time of differentiation of EBs into RBs. One report has suggested proteolytic degradation as a possible mechanism of Hc1 dispersal. A chlamydial gene, *euo*, coding for an early protease of 181 amino acids and 20.9 kDa, has been identified [46], and whose expression has led to selective degradation of the C-terminal extremity of Hc1 bound to DNA. This would elicit Hc1 dissociation, and hence, the decompaction of DNA necessary for the resumption of transcription and metabolism. However, very

recently, another report suggested a simple dissociation of Hc1 as a mechanism of chlamydial chromatin relaxation. Chlamydial 2-C-methylerythritol 2,4-cyclodiphosphate (MEC) has been found to dissociate Hc1 from chlamydial chromatin. MEC is a metabolite of the non-mevalonate methylerythritol phosphate (MEP) isoprenoid biosynthetic pathway. The precise mechanism is not known but MEC may be a competitive inhibitor or an allosteric effector of Hc1. In addition, a chlamydial IspE (CT804), an intermediate enzyme of the MEP pathway, is required in this process. Actually, chlamydial ispE expression in *Escherichial coli* removes the lethality resulting from chromatin compaction after Hc1 expression [47].

As mentioned above, disulfide bonds form at the time of de-differentiation, and it appears that cellular and chlamydial isomerases are required for their rupture. However, differentiation being a complex process, several other events likely occur. EBs could have all enzymes capable of mediating early events immediately after internalization, such as lysosomal fusion avoidance and differentiation of EBs into RBs. It would be interesting to determine the protein differential expression pattern in EBs and RBs to identify all the proteins necessary in these processes. For these reasons, RBs should be collected 18 h after infection, before de-differentiation, to ensure that the RB population is not contaminated by EBs.

1.4.4 *Chlamydia*-host cell interaction

Once within the host cell, *C. trachomatis* does not completely control host metabolism, but must modulate host reactions to develop suitable conditions for its growth. Thus, the intracellular pathogen must secrete compounds in the cytoplasm that have the ability to change host behaviour. *C. trachomatis* are found to express 2 eukaryote-like Ser/Thr kinases, Pkn1 (CT145) and PknD (CT301). The activity of these kinases may confer signals to regulate cellular functions [48]. In addition, the presence of the gene encoding PP2C-type protein phosphatase (CT259) in the chlamydial genome argues for a functional kinase-based signalling cascade in *Chlamydia* for interaction with host signalling pathways.

Among the chlamydial compounds released in the cell cytosol that interact with the host for maintenance of the infection process, *Chlamydia* protease/proteasome-like activity factor (CPAF) has been identified. This protein is synthesized as 70-kDa pro-CPAF that is gradually processed for a mature dimeric CPAF with 35- and 29-kDa fragments [49]. Active CPAF is found to degrade host transcription factors (RFX5) required for major histocompatibility complex gene activation. CPAF also degrades keratin-8, a key component of the intermediate filaments involved in cell integrity. This keratin degradation may allow increased cytosol fluidity, facilitating chlamydial inclusion expansion [50]. The way that CPAF is secreted from chlamydial inclusions to the host cytosol remains unknown.

To favour its growth, *C. trachomatis* also interacts with the host to inhibit programmed cell death [51]. Very recently, it has been discovered that *C. trachomatis* inhibits apoptosis by broad cleavage of proapoptotic proteins with Bcl-2 homology domain 3 (BH3-only proteins), among them Bad, Bmf, active Bid (tBid), Puma, Noxa, and Bim [52-54]. BH3-only proteins are required for the release of cytochrome c from mitochondria. Since proteasomal activity is needed in the degradation of some of these BH3-only proteins, CPAF may be involved in degradation of these compounds. Tse *et al.* [55] showed also that *C. trachomatis* inhibited apoptosis by sequestering protein kinase δ (PKCδ) in inclusions. By binding PKCδ through its C1 domain in the context of ancillary protein moieties, diacylglycerol directs the PKCδ in or near the chlamydial vacuole, away from the mitochondria. In fact, PKCδ, a lipid-dependent serine/threonine kinase, is a proapoptotic regulator. Once cleaved, this kinase releases a catalytically-active fragment that translocates to the mitochondria where it promotes the release of cytochrome c with subsequent activation of the apoptotic pathway. In contrast, late at the end of growth, *C. trachomatis* must induce apoptosis to favour EB propagation, probably through apoptotic bodies. *Chlamydia* protein associating with death domains (CADD) that is synthesized late in the developmental cycle has been found to modulate host cell apoptosis [56]. CADD has a death domain-like region that can interact with tumour necrosis factor family proteins and induce cell apoptosis. Since CADD seems to be an oxydoreductase, it can also induce cell necrosis [57]. Thus, *Chlamydia* interacts with the

host machinery through chlamydial-secreted compounds. About 130 cellular genes are induced after *C. trachomatis* infection [58].

The question is to determine the mechanism utilised by *C. trachomatis* to release some compounds modulating the host response when the inclusion membrane is impermeable, even to small molecules whose molecular weight is at least 0.52 kDa [59]. The answer to this question has come from chlamydial genome analysis, which has made it possible to predict the existence of the type III secretion system (TTSS). In general, the TTSS is used by Gram-negative bacteria for secreting and injecting pathogenicity proteins into the eukaryote cell cytosol. Whereas the genes coding for this system are typically linked in small pathogenicity islands containing an A+T-rich signature among enterobacteriaceae, the chlamydial orthologs are divided into 3 different loci in the genome and lack the A+T-rich signature [8].

While there is no doubt about the existence of the chlamydial TTSS, the identification of proteins forming this apparatus is still in progress. Some authors could provide experimental evidence for a functional TTSS in *C. trachomatis* by referring to the homology of structural proteins constituting this system among Gram-negative bacteria. Thus, by combining RT-PCR, immunoblotting and matrix-assisted laser desorption ionization time-of-flight (Maldi-Tof), Fields et al.[60, 61] successfully identified some of these proteins, including CdsJ (contact-dependent secretion J) and CopN (*Chlamydia* outer membrane protein N). CdsJ, a predicted lipoprotein homologous to *Yersinae* spp YscJ, is essential in the TTSS because of its inter-connections with the internal and external components of this system. By homology with *Yersinae* YopN, CopN is a regulating protein that could be localized on chlamydial surfaces where it would block TTSS pores, preventing protein secretion in the absence of a suitable signal. Recently, a chaperone of CopN, the chlamydial LcrH-2, has been proposed [62]. As mentioned elsewhere, Tarp is also secreted by the TTSS [30].

The anti-host proteins released have been identified according to the fact that proteins secreted by a type III machine of one pathogen can also be secreted by the heterologous

machine of another pathogen. Subtil *et al.* [63] have reported that proteins expressed by the hybrid genes *incB/cya* and *incC/cya* in wild type *Shigella flexneri* were excreted in the supernatant, while in the mutant *mxiD*, in which the TTSS is completely compromised, there was no excretion of these proteins in the supernatant. *Cya* is *Bordetella pertussis* gene encoding calmodulin-dependent adenylate cyclase used in this experiment as a reporter gene. This made it possible to state that inclusion proteins, such as IncA, IncB and IncC, pKn5 and CopD, were secreted by the TTSS in *Chlamydia*. Pkn5 may be a serine/threonine kinase or an aminoglycoside 3'phosphotransferase [48]. CopD, similar to *Yersina* YopD, may associate with another protein to form a porin-like structure in the membrane, facilitating the translocation of other type III secreted substrates [64].

In the genus *Chlamydia*, the TTSS appears to correspond physically to pili-like structures (Figure 5), discovered for the first time by Matsumoto [65] more than 20 years ago. Recently, Chang *et al.* [66] combined electron microscopy and computer image analysis, generating more information on the organization of these appendages. They noted that the pili-like structures are made up of helical arrangements of protein subunits having a periodicity of 50 Å. These appendices, 60-80 Å in diameter and 500 Å in length, leave the inner membrane, traverse the periplasmic space and pass in the outer membrane through a hexagonal ring. The proteins constituting the appendages have not yet been identified, but the possibility is not excluded those the proteins secreted, such as IncB, IncG or IncA, could be some of them.

Figure 5. Surface projections of *Chlamydia psittaci* elementary body. **Reproduced from A. Matsumoto. 1982. J Bacteriol. 151,1040-2 [67].**

1.4.5 Growth

Since several chlamydial metabolic pathways are incomplete, *C. trachomatis* must acquire different nutrients from host cells. Although chlamydial nutritive needs are known, there is no rich medium for its growth in the absence of host cells. Studies must be undertaken to determine if the inability *C. trachomatis* to grow in the absence of host cells results from the need for cellular signals allowing EB differentiation into RB, or from the need to transform nutrients at the level of the inclusion membrane to make them easier to assimilate by the pathogen. Since *C. trachomatis* does not control host metabolism, both host and parasite take up the same available nutrients. To date, no compound allowing *C. trachomatis* to compete efficiently with the host cell has been identified. This is why it is important to add cycloheximide to culture media to strengthen chlamydial growth rather than that of the host cell. *C. trachomatis* may have developed mechanisms allowing it to survive during food shortages. Indeed, it was noted that *C. trachomatis* developed an aberrant and persistent form when intracellular tryptophan pools were reduced in response to gamma interferon [68]. In addition, chlamydial genome sequence analysis supports the hypothesis of an ATP-limited biosynthesis pathway, probably an emergency one [8].

In regard to cellular division, it is difficult to conceive the chlamydial mechanism of septum formation, in the apparent absence of FtsZ protein. Indeed, FtsZ is a GTPase required for cellular division, because it participates in the ring-like structure formation essential to septal protein assembly. Until now, no chlamydial protein sharing significant homology with FtsZ has been identified. However, Brown and Rockey [70], using fluorescent-antibody labeling, detected a chlamydial antigen called SEP (for septum) in dividing RBs. SEP was localized to a ring-like structure at or near the plane of chlamydial division. According to these authors, this localization pattern corresponds to that of FtsZ in other bacterial species.

Moreover, even if the *C. trachomatis* genome contains all the genes necessary to encode proteins of the PG biosynthesis pathway, attempts to identify PG in RBs have been

unsuccessful. Just like FtsZ, PG plays a part in cellular division by allowing the formation of invaginations between 2 daughter cells during cytokinesis. Recent studies showed that the chlamydial *murA* gene was expressed, and the resulting enzyme was functional. Indeed, MurA is a uridine diphosphate N-acetylglucosamine enolpyruvyl transferase, an enzyme essential to the PG biosynthesis pathway [70]. The authors also noted that *C. trachomatis* serovar L2 MurA contains a cysteine-to-aspartate change at amino acid 119. This active site substitution alters the pH optimum for the enzyme. Chlamydial MurA enzyme has high activity at pH values less than 7.0, and loses activity at pH values greater than 7, whereas wild type MurA activity remains constant over a pH of 5.0 to 9.0. Considering the fact that the pH of inclusion is higher than 7.0, 20 h after chlamydial infection, McCoy *et al.*[70] suggested that PG could be synthesized only during the early stages of the *C. trachomatis* life cycle. According to them, the PG synthesized precociously could be recycled later to take part in cellular division. However, UDP-N-acetylmuramoylalanylglutamyl DAP ligase has been found in RBs. This enzyme is involved in the synthesis of the muramyl-peptide unit, the first stage in PG assembly. This finding suggests that PG biosynthesis occurs during RB growth and cell division [19]. Nevertheless, this PG and its precurseurs remain to be identified in chlamydial RBs.

1.4.6 EB exit

EBs coming from the de-differentiation of RBs must, at the cycle end, leave the infected host cell to infect new surrounding cells. The exit mechanisms deployed by EBs are still unknown. Electron microscopy studies showed that some serotypes from A to K can leave without causing host cell lysis. The overall inclusion would exit the cell by a process similar to exocytosis [71]. Other serotypes from L1 to L3 could be released after host cell lysis. It is not know if this lysis requires specific chlamydial lytic enzymes or if it results from the pressure exerted by the inclusion. Recently, Perfettini *et al.* [72] noted that *C. trachomatis* MoPn could use cell apoptosis to spread. Indeed, apoptotic bodies containing chlamydial particles, could be internalized by surrounding cells in which the particles, instead of being digested, perpetrate infection.

1.5 GENE EXPRESSION

Shaw *et al.* [73] have identified 3 classes of chlamydial developmental genes: early genes detected 2 h after internalization, that are necessary to complete the differentiation of EBs into RBs; mid-cycle genes appearing 6-12 h post-infection and required in RB metabolism and growth; and, finally, late genes that are transcribed 12-20 h post-infection and are important in the de-differentiation of RBs into EBs. Using the microarray approach to identify development stage-specific gene sets, Nicholson *et al.* [74] demonstrated that only 22% of the chlamydial genome was differentially expressed during the developmental cycle. The majority of other genes had constitutive expression in the basic functions of the chlamydial cell. Gene expression, according to chlamydial growth stages, means transcriptional regulation of development. However, until now, the signals and mechanisms allowing global gene expression regulation are unknown. Although 3 transcription factors (σ^{66}, σ^{54} and σ^{28}) have been identified in *Chlamydia*, nothing to date indicates that chlamydial gene expression control is similar to that allowing *Bacillus* spp. sporulation, and implies a cascade of sigma factors [75]. σ^{66} could be involved in some late gene expression such as *hctA* and *hctB*. The σ^{54} promoter has been identified for 2 chlamydial late genes of unknown function. σ^{28} encoded by *rspD* gene recognizes the promoter of *hctB*, that is, the gene encoding Hc2, a histone-like protein implicated in DNA compaction during back differentiation of RBs into EBs [76,77]. Recently, Koo and Stephens [78] discovered a unique 2-component regulation system which can play an important part in late gene expression. This single system is made up of a sensor kinase, CtcB (*Chlamydia* 2-component B), and a response regulator, CtcC, having homology to the σ^{54} activator. It appears that in the presence of Mg^{2+}, Mn^{2+} and Fe^{2+}, the CtcB sensor can autophosphorylate and then transfers its phosphoryl group to the activator CtcC. Phosphorylated CtcC becomes oligomerized and then activates σ^{54} holoenzyme. Since CtcB and CtcC are expressed late, it is possible that they have roles in the de-differentiation of RBs to EBs.

Methylation may also play a role in chlamydial gene regulation. Recently *C. trachomatis* serovar D protein release factor methylation (PrmC) has been identified. PrmC (CT024)

contains a hallmark sequence of an N^6-adenine-specific DNA methyl transferase. PrmC is constitutively expressed during the chlamydial intracellular developmental cycle and is able to methylate chlamydial release factor within the tryptic fragment containing the universally conserved motif glycine-glycine-glutamine (gly-gly-gln) [79]. The role of methylation in the regulation of gene expression and protein activity of *Chlamydia* remains to be revealed.

When the EB enters the host cell, its DNA is decompacted, allowing gene expression. One may wonder if DNA decompaction ends before transcription restarts. Indeed, it is possible that DNA relaxation happens gradually so that the genes to be transcribed are in the portion of relaxed DNA, whereas those of the condensed portion cannot be expressed. The decondensation origin point and the direction of this decondensation can influence transcription factor activity and thus determine the order in which the genes are transcribed. Further studies are required to explore the global regulation of chlamydial genes. This will constitute an important advance in understanding the biology of the pathogen.

1.6 CONCLUSION

The sequelae of chlamydial diseases result from a long process requiring multiple recurrences. New strategies besides antibiotics to prevent recurrences must be considered. Chlamydial biology studies can hasten the design of new anti-chlamydial approaches. Indeed, many aspects of *C. trachomatis* biology still remain obscure. Current or future research should focus on the following stages: the mechanisms of EB penetration and differentiation, the means of division used by RBs in the absence of FtsZ, the transcriptional regulation of chlamydial genes, the identification of anti-host proteins modulating host metabolism, the inclusions role in nutrition and chlamydial growth.

1.7 ACKNOWLEDGEMENTS

We thank Ovid Michael Da Silva for editing this manuscript. M.P. is the recipient of the"Programme Canadien des Bourses de la Francophonie" scholarship.

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CHAPITRE II: ARTICLE 2

CHLAMYDIA TRACHOMATIS PERSISTENCE: AN UPDATE

CHLAMYDIA TRACHOMATIS PERSISTENCE: AN UPDATE

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Published in Microbiological Research

[In press]

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2.1 SUMMARY

Chlamydial persistence is a reversible state generated during deleterious conditions to growth. In persistence, *C. trachomatis* remains viable but atypical, with an enlarged, aberrant form and quiescent metabolism. It favours chronic chlamydiosis, leading to serious sequelae. Although the mechanism of persistence formation is still unknown, more reliable molecular approaches tend to confirm that persistence occurs *in vivo*, lasting as long as 3 years. One approach consists of identifying unprocessed rRNA found only in viable *Chlamydia*, when infection is not apparent. Another approach, referring to the fact that immunity is type-specific, consists of showing by genotyping that multiple recurrences are due to the same genovar. At the molecular level, persistence is characterized by increased expression of *ct755,* 1 of the 3 Hsp60-coding genes. In addition, chromosomal replication occurs continuously and cell division is blocked possibly due to the repression of genes such as *ftsW* and *amiA*. At the immunological level, persistence reveals the failure of host-defence mechanisms because of reduction or suppression of pro-inflammatory or cytotoxic responses.

KEYWORDS

Chlamydia trachomatis; persistent infection; therapeutic failure; escape from host-defence

2.2 RÉSUMÉ

La persistance chlamydienne est un phénomène réversible qui survient lors des conditions de croissance inadaptées. *C. trachomatis* persistant est une particule viable, mais atypique, avec une morphologie élargie et aberrante, un métabolisme quiescent. La persistance favoriserait les chlamydioses chroniques souvent responsables des séquelles sérieuses. Bien que le mécanisme moléculaire de formation de la persistance demeure encore inconnu, les approches moléculaires plus fiables tendent à démontrer que *C. trachomatis* persiste *in vivo*, et même pendant plus de 3 ans. Une approche consiste à identifier les ARNr 16S non épissés qui sont uniquement exprimés par les chlamydies viables lorsque l'infection est inapparente. Une autre approche se réfère au fait que l'immunité est spécifique au type et consiste à montrer par genotypage que les récurrences multiples survenant sont dues au même génotype chlamydien. Au niveau moléculaire, la persistance apparaît avec une expression plus accrue du *ct755*, un des trois gènes codant pour la protéine du choc thermique de 60 kDa (hsp60). En plus, la réplication chromosomique se produit continuellement, alors que la division cellulaire est bloquée possiblement dû à la répression des gènes requis tels que *ftsw* et *amiA*. Au niveau immunologique, la persistance résulterait de l'échec des mécanismes de défense de l'hôte se traduisant par la suppression des réponses pro-inflammatoires et cytotoxiques.

MOTS-CLÉS

Chlamydia trachomatis, infection persistante, échec thérapeutique, échappement é la défense de l'hôte

2.3 INTRODUCTION

Chlamydia trachomatis, like all other chlamydiae, characterized by its obligate intracellular lifestyle and unique biphasic developmental cycle (Fig. 1). Phylogenetically distinct from bacteria, chlamydiae are members of the order chlamydiales. Until recently, the latter had had one family, the chlamydiaceae, containing a genus, *Chlamydia* subdivided into 4 species: *C. trachomatis*, *C. pneumoniae, C. psittaci* and *C. pecorum*. Recently, the chlamydiales classification was revised and it now has four families, *Chlamaydiaceae*, *Simkaniaceae*, *Parachlamydiaceae* and *Waddliaceae*. Likewise, the unique *Chlamydiaceae* genus, *Chlamydia*, is now subdivided into two genera: *Chlamydia* and *Chlamydophila*. The genus *Chlamydia* has three species, *C. trachomatis,* the human pathogen, *C. muridarum,* the mouse and hamster pathogen, and *C. suis* infects swine. The genus *Chlamydophila* has 6 species, among which the two most important are *C. pneumoniae,* causing human and mammalian respiratory tract infections, and *C. psittaci* that primary infects birds, but that can cause zoonotic diseases in humans (Everett et *al.*, 2000).

All chlamydiae have a unique biphasic developmental cycle during which the bacterium is found in two forms; the extracellular form named elementary body (EB) and the intracellular form, the reticulate body (RB) (Fig. 1). *C. trachomatis* EBs and RBs have a Gram-negative type wall with inner and outer membranes, but the peptidoglycan layer is not seen. Instead, a supramolecular structure containing disulfide-cross-linked proteins confers EB rigidity, but it is lost in RBs. EBs are osmotically-stable and metabolically-inactive particles specialized in cell infection. They are round, have a compacted nucleoid, and are small in size (0.2-0.6 μm in diameter). After attachment and penetration in cells by an unknown mechanism, EBs remain internalized in vacuole that escapes phagolysosomal fusion (Heinzen *et al.*, 1996). Within these vacuoles named inclusions, EBs differentiate into RBs after several transformations. Unlike EBs, RBs are larger (0.6-1.5 μm in diameter), less compacted, metabolically active and able to divide by binary fission. In *C. trachomatis*, around 18 h post-infection, RBs resulting by binary fission differentiate back into EBs that will afterwards be expelled from the cell, either by exocytosis, or cellular lysis 48-72 post infection (Wyrick, 2000).

C. trachomatis, which is subdivided into two biovars (trachoma and lymphogranuloma (LGV)) clustering 18 serovars, is responsible for a broad spectrum of diseases worldwide. Serovars A, B, Ba, and C of biovar trachoma infect conjunctive epithelium and lead to ocular infections that can progress to trachoma, the leading cause of preventable blindness. In developing countries, these serovars remain endemic. Today, it is estimated that there are about 162 million infected people worldwide, and 6 million of them are blind (Mabey and Fraser-Hurt, 2001; Mabey *et al.*, 2003). Serovars D-K, Da, Ia and genovariant Ja infect genital epithelium and cause urogenital tract infections. These serovars remain the major causes of sexually-transmitted diseases in developed and in developing countries, with about 92 million new infections each year ((Toth *et al.*, 2000; Bjartling *et al.*, 2000)). Some of these serovars are the etiologic agents of some kinds of rheumatoid arthritis. Finally, the LGV biovar has four invasive serovars (L1, L2, L2a and L3) that are able to infect not only genital epithelium, but also monocytes and lead to a systemic disease known as lymphogranuloma venereum (Mabey and Peeling, 2002). Serious sequelae (blindness, tubal infertility, ectopic pregnancy, etc.) due to chlamydial diseases are observed only if the diseases remain chronic with the resulting chronic inflammation being responsible for fibrosis and scaring that characterize all chlamydial diseases.

To explain chlamydial sequelae, several mechanisms have been proposed. Many authors suggest that because hypersensitivity mediated by the T helper type 2 (Th2) response is ineffective and long lasting, it could give rise to the observed tissue injury (Yang, 2001). In addition, multiple *C. trachomatis* re-infections evoke 60-kDa heat shock protein (hsp60) accumulation. As *C. trachomatis* hsp60 displays 48% identity to human hsp60, chlamydial hsp60 could break human tolerance to its own hsp60, inducing auto-immunity (Viale *et al.,* 1994; Yi *et al.*, 1997). Thus, by allowing chlamydial hsp60 accumulation in the host, recurrence due to persistence and re-infections would be at the origin of severe chlamydial disease.

To provoke chronic infection, *C. trachomatis* could adopt several strategies. One of these consists of being silent, resulting in asymptomatic infections that cannot be diagnosed at

that time. This promotes bacterial progression, even to the most internal tissues. In addition, *C. trachomatis* major outer membrane protein (MOMP) displays variable immunodominant antigenic epitopes. Variation in these epitopes explains the absence of strain-specific immunity and multiple re-infections by different serovars or by the same mutated serovar are still possible (Millman *et al.*, 2001). For these reasons, even if the initial infection is resolved, re-infections are possible and can lead to auto-pathological immune response induction (Beatty *et al.*, 1994d). Although re-infections occur, the refinement of chlamydial diagnostic methods will allow us to establish whether *C. trachomatis* can persist.

Chlamydial persistence refers to *C. trachomatis* possibly being in an atypical, intracellular and metabolically less active state that is difficult to resolve not only by the host defence system, but also by antibiotherapy. This review discusses *C. trachomatis* persistence. It precisely describes studies of chlamydial persistence identification, conditions promoting this situation, and the means used to reveal chlamydial persistence *in vivo* and *in vitro*. Finally, the review discusses the possible mechanisms that maintain persistence.

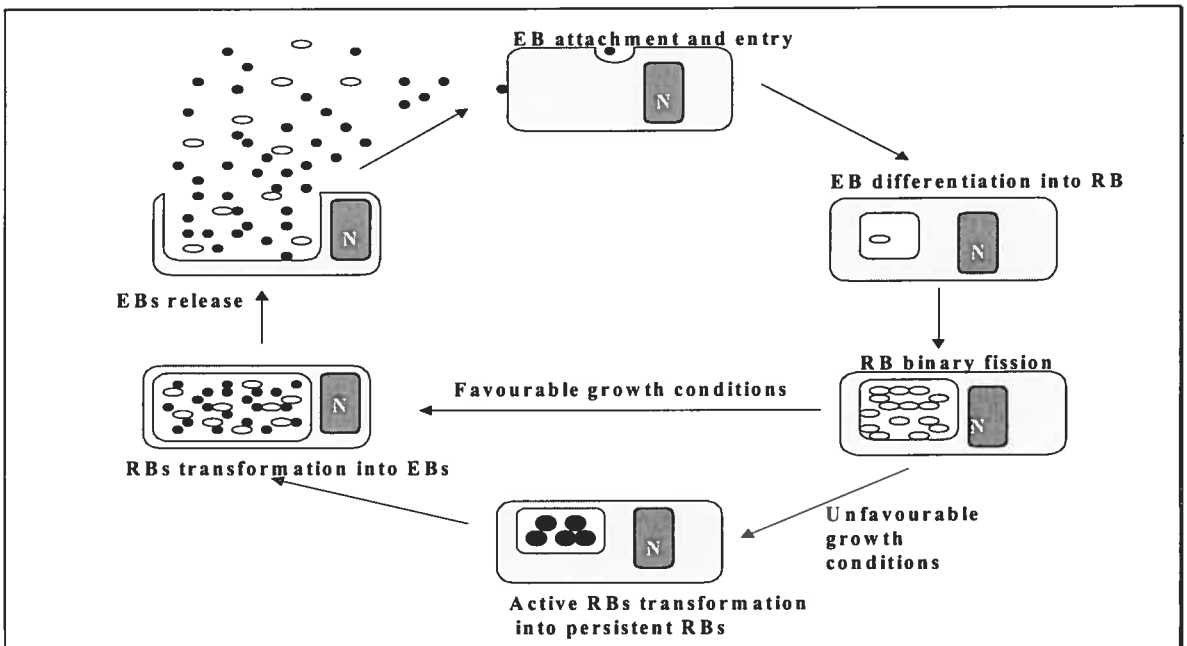

Figure 1: Chlamydial life cycle. N: nucleus; EB: elementary body; RB: reticulate body

2.4 THERAPY AND THERAPEUTIC FAILURE

In general, *C. trachomatis* infections are resolved by treatment with antibiotics that are able to pass through lipophilic plasma membranes to reach the RBs which are metabolically-active and so most susceptible to treatment. Cyclins (doxycycline and tetracycline), quinolones (ofloxacin and levofloxacin), and macrolides (erythromycin and azithromycin) are the drugs of choice in *C. trachomatis* infection treatments. Among ß-lactamins, only amoxicillin is used in chlamydial infection therapy, particularly in pregnant women (Guaschino and Ricci, 2002). The microbiology cure rate can vary from 90 to 100% according to administered drug used (Low and Cowan, 2002). However, despite these high cure rates, recurrent, exacerbating infections are reported.

A prospective study by Whittington and *al.* (2001) showed that therapeutic failures could occur. In fact, these authors noticed that among 792 patients struck by *C. trachomatis* genital tract infections, 50 remained infected 42 days after appropriate treatment with doxycycline or azithromycin. Multivariate analysis disclosed that several factors were independently associated with infection continuation, notably, age of the patient and the renewal of sexual intercourse. Among patients who remained infected, 7.3% began sexual intercourse again, while 3.5% abstained (Whittington *et al.*, 2001). Thus, antichlamydial antibiotherapy failure cannot be only explain by re-infection. Usually, factors like low compliance, drug pharmacokinetic and availability at the tissue level can also dictate failure. However, failure has been encountered even if azithromycin is given in a single dose. This finding excludes low compliance as a therapeutic failure factor in these cases. Work in our laboratory has shown that failure could occur in spite of drug availability. Actually, treatment of *C. trachomatis* serovar L2 infection in susceptible CF-1 mice with liposome-encapsulated doxycycline gave the best results, but not a microbiological cure rate of 100% (Sangare *et al.,* 2001). Even so, liposomes allow direct antibiotic transport to the infection site. This avoids drug loss due to interactions with proteins. Then, the antibiotic can find itself in a sufficient quantity at the infection site (Ravaoarinoro and Toma, 1993; Sangare *et al.*, 1998; Sangare *et al.,* 1999). To sum up,

these works show that factors other than low compliance and drug bio-availability are implicated in antibiotherapy failure.

Some studies suggest that chlamydial resistance could play a role in therapy failure. One was a case report (Somani *et al.,* 2000) of a pregnant woman and a couple who were respectively infected by *C. trachomatis* serovars E and F. In this report, the minimal concentrations of doxycycline, ofloxacin and azithromycin required to inhibit these 2 patient isolates were markedly higher than for susceptible control *C. trachomatis* strain. For example, the doxycycline minimal inhibitory concentrations were above 4 μg/ml for the 2 patient isolates but only 0.015μg/ml for the susceptible controls. Following this work the authors suggested that some *C. trachomatis* strains can exhibit heterotypic resistance. This notion holds that the chlamydial population contains both susceptible and resistant organisms. Studies on this subject have discovered that only a small percentage of organisms, around 1% of the population, is multi-resistant (Jones *et al.*, 1990; Lefevre *et al.,* 1997). The mechanism responsible for heterotypic resistance is unknown, but persistence may be suggested. In fact, it is reported that under some conditions *C. trachomatis* adopted an intracellular form that is metabolically less active and thus more insensitive to multiple antibiotics. This situation results in a lasting association between the pathogen and its host; hence, the term persistence.

2.5 CHALAMYDIAL PERSISTENCE STUDIES *IN VITRO*

Even if chlamydial persistence was suspected to occur *in vivo*, it was first reproduced and studied in *in vitro* models. In this context, several factors can interrupt chlamydial growth and induce the chlamydial persistent stage. The different factors that are likely to evoke *C. trachomatis* persistence are enumerated below.

2.5.1 Cytokine effects on *C. trachomatis*

The chlamydial stage of suspected persistence was studied in *C. trachomatis* infected-cells treated with tumor necrosis factor (TNF-α) or interferon-γ (IFN-γ) (Shemer-Avni *et al.,* 1988; Beatty *et al.*, 1995). However, to date, only IFN-γ-induced persistence has been best characterized. Electron microscopy of HeLa 229 cells infected with *C. trachomatis*

serovar A and post-treated with IFN-γ 0.2 μg/ml disclosed small inclusions containing enlarged, aberrant and non-infectious RBs. Removal of IFN-γ from the culture media allowed these RBs to recover their normal form and growth, to then differentiate further into infectious EBs. Based on this observation, it was suggested that these atypical RBs could represent a persistent stage (Beatty *et al.,* 1995). Immunochemistry and immunobloting revealed that these particles exhibited a lower expression of MOMP, but with a normal or increased hsp60 level. Since MOMP is an immuno-protective target, and hsp60 is an immunopathological target, the role of these atypical particles in chronic infection pathogenesis has been suggested (Beatty *et al.,* 1993; Beatty *et al.*, 1994a).

It appears that IFN-γ induces chlamydial persistence by allowing tryptophan depletion. Actually, IFN-γ activates indoleamine 2,3-dioxygenase (IDO), an enzyme that catalyzes the initial step of tryptophan degradation. As a result, the amount of this essential amino acid is reduced (Beatty *et al.,* 1994b; Beatty *et al.*, 1994e). Chlamydial genome sequencing has demonstrated that different *C. trachomatis* serovars do not have all the genes required for tryptophan synthesis. Unlike ocular serovars, oculo-genital serovars possess one gene, the one coding for tryptophan synthetase. This enzyme, which plays a part in the final step of tryptophan synthesis, converts indole formed beforehand into tryptophan (Fehlner-Gardiner *et al.,* 2002). Since indole can be synthesized by some bacteria infecting the genital tract, it is suggested that *Chlamydia* co-infection with these indole-producing bacteria are involved in persistent RB reactivation *in vivo* (Caldwell *et al.*, 2003) (Fig. 2.). The fact that persistent particles can revert to normal productive particles allows us to understand why persistent infections appear by multiple recurrences.

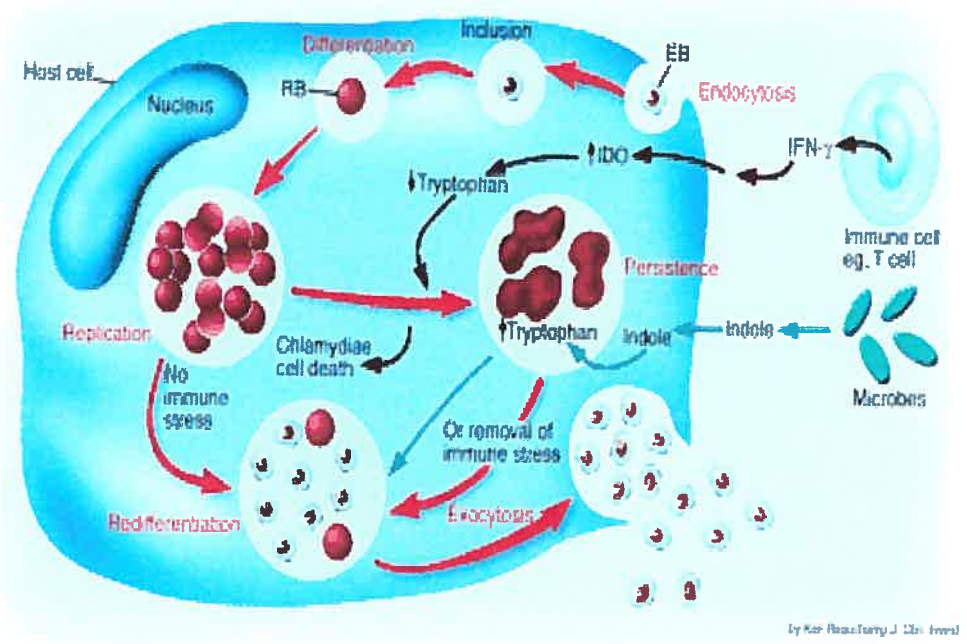

Fig. 2. Chlamydial active life cycle versus persistent cycle. IFN-γ induces cellular IDO, which results in a marked decrease in available tryptophan. This interrupts chlamydial active growth and induces chlamydial persistence. IFN-γ removal allows differentiation of persistent EBs into active EBs. Alternatively genital strains of *Chlamydia* that possess functional tryptophan synthetase may convert indole, produced by local microflora, into tryptophane and thus redifferentiate into active EBs. IFN-γ, interferon-gamma; IDO, indoleamine dioxygenase; EB, elementary body; RB, reticulate body. **Reproduced from R.P. Morrison. 2003. 111, 1647-9.**

2.5.2 Growth in non-permissive cells

C. trachomatis serovar E culture in non-permissive synovial cells results in the development of spontaneous persistence, without requiring any treatment (Hanada *et al.*, 2003). Similarly, growth of *C. trachomatis* serovar K in human peripheral blood monocytes or in human monocytic cell line U-937 culminates in persistence. Under these conditions, the resulting atypical RBs are enlarged, viable, but non-cultivable, and contain electron-dense material in their centre (Koehler *et al.*, 1997). Unlike persistent particles induced by IFN-γ, persistent RBs in monocytes do not reactivate into productive infections despite the addition of tryptophan or antibody against IFN-γ or TNF-α in culture media. Moreover, IDO activity is not found in persistently-infected monocytes. This considerable difference shows that persistence in monocytes is not due to tryptophan-depletion. Finally, the fact that *C. trachomatis* serovars K and E remain persistent in monocytes and in synovial cells, heightens the possibility that *Chlamydia* goes to the joints where it can harbour a chronic inflammatory response causing arthritic damage.

2.5.3 Nutritional deficiency effects

Nutrient restrictions can also induce *C. trachomatis* persistence. It has been noted that under iron restriction, *C. trachomatis* serovar E RBs become abnormal with loose and wavy outer membranes. Inclusions containing such abnormal RBs are small and surrounded by electron-dense material (Raulston, 1997). Harper *et al.* (2000) found that minor changes in amino acids and glucose deprivation in *Chlamydia* media led to the development of persistent particles similar to those identified by Beatty and colleagues (1994a). After studying *C. trachomatis* morphological changes under different conditions, Harper and collaborators suggested that the development of *Chlamydia* persistence is a general response to stress.

2.5.4 Antibiotic effects

Antibiotics like penicillin, ofloxacin and ciprofloxacin are listed among compounds that are liable to induce persistence (Johnson and Hobson, 1977; Segreti *et al.*, 1992; Dreses-Werringloer *et al.*, 2000). *C. trachomatis* exposure to penicillin leads to enlarged and .

aberrant RBs, the so-called "penicillin forms", that resume normal growth after penicillin removal. Although chlamydial wall peptidoglycan has not been already found, we know that *C. trachomatis* has important genes required in peptidoglycan synthesis (Stephens *et al.,* 1998). One of these genes, *murA*, is actually expressed (McCoy *et al.*, 2003). It might be then that penicillin binding to its 3 penicillin binding-proteins (PBP-1, -2 and -3) interferes with presupposed peptidoglycan synthesis (Storey and Chopra, 2001). This interference with chlamydial growth may explain its persistence. A recent study showed that *C. trachomatis* particles resistant after exposure to penicillin became phenotypically resistant to chlamydicidal concentrations of azithromycin (Wyrick and Knight, 2004).

To understand antibiotherapy failures, researchers became interested in the prolonged effect of 2 antibiotics against *C. trachomatis* serovar K, *in vitro* (Dreses-Werringloer *et al.*, 2000). The resulting studies disclosed that ciprofloxacin and ofloxacin, instead of resolving the infection, induced aberrant but viable particle formation, like those reported by Beatty *et al.* (1994a). Although ciprofloxacin and ofloxacin were dispensed in minimal bactericidal concentrations, 20 days after treatment, these particles remained in the culture media. Under certain conditions, antibiotics in chlamydial infection therapy could favour persistence, instead of resolving infection. This outcome could be in agreement with therapy failures reported clinically.

2.6 *IN VIVO* STUDIES OF CHLAMYDIAL PERSISTENCE

The capacity of *C. trachomatis* to persist *in vivo* was perceived thanks to the introduction of diagnostic tests based on nucleic acid amplification. These tests, including polymerase chain reaction (PCR) and ligase chain reaction, allowed us to pick out ambiguities that could misrepresent the persistence. For example, it was noted that in areas where trachoma rages in the holoendemic state, primary infection in young people was diagnosed by conventional culture methods. While chronic conjunctivitis was developing into blindness in elderly people, the etiologic agent, *C. trachomatis*, was detected only by PCR (Grayston and Wang, 1975). Likewise, in the case of primary genital infections, even if they are asymptomatic or subclinical, the pathogen is easily isolated in culture.

However, in some women with obstructive infertility, *C. trachomatis* remains barely isolatable, even if its corresponding DNA and RNA are revealed by PCR (Brunham *et al.,* 1985). The lack of sensitive conventional culture methods can explain these ambiguous findings. However, the presence of persistent non-cultivatable *C. trachomatis* can be also suggested in this failure of identification. Although the presumption of persistence is credible, indisputable proof of persistence remains to be provided *in vivo*. To demonstrate that *Chlamydia* persistence occurs *in vivo*, not only should its nucleic acid be detected, but the viability of the organism should also be shown. This is especially important as some studies rebut the possibility that *C. trachomatis* could become persistent and contribute to disease development with chronic pathologies. Although *Chlamydia* RBs are killed by azithromycin treatment, residual chlamydial envelopes can persist for at least 28 days and continue to harbour inflammatory responses (Wyrick *et al.,* 1999). According to this point of view, a sustained and harmful inflammatory response could be due to residual antigens, but not to chlamydial persistence.

The approach to demonstrate the presence and viability of *C. trachomatis* in persistently-infected patients consists of identifying rRNA primary transcripts. These unprocessed rRNAs include not only coding sequences (23S and 16S rRNAs), but also non-coding sequences flanked at 3' and 5' extremities. The maturation of unprocessed rRNA into processed rRNA requires removal of non-coding sequences. Unprocessed rRNA maturation is so fast that intron suppression at the 5' extremity is completed before RNA polymerase achieves transcription of whole operon. Considering the fast transition between unprocessed and processed rRNA transcripts, the presence of unprocessed transcripts in medium means that they are synthesized recently. This shows that the organism is metabolically active and thus viable (Gerard *et al.*, 1997). Thus, to conclude that *Chlamydia* is persistent, one approach is to study not only its abnormal morphology, but also its viability. With this approach, some authors found persistent *C. trachomatis* in synovial tissue from arthritis patients (Gerard *et al.,* 1998). This allowed them to hypothetize the possible role of persistence in the pathogenesis of arthritis.

Other proof of *C. trachomatis* persistence *in vivo* was be supplied recently by 2 independent studies. In both of them, the approach used allowed them to distinguish persistence from re-infection. This approach was based on the principle that antichlamydial immunity is type-specific. According to the principle, once the initial infection is resolved, re-infection is believed to be the result of exposure to another chlamydial type that differs from the initial infecting type. In contrast, persistent infections are those in which *C. trachomatis* has entered the metabolically-quiescent and non-infectious state. In this case, the chlamydial type in several infection recurrences is the same.

To demonstrate persistence *in vivo*, the genotype of the infecting strain must be determined at every recurrence. Genotyping has an advantage in that it can equally establish if re-infection is due to the same mutated type. Based on this approach, some authors identified several women with 3-10 recurrent chlamydial cervical infections by the same serovars over 2-5 years, despite suitable treatment with doxycycline and azithromycin. Among these women, 5 had recurrences with the same genotypes (D, I, Ia, H and Ja). This result shows that genotypes D, I, Ia, H and Ja can persist over several years, notwithstanding appropriate treatment (Dean *et al.,* 2000). Using the same approach, another group tracked trachoma development in a cohort of at least 100 women (Smith *et al.,* 2001). As in the previous study, these researchers noted that despite suitable treatment with azithromycin, 11 women among the 100 always remained infected with the same genotype, even after 3 years. This finding, according to the authors, is palpable proof of persistence *in vivo*. Although the two studies favour the concept of persistence *in vivo*, the identification of persistent particles in tissue from infected individuals is also considerable proof. Bragina *et al.* (2001) undertook electron microscopic examination of urethral and cervical material from patients who had been suitably treated with azithromycin. The results revealed the presence of persistent particles similar to those described by Beatty *et al.* (1994a).

2.7 MOLECULAR MECHANISM OF PERSISTENCE

The few studies performed until now did not dissect the molecular mechanism leading to persistence. Generally, it is suggested that deprivation of essential amino acids, like tryptophan and cysteine, can stop the expression of late proteins, such as MOMP as well as cysteine rich proteins. This leads to a halt in RB division and RB de-differentiation into EB. According to this viewpoint, the persistent *C. trachomatis* would be intracellular undifferentiated particles (Beatty et *al.,* 1994d).

It has been found by real-time reverse transcription (RT)-PCR that *dnaA*, *polA* and *mutS* genes are transcripted in persistent *C. trachomatis* serovar K induced by culture in non-permissive monocytes. Products of these genes are involved respectively in the initiation, replication and repair of chromosomal DNA. However, *ftsK* and *ftsW* engaged in cell division are not expressed 1 day after monocyte infection (Gerard *et al.,* 2001). Other authors have confirmed that DNA replication and segregation continue, but cell division is abrogated in persistent chlamydial particles (Belland *et al.,* 2003). In addition, these studies have tried to identify the cell division molecular step that is abrogated. They have demonstrated that in persistent *C. trachomatis* induced by IFN-γ treatment, *ftsW* and *amiA* transcription is suppressed, whereas *ftsK* transcription remains unchanged. Normally, FtsW is a chaperone of PBP-3 required in septal peptidoglycan synthesis, and AmiA is an amidase allowing segregation between the two daughter cells. FtsK is a protein recruited to the FtsZ ring before final septal peptidoglycan synthesis by BPB-3 and the separation of daughter cells by amidase action. Thus, in persistent *C. trachomatis*, septum synthesis does not occur because PBP-3 is not recruited. Also, among 3 hsp60-encoding genes (*groEL*, *ct604* and *ct755*), *ct755* expression predominates during productive infection, whereas *ct604* product is abundant during persistent infection. Thus, the *ct604* product may perform some functions and/or maintain of the persistence state (Gerard *et al.,* 2004).

In conclusion, these findings taken together allow us to say that persistent particles are viable entities in which gene transcription and regulation occurs. Since these persistent

particles have been found only under unfavourable conditions, it is logical to think that they are a means for *C. trachomatis* to avoid adverse conditions such as those generated by the host immune response.

As mentioned above, replication and transcription continue in chlamydial persistence particles. However, compounds blocking chromosomal replication, e.g. ciprofloxacin and ofloxacin, equally induce a persistence state (Dreses-Werringloer *et al.,* 2000). No studies have been undertaken to show if DNA replication also occurs in persistence particles induced in these situations. Actually, it would be interesting to know how persistent *C. trachomatis* continues its DNA replication despite the presence of quinolones that normally block transcription and replication. More investigations are required to characterize chlamydial persistence.

2.8 ESCAPE MECHANISMS TO AVOID THE HOST DEFENCE SYSTEM

To persist inside their hosts, micro-organisms must develop various strategies. For example, to persist, the herpes simplex virus must remain latent in epidermal demyelized neurones where it is not accessible to host immune cells. Equally, *C. trachomatis* persistence implies strategies allowing it to avoid the host immune system. Here, possible strategies that may be used by *C. trachomatis* to avoid host immune defence are suggested.

2.8.1 Insufficient antichlamydial immunity

Various studies have disclosed that the CD4+Th1 response is absolutely required to resolve primary infection, even when CD4+Th2 response is ineffective. CD4+Th1 response leads to antichlamydial immunity by allowing increased opsonization, cytotoxic T lymphocyte (CTL) activation, and above all, high production of chlamydistatic IFN-γ. However, in the CD4+Th2 response, IFN-γ production is too low to exert chlamydistatic effects. In addition, it has been reported that even with the transfer of CD4+Th2 clones in nude mice with genital infection resulting in antibodies against multiple chlamydial antigens, the infection is only reduced but not resolved (Hawkins *et al.,* 2002). Such a response is definitely regarded as ineffective because it does not dislodge RBs or

persistent intracellular particles. Cytokines play an important role in induced immune response polarization. IFN-γ and interleukin (IL)-12 favours Th1 responses, but inhibit Th2 responses. In contrast, IL-4 and IL-10 stimulate Th2 responses, but inhibit Th1 responses. For this reason, initial steps during infection are crucial in the induction of an appropriate response. Several authors have noticed that IFN-γ and IL-12 required to polarize the immune response towards the Th1 profile could come from natural killer cells and dendritic cells, respectively (Tseng and Rank, 1998; Matyszak *et al.*, 2002). However, the early source of IL-4 and IL-10 allowing the induction of Th2 responses is not known.

The question is: can persistent *C. trachomatis* modulate the host response so as to favour ineffective Th2 response. Until now, no studies have been conducted that correlate ineffective Th2 responses with chlamydial persistence. On the other hand, we know that some genetic and environmental factors can favour Th2 responses. Actually, a study in Gambia showed a significant association between IL-10-1082G and scar trachoma in an ethnic group. This allele favours IL-10 production that allows induction of Th2 responses (Mozzato-Chamay *et al.,* 2000). Likewise, some parasites and bacteria, including *Trichomonas vaginalis* and *Neisseria gonorrhoeae,* activate IL-10 synthesis (Jeremias *et al.,* 1998; Cohen *et al.*, 1999). Thus, *C. trachomatis* co-infection with such organisms rather favours Th2 responses. Finally, genetic factors and the genital environment may evoke or maintain chlamydial persistence. It is also possible that effective Th1 responses could be induced initially but subverted later. Actually, a chlamydial protease able to degrade host transcriptional factors required for major histocompatibility gene activation has been identified (Zhong *et al.,* 2001).

2.8.2 Low inflammatory response

Epithelial cells infected by *C. trachomatis* produce various pro-inflammatory cytokines including, IL-8, Groα, granulocytes/macrophages-colony stimulating factor (GM-CSF), IL-1α and IL-6. These cytokines could be important in initiating the immune response (Rasmussen *et al.,* 1997). The greater the chlamydial growth, the more significant is cytokine synthesis. Seeing that *C. trachomatis* remains in growth arrest in the persistence

state, it is logical that the amount of cytokines produced is reduced. As a result, the inflammatory response could be diminished. For example, *C. pneumoniae* (*Chlamydophila pneumoniae*) remained viable but latent 30 days after treatment with various antichlamydial compounds. However, under these conditions, the amount of secreted IL-6 and IL-8 were severely reduced compared to that occurring during productive infection (Kutlin *et al.*, 2002). Thus, the authors suggested that decreased cytokine synthesis is not due to antichlamydial compounds, but rather to a lower number of chlamydial particles in the host cell. Chlamydial persistence, which is expressed by reduced metabolic activities, could lead to cytokine secretion diminution with a decline of inflammation intensity and, consequently, other immune responses. These could contribute to persistence.

2.8.3 Apoptosis modulation

Since persistent chlamydial particles are hidden in host-infected cells, they avoid humoral-mediated immunity. For that reason, CD8+ T cells are required to kill infected cells in order to dislodge persistent *Chlamydia*. Actually, it has been shown that persistently-infected L cells are recognized and lysed by CD8+ T cells *in vitro* (Beatty and Stephens, 1994; Rasmussen *et al.,* 1996). Infected cells can also be killed by apoptosis. Thus, persistence occurs because CD8+ T cells and apoptosis fail to kill persistently-infected cells *in vivo*.

This review of the literature allows us to suggest the possibility that *C. trachomatis* modulates apoptosis in order to persist. Actually, it has been reported that *C. trachomatis* could induce CTL programmed death to suppress cytotoxic activities. Some studies have shown that macrophages persistently infected with *C. trachomatis* serovar K elicit lymphocyte apoptosis. On the other hand, if macrophages are infected with irradiated EBs, they do not provoke lymphocyte death (Jendro *et al.,* 2000). It appears that several mechanisms are implicated in lymphocyte apoptosis induction by infected macrophages. An humoral mechanism, through TNF-α, permits lymphocyte apoptosis at a distance, before these lymphocytes interact with infected cells. In addition, lymphocytes that

already interact with persistently-infected cells can be killed by apoptosis (Jendro *et al.*, 2004).

In addition to escape the CTL, persistent *C. trachomatis* must remain inside the host cell to avoid humoral-mediated immunity. For that reason, persistent chlamydial particles must block infected cell apoptosis. Actually, it has been demonstrated that epithelial cells persistently infected with *C. trachomatis* serovar A after IFN-γ treatment are resistant to apoptosis induced by different agents, such as ectoposide and stauroporin (Dean and Powers, 2001). Reversion of persistent chlamydial particles into normal particles, after removal of IFN-γ from the culture medium, ultimately leads to epithelial cell apoptosis. This means that persistent chlamydial particles, but not RBs, block the programmed death of infected cells. In contrast, to spread, active RBs may induce apoptosis of infected cells (Perfettini *et al.*, 2003). The question is: how does persistent *C. trachomatis* elicit lymphocyte apoptosis and simultaneously block infected cell apoptosis. This possibility suggests that a chlamydial compound could be translocated from infected cells towards the extracellular medium to prompt just lymphocyte apoptosis. However, the translocation mechanism required in this process is not known.

2.8.5 Privileged tissue localization

A specific microenvironment can also favour persistence. In the eyes, *C. trachomatis* infects the conjunctival epithelium. However, infected tarsal conjunctiva cover the ocular bulb, including the cornea, every time the eyes are closed. Thus, the corneal epithelium and even stem cells in the limbus could be equally infected. If this is the case, one can understand why persistence is maintained. Actually, the cornea does not have vessels and is inaccessible to immune system cells. Nothing stops us from suggesting that chlamydial tissue in the genital tract is not reached by immune cells.

2.9 CONCLUSION

Clinical reports, *in vitro* and *in vivo* studies offer new ways of investigating chlamydial persistence to better understand *C. trachomatis* disease pathogenesis. Although *in vitro* models offer means of examining persistent particles, they do not adequately reflect the

complex *in vivo* environment to allow the establishment of different interactions that lead to development of persistence. Induced immune responses, nutritional deficiency, environmental changes due to hormonal fluctuations and antibiotic pressure are multiple factors to which *C. trachomatis* is bound *in vivo* and which can influence its growth. However, even if chlamydiologists agree that *C. trachomatis* can become persistent, there is still a lot to do to understand its features of persistence. The role of the persistent stage in the *C. trachomatis* developmental cycle as well as the molecular mechanism allowing persistence formation remains to be determined. Understanding these different mechanisms will open up new therapeutic avenues that could be deployed against diseases due to this intracellular bacterium.

2.10 ACKNOWLEDGEMENTS

We thank Ovid Michael Da Silva for editing this manuscript. We precise that M.P. is the recipient of a"Programme Canadien des Bourses de la Francophonie" scholarship.

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CHAPITRE III:

LES MALADIES CAUSÉES PAR *CHLAMYDIA TRACHOMATIS*

3.1 INTRODUCTION

La bactérie intracellulaire obligatoire et pathogène de l'humain, *C. trachomatis*, est responsable de différentes infections sévissant dans le monde entier. Ces maladies, généralement désignées chlamydioses, regroupent la conjonctivite, la lymphogranulome vénérienne, la cervicite chez la femme, l'urétrite non gonococcique chez l'homme, l'ophtalmie néonatale. Le plus souvent asymptomatiques, les chlamydioses aiguës peuvent spontanément être résolues grâce à une réponse immunitaire générée efficace et/ou à la suite de l'antibiothérapie. Cependant, non résolues, les chlamydioses progressent et occasionnent des pathologies chroniques plus graves causant les séquelles souvent indélébiles.

C. trachomatis est subdivisé en 19 serovars de tropisme différent. Les sérovars oculaires, A à C, infectent l'épithélium conjonctival et conduisent à une conjonctivite évolutive causant le trachome, responsable de la cécité curable. Les serovars oculo-genitales, D à K, occasionnent non seulement les infections génitales, mais aussi oculaires. Les infections génitales résultantes évoluent souvent en maladies inflammatoires pelviennes chroniques pouvant altérer la fonction reproductive de l'appareil génital de la femme. Enfin, les sérovars genitaux, L1-L3, demeurent capables de proliférer dans les monocytes et ainsi occasionner l'infection systémique, la lymphogranulome vénérienne. Ce chapitre décrit brièvement les chlamydioses et les mécanismes de pathogenèse impliqués. Les approches thérapeutique et vaccinale y sont également succinctement décrites.

3.2 LES CHLAMYDIOSES

3.2.1 Le trachome

Le trachome est une kérationoconjonctive causée par les infections répétées aux sérovars A, Ba, B et C de *C. trachomatis*. Le trachome se développe en plusieurs stades. Le stade actif prévaut plus chez les jeunes individus, âgés de moins de 25 ans. Ce stade est caractérisé par une infiltration de mononucléaires et de polymorphonucléaires organisés en follicules, au niveau de la conjonctive. Une vascularisation plus prononcée de la cornée supérieure (Pannus) y est également observée. Après plusieurs années de réinfections, une inflammation chronique conduisant aux lésions des tissus s'installe. La réparation du tissu lésé conduit finalement à la cicatrisation caractérisant des infections à *C. trachomatis*. Le tissu cicatriciel plus rigide le long de la membrane basale conjonctivale, est composé de collagène de type IV et V, au lieu du collagène de type I et III plus lâche, normalement trouvé au niveau du trauma. À mesure que la cicatrisation progresse, il s'ensuit l'éversion de la paupière supérieure (entropion). Il en résulte également l'affaiblissement des glandes lacrymales accessoires et l'abrasion de l'oeil par les cils, ce qui a pour conséquence la sécheresse oculaire (trichiasis). Ceci, au bout du compte, conduit à l'opacité de la cornée, d'où la cécité résultante (Mabey et Fraser-Hurt, 2001).

Les événements pathologiques responsables des dommages de l'épithélium conjonctival et leur cicatrisation ne sont pas tout à fait connus. Les cytokines qui médient les réponses immunitaires semblent jouer un rôle. Les cytokines fibrogènes dont l'IL-1, le TNF-α et le PDGF semblent, en effet, intervenir dans la formation des fibroses du trachome (Abu El-Asrar et *al.*, 1998; Abu El-Asrar et al., 2001). En stimulant l'expression du facteur de croissance du tissu connectif (CTGF), le TGF-β serait également impliqué dans l'immunopathogenèse du trachome. Le CTGF induit la synthèse du collagène par les fibroblastes de la cornée (Blalock et *al.*, 2003). La gélatinase B, retrouvée dans les tissus de biopsie des individus souffrant du trachome, est probablement sécrétée par les macrophages et pourrait être à l'origine de la dégradation de la matrice des tissus. Également, le peptide antimicrobien, la β-défensine, exprimée de manière chronique dans les tissus intraoculaires, peut promouvoir la prolifération des cellules et la formation de la

fibrine (Haynes *et al.*, 2000). Le processus de cicatrisation se produit certainement en plusieurs étapes et doit requérir plusieurs composés. Des études supplémentaires sont requises pour étayer le processus.

Si aujourd'hui le trachome est quasi-éradiqué de l'Amérique du nord et de l'Europe, la maladie continue de sévir dans les pays pauvres. En effet, elle demeure encore endémique dans 48 pays, dont les régions d'Afrique sub-sahélienne, le sud et le centre de l'Asie, l'Amérique latine et l'Australasie. Environ 150 millions de personnes, dans le monde entier, en sont atteintes, soit 10 millions atteints de trichiasis et 6 millions atteints de cécité. La maladie est transmise de l'œil à l'œil, par les doigts, les vêtements souillés, la toux et les éternuements, et par l'espèce de mouche *Musca sorbens*. Pour éliminer le trachome d'ici 2020, en tant que problème de santé publique, l'organisation mondiale de santé (OMS) a proposé la stratégie SAFE en Anglais ou CHANCE en Français. Par l'acronyme SAFE, il est décrit les moyens à adopter dans une communauté pour traiter les différents stades du trachome et réduire les risques de transmission de la maladie. La stratégie CHANCE (SAFE) promouvoit la **ch**irurgie (**s**urgery) pour remédier à l'entropion et la trichiasis, les **a**ntibiotiques (**a**ntibiotics) pour traiter la maladie active, le **n**ettoyage facial (face washing), le **c**hangement de l'**e**nvironnement (**e**nvironnemental improvement) par l'éducation et le développement de l' économie locale.

3.2.2 Les infections oculo-génitales

3.2.2.1 Chez la femme

Malgré les progrès de la médecine, les sérotypes chlamydiens D à K demeurent les causes majeures des maladies sexuellement transmissibles dans le monde. L'organisation mondiale de santé (OMS) estime qu'environ 92 millions de personnes, incluant les femmes et les hommes, en sont infectées chaque année dont 4 millions en Amérique du nord, 15 millions en Afrique et plus de 45 millions en Asie du sud. 70 à 80% des infections génitales à *C. trachomatis* chez la femme demeurent asymptomatiques. 50% de ces infections sont spontanément résolues, alors que 10% des infections non résolues

progressent de l'épithélium cervical jusqu'au tractus génital supérieur et induisent les maladies inflammatoires pelviennes (PIDs). Chez la femme, *C. trachomatis* est responsable de plus de la moitié de cas de PIDs répertoriées (Paavonen et Lehtinen, 1996). Comme dans le cas du trachome, les PIDs chroniques induisent les fibroses et la cicatrisation des épithéliums, endommagent l'appareil génital. Il peut alors en résulter l'infertilité tubaire ou les grossesses ectopiques (Toth et *al.*, 2000; Bjartling et *al.*, 2000). L'étude classique de Westrom (1995) indique que l'incidence du facteur tubaire de sub-fertilité est approximativement de 10% après un épisode de PID, environ 20% après 2 épisodes de PID et 40% après 3 épisodes. En effet, comme *C. trachomatis* envahit en particulier l'épithélium prismatique, le segment ampullaire densément cilié des trompes de fallopes en est susceptible. Par cette voie, les PIDs peuvent occasionner l'occlusion tubaire distale et l'infertilité subséquente, ou l'occlusion distale partielle avec un risque plus élevé de grossesses ectopiques (Figure 1). Si les infections silencieuses ascendantes ou récurrentes sont impliquées dans la progression des chlamydioses, il est également constaté la progression rapide post-partum ou post-avortement du pathogène, de l'épithélium cervical au tube utérin (Barbacci et *al.*, 1986; Wölner-Hansen et *al.*, 1981). Le risque des infections ascendantes est également plus élevé durant l'instrumentation utérine comme lors de l'hystérosalpingographie et de la laparoscopie avec hydrotubation, qui constituent les moyens utilisés pour détecter les pathologies tubaires chez les femmes sub-fertiles. Les infections ascendantes occasionnent diverses autres pathologies dont la salpingite, la péri-hépatite (syndrome de Fitz-Hugh-Curtis), la péritonite (Mårdh et *al.*, 2004). Les infections génitales chlamydiennes seraient également impliquées dans le cancer utérin.

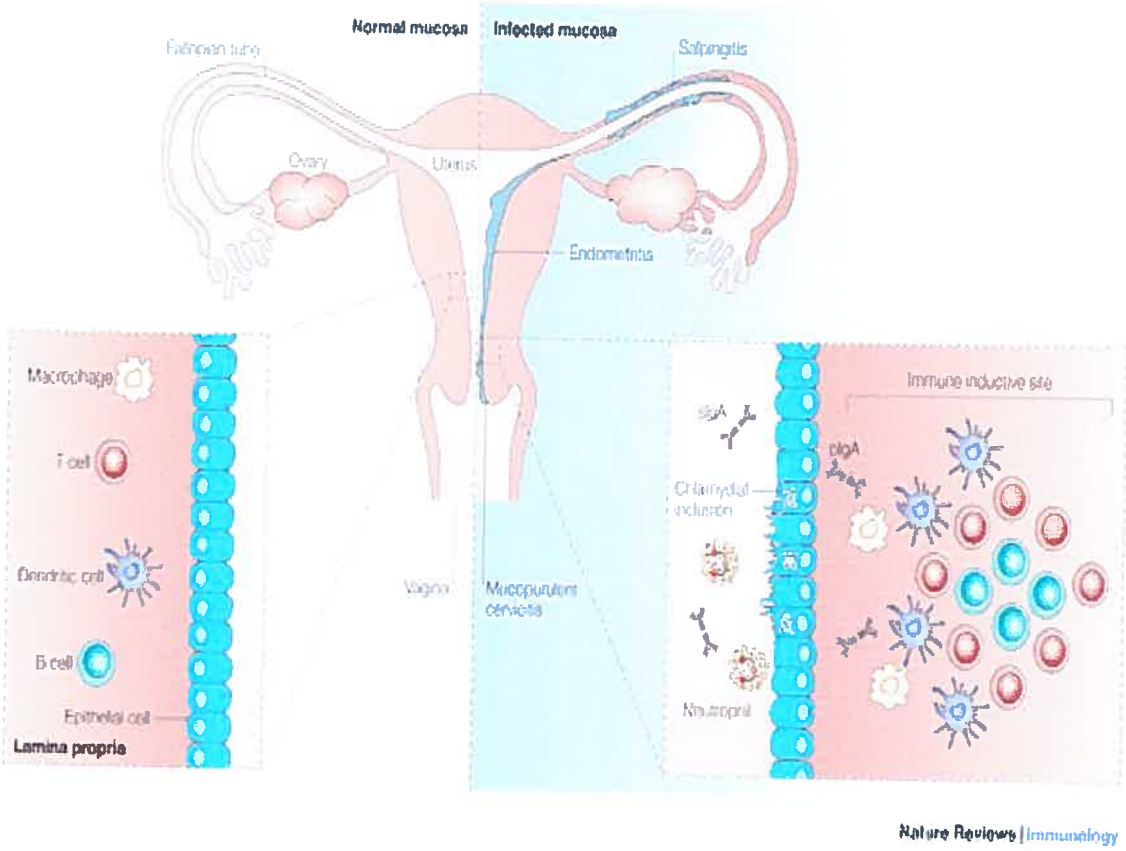

Figure 1. Infection du tractus génital de la femme par *Chlamydia trachomatis*. Les corps élémentaires de *C. trachomatis* infectent les cellules prismatiques cervicales. Souvent asymptomatiques, de telles infections peuvent progresser jusqu'à l'endomètre et aux tubes de Fallope, causant les maladies pelviennes inflammatoires et l'inflammation tubaire (salpingite), la cicatrisation et l'occlusion tubaires responsables de l'infertilité et de grossesses ectopiques. La réaction inflammatoire est caractérisée par l'influx des macrophages et des neutrophiles, la formation des sites inductifs des cellules immunitaires. Ces sites qui contiennent les cellules B, T et dendritiques, cordonnent l'initiation de la réponse immunitaire acquise incluant le déploiement des IgA sécrétrices, pIgA, polymérique IgA. **Tiré de R.C. Brunham and J. Rey-Ladino. 2005. Nat. Rev. Immunol. 5, 149-61.**

3.2.2.2 Chez le nouveau-né et le nourrisson

C. trachomatis demeure le pathogène le plus couramment retrouvé dans le tractus génital de la femme enceinte. La contamination transplacentaire est exceptionnelle, mais peut survenir et aboutir à différents dommages: l'avortement spontané (Novikova, 2000), la mort fœtale, la rupture prématurée des membranes et la prématurité (Kovacs et *al.*, 1998). Le mécanisme par lequel *C. trachomatis* cause les dommages sur le fœtus n'est pas clair. Hsp60 chlamydien pourrait jouer un rôle crucial dans le processus de l'inflammation chronique. L'exposition prolongée de Hsp60 chlamydien concomitamment à Hsp60 humain induirait une réponse immunopathologique endommageant les tubes de Faloppe. Or, Hsp60 est la première protéine synthétisée par le zygote après fécondation et est également exprimée par la décidue maternelle (Grigore et Indrei, 2001). Les lymphocytes anti-Hsp60 auto-réactives induites dans ce contexte pourraient alors causer les dommages aussi bien sur le zygote que sur le fœtus (Kligman et *al.*, 1998; Ziegert et *al.*, 1999; Sziller et *al.*, 1998). De même, Hsp10 de *C. trachomatis* serait impliquée dans l'inflammation chronique et les séquelles résultantes. Hsp10 chlamydien exhibe, en effet, 33.3% d'homologie avec le facteur précoce de grossesse ayant à la fois une immunosuppressive et une activité de croissance. Chez la souris, l'inhibition de ce facteur de croissance retarde le développement de l'embryon et/ou l'inhibition de l'implantation. Betsou et *al.* (2003) ont montré que les anticorps anti-hsp10 peuvent reconnaître le facteur précoce de grossesse. De plus les anticorps dirigés contre le facteur de grossesse précoce ont été retrouvés chez la femme enceinte ayant déjà été infectée dans le passé par *C. trachomatis*.

L'infection *in utero* demeure peu probable (Numazaki et *al.*, 2003). Le pathogène peut néanmoins se transmettre au nouveau-né lors du passage dans le canal vaginal. De tels nouveaux-nés sont parfois sujets à la conjonctivite ou à la pneumonie néonatales (Mårdh, 2002) et développer plus tard des séquelles graves parmi lesquelles se trouve l'obstruction chronique des poumons (Harrisson et *al.*, 1982). La possibilité que *Chlamydia* puisse être à l'origine de cas de syndromes de la mort subite du nouveau-né reste encore à démontrer (Lundemose et *al.*, 1990).

3.2.2.3 Chez l'homme

Les infections génitales à *C. trachomatis,* chez l'homme, se présentent sous forme d'urétrite. Environ 50% de ces infections demeurent asymptomatiques et peuvent engendrer l'épididymite et la prostate. Bien que *C. trachomatis* soit soupçonné de coloniser le tractus génital supérieur de l'homme, il n'y a aucune évidence que cela entraîne l'occlusion du système canaliculaire génital et des dommages aux cellules épithéliales requises dans la spermatogenèse. En fait, le rôle de *C. trachomatis* dans l'infertilité masculine demeure très controversé. *C. trachomatis* pourrait entraîner l'infertilité chez l'homme en occasionnant le dysfonctionnement des gamètes. En effet, *C. trachomatis* sérovar E pourrait s'attacher à travers leurs LPS aux spermatozoïdes et provoquer la phosphorylation de la tyrosine des protéines de surface du gamète. Ceci aurait pour conséquences la capacitation précoce, la réduction de la motilité ou la mort du gamète (Hosseinzadeh et *al.*, 2000; Hosseinzadeh et *al.*, 2001; Hosseinzadeh et *al.*, 2003). *C. trachomatis* induirait également la formation de l'oxygène réactif qui peroxyderait les lipides membranaires, infligeant ainsi les dommages à la membrane plasmique du gamète mâle et altérant les enzymes présentes (Segnini et *al.*, 2003). Bien que *C. trachomatis* semble endommager les gamètes mâles *in vitro*, cela pourrait ne pas refléter la réalité *in vivo*. Les travaux récents d'Hosseinzadeh et *al.* (2004) ont montré que la qualité et motilité des gamètes des individus infectés à *C. trachomatis* n'étaient nullement affectées. Il a été également suggéré que les anticorps anti-hsp60 humains induits en réponse à l'infection chlamydienne puissent neutraliser les spermatozoïdes humains et empêcher leur fonction fécondatrice. Cependant, les travaux de Eggert-Kruse (2002) montrent que bien que les IgA et les IgG anti-Hsp60 soient présents dans le sperme des individus ayant une infection chlamydienne asymptomatique, l'intégrité et la fonction des spermatozoïdes ne sont pas altérées. Au vu de ces différentes études il est suggéré que la réduction de taux de grossesses constatée dans les couples infectés par *C. trachomatis* puissent être les résultats des effets occasionnés uniquement au niveau de l'appareil reproductif de la femme (Idahl et *al.*, 2004). D'autres approches expérimentales doivent être adoptées afin de conclure définitivement les effets de *C. trachomatis* sur le système reproducteur de l'homme.

Chez l'homme, 1-3% des infections chlamydiennes progresseraient en arthrite réactive (Wollenhaupt et Zeidler, 1990). Étant donné que différents sérotypes dont le K peuvent infecter les monocytes et y demeurer dans un état latent, Koehler et *al.* ont suggéré que les monocytes ainsi infectés puissent se rendre dans les jointures et occasionner une multitude d'événements responsables de l'arthrite (Koehler et *al.*, 1997). *C. trachomatis* est, de plus, capable d'infecter les fibroblastes synoviaux et y demeurer latent *in vitro* (Hanada et *al.*, 2003). Parmi les événements induits occasionnant l'arthrite, il est proposé que *C. trachomatis* puisse rompre la tolérance à l'allèle HLA B27, ce qui expliquerait l'association de l'arthrite avec la présence de cet allèle (Popov et *al.*, 2002).

3.2.3 La lymphogranulome vénérienne

La lymphogranulome vénérienne (LVG), ou maladie de Nicolas-Favre, est une affection sexuellement transmissible relativement rare, y compris dans les zones d'endémicité actuelle dont l'Inde, l'Afrique sub-saharienne, l'Amérique du sud et les Caraïbes. Il est de plus en plus rapporté l'apparition d'une forme speciale de la maladie, la LVG rectale, chez les hommes homosexuels, dans les pays industrialisés (Etats-Unis, Royaumes unis, France) (Weir, 2005). La LGV représenterait selon les régions 1 à 10% des étiologies des ulcères génitaux. Les sérotypes de *C. trachomatis* L1-L3, responsables de la LVG, infectent les cellules sub-mucosales, traversent la matrice extracellulaire pour infecter les monocytes et les macrophages (Davis et Wyrick, 1997). Dès lors, le pathogène se propage dans les nœuds lymphatiques régionaux et occasionne la LVG dont les signes cliniques sont subdivisés en 3 stades. 3 à 40 jours après l'inoculation, un chancre primaire, petit et herpétiforme, qui guérit spontanément apparaît au niveau du site de l'inoculation (prépuce, vulve...). Le stade secondaire aiguë apparaît 2 à 6 semaines plus tard et s'exprime par l'adénopathie inguinale et/ou crurale confluente, réalisant un bubon. Non ou mal traitée, la maladie évolue en un stade tardif ou tertiaire. Dans cette situation les fibroses se forment et peuvent causer l'obstruction lymphatique, occasionnant l'éléphantiasis des organes génitaux. La participation rectale peut mener à la formation des strictures et des fistules. Cet ensemble de manifestations est connu chez la femme comme étant le syndrome esthiomiène (Mabey et Peeling, 2002).

3.3 IMMUNITÉ ANTI-CHLAMYDIENNE

Des infections à *C. trachomatis* peuvent en réalité être résolues spontanément, sans requérir un traitement thérapeutique quelconque (Morre et *al.*, 2002; Joyner et *al.*, 2001). La résolution spontanée de telles infections suggère une immunité antichlamydienne vigoureuse et protectrice, induite à la suite d'une infection chlamydienne. Pour caractériser la réponse immunitaire protectrice, les modèles animaux ont souvent été utilisés. Chez la souris par exemple, la réponse initiale induite, survenant 1 à 2 jours post-infection, est caractérisée par l'inflammation avec l'infiltration mucosale des neutrophiles, des lymphocytes T et des monocytes (Morrison et Morrison, 2000). Les neutrophiles abondamment recrutés au site de l'infection jouent un rôle prépondérant puisqu'ils détruisent les CEs accessibles (Register et *al.*, 1986; Barteneva et *al.*, 1996). Si les neutrophiles ne permettent pas l'éradication du pathogènes, ils limitent néanmoins la propagation de l'agent infectieux. Dans ce processus de défense non spécifique, les cellules tueuses naturelles (NK) jouent également un rôle important dans le contrôle de l'infection initiale. Si la déplétion des cellules NK chez les souris, à l'aide d'anticorps anti-asialo GM1, ne réduit pas immédiatement le nombre de particules chlamydiennes infectantes dans le tractus génital, elle retarderait la résolution de l'infection (Tseng et Rank, 1998). Les capacités défensives des cellules NK ne proviennent pas uniquement de leurs effets cytotoxiques, mais également de leur contribution au développement de la réponse Th1 robuste grâce à l'IFN-γ et l'IL-12 qu'elles produisent. En effet, l'éradication de *C. trachomatis* requiert absolument et précisément la réponse spécifique de type Th1.

Les cellules dendritiques (DC) jouent un rôle primordial dans l'induction de la réponse acquise. Elles sont, en fait, les seules parmi les cellules présentatrices d'antigènes, capables une fois activées à la suite de leur internalisation antigénique, de migrer dans les différents ganglions lymphatiques afin d'initier la réponse Th1. Les DC différenciés à partir des PBMC humains étaient capables de présenter efficacement les antigènes de *C. trachomatis* sérovar L2 en association avec le CMH de classe I et II et pouvaient ainsi induire une réponse antichlamydienne de CD4+ et CD8+ spécifique, *in vitro* (Matyszak et *al.*, 2002). Ainsi activées, les DC produisaient le TNF-α et l'IL-12, mais pas l'IL-10, ce qui favoriserait plus la réponse Th1, plutôt que Th2 (Matyszak et *al.*, 2002).

Toutes les études menées ces dernières années confirment que les cellules Th1 CD4+, non pas les cellules Th2 CD4+ et les cellules T CD8+, sont requises pour la résolution des infections chlamydiennes primaires. Les souris n'exprimant pas la molécule de CMH de type II sont, en effet, incapables de résoudre l'infection chlamydienne génitale initiale (Morrison et *al.*, 1995). En général, La réponse CD4+Th1 aboutit à l'immunité à médiation cellulaire en permettant l'expression par les cellules B des IgG2b opsonisants et l'activation des cellules T CD8+ cytotoxiques. À l'opposé, les cellules CD4+ Th2 jouent un rôle important dans l'immunité humorale en conférant une aide aux cellules B afin qu'elles expriment différents anticorps non opsonisants. Bien que la réponse Th1 permette l'expression des CTL, sa capacité à remédier l'infection chlamydienne est plutôt attribuée à l'IFN-γ produit. L'IFN-γ exhibe des effets divers chlamydiastatiques parmi lesquels se trouve sa capacité à induire l'indoléamine 2,3-dioxygénase, une enzyme qui cause la déplétion en tryptophane. Ceci a pour conséquence, l'inhibition de la croissance de *Chlamydia* qui est auxotrophe pour cet acide aminé (Byrne et *al.*, 1989). L'IFN-γ permet aussi l'activation des macrophages. Cependant, un autre mécanisme d'éradication des infections chlamydiennes primaires, indépendant de l'IFN-γ est également soupçonné mais demeure à identifier (Perry et *al.*, 1997).

Jusqu'à présent, le rôle des cellules T CD8+ dans l'immunité antichlamydienne demeure controversé. Pourtant, *C. trachomatis* est capable d'induire une population de CTL CD8+ actives (Beatty et *al.*, 1994; Fling et *al.*, 2001). Les protéines de l'inclusion chlamydienne, Cap1 (31 kDa) et CrpA (15 kDa) injectées intraveineusement aux souris induisent la réponse de cellules T CD8+, partiellement protectrice (Fling et *al.*, 2001; Starnbach et al., 2003). Au contraire, les travaux de Morisson et *al.* (2000) ont constaté que la déplétion des cellules TCD8+ par l'injection des anticorps anti-CD8+ chez la souris C57BL/6, n'avait aucun effet sur l'évolution de l'infection. Ces résultats quelque peu discordants pourraient s'expliquer par l'abolition de la réponse T CD8+ induite. Une protéase chlamydienne capable de dégrader les facteurs de transcription de l'hôte requis pour l'activation des gènes du CMH a été, en effet, récemment identifiée (Zhong et *al.*, 2001). S'il est sensé que les cellules T CD8+ puissent participer à l'immunité anti-chlamydienne, les investigations doivent être plus poussées afin de clarifier leur contribution dans l'immunité antichlamydienne.

À la différence de la réponse Th1, la réponse Th2 serait inefficace dans la résolution des infections chlamydiennes primaires. Chez les souris nues ayant reçu les clones de cellules CD4+Th2, les IgA et les IgG1 produits atténuaient l'infection, mais ne la résolvaient pas (Hawkins et *al.*, 2002). De plus, l'adressage (homing) de ces cellules au niveau de la muqueuse du tractus génital était réduit, à cause de leur faible expression du ligand de P-selectin. La réponse Th2 induite durant une première infection est définitivement perçue comme inefficace, car elle ne confère aucune possibilité de déloger les CRs intracellulaires. Non résolue, l'infection primaire prolongée deviendrait chronique et dommageable. C'est dans ce sens que la réponse Th2 est qualifiée de pathologique. Aussi, les IgA et les IgG1 uniquement produits sous le profil de la réponse Th2, sont considérés comme les indicateurs de l'infection chronique (Mouton et *al.*, 2002).

La réponse Th2 est néanmoins importante durant les réinfections. Grâce à l'aide procurée par les cellules Th2 CD4+, les cellules B pourraient produire les anticorps qui avec les cellules Th1 CD4+ participeraient à la résolution de la réinfection. Morrison et *al.* (2001) ont montré que les souris déplétives en cellules CD4+ et CD8+, résistent aux réinfections aussi bien que ne le font les souris immunocompétentes. La fonction effectrice des anticorps dans ce cas-ci serait médiée par le récepteur Fc (FcR). Moore et *al.* (2002) ont montré que l'infection primaire chez la souris sauvage et chez celle mutante FcRKO est identique, alors que l'infection secondaire n'est plus intense que chez la souris FcRKO. Il semble que la cytoxicité dépendant des anticorps (ADCC) et la présentation des antigènes chlamydiens soit plus accrue durant les réinfections.

3.4 L'IMMUNOPATHOGÉNÈSE

Les différentes études épidémiologiques révèlent que les épisodes multiples de PID prédisposent les femmes aux séquelles graves dont les dommages tissulaires et l'infertilité. De plus, chez les primates, les séquelles ne sont observées qu'à la suite des récurrences multiples (Patton et *al.*, 1987). Les infections chlamydiennes chroniques ou récurrentes sont associées aux séquelles graves observées. Plusieurs mécanismes sont émis pour expliquer l'immunopathologie associée aux maladies dues à *C. trachomatis*.

Le premier mécanisme stipule que parce que la réponse Th2 est inefficace, son induction lors d'une inoculation initiale prolonge l'infection et conduit à une inflammation prolongée et donc délétère. Yang (2001), utilisant un modèle murin d'infection de poumon, a identifié deux type d'hypersensibilité de type retardé (DTH) induits suite à l'infection par *Chlamydia*: le DTH de type Th1 protectrice et le DTH de type Th2 pathologique. Durant le DTH de type Th2, l'inflammation est caractérisée par une infiltration de mononucléaire uniquement, notamment les neutrophiles et les éosinophiles. Les neutrophiles jouent un rôle très important dans le développement des pathologies et de l'infertilité, car ils causent les dommages potentiels à travers la libération des protéinases et les hydrolases. De plus, les neutrophiles lorsque exposés au GM-CSF, à l'IL-8 et au TNF-α, produisent de quantités plus importantes d'intermédiaires d'oxygène réactive et de granules enzymatiques très cytotoxiques.

Un autre mécanisme implique la protéine du choc thermique de 60 kDa (Hsp60). Plus récemment, Hsp10 a été aussi associée au facteur tubaire de fertilité (LaVerda et al., 2000). Les Hsp forment un groupe de chaperonnes de 15 à 110 kDa qui sont produites en réponse à différentes sortes de stress notamment la chaleur. Elles sont importantes dans la survie cellulaire prévenant la dénaturation ou l'agrégation anormale des protéines en préservant leur conformation. Hsp60 est une protéine phylogénétiquement conservée et retrouvée chez tous les organismes allant des bactéries aux humains. Chez les *Chlamydia*, Hsp60 est requise dans l'assemblage de la protéine majeure de la membrane externe (MOMP) durant la dédifférenciation des CRs en CEs. Les Hsp60 humaine et chlamydienne exhibent environ 48% d'homologie (Viale et *al.*, 1994). Différentes données montrent que 16-25% de femmes fertiles possèdent les anticorps anti-Hsp60, comparé à 36-44% chez les femmes ayant une cervicite à *C. trachomatis*, 48-60% chez les femmes ayant la maladie inflammatoire pelvienne chlamydienne et 81-90% chez les femmes ayant les dommages tubaires dû par *C. trachomatis* (Toye et *al.*, 1993; Brunham et *al.*, 1992; Eckert et *al.*, 1997). Si ces données suggèrent fortement le rôle de Hsp60 dans la pathogenèse de maladies à *C. trachomatis*, les mécanismes précis impliqués demeurent encore à déterminer.

L'induction de la réponse non spécifique par différentes Hsp60 pourrait constituer un mécanisme potentiel par lequel les infections chlamydiennes induisent les maladies auto-immunes souvent caractérisées par une inflammation chronique. En effet, en situation de stress mutuel les particules chlamydiennes et les cellules-hôte infectées expriment à leur surface leurs hsp60 correspondantes. De plus, avec les cycles lytiques, les molécules de hsp60 aussi bien humaines que chlamydiennes, habituellement intracellulaires, sont libérées de la cellule. L'interaction de ces protéines avec les monocytes, les macrophages et les cellules dendritiques via les récepteurs Toll-like (TLR 2 et 4) occasionne la libération de différentes cytokines (TNF-α, IL-1, IL-6 et IL-12) et l'oxyde nitrique (NO) (Chen et *al.*, 1999; Bulut et *al.*, 2002 . À travers leur fonction d'induction de cytokines pré-inflammatoires, les Hsp60, même humaines, sont considérées comme un signal dangereux pour le système immunitaire inné. Cependant, bien plus d'études sont requises pour démontrer si l'induction de cytokines observée est véritablement due aux molécules de Hsp60 et non pas aux LPS contaminant (Gao et Tsan, 2003).

Bien que les réactions immunitaires spécifiques induites par Hsp60 chlamydienne au cours de l'infection ne soient pas exactement identifiées, on sait qu'elles pourraient exercer des réactions croisées et participer ainsi à la pathogenèse des maladies à *C. trachomatis*. Les clones de cellules T spécifiques à *Chlamydia* isolés des plaques sclérotiques se sont révélés réactives aussi bien avec un épitope de Hsp60 chlamydienne qu'humaine (Mosorin et *al.*, 2000). Perschinka et *al.* (2003) ont identifié 8 épitopes distincts retrouvés sur toutes les Hsp60/65 de l'humain, de *C. trachomatis*, d'*Escherichia coli* et de *Mycobacterium tuberculosis*. Les anticorps contre les épitopes de Hsp60 humaine réagissent non seulement avec Hsp60 humaine, mais également avec toutes les autres Hsp60 microbiennes. Ces épitopes communs peuvent servir de cibles auto-immunes dans les désordres auto-immunes tels que l'arthrite rheumatoïdes dont l'un des agents étiologiques semble être *C. trachomatis*.

De même, l'homologie entre les hsp60 humaines et chlamydiennes pourrait permettre la rupture de la tolérance de l'hôte à ses propres Hsp60. Selon le mécanisme Janeway, la tolérance à un immunogène du soi peut être rompue si l'on est co-immunisé avec 2

immunogènes hétérologues (Mamula et *al.*, 1992). Yi et *al.* (1997) ont constaté qu'une co-immunisation de la souris CBA avec Hsp60 de souris et celle de *Chlamydia* entraînait la rupture de la tolérance de la souris à ses propres Hsp60. Les auteurs ont également constaté que cette rupture de tolérance s'accompagnait d'un shift de la production de l'IL-10 vers celle de l'IFN-γ. Cette constatation montre qu'une rupture de la tolérance s'accompagne d'un shift de la réponse Th2 vers celle Th1. Certains auteurs pensent que la réponse Th2 au lieu d'être toujours considérée comme immunopathologique, pourrait être considérée comme un moyen d'immunorégulation protectrice (Van Eden et *al.*, 1998). La cytokine anti-inflammatoire IL-10, produite au cours de la réponse Th2, permet en effet de réduire l'inflammatoire agressive produite au cours de la réponse Th1 et de maintenir également des lymphocytes auto-réactifs dans un état anergique (Sundstedt et *al.*, 2003; Seewaldt et *al.*, 2002).

3.5 APPROCHE VACCINALE

Confronté à d'énormes obstacles, le développement d'un vaccin contre les infections à *Chlamydia* demeure un véritable défi. Certaines de ces difficultés étaient déjà pressenties durant les premiers essais vaccinaux. Chez certains humains vaccinés contre le trachome, l'immunité résultante était de courte durée et induisait les effets immunopathologiques plus sévères que chez les individus non vaccinés. Afin de développer un vaccin sain et protecteur, plusieurs stratégies sont adoptées incluant la recherche des antigènes immunodominants protecteurs et immunopathologiques, la caractérisation de la réponse protectrice effectrice induite. Les données obtenues sur les modèles animaux et humains suggèrent qu'un vaccin idéal devrait induire une forte production d'anticorps mucosaux (IgG et IgA) et une forte réponse de cellules Th1 CD4+. À ce jour, l'immunogène immunodominant protecteur le plus étudié est la MOMP chlamydienne codée par le gène *omp1*. MOMP est une protéine complexe de 40 à 44 kDa, la plus abondante à la surface des CEs et des CRs où elle pourrait agir comme porine. La molécule MOMP est constituée de 4 domaines séro-variables espacés de domaines constants. Elle induit la production d'anticorps dirigés contre les domaines variables et donc spécifiques aux sérovars. Cependant la réponse Th importante pour stimuler et maintenir la réponse de

cellules B est tout d'abord élicitée par les domaines constants et seulement un domaine variable (Oritz et *al.*, 2000). La MOMP recombinante, les peptides synthétiques du MOMP, le vaccin d'ADN codant pour la MOMP et le transfert passif des antigènes monoclonaux spécifiques au MOMP ont été évalués pour leur efficacité protectrice. Cependant les résultats ont été décevants (Cotter et *al.*, 1995; Su et *al.*, 1995; Pal et *al.*, 1997; Pal et *al.*, 1999). Face à ces échecs plusieurs raisons ont été avancées, notamment la nécessité des adjuvants appropriés et la voie d'introduction du vaccin. Les études entreprises récemment tiennent compte de ces raisons d'échec possible. Ainsi on a pu constater que l'immunisation par voie mucosale (orale, intra-nasale et vaginale) confère une réponse protectrice comparée à l'injection sub-cutanée (Kelly et *al.*, 1996). En effet, l'immunisation par voie parentérale induit la réponse Th2 alors que celle par les voies mucosales induit celle Th1 requise. De plus, la voie intranasale produit une réponse plus importante d'IgG et d'IgA par rapport aux autres voies (Igietseme et *al.*, 1998, Rudin et *al.*, 1998). L'utilisation des adjuvants appropriés peut permettre également d'induire une immunité. L'immunisation transcutanée des souris avec la MOMP en combinaison avec la toxine cholérique et les oligodésoxynuclétides CpG induit la production des IgG et IgA mucosaux spécifiques et une réponse Th1 spécifique requises pour la résolution de l'infection (Berry et *al.*, 2004). Cependant, même si ces résultats sont encourageants, il n'en demeure pas moins que l'immunité induite par cette protéine majeure est partielle car elle est plus faible que celle engendrée par l'infection elle-même. De plus, la MOMP étant une protéine polymorphique parmi les souches de *C. trachomatis*, l'immunité engendrée pourrait être spécifique à l'immunotype. Ceci signifie qu'un vaccin à ADN ou à base de sous-unités protéiques devrait requérir aussi d'autre antigènes en plus du MOMP afin d'améliorer le degré de l'immunité. À cet effet, plusieurs autres candidats ont été suggérés. Les travaux de Goodall et *al.* (2004) montrent que d'autres antigènes chlamydiens dont l'enzyme glycolytique énolase, la protéine putative de la membrane externe pmpD et une protéine de fonction inconnue ct579 peuvent induire une réponse de cellules T CD4+. D'aucun pense également que les protéines structurales du système de sécrétion de type III ainsi que les protéines effectrice qui y sont sécrétées, les protéines de l'inclusion Inc, les pmp seraient des candidats dans le développement d'un vaccin (Stephens, 2000). En fait, il reste à identifier les différents immunogènes chlamydiens et

à tester si l'administration de multiples gènes ou protéines ensemble induit une immunité protectrice suffisante.

Une immunité protectrice notable et égalant celle générée lors d'une infection primaire a été produite à la suite d'un transfert adoptif chez les souris de cellules dendritiques pulsées *ex vivo* avec des CEs de *C. trachomatis* inactivés (Shaw et *al.*, 2001; Su et *al.*, 1998). Une telle immunisation induit les anticorps spécifiques et la réponse Th1 CD4+. Cependant cette approche non conventionnelle et attrayante ne peut être applicable chez l'homme dû aux réactions alloréactives pouvant être induites.

Nombreux sont des chercheurs qui pensent qu'un vaccin vivant atténué serait approprié dans la prophylaxie des infections chlamydiennes (Brunham et *al.*, 2000; Zhang et *al.*, 1999). Une souche de *C. trachomatis* atténuée et vivante permettra, en effet, d'exprimer différents immunoantigènes durant tous les stades du cycle de développement. Ceci offre l'opportunité d'induire l'immunité protectrice contre plusieurs antigènes et donc contre plusieurs sérovars. Cependant cette approche demeure difficile à réaliser tant que les capacités métaboliques de base et les facteurs de virulence chlamydiens ne sont pas déterminées. De plus il faudrait également identifier toutes les protéines chlamydiennes induisant les réponses de l'hôte auto-pathologiques afin de permettre leur délétion spécifique si elles ne sont pas essentielles. Finalement, maintenant que la technique de clonage par plaque récemment développées offre la possibilité de sélectionner les lignées de clones (Matsumoto et *al.*, 1998), il reste à développer un système de transfert de gène chez *C. trachomatis* afin de pouvoir générer les mutants.

Un autre des obstacles pouvant freiner le développement d'un vaccin est le fait que l'immunité générée est de courte durée. Rank et *al.* (1988) ont inoculé les souris et les cobayes à de temps variés, après une infection primaire. Ils ont constaté que l'immunisation contre les re-infections écourte l'infection si les cobayes sont inoculés 30 jours après l'infection primaire. Cependant, si les cobayes sont inoculés 77 jours après l'infection primaire, le niveau d'immunisation est réduite. De même, les souris sont protégées si elles sont re-infectées 100 jours après l'infection primaire, mais pas après

155 jours (Ramsey et *al.*, 1991). Les raisons expliquant la durée de vie courte de l'immunité ne sont pas connues. Pourtant, les lymphocytes CD4+ mémoires spécifiques aux antigènes chlamydiens apparaissent être présents pendant un long moment dans les tissus lymphoïdes secondaires (Su and Caldwell, 1995). Les raisons possibles sont la réduction de la réponse proliférative dans les tissus génitaux (Igietseme et Rank, 1991) ou un défaut dans le recrutement de lymphocytes mémoires dans la muqueuse du tractus génital (Kelly, 2003).

3.6 LES TRAITEMENT THÉRAPEUTIQUES

3.6.1 Les cyclines

Les cyclines, molécules constituées de 4 cycles sur lesquels sont greffés différents groupes, comptent parmi elles les antichlamydiens : la doxycycline (Dox), la tétracycline (Tet), la minocycline et la lymecycline. La Dox et la Tet sont depuis plus de 25 ans largement utilisés dans la thérapie des maladies à *C. trachomatis*. Le guide de traitement du centre de contrôle de maladies (CDC) d'Atlanta, recommande la Dox comme premier choix dans la thérapie des maladies chlamydiennes. L'usage oral des cyclines est toutefois contre-indiqué chez l'enfant de moins de 8 ans. Grâce à leur liposolubilité, ces antibiotiques, la Dox en particulier, sont doués d'une excellente capacité de pénétration intracellulaire et intrabactérienne et donc d'une bonne diffusion tissulaire. Les cyclines sont bactériostatiques et interfèrent avec la synthèse protéique en se fixant sur la petite sous-unité ribosomale et en inhibant la mise en place de l'aminoacyl RNAt au niveau du site A. La Dox est utilisée à la posologie de 100 mg 3 fois par jour. La durée du traitement dépend du l'infection en présence: 14 jours s'il s'agit du trachome, 7 s'il s'agit de l'urétrite ou la cervicite et 21 jours s'il s'agit de la lymphogranulome vénérienne (Chiu et Amsden, 2002, Guashimo et Ricci, 2002).

3.6.2 Les macrolides

Les macrolides, particulièrement l'érythromycine (Ery), représentent une alternative aux cyclines. L'Ery est synthétisée par *Streptomyces erythraeus* et est constituée d'un cycle

lactone de 14 carbones sur lequel sont attachés 2 sucres, la désosamine et la cladinose. Comme la Dox, l'Ery inhibe la synthèse protéique, mais en se liant à l'ARNr 23S de la sous-unité 50S du ribosome chlamydien et/ou en provoquant la dissociation d peptidyl de transfert (ARNt) des ribosomes lors de la phase d'élongation. Malgré ses effets négatifs sur le système gastro-intestinal, l'Ery est prescrite dans le traitement des infections chlamydiennes chez la femme enceinte. Pour traiter la cervicite ou l'urétrite, le CDC recommande l'Ery base ou éthylsuccinate aux posologies respectives de 500mg et 800mg 4 fois par jour pendant 7 jours. Bien que les taux d'éradication de *C. trachomais* obtenus approchent les 100%, les taux de guérison clinique ne sont approximativement que de l'ordre 80%. Parmi les autres macrolides, la clarithromycine et la josamycine pourraient également être considérées, compte tenu de leur activité anti-chlamydienne, mais les posologies appropriées demeurent encore à établir.

L'introduction du traitement à dose unique d'azithromycine constitue une avancée importante dans la thérapie des maladies chlamydiennes (Duran and Amsden, 2000). L'azithromycine est un macrolide azalidé chimiquement apparenté à l'érythromycine, mais dont le cycle lactone contient un groupe aminé (Alvarez-Elcoro and Enzler, 1999). Cette modification confère à la molécule une stabilité en milieu acide, une capacité de pénétration tissulaire importante, une concentration plasmatique faible et une demi-vie élevée de 50-68 h. C'est donc un antibiotique biodisponible permettant la résolution de chlamydioses avec seulement une dose unique de 1g. Ceci a permis de pallier au problème d'adhérence médicamenteuse (compliance) altérant l'efficacité d'un traitement tel qu'observé dans le traitement de chlamydiose avec de multiples prises de Dox. Les résultats thérapeutiques du traitement à dose unique de l'azithromycine et à doses multiples de la Dox sont pourtant semblables: le taux de guérison microbiologique de l'azitromycine est de 95-100% comparé à 88-100% pour la Dox, des taux de guérison clinique de 89-100% versus 94-100 pour la Dox. Ainsi, comme la Dox, l'azithromycine est recommandée en thérapie de 1ère intention des maladies à *C. trachomatis*. Le seul inconvénient de l'azithromycine par rapport à la Dox demeure son coût élevé (Guaschimo and Ricci, 2002).

3.6.3 Les fluoroquinolones

Les fluoroquinolones, antibiotique synthétique comportant un noyau 4-quinolone et une molécule de fluore, dérivent de l'acide nalidixique. Grâce à leur capacité à pénétrer les cellules et leurs activités bactéricides, les fluoroquinolones parmi lesquelles la ciprofloxacine, l'ofloxacine et la sparfloxacin, sont utiles dans la thérapie des chlamydioses. Ce sont des molécules capables de se lier aux complexes ADN-gyrase (topoisomerase II) et ainsi perturber la réplication et la réparation de l'ADN, la transcription et la ségrégation chromosomique durant la division. Parmi ces molécules, la ciprofloxacine, pourtant efficace au départ, s'est révélé inutile par la suite due aux nombreuses récurrences observées. Jusqu'à présent, l'ofloxacine semble maintenir son efficacité procurant de taux de guérison microbiologique et clinique pouvant atteindre 100%. Selon les recommandations du CDC, l'ofloxacin est utilisée en 2nd intention dans le traitement des chlamydioses génitales à la posologie de 300 mg oralement 3 fois par jour pendant 7 jours. La levofloxacin est aussi utilisée en 2nd intention à la posologie de 500mg oralement pendant 7 jours. La sparfloxacin pourrait éventuellement être considérée dans la thérapie des chlamydioses (Rigway, 1997; Gusaschimo et Ricci, 2002).

3.6.4 Les bêta-lactamines

Bien que les bêta-lactamines puissent altérer la croissance de *C. trachomatis* et induire la formation de particules aberrantes persistantes, elles ne sont pas suffisamment actives et ne sont de ce fait pas utilisées dans la thérapie de chlamydioses. Exceptionnellement, l'amoxicilline est recommandée en 1ère intention dans le traitement des chlamydioses chez la femme enceinte. Les études cliniques montrent l'efficacité de l'amoxicillin dans le traitement de l'urétrite chez les hommes et la cervicite chez les femmes (Csango et *al.*, 1989; Paavonen et *al.*, 1989). Bien que la paroi chlamydienne soit dépourvue de peptidoglycane, il est probable que *C. trachomatis* puisse requérir les peptidoglycane ou ses précurseurs pour son métabolisme. Ceci expliquerait la sensibilité chlamydienne à l'amoxicillin qui inhibe la synthèse du peptidoglycane.

3.7 LA RÉSISTANCE AUX ANTICHLAMYDIENS

Malgré la disponibilité de l'antibiothérapie, les infections à *C. trachomatis* continuent d'être exacerbantes. Les infections récurrentes sont en effet parfois constatées même après l'observance de l'antibiothérapie appropriée, ce qui peut suggérer l'échec de la thérapie. La résistance multiple aux antichlamydiens peut être une des causes des échecs thérapeutiques, puisqu'une résistance relative aux isolats de *C. trachomatis* à la tétracycline et à l'érythromycine a été reporté (Mourad et *al.*, 1980; Jones et *al.*, 1990; Somani et *al.*, 2000). Pourtant de sérieux doutes subsistent quant à l'apparition de possible cas résistance *in vivo*. En effet, dans la plupart des cas, les souches de *C. trachomatis* résistantes n'ont été obtenues que *in vitro*, sous la pression d'antibiotiques. Dessus-Babus et *al.* (1998) ont ainsi obtenu des souches de *C. trachomatis* résistant à l'ofloxacine et à sparfloxacine, résultant d'une mutation du gène gyrA codant pour la sous-unité A de la gyrase. Cependant, la preuve de la résistance *in vivo* a été apportée par Misyurina et *al.*(2004) en identifiant des mutations dans les deux gènes de l'ARNr 23S chez les souches de *C. trachomatis* multi-résistantes aux macrolides provenant de patients.

3.8 LES CYTOKINES PRO-INFLAMMATOIRES

Les infections chlamydiennes génitales ou oculaires sont toutes caractérisées par une inflammation prolongée qui serait responsable des dommages tissulaires graves. Puisque les cytokines pro-inflammatoires jouent un rôle important dans l'induction et la promotion de l'inflammation, il serait important d'étudier leur profil d'expression au cours des maladies auto-immunes inflammatoires telles celles occasionnées par *C. trachomatis*. De ce fait, la structure, la fonction et la régulation des cytokines pro-inflammatoires doivent être connues.

3.8.1 L'IL-12

L'IL-12 bio-active, IL-12p70, est une protéine hétérodimérique composée de sous-unités p35 (chaîne α) et p40 (chaîne β) liées entre elles par des liens disulfides. Alors que p35

est ubiquitaire, l'expression de p40 est limitée et fortement régulée. L'IL-12 est produite par une variété de cellules, mais les producteurs majeurs sont les cellules présentatrices d'antigène (APC) tels que les monocytes, les macrophages, les cellules dendritiques (DC). Le récepteur de haute affinité de l'IL-12 est composé de deux sous-unités ($\beta1\beta2$) co-exprimées sur les cellules T, NK et DC. L'IL-12p40 interagit avec la sous-unité du récepteur $\beta1$ alors p35 interagit préférentiellement avec la sous-unité $\beta2$ qui, en plus, constitue la chaîne du récepteur traduisant les signaux. Après liaison de l'IL-12, les Janus kinase, Jak2 et Tyk2, respectivement associées aux chaînes $\beta1$ et $\beta2$, sont activées par transphosphorylation. Ces JAKs activées en retour phosphorylent les groupes tyrosines présents dans le domaine cytoplasmique de $\beta2$, il en résulte la formation des sites d'arrimage pour les STATs (signal transducers and activators of transcription), particulièrement STAT4. Les STATs qui se lient aux récepteurs phosphorylés via le domaine d'homologie SRC (SH2) sont subséquemment phosphorylés par les JAKs au niveau de leur tyrosine. Les STATs phosphorylés forment alors les homo- ou les hétéro-dimères qui peuvent alors transloquer au noyau où ils se lient à l'ADN pour activer la transcription de certains gènes (Leonard, 1998).

L'expression du récepteur de l'IL-12 sur les cellules T est fortement régulée. L'IL-12 est une cytokine critique qui régule la différenciation de cellules naïves T CD4+ en cellules T helper 1. Les cellules Th1 expriment le complexe du récepteur de haute affinité ($\beta1$ et $\beta2$) alors que les cellules Th2 ne peuvent pas exprimer la chaîne $\beta2$. De ce fait l'IL-12 agissant via son récepteur favorise plutôt l'engagement du phénotype Th1. Ainsi, la régulation du récepteur de l'IL-12 est un important facteur qui gouverne la différenciation de cellules Th1 et Th2. L'IFN-γ augmente la production du facteur de transcription T-bet lequel maintient l'expression de l'IL-12R$\beta2$, ce qui favorise la réponse Th1. Au contraire l'IL-4 inhibe l'expression de l'IL-12R$\beta2$, ce qui favorise la réponse Th2. Les cellules Th1 produisent l'IFN-γ et promouvoit la réponse à médiation cellulaire essentielle pour la lutte contre les pathogènes intracellulaires. L'IFN-γ produit par les cellules stimulées par IL-12 favorise l'activité bactéricide des phagocytes et augmente la réponse immunitaire innée. Étant donné le rôle primordial de l'IL-12 dans le développement de la réponse Th1, il est suggéré que l'IL-12 endogène puisse jouer un rôle critique dans la pathogenèse

des désordres auto-immunes médiée par les cellules Th1, telles que les maladies inflammatoires de l'intestin, l'arthrite rheumatoïde (Athie-Morales et *al.*, 2004 ; Vandenbroeck et *al.*, 2004)

3.8.2 L'IL-6

L'IL-6 est une cytokine aux effets pléitropiques et redondants produite par différents types de cellules dont les lymphocytes T et B, les monocytes, les fibroblastes, les kératinocytes, les cellules mésangiales et tumorales. Structuralement, l'IL-6 subit un repliement peptidique de manière que la forme tertiaire soit caractérisée par la présence de 4 longues hélices-α (A-D) arrangées de telle sorte que les deux premières hélices d'une part et les deux autres hélices d'autre part soient presque parallèles entre elles.

Le récepteur de l'IL-6 (IL-6R) exprimé par différents types de cellules comprend la chaîne α trans-membranaire de 80-kDa et la chaîne β, une glycoprotéine transmembranaire de 130-kDa désignée gp130. L'IL-6 se lie à son ligand, l'IL-6Rα qui peut être transmembranaire ou soluble, pour former un complexe de forte affinité pour gp130. Cette dernière est une protéine possédant 2 motifs Box1 et Box 2 et 6 résidus de tyrosine cytoplasmiques lui permettant de générer les signaux. Ainsi, suite à l'interaction IL-6/IL-6R/gp130, les JAKs dont JAK1, JAK2 et Tky-2 présentes au niveau des motifs Box1 et Box2, sont activées et phosphorylées. Il en résulte l'activation des STATs, possiblement STAT1, STA3 et STAT5. En plus, la voie de MAP kinase peut être aussi activée. La voie de traduction des signaux de l'IL-6 peut être négativement régulée par différentes protéines inhibitrices telles celles de la famille des SOCS (suppressor of cytokine signaling). La fonction biologique de l'IL-6 engendrée dépend de la région cytoplasmique de gp130 d'où est généré le signal et du facteur de transcription activé. L'IL-6 induit, en effet, plusieurs fonctions. Elle occasionne la croissance des cellules T et la différenciation de cellules T cytotoxiques en augmentant l'expression du récepteur de l'IL-2 et la production de l'IL-2. En synergie avec l'IL-3, l'IL-6 supporte la formation des colonies des cellules blastes multi-linéages de l'hématopoïèse. L'IL-6 induit aussi la différenciation de macrophages, de mégacaryocytes et des ostéoclastes. Dans la réaction de la phase aiguë, IL-6 stimule les hépatocytes à produire différentes protéines et

simultanément supprime la production de l'albumine. Lorsque administrée, l'IL-6 peut causer la fièvre et agir comme facteur de croissance de différentes types de cellules telles que les cellules tumorales. Une dérégulation de la synthèse de l'IL-6 peut entraîner différents désordres auto-immuns parmi lesquels l'arthrite rheumatoïde (Kaplanski et *al.*, 2003 ; Leonard, 1998 ; Naka et *al.*, 2002)

3.8.3 TNF-α

Le TNF-α est le prototype des cytokines de la famille de TNF. Le TNF-α humain est produite comme un peptide non glycosylé trans-membranaire de 233 acides aminés, 26kDa. La forme soluble de 157 acides aminés est dérivée du précurseur trans-membranaire suite au clivage du peptide signal par l'activité des métalloprotéinases spécifiques (TNF converting enzyme ou TACE). Le TNF-α soluble active est un homotrimère, formant un sandwich allongé de feuillets plissés β antiparallèles avec une topologie « jelly-roll ». Les monomères sont intimement associés autour d'une symétrie à triple axe pour former un trimère compacté en forme de cloche. Les phagocytes mononucléaires sont la principale source du TNF-α, cependant d'autres cellules telles que lymphocytes T (Th1 et Tc) et B, les cellules NK et bien d'autres peuvent produire le TNF-α après activation. Les cellules répondant au TNF-α sont celles qui possèdent les récepteurs appropriés, c'est-à-dire une des deux protéines trans-membranaires de type I, TNFRI et TNFRII, caractérisées par la présence de régions répétées riches en cystéine dans leur domaine extracellulaire. TNF-RI contient aussi le domaine de la mort cytosolique de 80 acides aminés. Les agents qui stimulent la synthèse du TNF, tels que les LPS, induisent aussi l'IL-1. L'interaction du TNF-α avec son récepteur conduit à l'homoagrégation de 2 ou 3 récepteurs, ceci permet l'activation et le recrutement de différentes protéines signales parmi lesquelles les TRAF (TNF receptor-associated factors). Il en résulte une cascade d'événements aboutissant à l'activation de NF-κB. Le recrutement d'autres protéines signales tels que TRADD, FADD/MORT, RIP ou RAIDD, interagissant avec le domaine de la mort de TNF-RI peut être aussi constaté. Ces protéines peuvent soit s'associer avec la caspase 8 ou 2 et déclencher l'apoptose, soit interagir avec TRAF-2 et promouvoir l'activation de NF-κB. Une myriade d'effets du TNF-α est rapportée dans la littérature. Il semble que les protéines de la famille de TNF

et leurs récepteurs sont requis dans le développement des organes lymphoïdes périphériques et dans le développement de la réponse humorale adaptive (Krakauer, 1998). Le TNF-α contribue à la résistance aux pathogènes intracellulaires, tels que *Listeria monocystogenes*. Les macrophages activés produisent du TNF-α et IL-12 qui, ensemble, stimulent la synthèse de IFN-γ par les cellules NK. Le TNF-α et IFN-γ produits peuvent alors stimuler les macrophages à produire les composés microbicides comme l'oxyde nitrique (NO). Le TNF-α est également impliqué dans les effets pathologiques, notamment le développement du choc septique, de la malaria cérébrale, des maladies auto-immunes telles que l'arthrite rheumatoïde (Pfeffer, 2003).

3.8.4 L'IL-1β

L'IL-1β avec l'IL-1α et l'IL-1ra forment les cytokines de la famille de l'IL-1. Alors que l'IL-1 (α et β) induit les réponses biologiques, l'IL-1ra contrôle cette activité en agissant comme un antagoniste naturel du récepteur de l'IL-1. L'IL-1β est produite comme précurseur inactif, le pro-IL1β (31 kDa, 269 acides aminés), lequel est clivé par l'enzyme convertissant l'IL-1β (ICE) pour générer l'IL-1β mature de 17 kDa. Les phagocytes mononucléaires sont la source majeure de l'IL-1β, mais toutes les autres cellules nucléées sont également capables d'en produire. L'étude cristallographique montre que l'IL-1β est une protéine globulaire ressemblant à un tétraèdre dont les faces triangulaires sont formées de 12 brins β antiparallèles connectés par une boucle hydrophilique bien exposée à la surface. L'IL-1β reconnaît deux types de récepteurs IL-1RI et l'IL-1RII. L'activité biologique est médiée par l'IL-1RI, alors que l'IL-1RII inhibe l'activité de l'IL-1 en agissant comme récepteur de leurre. Après la liaison de l'IL-1 à l'IL-1RI, la kinase associée au récepteur de l'IL-1 (IRAK) est rapidement activée et s'associe avec la portion cytoplasmique de IL-1RI. De cette façon, IRAK peut s'associer avec TRAF6. Le complexe ainsi formé peut alors interagir avec une autre protéine trans-membranaire, l'IL-1R-AcP (protéine accessoire du récepteur de l'IL-1), requise dans l'émission des signaux. Il s'ensuit une cascade d'événements conduisant à l'activation de NF-κB et des gènes de cytokines pro-inflammatoires. IL-1β et l'IL-1α jouent un rôle important dans la défense de l'hôte aux infections en agissant à différents niveaux du système de défense de l'hôte. Au niveau de la réponse innée, l'IL-1 participe au recrutement des leucocytes aux

sites de l'infection en induisant l'expression de molécules d'adhésion (ICAM, ELAM et VCAM) sur l'endothélium vasculaire et l'expression d'un certain nombre de chémokines telles que l'IL-8 qui attirent les neutrophiles. L'IL-1 stimule les monocytes et les neutrophiles à synthétiser différentes cytokines pro-inflammatoires dont IL-6, le TNF-α et l'IL-8. En permettant l'expression accrue de l'IL-2 et de son récepteur, l'IL-1 constitue un co-mitogène pour les cellules T. L'IL-1 agit préférentiellement comme co-stimulateur des cellules Th2. L'IL-1 peut aussi promouvoir la maturation et la différenciation des cellules B en induisant la synthèse de la chaîne légère κ sur les cellules pré-B. Cependant, lorsque produite en grande quantité dans la circulation, l'IL-1 agit de manière systémique et exerce les effets endocrines létaux tels que l'activation du centre de la fièvre, l'axe hypothalamo-hypophysaire-adrénaline. IL-1 peut aussi stimuler la résorption de l'os en induisant la sécrétion des metalloprotéinases par les chondrocytes occasionnant la dégradation du cartilage et la libération des protéoglycanes. Finalement, l'IL-1 stimule aussi les macrophages à produire les gélatinases qui participent à la dégradation de la matrice extracellulaire, et agit sur les fibroblastes en permettant leur prolifération et la sécrétion des collagénases (Krakauer, 1998; Dinerallo, 2000).

3.8.5 L'IL-8

L'IL-8 est une protéine, membre de la famille des chémokines CXC possédant la séquence ELR (glu-leu-arg) N-proximal. L'IL-8 est produite sous deux formes par des cellules variées en réponse à différents stimuli. Une forme de 77 acides aminés est générée par les cellules tissulaires comme les cellules endothéliales et les fibroblastes, une autre forme de 72 acides aminés, la plus active, est produite par les leucocytes. L'IL-8 peut se lier avec la même affinité à deux récepteurs différente: CXCR1 et CXCR2. Les protéines CXCR appartiennent à la famille des récepteurs 7-transmembranaires qui sont couplées à la protéine G. La liaison de la chémokine à son récepteur conduit à la dissociation des sous-unités de la protéine G. Ces sous-unités peuvent activer alors la phospholipase C β (PLCβ) et conduire à l'hydrolyse de la phosphatidylinositol 4,5 biphosphates (PIP_2), ce qui produit l'inositol triphosphate (IP_3) et le diacyl-glycérol (DAG). L'IP_3 permet la mobilisation du Ca^{2+} intracellulaire. Le DAG stimule la phospho-kinase C (PKC) qui phosphoryle et active un certain nombre d'effecteurs tels que la

MAP kinase. Le signal émis peut aussi conduire à la polymérisation de l'actine, la reconfiguration des protéines d'adhésion et d'autres réponses cellulaires contribuant à la migration des cellules (Goldsby, 2000; Bickel, 1993).

L'IL-8 joue un rôle majeur aussi bien dans l'inflammation aiguë que chronique en activant et en chemoattractant des neutrophiles. De plus l'IL-8 occasionne la degranulation des neutrophiles et donc la libération de la défensine, de la cathepsine G, et du CAP3/azurocidine qui recrutent les cellules T, les monocytes et les neutrophiles additionnels. La défensine et la cathepsine G libérées sont respectivement comitogènes et mitogènes pour les cellules T, induisent la production de IFN-γ et de l'IL-4, promouvoient la production d'anticorps en réponse à un antigène. Ainsi, l'IL-8, en degranulant les neutrophiles, initie une cascade immunostimulant qui convertit la réponse innée en une réponse adaptée. L'IL-8 est aussi produite par différentes cellules des tumeurs humaines. Il semble qu'à cause de ses effets mitogènes, angiogènes et motogènes, l'IL-8 jouerait un rôle dans la progression des cancers et le développement des métastases (Xie, 2001; Krakauer, 1998).

3.8.6 L'IL-10

L'IL-10, une cytokine anti-inflammatoire homodimérique de 37 kDa, est produite *in vivo* par les monocytes/macrophages, les cellules T (Th2, Tc2 et Tr1) et B, et peut-être les kératinocytes. Les deux monomères identiques, de 160 acides aminés et 18.5kDa chacun, s'entrelacent et se replient de façon à produire 2 domaines formant un V. Chaque domaine possède 6 hélices, 4 (A-D) sur un monomère, 2 autres (E et C) sur l'autre. Le gène codant pour l'IL-10 (5 exons, 5.1 Kb) localisé sur le chromosome 1 est fortement polymorphique. Le récepteur de l'IL-10, présent sur divers types de cellules est constitué de 2 chaînes différentes, α et β, appartenant à la famille de récepteurs de cytokines de type II. L'IL-10 se lie à la chaîne α, le complexe formé peut alors interagir avec la chaîne β, importante pour médier les effets de l'IL-10. Cette interaction active les tyrosines kinases Jak1 et Tyk2, lesquels sont respectivement attachés à l'IL-10Rα et l'IL-10Rβ. L'engagement du récepteur et la phosphorylation des tyrosines activent les facteurs de

transcriptions cytoplasmiques STAT 1, 3 et 5, il en résulte leur translocation au noyau, d'où l'activation des gènes. En s'opposant à différentes actions privilégiant la réponse Th1, l'IL-10 promouvoit la réponse Th2. L'IL-10 supprime substantiellement la réponse Th1 en inhibant la production de l'IFN-γ par les cellules T, particulièrement via la suppression de la synthèse de l'IL-12 par les cellules accessoires. Également, l'IL-10 interfère avec la capacité des APC telles les monocytes à présenter les antigènes aux cellules T en inhibant l'expression du CMH de classe II et les molécules de co-stimulation tel que le CD86. De plus, l'IL-10 prévient la maturation des monocytes en DC, plutôt favorise la maturation des monocytes en macrophage. IL-10 inhibe aussi la production de différentes cytokines pro-inflammatoires, IL-1β, IL-6, IL-8, G-CSF, GM-CSF et TNF-α. Plutôt, cette cytokine anti-inflammatoire augmente la production des médiateurs anti-inflammatoires tels que l'IL-ra et le récepteur soluble du TNF-α. Puisque toutes les protéines pro-inflammatoires sont transcriptionellement contrôlées par NF-κB, il est suggéré que l'IL-10 puisse exercer ses effets anti-inflammatoires en inhibant la translocation nucléaire de NF-κB, ou en inhibant la liaison de NF-κB déjà présent au noyau, à l'ADN. Définitivement, l'IL-10 possède les effets protecteurs en prévenant une réponse inflammatoire incontrôlée. Cependant, une expression plus élevée peut inhiber la réponse protectrice aux micro-organismes intracellulaires. D'ailleurs, certains virus parmi lesquels le virus Epstein-Barr (EBV) produisent une protéine homologue à l'IL-10, d'autres virus tel que le virus respiratoire syncytial ont développé des stratégies pour induire l'IL-10 cellulaire, tout ceci afin de supprimer la réponse inflammatoire locale. De plus étant donné le polymorphisme du promoteur et du gène de l'IL-10, il est possible que certains haplotypes soient liés à la sévérité ou susceptibilité de la maladie (Fickenscher et *al.*, 2002 ; Li et He, 2004 ; Mocellin et *al.*, 2003).

3.9 CONCLUSION

Bien que les maladies causées par *C. trachomatis* ne soient pas véritablement mortelles, elles occasionnent à la longue des séquelles graves. De ce fait, les dépistages réguliers même en absence de quelconque signe doivent être prescrits.

3.10 PROBLÉMATIQUE ET OBJECTIFS

Les diverses maladies causées par *Chlamydia trachomatis* ont la particularité d'être d'abord aiguës, puis évolué sous formes chroniques caractérisées par une inflammation continue responsable des séquelles graves. Chez la femme, les infections génitales chroniques endommagent le système de reproduction et conduisent aux grossesses ectopiques ou à l'infertilité tubaire. Concernant les infections oculaires durables, elles peuvent se développer en trachome et ainsi entraîner la cécité. Puisque ces infections sont le plus souvent asymptomatiques, elles demeurent alors non dépistées et donc non résolues par des antibiotiques disponibles, d'où l'installation des formes d'infections chroniques et sérieuses. Différentes études rapportent néanmoins que même traitées convenablement, les infections à *C. trachomatis* peuvent tout de même demeurer non résolues et conduire subséquemment aux infections chroniques sévères. Le rôle de la persistance dans les échecs thérapeutiques rapportés n'est vraiment pas clair. Il est, en effet, constaté qu'en présence de conditions non propices à de croissance, *C. trachomatis* adopterait une forme de latence, intracellulaire, non infectieuse, morphologiquement aberrante, perdurant dans l'organisme. Les échecs thérapeutiques ont été constatés immédiatement après la thérapie, même chez les patients n'ayant pas pu être réinfectés en évitant tout rapports sexuels. Ceci laisse pressentir le développement de la persistance durant la thérapie.

Le 1er objectif de ce projet de recherche est de montrer si le traitement des infections chlamydiennes *in vitro* par des antibiotiques habituellement utilisés dans la thérapie des infections chlamydiennes conduit à l'infection persistante à la place de la résoudre.

On sait maintenant que les séquelles résultant des infections à *C. trachomatis* chroniques sont proprement occasionnées, non pas par la bactérie infectieuse elle-même, mais par la réponse immunitaire induite. Une telle réponse délétère n'a pas encore été définitivement déterminée, mais semble être associée à l'infection persistante, plutôt que productive. Il est possible que la réponse T-helper de type 2 générée, inefficace et donc prolongée, puisse en fin de compte s'avérer dommageable, alors que la réponse Th1 serait requise pour la résolution de l'infection. Il est également possible que l'inflammation continue et

les cytokines cytotoxiques continuellement produites puissent concourir aux dommages. Grâce à leurs fonctions d'inducteur et de modulateur de la réponse immunitaire, les cytokines pro-inflammatoires initialement induites joueraient un rôle déterminant dans la polarité et le maintien de la réponse immunitaire.

Le 2^{ième} objectif de ce projet de recherche est de déterminer le profil d'expression différentielle de cytokines pro-inflammatoires selon que l'infection est productive ou persistante.

DEUXIÈME PARTIE:
MATÉRIELS, MÉTHODES ET RÉSULATS

CHAPITRE IV: ARTICLE 3

EFFECTS OF SUSTAINED ANTIBIOTIC BACTERICIDAL TREATMENT ON *CHLAMYDIA TRACHOMATIS*-INFECTED EPITHELIAL-LIKE CELLS (HeLa) AND MONOCYTE-LIKE CELLS (THP-1 AND U-937)

Effects of sustained antibiotic bactericidal treatment on *Chlamydia trachomatis*-infected epithelial-like cells (HeLa) and monocyte-like cells (THP-1 and U-937)

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Accepted for publication in
International Journal of Antimicrobial Agents

4.1 ABSTRACT

Background: *Chlamydia trachomatis* is a human pathogen that causes multiple diseases worldwide. Despite appropriate therapy with existing antichlamydial antibiotics, chronic exacerbated diseases often occur and lead to serious sequelae. Since *C. trachomatis* has been found to enter a persistent state after exposure to deleterious conditions, the role of persistence in the failure of chlamydial antibiotherapy is questioned.

Methods: HeLa, THP-1 and U-937 cells were infected with 10^4 *C. trachomatis* serovar L2 infectious particles. 3 days later, the infected cells were treated with minimal bactericidal concentrations of doxycycline (Dox), erythromycin (Ery) or tetracycline (Tet) for 24 or 30 days. Antibiotic efficacy was assessed by measuring chlamydial inclusion and infectious particles, by investigating the resumption of chlamydial growth after antibiotic removal, and by testing *Chlamydia* viability using reverse transcriptase-polymerase chain reaction targeting unprocessed 16S rRNA, processed 16S rRNA and *omp1* mRNA.

Results: Treatment of infected HeLa cells with the usual antichlamydial antibiotics suppressed chlamydial active growth. The infection remained unapparent. However, 24 days post-treatment, the bacterium was found to be viable, as proved by continued expression of unprocessed and processed 16S rRNA and *omp-1* mRNA. This inactive unapparent chlamydial state is not infectious, suggesting *Chlamydia* persistence.
In both permissive THP-1 and non-permissive U-937 cells, *C. trachomatis* also developed persistence. Unlike in HeLa cells, persistent chlamydial infection in THP-1 and U-937 cells was resolved after 30-days Dox treatment. Of interest, we noticed that only THP-1 and U-937 cells, which were persistently infected following their interaction with infected HeLa cells, remained capable of transmitting active infection to HeLa cells.

Conclusion: These findings suggest that Dox, Tet and Ery, usually administered to combat chlamydial diseases, fail to resolve persistent infection occurring during treatment in non-immune HeLa cells. However, in immune THP-1 and U-937 cells, the

persistent infection is resolved by therapy with Dox. Epithelial cells could be the reservoir of persistent chlamydial particles.

Key words: persistent infection, therapy failure, doxycycline, erythromycin, tetracycline

4.2 RÉSUMÉ

Problématique: *Chlamydia trachomatis* est une bactérie pathogène de l'humain, responsable de nombreuses maladies dans le monde entier. Malgré la thérapie appropriée avec des antibiotiques disponibles, il survient souvent les maladies chroniques exacerbant avec des séquelles graves. La forme chlamydienne persistante se développant dans les conditions délétères pourrait jouer un rôle dans les échecs thérapeutiques rapportés.

Méthodes: Les cellules HeLa, THP-1 et U-937 on été infectées avec 10^4 particules de *C. trachomatis* serovar L2. 3 jours plus tard, ces cellules ont été traitées avec la doxycycline (Dox), tétracycline (Tet) et érythromycine (Ery) aux concentrations minimales bactericides, pendant 24 à 30 jours. L'efficacité thérapeutique de ces 3 antibiotiques a été évaluée de plusieurs façons: (1) mesure des inclusions chlamydiennes présentes et des particules infectieuses libérées, (2) étude de la reprise de l'infection après suppression de l'antibiotique du milieu de culture, (3) test de la viabilité de *Chlamydia* par la réaction de transcription inverse- polymérisation en chaîne ciblant les ARNr 16S matures ou non matures et les ARNm de *omp1*.

Résultats: le traitement de cellules HeLa avec des antibiotiques antichlamydiens usuels supprime la croissance active chlamydienne. Ainsi, l'infection demeure inapparente. Cependant, 24 jours après le traitement, la bactérie est toujours retrouvée viable, tel que prouvé par l'expression continue des ARNr 16S matures ou non matures et des ARNm du gène *omp1*. Cet état chlamydien inapparent et non infectieux suggère la persistance.
Dans les cellules moncytiques permissives THP-1 et non permissives U-937, *C. trachomatis* peut y demeurer aussi persistant. À la différence de ce qui se produit dans les cellules HeLa, l'infection chlamydienne dans les cellules THP-1 et U-937 est résolue 30 jours après le traitement à la Dox. Fait intéressant, seulement les cellules THP-1 et U-937 qui ont acquis la persistance à la suite de leur interaction avec les cellules HeLa, demeurent capables de transmettre l'infection productive aux cellules HeLa saines.

Conclusion: ces résultats suggèrent que la Dox, la Tet et l'ery, habituellement administrés pour combattre les infections chlamydiennes, échouent à résoudre l'infection

persistante survenant durant le traitement des cellules non immunitaires HeLa. Au contraire, dans les cellules immunitaires THP-1 et U-937, l'infection persistante est résolue par la thérapie à base de la Dox. Ainsi, les cellules épithéliales pourraient constituer un réservoir de particules chlamydiennes persistantes.

Mots-clés: doxycycline, érythromycine, infection persistante, tetracycline, therapy failure

4.3 INTRODUCTION

The obligate intracellular bacterium *Chlamydia trachomatis* is the leading cause of ocular and genital infections worldwide. Usually asymptomatic, unresolved diseases sometimes progress to chronic infections with serious sequelae. Chronic ocular infections due to serovars A to C result in trachoma, the second highest cause of preventable blindness [1, 2]. The more prevalent chronic genital infections, with serovars D to K, are responsible for pelvic inflammatory diseases, ectopic pregnancy and tubal infertility in women [3, 4]. With serovars L1-L2, *C. trachomatis* causes systemic diseases, such as lymphogranuloma venereum [5]. *C. trachomatis* is found to increase HIV transmission and is also suspected to contribute to cervical cancer development due to human papillomavirus [6-9].

Among bacteria, *C. trachomatis* is recognized by its unique biphasic developmental cycle in which it alternates between 2 forms: extracellular particles known as elementary bodies (EBs, 200-300 nm in diameter), and intracellular forms or reticulate bodies (RBs, 1,000-1,500 nm in diameter). The dense, metabolically-inactive EBs are specialized in cell infection. After entry into cells via an unknown mechanism, they reside within the phagosome, often called inclusion that never fuses with lysosomes [41]. Within the phagosome, the EBs differentiate into RBs having an active metabolism and ensuring growth by binary fission. Further, daughter RBs differentiate back into infectious EBs that, expelled in cells, ensure new rounds of infection [11]. This *Chlamydia* life cycle can be altered or interrupted by deleterious conditions. Beatty et *al.* [12] found that following interferon-gamma (IFN-γ) treatment, *C. trachomatis* acquires persistence or latency, in which the bacterium remains non-infectious with an enlarged aberrant form and a low metabolism. In addition, this persistent form exhibits an abnormal antigenic profile with increased level of 60-kD heat shock protein, decreased level of major outer membrane protein (MOMP), but unchanged lipopolysaccharide [13, 14]. Tumor necrosis factor-alpha (TNF-α), penicillin and nutrient deprivation, e.g. iron, glucose and amino acid deficiency, are also known to induce *C. trachomatis* persistence [15-19]. It has been suggested that persistence contributes to the pathogenesis of chronic diseases [20]. Actually, *C. trachomatis* can be easily isolated in culture from patients with active disease. However in patients with chronic disease, although the presence of *Chlamydia* is

proved by molecular tests (ligase chain reaction, polymerase chain reaction (PCR)), it remains non-cultivable, suggesting a persistent, non-infectious state [21, 22]. In addition, by studying the natural history of chlamydial infection, Joyner et *al.* [23] and Morre et *al.* [24] found that infections with *Chlamydia* can be resolved spontaneously, but infections sometimes persist and can lead to pelvic inflammatory diseases capable of inducing serious sequelae.

Different antibiotics are recommended for the effective treatment of chlamydial diseases. The most important ones are doxycycline (Dox), tetracycline (Tet), ofloxacine, ciprofloxacin, azythromycin. Erythromycin (Ery) and amoxicillin are often used to treat pregnant women [25, 26]. Although these antibiotics are effective, exacerbated recurrent diseases are often reported [27]. Some patients are found to remain infected after adequate treatment. Since some of these patients do not renew sexual intercourse, persistence rather than re-infection has been implicated in therapy failure, resulting in chronic chlamydial disease development [28].

In this study, *in vitro* chlamydial-infected cell models were tested to establish if the usual antichlamydial antibiotics fail to resolve diseases by inducing persistence. The data show that HeLa cells remain persistently infected after long-lasting bactericidal treatment with Dox, Tet and Ery. However, persistent chlamydial infection in THP-1 and U-937 cells remains resolvable without any treatment, but resolution is faster with treatment.

4.4 MATERIALS AND METHODS

4.4.1 Cell culture

The human cervical adenocarcinoma cell line HeLa 229 was obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). The cells were cultured in minimal essential medium with Hank's salts (MEM/H) (Sigma, St. Louis, USA), containing no supplementary essential amino acids and L-glutamine, but supplemented with 10% fetal bovine serum (FBS). The human acute monocytic leukemia cell line THP-1 was purchased from the ATCC. The histolytic lymphoma cell line U-937 (ATCC CRL-1593.2) was donated by Dr Yves Raymond (Laboratoire d'auto-immunité, CHUM-Hôpital Notre-Dame, Montréal, QC, Canada). These cell lines were maintained in RPMI 1640 medium containing 2 mM L-glutamine, 50 µg/ml gentamycin, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM Hepes, 1 mM sodium pyruvate, and supplemented with 10% FBS. Optimal growth of HeLa 229, THP-1 and U-937 cells occurs in a humidified incubator at 37°C under 5% of CO_2.

4.4.2 Chlamydial organism

Chlamydia trachomatis serovar L2/434/Bu was provided by the " Laboratoire de Santé Publique du Québec". *C. trachomatis* L2 was propagated in HeLa 229 cell monolayers, in MEM/H media containing cycloheximide 50 µg/ml (Sigma), in addition to the other supplements enumerated above. *C. trachomatis* EBs were purified by ultracentrifugation on sodium diatrizoate (Sigma), suspended in sucrose-phosphate-glutamic acid (SPG) buffer, and stored in aliquots at -80°C [29].

For EB titration, 2.5 10^5 HeLa cells were seeded on sterile glass coverlips contained in 24-well plates, and incubated at 37°C under 5% of CO_2. 18-24 h after incubation, the culture medium was replaced by different dilutions of EBs in SPG buffer. The plates were then centrifuged for 1h at 500 x g, 25°C. After centrifugation, the inocula were replaced by MEM/H containing cycloheximide 0.5 µg/ml and the plates were incubated at 37°C under 5% CO_2. 48 h post-infection, the cells were fixed with methanol and

exposed to May-Grünwald-Giemsa stain. Inclusions were visualized by light microscopy and enumerated as viable inclusion-forming units (IFUs).

4.4.3 Antibiotics, minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) determination

Dox, Tet and Ery, purchased from Sigma, were supplied as powder. Each powder was solubilized in sterile distilled water as recommended by the manufacturer. The MIC and MBC of each antibiotic were determined [30, 31]. Briefly, HeLa cells were infected by *C. trachomatis* L2 at the multiplicity of infection (MOI) of 0.05 and treated with different antibiotic concentrations. The lowest antibiotic concentration that inhibits chlamydial inclusion formation 48 post-treatment is the MIC. However, the lowest antibiotic concentration that does not allow *Chlamydia* growth after 2 passages on antibiotic-free media is the MBC.

4.4.4 *C. trachomatis*-infected cells treated with antibiotics

HeLa cells, seeded 18-24 h earlier on sterile glass coverslips in 24-well plates, were infected with *C. trachomatis* L2 at a MOI of 0.1. These infected cells are fed with antibiotic-free MEM/H supplemented with 10% of FBS and cycloheximide 0.5 µg/ml. 3 days post-infection, the supernatant media were removed and replaced by 10% FBS-supplemented MEM/H without cycloheximide, but with Dox, Tet or Ery at MBC. Medium was supplied every 4 days for 24 days. Every 2 days, *Chlamydia*-infected cells, stained with May-Grünwald-Giemsa, were examined by light microscopy and the numbers of inclusions were expressed as IFUs.

4.4.5 Non-molecular viability test

To check if *C. trachomatis* was killed by antibiotic treatment, we studied bacterial growth renewal after stopping antibiotic treatment. Thus, 2, 4, 6, 8, 10 and 16 days post-treatment, antibiotic-containing media were replaced by antibiotic-free media with 0.5 µg/ml cycloheximide. The plates were then incubated under optimal conditions for 10 days, and the cells were observed regularly by light microscopy to assess chlamydial growth. At the same time, we measured *C. trachomatis* infectious particles present in

media when antibiotic treatment was stopped and 10 days after treatment suppression. Finally, we wanted to know if infected cells treated for 16 days still harbor infectious particles. We used these antibiotic-treated, infected cells as inoculum to infect healthy HeLa monolayers and chlamydial growth was assessed by light microscopy.

4.4.6 Chlamydial persistence in THP-1 and U-937 cells and their treatment with Dox

Persistence was established by infecting THP-1 and U-937 cells with *C. trachomatis* serovar L2. These monocyte-like cells were infected either after co-culture with infected HeLa cells or after direct inoculation. HeLa, THP-1 and U-937 cells, in 24-well plates, were inoculated separately with 10^4 EB IFUs in culture media containing cycloheximide. Cell infection was intensified by centrifugation at 500xg for 1 h. The cells were then incubated under optimal growth conditions. 3 days post-infection, with regard to straight infected THP-1 and U-937 cells, the inoculum was removed and replaced by fresh medium that did not contain cycloheximide, but with or without Dox 1 μg/ml. For co-culture, healthy THP-1 or U-937 cells in fresh medium with or without Dox 1 μg/ml were added to infected HeLa cells. Non-infected THP-1 and U-937 cells were also cultured as controls. From this day on and every 3 days, the cells were collected to measure infection by enumeration under light microscopy. 30 days later, one-half of the cells for each treatment were used to detect *C. trachomatis* by reverse transcriptase (RT)-PCR as described below. The remaining one-half of the cells served as inocula to infect healthy HeLa monolayers. This was done to determine if persistent *C. trachomatis* inside monocytes release infectious particles and infected permissive HeLa cells. To do that, U-937 and THP-1 cells were washed several times in phosphate-buffered saline (pH 7.0) to remove free infectious particles and cultured in fresh medium. 1 week later, 1.5 x 10^5 of each kind of cells, the cell lysate and culture medium were incubated on HeLa monolayers for 10 days.

4.4.7 Viability test by RT-PCR

Total RNA was prepared from cells in suspension (THP-1 and U-937) and from HeLa cell monolayers. Cells in suspension were harvested by centrifugation. Total RNA was extracted by RNeasy mini kit (Qiagen, Mississauga, ON, Canada) according to the

manufacturer's instructions. From HeLa cell monolayers, total RNA was collected by scraping after cell lysis. During extraction, total RNA fixed on columns was treated with RNase-free DNase I (Invitrogen, Burlington, ON, Canada). Purified RNA was quantified in a BioSpec-Mini apparatus (Shimadzu). The first strand of cDNA was reverse transcribed in a total reaction volume of 20 μl, containing 200 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen), 250 ng of random primer oligonucleotides (Invitrogen), 200 U of RNaseOUT (Invitrogen), 0.5mM of each dNTP and about 1 μg of purified total RNAs.

First-strand cDNA was amplified in a total PCR mixture of 25 μl. Each mixture contained 20 mM of Tris-HCl, 50mM of KCl, 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each specific primer, 1U of *Taq* DNA polymerase and 50 ng of cDNA. Amplification was carried out in a Perkin-Elmer 2400 thermocycler. The following primers were used for amplification of each gene [32]:

- processed 16S rRNA:

  5'-CTGCAGCCTCCGTAGAGTCTGGGCAGTGTC-3' dowstream,
  5'TTCAGATTGAACGCTGGCGGCGTGGATG-3' upstream;

- unprocessed 16S rRNA:

  5'-CTGCAGCCTCCGTAGAGTCTGGGCAGTGTC-3' dowstream,

  5'-GCCAGTATAGATGCTTGTGAGGA-3' upstream;

- *ompl* gene:

  5'-GCCGCTTTGAGTTCTGCTTCCTC-3' upstream,

  5'-ACCTTGCTTGCCA(T/C)TCATGGT-3' dowstream.

Each amplification began with an initial denaturing step at 92°C for 2 min. For unprocessed 16S RNA, 35 cycles were carried out as follows: 45 sec of denaturation at 94°C, 45 sec of annealing at 60°C and 1 min of amplification at 72°C. For processed 16S rRNA PCR, 25 cycles were performed with annealing at 65°C. For *ompl* PCR, 35 cycles

were undertaken with annealing at 55°C. 8 μl of amplification products were analyzed by electrophoresis in a 2% agarose gel and stained with ethidium bromide.

4.5 RESULTS

4.5.1 Determination of MICs and MBCs

MICs and MBCs found were respectively 0.06 and 1 µg/ml for Dox, 0.128 and 3 µg/ml for Tet, 0.12 and 2 µg/ml for Ery.

4.5.2 Effects of Dox, Tet and Ery on *C. trachomatis* growth in HeLa cells

HeLa cells were infected with *C. trachomatis* infectious particles at MOI of 0.1. 3 days post-infection, antibiotics at MBC were applied on the infected cells. To assess the efficiency of antichlamydial therapy, inclusions were enumerated every 2 days. The results are represented in Figure 1. For each antibiotic, the highest inclusion number was found at day 2 post-treatment. Later, the numbers of inclusions diminished drastically. From day 6 onwards, the few inclusions enumerated were empty. These empty inclusions were observed until day 10. After this, no inclusions were seen.

At the same time, the effects of the 3 antibiotics on EB release were assessed by titration in supernatant media. At day 2 post-treatment, only a few EBs were released. At day 6, no EBs were found in supernatant media (data not shown).

4.5.3 Death or viability of *C. trachomatis* after antibiotic treatment

To make sure that antichlamydial bactericidal treatment with Dox, Tet and Ery ultimately killed *C. trachomatis*, we measured chlamydial growth resumption microscopically after antibiotic removal from the culture media. In addition, after antibiotic removal, cycloheximide was added to culture media to help chlamydial growth under optimal conditions. We noted that *C. trachomatis* growth resumed if treatment lasted 2 to 8 days for Dox and Tet, and 2 to 10 days for Ery (Table 1). Growth resumption was not seen if treatment lasted more than 10 days, and even 10 days after antibiotic removal.

To continue to make sure that these antichlamydial treatments really resolved chlamydial infection in HeLa cells, treatment was prolonged until day 16. The cells were then collected at this moment and 10 days after antibiotic removal. The collected cells were

lysed and used to inoculate HeLa cell monolayers, in order to verify if such cells harbored chlamydial infectious particles. We never found infectious particles in these cells. In addition, we never observed that such cells released infectious particles in culture media, even 10 days after antibiotic removal. These results suggest that 10 days of treatment with Dox, Tet and Ery, killed *C. trachomatis* cultured in HeLa cells.

4.5.4 Viability test by RT-PCR

The molecular approach based on RT-PCR of 16S unprocessed rRNA was taken to make definite conclusions about death or viability of *C. trachomatis* after antichlamydial treatment. This approach was first tried by Gerard et *al.* [33, 34] to show the viability of persistent non-cultivatable *Chlamydia* in patients with rheumatoid arthritis or ectopic pregnancy. Because unprocessed 16S rRNAs are extremely short-lived, they are only found in viable but not in dead *Chlamydia*. At the same time, we checked for the expression of processed 16S rRNA and *omp1* mRNA. The *omp1* gene encoded MOMP usually used to diagnose chlamydial diseases. The results showed that unprocessed 16S rRNAs continued to be expressed, even after 24 days of bactericidal treatment. Processed 16S RNA and *omp1* mRNA also continued to be expressed (Figures 2, 3 and 4). Thus *C. trachomatis* did not appear to be killed by bactericidal treatment with Dox, Tet and Ery.

In conclusion, long-lasting antichlamydial therapy of infected HeLa cells led to unapparent disease: infection continued, but was not overt and was revealed only by assessing the continued expression of unprocessed 16S rRNA. Thus, antibiotics in antichlamydial therapy could fail to resolve *Chlamydia* infection of epithelial cells. Since these *C. trachomatis* particles that had remained viable in epithelial cells were non-cultivatable, we concluded that these tests showed *C. trachomatis* persistence.

4.5.5 *C. trachomatis* L2 persistence in THP-1 and U-937 cells

In vivo, mucosal epithelial cells are the first target of *C. trachomatis*. However, serovar L2 can grow actively, in addition, in monocytes, and serovars of biovar trachoma (e.g E and K) are reported to infect monocytes. Here, we wanted to establish persistent infection

in THP-1 and U-937 cells. Thus, we infected these monocyte-like cells in 2 ways as described in Materials and Methods: (1) monocytes were directly inoculated with *C. trachomatis*; (2) monocytes became infected via their interaction with infected HeLa cells. We noted that *C. trachomatis* grew actively in THP-1 cells but not in U-937 cells. Typical chlamydial inclusions were seen only in THP-1 cells but not in U-937 cells (data not shown). However, although THP-1 cells were permissive to *C. trachomatis* growth, some of them did not harbor typical inclusions, survived chlamydial infection and resumed growth. For directly-infected THP-1 cells, renewed growth was observed from day 18. For THP-1 cells that had interacted with infected HeLa cells, renewed growth began slowly from day 24 (Figure 5A). To establish if the survival of infected THP-1 cells was due to insufficiency of infectious particles, we titrated infectious chlamydial particles at days 3, 6 and 9. For directly-infected THP-1 cells, we noticed that at day 9 there were 65 times more infectious particles than THP-1 cells number (Table 2). Thus, survival of THP-1 cells was not due to the insufficiency of infectious particles.

U-937 cells were not permissive to *C. trachomatis* growth. Typical inclusions were not observed in infected U-937 cells. In addition, directly-infected U-937 cells exhibited nearly the same growth curve as healthy U-937 cells (Figure 5B). When we titrated EBs released in the supernatant medium at days 3, 6 and 9, increased chlamydial progeny were not seen (data not shown). However, the growth of U-937 cells interacting with infected HeLa cells was inhibited until day 15. This inhibition was not due to *C. trachomatis* infection. We found that interaction of U-937 cells, even with healthy HeLa cells, inhibited U-937 cell growth. When infected HeLa monolayers were completely degraded, this inhibition was removed, and U-937 cell growth resumed (Figure 5B).

4.5.6 *C. trachomatis* persistence in permissive THP-1 cells and non-permissive U-937 cells

As described below, THP-1 cells survived chlamydial infection, and U-937 cells remained non-permissive to chlamydial growth. Nevertheless, we were interested to know if they harbor viable and persistent *C. trachomatis*. Thus, 33 days post-infection,

monocyte-like cells were collected, and total RNA was extracted. RT-PCR targeting unprocessed 16S rRNA was undertaken to assess the presence of viable *C. trachomatis* in these cells. We discovered that the cells harbor viable *C. trachomatis,* as revealed by the expression of unprocessed 16S rRNA (Figure 6). Thus, unapparent, persistent infection occurred in permissive and non-permissive monocyte-like cells.

At the same time, we wanted to know if, as in HeLa cells, *C. trachomatis* can persist in THP-1 and U-937 cells despite bactericidal treatment with Dox. Infected THP-1, U-937 and HeLa cells were then treated 3 days post-infection with Dox at MBC. RT-PCR showed that 30 days post-treatment, unprocessed 16S rRNA was not expressed anymore in THP-1 and in U-937 cells. However, unprocessed 16S rRNA continued to be expressed in HeLa cells 30 days after Dox treatment (Figure 6). In addition, we noticed that 2 mounths later, non-treated, persistently-infected THP-1 and U-937 cells resolved their persistent infection without requiring any treatment.

In conclusion, these results demonstrated that Dox was able to resolve persistent *C. trachomatis* infection in monocyte-like cells (THP-1 and U-937), but not in epithelial-like cells (HeLa).

4.5.7 Infectious EB release from THP-1 and U-937 cells harboring persistent *C. trachomatis*

We considered that persistent *C. trachomatis* in monocyte-like cells is capable of changing or reverting to infectious EBs that can cause an overt infection in permissive cells. To verify this hypothesis, 30 days after infection, or after interaction with infected HeLa cells, monocyte-like cells was collected, washed several times and sub-cultured. 8 days after subculture, supernatant media were collected and stored; the cells were washed several times to eliminate free EBs. Supernatant media, cell lysates and viable cells were used to inoculate HeLa cell monolayers. EBs infecting HeLa cells were released only from THP-1 and U-937 cells that became persistently infected after interaction with infected HeLa cells, and not from persistently-infected THP-1 and U-937 cells that never interacted with HeLa cells (Table 3). In persistently-infected THP-1 cells that had

interacted with HeLa cells, infectious EBs were found also in the cytoplasm, since cell lysates produced infection of HeLa cells. In U-937 cells, there were no infectious particles in the cytoplasm, probably because they were non-permissive cells; they harbored only non-infectious persistent particles, and infectious particles emitted might be rapidly released outside the cells.

4.6 DISCUSSION

Although antichlamydial antibiotics are effective, as proved by their low MIC, they sometimes fail to resolve chlamydial infection [28]. Moreover, persistent infections recover after appropriate therapy, suggesting the role of antibiotics in persistence development [35-37]. The present *in vitro* study shows that long-lasting bactericidal treatments with Dox, Tet and Ery, antibiotics usually used in chlamydial infection therapy, do not resolve infection of HeLa cells by *C. trachomatis* serovar L2. After 24 days of treatment, the infection becomes unapparent, but *C. trachomatis* remains alive, as shown by the continuous expression of 16S unprocessed rRNA, 16S processed rRNA, and the *ompl* gene mRNA. Tetracyclines (Dox and TET) and macrolides (Ery) are normally bacteriostatic, inhibiting protein synthesis by settling on 30S rRNA and 23S rRNA, respectively. Here, we used MBCs that normally kill *C. trachomatis*. However, since infection remains unresolved and is not overt, we conclude that it is persistent or latent. Azithromycin, ciprofloxacin and ofloxacin, which are considered as bactericidal, are also found to induce *C. trachomatis* persistence instead of resolving infection [38, 39]. Chlamydial persistence has been also noticed after treatment of infected epithelial cells with cytokines (TNF-α and IFN-γ) or penicillin [12, 18, 19]. Chlamydial persistence has been developed in nutriment deficient-culture or in non-permissive cells such as fibroblasts [40]. Because persistent chlamydial particles remain inactive and are thus insensitive to antibiotics, the development of persistence during antichlamydial therapy is worrisome, since it may lead to therapy failure.

The mechanism of antibiotic-induced persistence is not known. It is not clear if persistent particles are newly-induced or intermediate forms between EB and RB that are naturally persistent. By way of a reminder, *C. trachomatis* is found as EBs and RBs. After entry into cells, extracellular EBs differentiate into RBs that ensure growth by binary fission. Since EBs are metabolically inactive, they must be naturally resistant to antibiotics that act only in metabolic steps. However, RBs that are metabolically active must be highly sensitive to antibiotics and other antichlamydial compounds. Consequently, natural EB resistance decreases during differentiation into RBs, while RB sensitivity decline with de-differentiation into EBs. Thus, heterogeneous particles with different morphologies and

sensitivities to antibiotics may be found in chlamydial inclusions: EBs, RBs and intermediate forms between EBs and RBs. However, the antibiotic sensitivity of these 3 kinds of particles has not yet been determined. Persistent particles may be one of these 3 kinds of particles or newly-induced particles. It has been found that the bactericidal concentration of Ery or Tet blocks the conversion of EBs into RBs [41, 42]. Does lasting bactericidal treatment kill naturally-resistant intracellular EBs and intermediate forms? This remains to be determined. It would be easier to think that persistent particles are intracellular EBs or intermediate particles that exhibit natural resistance. This would easily explain why very different conditions induce persistence. However, until now, different studies have hypothesized that persistent particles may be enlarged RBs that stop their division at the segregation level and acquire metabolic quiescence after antimicrobial treatment [12, 43, 44]. Ery and Dox are found to block RB binary fission, producing ghost bodies and enlarged RBs [41, 45]. In this case, it is difficult to envision a mechanism that allows sensitive RBs to acquire persistence. In regard to IFN-γ-induced persistence, Beatty et *al.* [13] found that induced indoleamine dioxygenase (IDO), by decyclising tryptophan, led to a shortfall of this essential amino acid. In this condition, chlamydial particles enter in a persistent state by arresting growth. However, this mechanism cannot be generalized. IDO has not been found in monocyte-like U-937 cells within which *Chlamydia* remains persistent [46, 47]. Harper et *al.* [48] reported that minor changes in nutriments cause a persistent state. Thus, it is possible that Dox, Tet and Ery, by interfering with protein synthesis, indirectly cause nutriment deficiency, leading to a persistent state at the same time. However, more molecular investigations are needed to understand the mechanism leading to persistence.

Suppression of treatment before 10 days culminates in overt chlamydial re-growth. This growth renewal may be ensured by normal RBs that are still alive. However, no active growth is observed if treatment lasts more than 10 days, even if *C. trachomatis* remains viable. We think that after 10 days of treatment, it remains in culture only as persistent particles. Thus, these remaining persistent particles have been unable to revert to normal EBs or RBs. Even so, most studies report that persistent particles revert to active particles once conditions again become favorable [49]. In our study, we did not find that. This may

be explained by differences in the concentration used. Beatty et *al.* [14] noted that persistent particles induced by INF-γ at 0.2 ng/ml were able to revert, but persistent particles induced by IFN-γ at 2 ng/ml were unable to revert to active particles.

C. trachomatis serovar L2 persisted also in monocyte cells line THP-1 and U-937. However, about 2 mounths post-infection, persistent infection in monocytes was resolved without any treatment. This infection resolution was faster – no more than 30 days– when Dox treatment was supplied. In highly-permissive HeLa cells, persistence remained even after 30 days of Dox treatment. This differential outcome of persistent infections might be due to differences in microbiocide activities within monocyte/macrophage immune cells and epithelial (HeLa) non-immune cells. Unlike epithelial cells, macrophages produce IFN-γ that is absolutely required to resolve chlamydial infection. Rothfuchs et *al.* [50] showed that by synthesizing IFN-γ, macrophages were able to protect RAG-$1^{-/-}$/INF-$\gamma^{-/-}$ mice (deficient in B and T cells) against *C. pneumoniae* infection. IFN-γ allowed different chlamydicidal activities, such as induction of nitric oxide synthase (NOS) that catalyzed the production of reactive nitrogen intermediates. Ramsey et *al.* [50] found that NOS deficiency led to exacerbated, persistent chlamydial infection. Thus, reactive nitrogen intermediates, produced more highly in macrophages than in epithelial cells, might take part in chlamydicidal activities in macrophages. Differences in the immune response might explain different outcomes of chlamydial diseases seen among people, some of whom are found to resolve infection spontaneously without requiring antimicrobial treatment, while others remain persistently infected [26, 47].

It was demonstrated that phagosomes leading to inclusions were, at all times, unable to fuse with lysosomes inside HeLa cells, thus avoiding *Chlamydia* particle lysis by lysosomal microbicide compounds [10]. However, phagolysosomal fusion has been found to occur in monocytic cell lines [51], partly explaining persistent chlamydial particle clearance. Although persistent infection in monocytes was resolved, it was not excluded that *in vivo*, monocytes remained continuously infected after their interaction with reservoir epithelial cells which continuously harbored persistent infection. These

persistently-infected monocytes might continue to go to the joints where they promoted arthritic damage as suggested by Koehler et *al.* [46].

This study revealed 2 outcomes of *C. trachomatis* serovar L2 infection inside THP-1 cells: either the infection was productive and led to cell death after bursting or the infection remained non-productive and persistent. The reasons for this result are not known. Did *C. trachomatis* persistence occur naturally during its growth? To answer this question, it is necessary to show if THP-1 cells that permit productive growth and those that allow persistence are genotypically and phenotypically the same.

Although monocyte-like THP-1 and U-937 cells remained persistently infected, they had been unable to produce normal infectious particles. This occurred only if THP-1 and U-937 cells had acquired their persistent infection after their interaction with productively-infected HeLa cells. These results suggested that communication with epithelial cells during acquisition of persistence might confer a signal or trigger the synthesis of a compound in monocytes that are required for reversion of persistent particles into infectious particles. Actually, it is possible that persistent chlamydial particles, in addition to the restoration of favorable conditions, require specific stimuli to revert into a normal form.

In conclusion, persistent *C. trachomatis* serovar L2 was harbored by HeLa cells that were deficient in defense responses, while immune THP-1 and U-937 cells by themselves resolved the infection. However, interaction between infected HeLa cells with THP-1 or U-937 cells might generate a stimulus that is able to induce persistent particles within monocytes to revert into infectious EBs. Elucidating the mechanism of persistence might help to develop new strategies in therapy of diseases due to *C. trachomatis*.

4.7 AUTHORS' CONTRIBUTIONS

PM carried out the experiments, analyzed and interpreted data, drafted the manuscript. MR conceived the study, participated in its design and coordination, helped to draft the manuscript. All authors read and approved the final manuscript.

4.8 ACKNOWLEDGEMENT

We are grateful to Dr Yves Raymond (CHUM- Hôpital Notre-Dame, Montreal, QC, Canada) for providing U-937 cells. We also thank Ovid Da Silva for editing this manuscript. MP is the recipient of a "Programme Canadien des Bourses de la Francophonie" scholarship.

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4.10 TABLES

Table 1. Resumption of *C. trachomatis* growth after antibiotic removal from culture media[a]

Days	Growth resumption		
	Dox	Tet	Ery
2	Yes	Yes	Yes
4	Yes	Yes	Yes
6	Yes	Yes	Yes
8	Yes	Yes	Yes
10	No	No	Yes
12	No	No	No

[a] Monolayers of HeLa cells in 24-well plates were infected with *C. trachomatis* serovar L2 at MOI of 0.1. 3 days post-infection, Dox, Tet and Ery at MBC (1.0, 3.0 and 2.0 µg/ml, respectively) were applied. After different days, antibiotic was suppressed, cycloheximide was added to the culture media and the cells were then incubated for more than 10 days to be sure that growth does not resume. Growth was measured at light microscopy. This experiment was performed in duplicate.

Table 2. *C. trachomatis* serovar L2 infectious particles (IFUs) compared to THP-1 cells number in culture media[a]

Time points	THP-1 interacting with infected HeLa cells			Straight infected THP-1 cells		
	IFUs x 10^4	THP-1 cells x 10^4	Ratio	IFUs x 10^4	THP-1 cells x 10^4	Ratio
3	29.81 ± 0.75	2.29 ± 0.70	13	1.35 ± 0.26	3.61 ± 0.71	0.37
6	25.03 ± 1.72	0.12 ± 0.03	208	38.48 ± 0.0 4	6.65 ± 1.25	5.79
9	17.76 ± 0.72	0.06 ± 0.03	296	20.41 ± 1.29	0.31 ± 0.15	65.84

[a] 10^5 HeLa cells or $1.5x10^5$ THP-1 cells in 24-well plates were infected with 10^4 *C. trachomatis* serovar L2 IFUs. 3 days later, co-culture was established with the addition of $1.5x10^5$ THP-1 cells on infected HeLa monolayer. Every 3 days, THP-1 cells were enumerated and *C. trachomatis* IFUs were titrated. Values represent the means of 2 experiments

TABLE 3. Transmission of infection to HeLa cells monolayer by THP-1 and U-937 persistently infected with *C. trachomatis* serovar L2[a]

Source of inoculum	THP-1 cells that interacted with infected HeLa cells	THP-1 cells infected by *Chlamydia*	U-937 cells that interacted with infected HeLa cells	U-937 cells infected by *Chlamydia*
Medium supernatant	+[b]	-[c]	+	-
Cell lysates	+	-	-	-
Viable cells	+	-	+	-

[a] 10^5 HeLa cells or $1.5x10^5$ THP-1 cells in 24-well plates were infected with 10^4 *C. trachomatis* serovar L2 IFUs. 3 days later, co-culture was established by adding $1.5x10^5$ THP-1 cells in infected HeLa monlayer. 30 days after interaction with infected HeLa cells or after chlamydial infection, THP-1 and U-937 cells were collected and washed several times in PBS. The cells were then cultured in fresh medium. After 2 subcultures, viable cells, cell lysates or supernatant were used as inocula to infect healthy HeLa monolayers.

[b] Presence of infectious particles infecting HeLa cells monolayers

[c] No infectious particles with no infection of HeLa cell monolayers

4.11 FIGURES

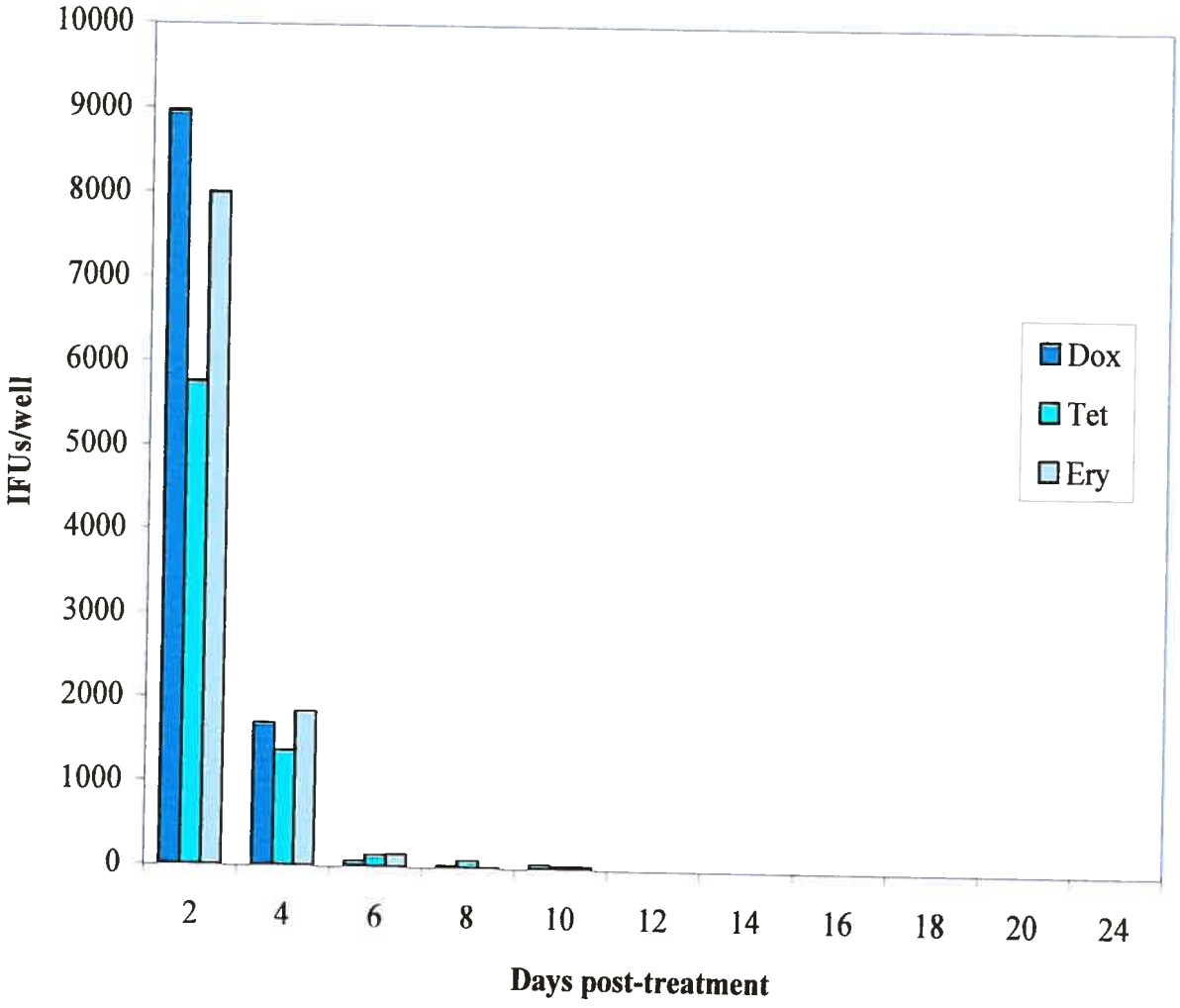

10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
IFUs/well
2
4
6
8
10
12
14
16
18
20
24
Days post-treatment
Dox
Tet
Ery

Figure 1. Effects of Dox, Tet and Ery on *C. trachomatis* inclusions in HeLa cells. 3 days post-infection of HeLa cells at MOI of 0.1, Dox, Tet and Ery at MBC (1.0, 3.0 and 2.0 μg/ml, respectively) were applied. Then, every 2 days for 24 days, the cells were exposed to May-Grünwald-Giemsa stain. Inclusions were visualized and enumerated by light microscopy. The data represented are means ± standard deviation (error bars) of 3 independent experiments and are expressed as IFUs.

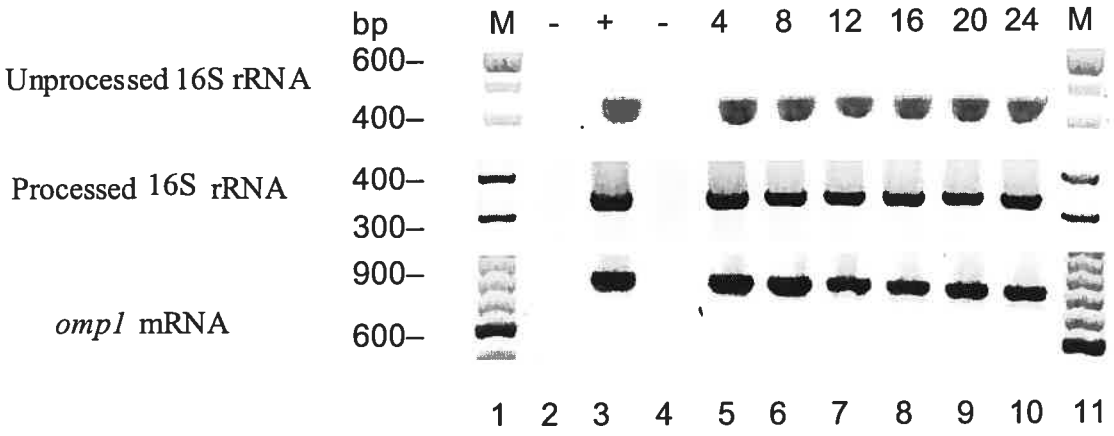

bp
M
-
+
-
4
8
12
16
20
24
M
Unprocessed 16S rRNA
600–
400–
Processed 16S rRNA
400–
300–
ompl mRNA
900–
600–
1
2
3
4
5
6
7
8
9
10
11

Figure 2. *C. trachomatis* L2 viability after treatment with Dox assessed by RT-PCR. HeLa cells were infected at MOI of 0.1 and treated with Dox at MBC (1 μg/ml). From day 3 post-infection, total RNAs were extracted every 4 days. Unprocessed and processed 16S rRNA, *omp1* transcripts were targeted by RT-PCR. Lanes: 1 and 11, DNA markers; 2, uninfected HeLa cells; 3, HeLa cells infected for 3 days; 4, HeLa cells infected with heat-killed *Chlamydia* for 3 days; 5-10, infected HeLa cells treated with Dox for 4, 8, 12, 16, 20, and 24 days.

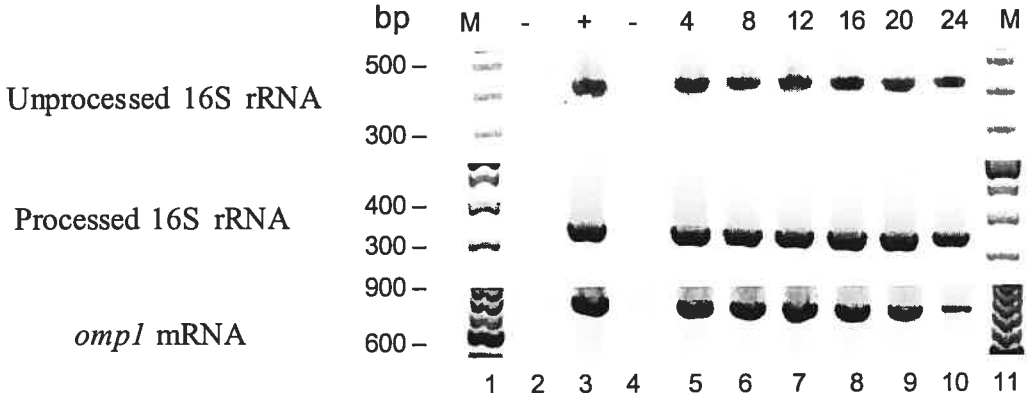

Unprocessed 16S rRNA
Processed 16S rRNA
omp1 mRNA
bp
M
-
+
-
4
8
12
16
20
24
M
500 –
300 –
400 –
300 –
900 –
600 –
1 2 3 4 5 6 7 8 9 10 11

Figure 3. *C. trachomatis* L2 viability after treatment with Tet assessed by RT-PCR. HeLa cells were infected at MOI of 0.1 and treated with Tet at MBC (3 μg/ml). From day 3 post-infection, total RNA were extracted every 4 days. Unprocessed and processed 16S rRNA, *omp1* transcripts were targeted by RT-PCR. Lanes: 1 and 11, DNA markers; 2, uninfected HeLa cells; 3, HeLa cells infected for 3 days; 4, HeLa cells infected with heat-killed *Chlamydia* for 3 days; 5-10, infected HeLa cells treated with Tet for 4, 8, 12, 16, 20, and 24 days.

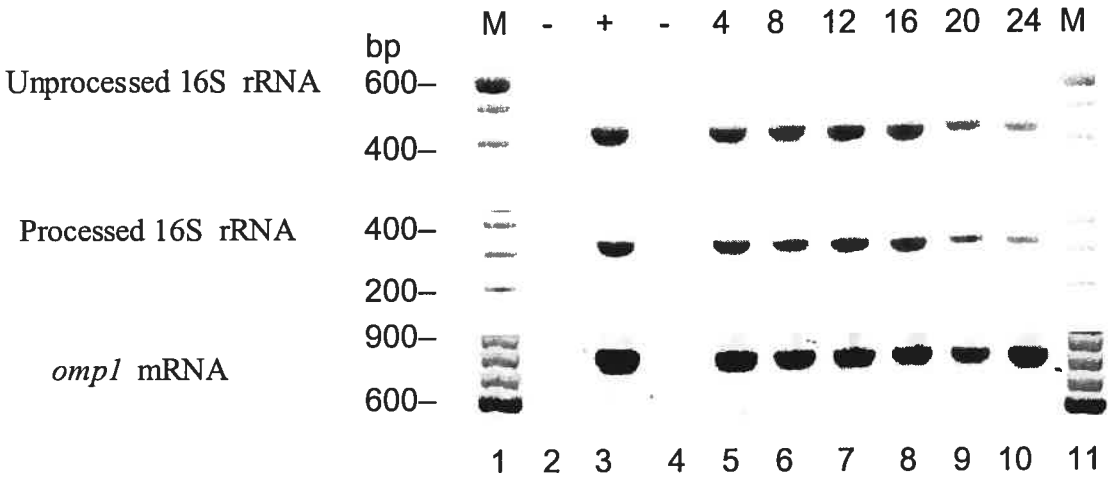
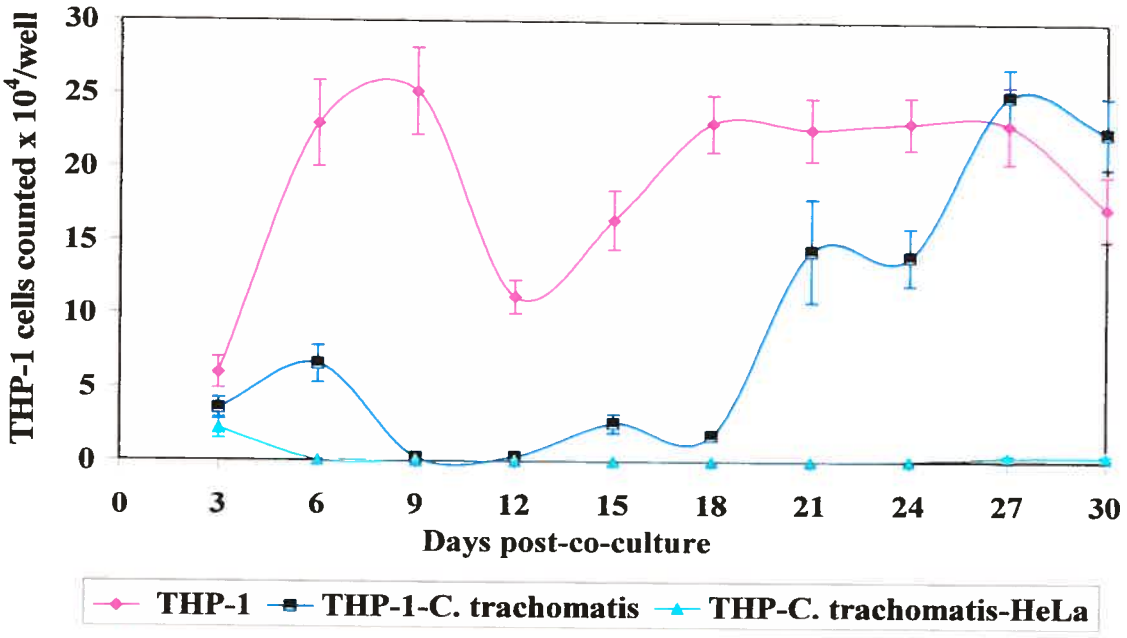

M - + - 4 8 12 16 20 24 M
bp
Unprocessed 16S rRNA
600–
400–
Processed 16S rRNA
400–
200–
900–
ompl mRNA
600–
1 2 3 4 5 6 7 8 9 10 11

Figure 4. *C. trachomatis* L2 viability after treatment with Ery assessed by RT-PCR. HeLa cells were infected at MOI of 0.1 and treated with Ery at MBC (2 μg/ml). From the day 3 post-infection, total RNAs were extracted every 4 days. Unprocessed and processed 16S rRNA, *omp1* transcripts were targeted by RT-PCR. Lanes: 1 and 11, DNA markers; 2, uninfected HeLa cells; 3, HeLa cells infected for 3 days; 4, HeLa cells infected with heat-killed *Chlamydia* for 3 days; 5-10, infected HeLa cells treated with Ery for 4, 8, 12, 16, 20, and 24 days.

A


THP-1 cells counted x 10^4/well
30
25
20
15
10
5
0
0
3
6
9
12
15
18
21
24
27
30
Days post-co-culture
THP-1
THP-1-C. trachomatis
THP-C. trachomatis-HeLa

B

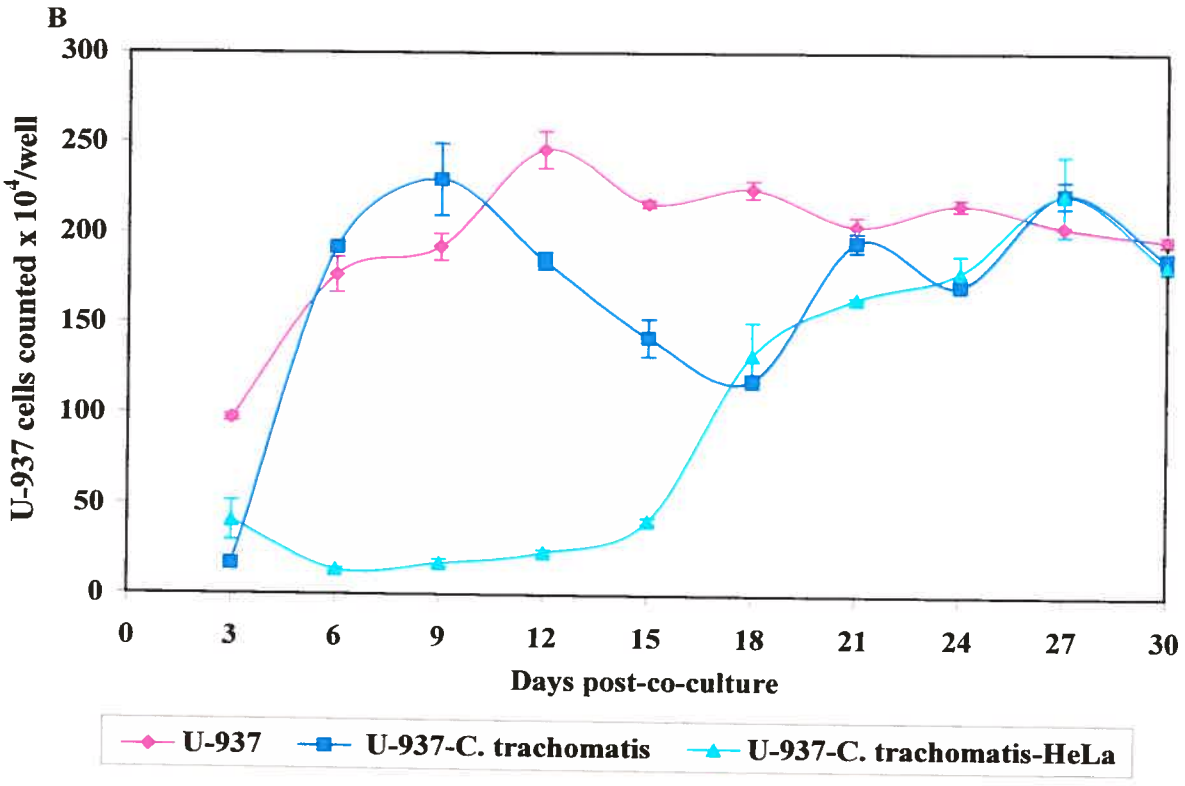
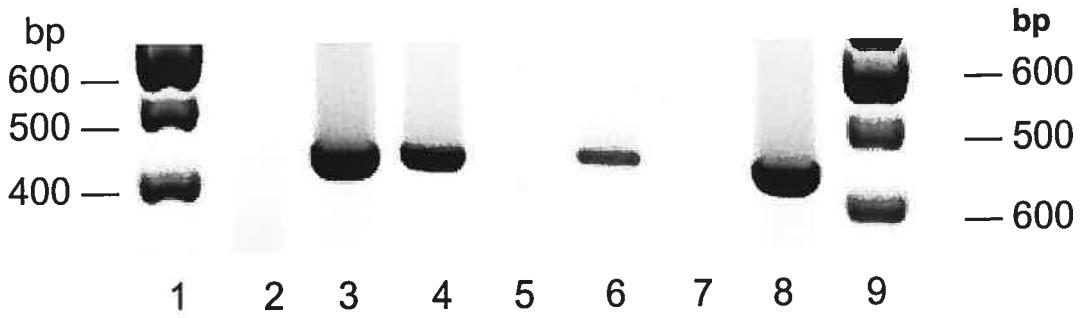

U-937 cells counted x 10^4/well
300
250
200
150
100
50
0
0
3
6
9
12
15
18
21
24
27
30
Days post-co-culture
U-937
U-937-C. trachomatis
U-937-C. trachomatis-HeLa

Figure 5. Growth curves of *C. trachomatis*-infected THP-1 (A) and U-937 (B) cells. 1.5 x 10^5 of these 2 kinds of cells were infected in 2 ways: either following straight inoculation with *C. trachomatis* EBs, or following co-culture with HeLa cells infected 3 days earlier with 10^4 EBs particles. Every 3 days for 30 days, viable THP-1 and U-937 cells were measured microscopically by the trypan blue dye exclusion method.


bp
600
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600

Figure 6. *C. trachomatis* serovar L2 viability in HeLa, THP-1 and U-937 cells after treatment with Dox assessed by RT-PCR. 10^5 HeLa cells, 1.5 x 10^5 THP-1 and U-937 cells were infected with 10^4 *C. trachomatis* L2 IFUs. 3 days post-infection, the cells were treated with Dox at MBC (1 μg/ml). 30 days later, total RNAs were extracted and analyzed by RT-PCR. Lanes: 1 and 9, DNA markers; 2, uninfected HeLa cells; 3, HeLa cells infected for 3 days; 4, THP-1 cells persistently infected with *C. trchomatis* L2 for 33 days; 5, *C. trachomatis*-infected THP-1 cells treated with Dox for 30 days; 6, U-937 cells persistently infected with *C. trachomatis* L2 for 33 days; 7, *C. trachomatis*-infected U-937 cells treated with Dox for 30 days; 8, *C. trachomatis*-infected HeLa cells treated with Dox for 30 days.

CHAPITRE V: ARTICLE 4

SUSTAINED INTERLEUKIN-6 AND INTERLEUKIN-8 EXPRESSION FOLLOWING INFECTION WITH *CHLAMYDIA TRACHOMATIS* SEROVAR L2 IN A HeLa/THP-1 CELL CO-CULTURE MODEL

Sustained interleukin-6 and interleukin-8 expression following infection with *Chlamydia trachomatis* serovar L2 in a HeLa/THP-1 cell co-culture model

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Submitted to

Scandinavian Journal of Immunology

5.1 ABSTRACT

Chlamydia trachomatis, an intracellular obligate bacterium, remains responsible for a large spectrum of disorders that can progress to chronic diseases, resulting in severe sequelae, such as tubal infertility and blindness. These sequelae may be due to deleterious immune responses that are induced by repeated or persistent infections. By initiating and regulating inflammation and immune responses, pro-inflammatory cytokines secreted by local infected epithelial and immune cells, such as monocytes, may play an essential role in immunity and the immunopathogenesis of chlamydial diseases. In this study, we mimicked the *in vivo* interaction between epithelial cells and monocytes by co-culturing epithelial-like HeLa cells with monocyte-like THP-1 cells. A multiplexed cytometric bead array assay was used to measure pro-inflammatory cytokines (interleukin-1beta [IL-1β], IL-6, IL-8, IL-10, IL-12p70 and tumor necrosis factor-alpha [TNF- α]) over a period of 18 days. We observed that pro-inflammatory cytokine secretion was augmented after *C. trachomatis* infection in HeLa and THP-1 cells. However, this heightened secretion was subsequently reduced. When infected HeLa cells were co-cultured with THP-1 cells, IL-6 and IL-8 secretion was sustained, IL-1β expression followed a bell-shaped curve, and IL-10, IL-12p70 and TNF-α synthesis was down-regulated. IL-6 and IL-8 may be involved in immunopathogenesis of chronic chlamydial infections. We also observed that throughout *C. trachomatis* persistence induced by doxycycline treatment, IL-1β, IL-6, IL-8 and TNF-α expression was reduced, whereas the synthesis of IL-10 and IL-12p70 remained unchanged, but not sustained. Thus, during chlamydial perisistent infection induced by treatment with Dox, none of tested cytokine has sustained expression.

Keywords: pro-inflammatory cytokines, doxycycline, persistence induction, chronic inflammation

5.2 RÉSUMÉ

La bactérie intracellulaire obligatoire, *Chlamydia trachomatis*, est responsable d'un large spectre de maladies qui peuvent progresser en pathologies chroniques et engendrer des séquelles sévères telles que les grossesses ectopiques et la cécité. Ces séquelles seraient dues aux réponses immunitaires délétères induites par des infections repétées ou persistantes. En initiant et en modulant l'inflammation et les réponses immunitaires, les cytokines pro-inflammatoires sécrétées par les cellules épithéliales locales et les cellules immunitaires telles que les monocytes, joueraient un rôle essentiel dans l'immunité ou l'immunopathogénèse des maladies chlamydiennes. Dans cette étude l'interaction *in vivo* entre les cellules épithéliales et les monocytes a été mimée en co-cultivant les cellules épithéliales cervicales HeLa avec la lignée de cellules moncytiques THP-1. L'essaie de cytométrie en flux multiplexe a été utilisé pour détecter et mésurer simultanément 6 cytokines pro-inflammatoires (interleukine-1beta [IL-1ß], IL-6, IL-8, IL-10, IL-12p70 et le facteur de nécrose tumoral-alpha [TNF-α]) durant 18 jours. La synthèse accrue de toutes les cytokines pro-inflammatoires après l'infection à *C. trachomatis* a été observée. Cependant, cette synthèse accrue décline subséquement dans les cellules HeLa et THP-1 séparemment. Pour ce qui est des cellules HeLa infectée et co-cultivées avec les cellules THP-1, l'expression de l'IL-1ß suit un profile en forme de cloche, la production accrue de l'IL-6 et l'IL8 demeure soutenue, alors que celle de l'IL-10, IL-12p70 et TNF-α est réduite. L'IL-6 and IL-8 pourraient jouer un rôle dans la pathogenèse des infections chlamydiennes chroniques. Fait intéressant, lors de l'infection persistante à *C. trachomatis* dans la co-culture de cellules HeLa/THP, induite à la suite du traitement à la doxycycline, la synthèse de l'IL-1β, l'IL-6, IL-8 et du TNF-α est réprimée. Bien que l'expression de l'IL-10 et l'IL-12p70 n'est pas affectée, elle n'est pas soutenue. Definitivement, durant une telle infection persistante aucune des cytokines testées n'est produite de manière prolongée.

Mots-clés: cytokines pro-inflammatoires, doxycycline, inflammation chronique, persistance.

5.3 INTRODUCTION

Chlamydia trachomatis, an atypical Gram-negative bacterium, is distinguishable from other eubacteria by its wall which lacks a peptidoglycan layer and by its obligate intracellular lifestyle that gives it a unique developmental cycle. To achieve its growth, *C. trachomatis* must successively adopt 2 forms: infectious extracellular elementary bodies (EBs) and metabolically-active reticulate bodies that are specialized in growth by binary scission in intracellular vacuoles, termed inclusions, that escape phagolysosomal fusion [31, 37]. *C. trachomatis* is subdivided into multiple serovars that cause a broad spectrum of diseases worldwide with severe sequelae. Serovars A to C are responsible for ocular diseases that can progress to trachoma, the primary cause of preventable blindness. Serovars D to K cause urogenital infections that damage the genital tract and lead to preventable infertility in women. These serovars can also induce reactive arthritis and perihepatitis. Serovars L1 to L3 are implicated in lymphogranuloma venereum, a systemic sexually-transmitted disease [19]. These different maladies are all characterized by chronic inflammation that evokes tissue damage, scarring and fibrosis, resulting in the severe sequelae enumerated.

Numerous studies suggest that the development of these sequelae is favoured by repeated or unresolved, persistent infections that induce deleterious, chronic immune responses [2, 22, 34]. Little is known about the auto-immunopathogenetic mechanisms that mediate severe tissue damage. It has been shown that cellular responses with high interferon-gamma (IFN-γ) production, like T-helper 1 (Th1) delayed type hypersensitivity (DTH) reactions, are protective against chlamydial infections, but Th2 DTH itself can elicit chronic pathologies [28, 39]. Other studies have revealed the important role played by chlamydial 60-kDa heat shock protein (Hsp60) in tissue damage during chronic infection [7, 15]. Hsp60 belongs to a family of related proteins which have been conserved during evolution. Chlamydial Hsp60 induces autoimmunity by evoking intense inflammation and by breaking host tolerance to its self-Hsp60 [5]. By modulating and promoting immune responses, pro-inflammatory proteins are major factor in chlamydial immunity and autoimmunity.

In vivo experiments, in mice infected with *C. trachomatis* MoPn have documented that cytokines synthesized at the site of infection determine the course of primary chlamydial genital infection [9]. These investigations have disclosed that the early, increased pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-α) and interferon-1beta (IL-1β) allow early eradication of infection, whereas elevated levels of macrophage inflammatory protein-2 (the equivalent of IL-8 in mice) contribute to pathogenicity. The heightened expression of pro-inflammatory cytokines, also found in the eyes of patients with active trachoma, might contribute to the development of scarring [6]. Since *Chlamydia* is at first a mucosal pathogen, target epithelial cells are pivotal in induction of immunity. *In vitro* studies have demonstrated that endocervical epithelial cells infected by *C. trachomatis* release pro-inflammatory cytokines, particularly IL-1α, IL-6, IL-8, Groα and granulocyte-macrophage colony-stimulating factor (GM-CSF). This release is required to initiate immune responses [24]. Other immune cells, such as monocyte-derived macrophages, can also contribute to immunity and auto-immunity. Monocytes that are attracted to the infected epithelium site help to resolve infection by IFN-γ release. The latter not only inhibits *C. trachomatis* growth, but is also required to induce Th1 responses [28]. Yet, monocytes/macrophages can harbour persistent *C. trachomatis* and act as carriers for *Chlamydia* to move from the genital epithelium to the synovium. There, they contribute to the pathogenesis of arthritis by maintaining chronic, sustained inflammation followed by continuous release of a broad spectrum of destructive pro-inflammatory cytokines [12, 16].

Since pro-inflammatory cytokines are important in the initiation and regulation of immune responses, and thus in the outcome of diseases, it is essential to characterize their expression profiles in different types of cells. In this paper, we report on IL-1β, IL-6, IL-8, TNF-α, IL-10 and IL-12p70 expression in HeLa and monocyte cell lines infected with *C. trachomatis serovar* L2. In addition, we have tried to partially mimic *in vivo* conditions by co-culturing infected HeLa cells with monocyte-like THP-1 cells and studied cytokine expression. Finally, we showed that these cytokine profiles can be altered by persistent *C. trachomatis* after doxycycline (Dox) treatment.

5.4 MATERIALS AND METHODS

5.4.1 Cell culture

HeLa cells obtained from the American Type Culture Collection (Rockville, MD) were cultured in minimal essential medium (MEM) with Hank's salts containing no essential amino acids and L-glutamine (MEM) (Sigma, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen, Burlington, Ontario, Canada). THP-1 purchased from ATCC (TIB-202) were cultured in RPMI 1640 medium containing 2 mM L-glutamine, 50 µg/ml gentamycine, 1,5 g/L sodium bicarbonate, 4.5g/L glucose, 10mM Hepes, 1 mM sodium pyruvate and supplemented with 10% FBS. HeLa and THP-1 cultures were maintained at 35-37°C in a humidified incubator containing 5% CO_2.

6.4.2 Bacterial strain, purification and titration

C. trachomatis serovar L2/434/Bu was provided by Laboratoire de Santé Publique du Québec (Montreal, Quebec, Canada). This bacterium was propagated in HeLa cell monolayers and fed with MEM/H supplemented with 10% FBS and 0.5 µg/ml cycloheximide (Sigma). The resulting EBs were purified by ultracentrifugation on sodium diatrizoate (Sigma) as described previously [33]. In brief, infected, confluent HeLa monolayers were collected in SPG buffer (0.01 M sodium phosphate [pH 7.2], 0.25 M sucrose, 5 mM L-glutamic acid) with a scraper. Infectious particles were released from the cells by sonication. First, the EBs were semi-purified by centrifugation at 500xg for 15 min (RC2-B Sorvall, rotor SS34) to remove cellular debris. Then, the collected supernatants were centrifuged at 15,000 rpm for 1 h at 4°C. Finally, the resulting pellets were suspended in SPG buffer and layered over 35% sodium diatrizoate for purification by ultracentrifugation at 43,000xg for 30 min at 4°C in a Ti-70 rotor (Beckmann Instruments, Palo Alto, CA, USA). EB bands were harvested and washed 3 times in SPG buffer by successive centrifugations, each at 43,000xg for 30 min at 4°C. Finally, purified *C. trachomatis* EBs were collected and suspended in SPG buffer, aliquotted and stored at -80°C.

EB stock was titrated by enumerating inclusion-forming units (IFUs). Briefly, 2.5 x 10^5 HeLa cells were seeded on sterile glass coverslips contained in 24-well plates. After 18-24 h, the culture medium was replaced by different dilutions of EBs in SPG buffer. The plates were then centrifuged for 1 h at 500xg and at 25°C. After centrifugation, inocula were replaced by MEM/H supplemented with 10% FBS and 0.5 μg/ml cycloheximide. 48 h post-infection, the cells were fixed with methanol and subjected to May-Grünwald-Giemsa staining. Inclusions were visualized by light microscopy and enumerated as viable IFUs [29].

5.4.3 Sample preparation for cytokine quantification

THP-1 cells and trypsinized HeLa cells were centrifuged at 500xg for 5 min. The supernatants were then discarded and cells in RPMI 1640 were counted and distributed in 24-well plates containing 1.5 x 10^5 and 10^5 per well of THP-1 and HeLa cells, respectively. 18-24 h later, the cultured cells were inoculated with 10^4 *C. trachomatis* serovar L2 IFUs in SPG buffer and centrifuged at 500xg for 1h at 25°C to facilitate infection. After centrifugation, SPG buffer containing the inoculum was discarded and replaced by complete RPMI medium containing 0.5 μg/ml cycloheximide. The cells were then incubated at 35-37°C under 5% CO_2. Co-culture was established 3 days post-infection. To do that, 1.5 x 10^5 fresh THP-1 cells in 1 ml of complete RPMI 1640 medium without cycloheximide were added to infected and non-infected HeLa cell monolayers. For control and infected HeLa and THP-1 cells, only 1 ml of culture media was added. From that moment on, culture supernatants were collected every 3 days, and stored at -70°C for cytokine quantification by cytometric bead array assay.

5.4.5 THP-1 growth curves

To check THP-1 viability throughout the experiment, THP-1 growth curves were charted. THP-1 cells were counted while samples for cytokine quantification were collected and stored. The trypan blue dye exclusion test was used to enumerate viable THP-1 cells.

5.4.6 Effects of Doxycycline on cytokine secretion

To determine if antichlamydial therapy with Dox affects cytokine secretion, experiments were performed as above. Dox purchased from Sigma was supplied as a powder. This powder was then solubilized into sterile water, as recommended. The minimal bactericidal concentration (MBC) of Dox in culture media was determined to be 1.0 μg/ml [30].

5.4.7 *Chlamydia* growth

HeLa cells cultured on sterile glass coverslips contained in 24-wells plates were infected with *C. trachomatis* serovar L2 at a multiplicity of infection (MOI) of 0.1 as described above. Infection was measured by counting inclusions every 2 days. Cells on coverslips were exposed to May-Grünwald-Giemsa staining. Inclusions were directly enumerated by light microscopy.

5.4.8 Cytometric bead array analysis

The BD™ human inflammation cytometric bead array kit (BD Biosciences, Mississauga, Ontario, Canada) was used according to the manufacturer's instructions to simultaneously quantify IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF-α in stored samples. Briefly, 6 sets of capture beads, each coated with specific capture antibodies, were mixed at the same proportion. 50 μl of the mixed capture beads, of the test sample and of phycoerythrin-conjugated specific antibodies were mixed for detection. The mixture, protected from light exposure, was incubated for 3 h at room temperature. After incubation, the mixture was washed with the addition of wash buffer followed by centrifugation at 200xg for 5 min. Finally, the supernatant was discarded and the pellet suspended in wash buffer for analysis by calibrated flow cytometer (FACSCalibur, BD Biosciences). Sensitivity for IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF-α was 7.2, 2.5, 3.6, 3.3, 1.9 and 3.7 pg/ml, respectively.

5.4.9 Statistical analysis

One-way ANOVA for repeated measures was conducted, with treatments and time points as the sources of variation. Post hoc Tukey testing was performed to calculate the

significance level between different groups. P values<0.05 were interpreted as being statistically significant.

5.5 RESULTS

5.5.1 Infected THP-1 cell growth

THP-1 cells were counted by trypan blue staining. As shown in Figure 1, THP-1 cells interacting with control HeLa cells did not proliferate during the 30-day culture period. Possibly, this interaction allowed monocyte differentiation into macrophage as suggested by Stříž et *al.* [35]. In our study, we had no interest in proving monocyte differentiation into macrophages. We noticed that THP-1 cells supported *C. trachomatis* serovar L2 growth. Light microscopy revealed typical chlamydial inclusions in the THP-1 cell cytoplasm (data not shown). Chlamydial growth ultimately led to cell lysis, explaining why the growth of infected THP-1 cells and THP-1 cells interacting with infected HeLa cells was much slower than that of non-infected THP-1 cells (Fig. 1). However, a few cells remained and survived chlamydial infection. In infected THP-1 cells, all those cells that harboured active chlamydial growth were lysed between days 9 and 12; only about 31,000 cells of the original 1.5×10^5 cells escaped active infection. After day 12, these surviving cells showed renewed high growth as seen in Figure 1. Among THP-1 cells interacting with infected HeLa cells, only only 3,400 cells survived between days 6 and 24; all actively-infected cells were lysed. Growth was slowly renewed after 24 days. The escape of THP-1 cells from active chlamydial infection was not due to EBs insufficiency. EBs in THP-1 cells interacting with infected HeLa cells were titrated. At day 9, 246 times more EBs than THP-1 cells were observed. In addition, despite the absence of active infection, surviving cells harbored *C. trachomatis* growth, proven by the presence of unprocessed chlamydial 16s rRNA (data not included). This observation suggests that *C. trachomatis* remained persistent in these cells.

5.5.2 *C. trachomatis* serovar L2 growth in HeLa cells

Chlamydia development in HeLa cells is high from the day 2 to the day 12, that is to say 2.45×10^4 to 4.06×10^4 of chlamydial IFUs. From the day 12 on, chlamydial growth decreased due to HeLa monolayer degradation (Table 1).

5.5.3 Cytokine synthesis in *C. trachomatis* serovar L- infected HeLa cells

We studied expression profiles of 5 pro-inflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α, and IL-12p70) and one anti-inflammatory cytokine (IL-10) in HeLa cells. As illustrated in Figure 2, IL-1β, IL-6, IL-8, TNF-α, IL-10 and IL-12p70 were all synthesized in control HeLa cells. This synthesis was in steady state except for IL-8 (Fig. 2c) which increased slightly with time, likely because of the increment of viable HeLa cells. Infection with *C. trachomatis* serovar L2 markedly augmented cytokine secretion. For example, the IL-1β level was 2 to 10 times higher than that produced in control HeLa cells (Fig. 2a). Statistical analysis disclosed significant differences between the controls and infected HeLa cells in all cases ($P<0.05$).

Three similar cytokine synthesis profiles were identified. IL-1β, TNF-α, IL-10 and IL-12p70 synthesis was characterized by increases alternating with decreases (Fig. 2a, 2d, 2e and 2f). After day 12, no increment was observed, probably because at this time the HeLa monolayers were damaged. The periods of increase and decrease may be linked to the chlamydial developmental cycle characterized by periods of lysis and regrowth. The IL-6 synthesis profile in HeLa cells was completely different (Fig. 2b). At the beginning, the rise in IL-6 was slow, only 12 times higher than control values. This amount continued to increase with time. At day 12, there was 995 times more IL-6 in infected cells than in the controls. However, from this day on, when the HeLa cell monolayers began to be degraded, IL-6 decreased but remained higher than in the controls. Regarding IL-8 (Fig. 2c), at the beginning, the synthesis was high, namely 80 and 173 times more than in the controls at days 3 and 6. From day 6, IL-8 synthesis decreased progressively. Thus, IL-6 increased in *C. trachomatis*-infected HeLa cells, while IL-8 synthesis was repressed.

5.5.4 Cytokine synthesis in *C. trachomatis* serovar L2- infected THP-1 cells

Pro-inflammatory cytokines in monocyte-like THP-1 cells were also measured (Fig. 3). It is suggested that monocytes harboring persistent chlamydial infection could go to the joints where they lead to arthritic damage. Pro-inflammatory cytokine synthesis was increased in infected THP-1 cells, as it was in HeLa cells. However, this synthesis

eventually decreased with time. IL-1β and TNF-α expression was only high at days 3 and 6 (Fig. 3a and 3d). IL-1β and TNF-α expression in infected THP-1 cell is not significantly different from expression in the controls ($P>0.05$). IL-6 expression increased with fluctuations and peaked at day 12, then declined (Fig. 3b). The IL-8 expression profile in infected THP-1 cells and in infected HeLa cells seems similar ($P>0.05$), even if the expression in infected THP-1 cells was 4 to 5 times higher (Figures 2c and 3c). At day 3, IL-12p70 expression is increased, about 6 times more than in controle (Fig. 3f). From day 3 to day 6, IL-12 expression decline before increasing again until day 12. After day 12, this expression decline until controle level. Synthesis of the anti-inflammatory cytokine IL-10 was high at the beginning and fell progressively with time until it reached the control level at day 12 (Fig. 3e).

Cytokine decreases may not be explained by reduction of whole THP-1 cell concentration, but only by the depletion of those cells that harboured active infection. We did not measure cytokine expression according to the number of *Chlamydia* IFUs, but previous studies have shown that it depends on chlamydial growth [24]. Cells that harbor active *C. trachomatis* growth were lysed; thus, only THP-1 cells harboring latent *Chlamydia* were left in the culture media.

5.5.5 Cytokine synthesis in *C. trachomatis* serovar L2- infected co-cultured HeLa/THP-1 cells

When epithelial infection occurs *in vivo*, inflammation is induced. Then, mononuclear cells such as monocytes and polynuclear cells are recruited to limit bacterial spread. To mimic the interaction *in vivo* between the epithelium and monocytes, infected HeLa cells were co-cultured with THP-1 cells and pro-inflammatory cytokines induction was quantified. IL-6 and IL-8 synthesis remained high over the entire period of 18 days, despite progressive degradation of the HeLa cell monolayers and the lysis of THP-1 cells (Figures 4b and 4c). IL-8 synthesis by infected co-cultured HeLa/THP-1 cells, versus synthesis by infected HeLa or THP-1 cells, remained the highest ($P<0.05$). IL-6 expression remains also hight in co-culture, but not significantly dofferent from synthesis by infected HeLa cells.

IL-1β synthesis followed a bell-shape curve: the high cytokine increase reached a peak at day 9 and then decreased gradually (Fig. 4a). Its expression profile suggests cytokine regulation in this co-culture system. Statistical analysis revealed that IL-1ß synthesis by co-cultured HeLa/THP-1 cells was significantly different from synthesis by infected HeLa cells and by infected THP-1 cells. Slight IL-1β expression in control cells was noted. This IL-1β increase could have resulted from release by dead cells, as IL-1β could be synthesized and pre-stored in the cell cytoplasm. In addition, unlike HeLa cells, monocyte-like cells are unstable cells that could differentiate by dead cells releasing compounds.

The IL-12p70, IL-10 and TNF-α expression profiles in HeLa, THP-1 and co-cultured HeLa/THP-1 cells were not significantly different ($P>0.05$). Apparently, however, IL-10 synthesis in infected HeLa cells sometimes appeared to be higher than in infected co-cultured HeLa/THP-1 cells (Fig. 2e and 4e). This suggests that the highest production of IL-6 and IL-8 in infected co-cultured HeLa/THP-1 cells did not result from additive effects, but from a real up-regulation. Thus, since the expression of TNF-α, IL-10 and IL-12p70 in infected co-cultured HeLa/THP-1 cells was similar to that in infected HeLa or THP-1 cells, they appear to be down-regulated in infected co-cultured cells.

5.5.6 Effects of Dox on cytokine expression

Our studies revealed that Dox treatment of HeLa cells at the MBC of 1 μg/ml, even for 24 days, suppressed active *C. trachomatis* growth, but did not resolve the infection. It remained in a persistent state, as disclosed by continuous chlamydial 16s rRNA expression (data not shown). We may question whether the transition between active and persistent infection come with altered pro-inflammatory cytokine expression. Dox treatment significantly inhibited IL-1β, IL-6, TNF-α synthesis (Table 2 and not shown). There were no significant differences between control and infected cells treated with Dox ($P<0.05$). Decreased cytokine synthesis was not due to Dox's anti-inflammatory action. A previous study has demonstrated that Dox can only inhibit cytokine synthesis from 0.01 mM of concentration [17]. In the present investigation, Dox was used at 0.002 mM. It did

not completely inhibit IL-8 synthesis. IL-8 synthesis in infected co-cultured cells treated with Dox was lower than in untreated infected co-cultured cells, but remained higher than in the controls. There were significant differences between the 3 groups ($P<0.05$) (Table 2). But this expression is not sustained. Dox treatment did not alter IL-10 and IL-12p70 synthesis, no significant difference compared with infected control, but this expression is not also sustained. In conclusion, none of tested cytokines has sustained expression during *C. trachomatis* serovar L2-persistant infection induced by treatment with Dox.

5.6 DISCUSSION

Severe sequelae, such as blindness, infertility and ectopic pregnancy resulting in chronic chlamydial diseases, are mediated by induced deleterious immune responses. Cytokines are not only proteins regulating immune responses, but some of them, notably IL-1 and TNF-α are proven to be cytotoxic. The present study examined pro-inflammatory cytokine expression profiles during *C. trachomatis* serovar L2 growth. *In vivo*, infected epithelial cells interacted intimately not only with resident macrophages and dendritic cells, but also with neutrophils, monocytes and natural killer cells that arrived at the infection site after inflammatory influx. To mimic one of these multiple interactions, epithelial-like cells (HeLa) were co-cultured with monocyte-like cells (THP-1), and induced pro-inflammatory cytokines were measured.

In vitro studies of the cytokine expression profiles induced by *Chlamydia* in permissive cells can be disadvantageous. *C. trachomatis* serovar L2 actively infects and grows in HeLa and THP-1 cells. This growth ultimately leads to the lysis of both cell types. At the same time, increased IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF-α expression is observed, but decreases with time. In this context, it is difficult to conclude that they are down-regulated. Some studies have shown correlations between the amount of secreted cytokines and *Chlamydia* growth [24]. In this case, the decrease of infected cells leads to the diminished cytokine release. However, under these conditions, if the amount of cytokine remains high when the number of cells is reduced, this event could be attributed to up-regulation.

IL-6 and IL-8 expression remained high in our co-culture model of HeLa and THP-1 cells during the 18-day study period. Since the secreting cell number was reduced after HeLa monolayer degradation and THP-1 cell lysis, we conclude that cytokine synthesis intensified. Several studies [18, 24] have established increased expression of IL-6 and IL-8 after infection with *C. trachomatis*, but in the present work IL-6 and IL-8 expression was found to be sustained. The role of IL-6 in chlamydial immunity is not clear. Williams et al. [36] reported that IL-6 is needed for an early optimal host response, probably by initiating or maintaining the Th1 response required to resolve chlamydial infection. IL-6

can partly block the suppressor activity of $CD4^+CD25^+$ regulatory T cells to allow the activation of pathogen-specific adaptive immune responses [21]. Because IL-6 is identified as a pleiotropic cytokine, its chronic overproduction is deleterious. It stimulates hepatocytes to produce acute-phase proteins, such as fibrinogen, and it can play an important role in the development of fibrosis during chronic chlamydial disease. In addition, IL-6 is an important B-cell growth factor, which links it with arthritis. Synoviocytes from arthritic patients have been shown to synthesize IL-6 [27]. *In vitro* studies also have documented increased IL-6 synthesis in *C. trachomatis*-infected synoviocytes [26]. Our results confirmed the possible role of IL-6 in immunity or in the pathogenesis of chlamydial diseases.

As mentioned above, IL-8 expression also is enhanced and sustained over time. IL-8, a CXC chemokine, may be an important component for local immunity by chemoattracting and activating neutrophils. However, it may be involved in chronic inflammation [3, 8, 10]. IL-8 production is enhanced in chronic, but not in early ileal lesions of Crohn's disease [4]. Some studies have reported correlations between IL-8 levels and polymorphonuclear infiltration [20]. IL-8 RNA is increased in the joints of patients with chronic arthritis and infected with *C. trachomatis* or *C. pneumoniae* [13]. In fact, continuous IL-8 production can promote the infiltration of neutrophils that are not only inefficient in resolving chlamydial infections, but can release proteases that damage cells.

IL-1β production increases markedly, peaks and finally decreases. This expression profile suggests its regulation. IL-1β release after *C. trachomatis* growth seems to be required to sensitize the host thereby triggering immune responses [24]. IL-1β release elicits pro-inflammatory cytokine secretion, including that of IL-6, IL-8 and GM-CSF. However, lasting IL-1β synthesis can be deleterious. Damage, for example, cartilage deterioration and fibrosis in patients with arthritis and trachoma, respectively, has been attributed to high IL-1β expression [6, 14]. Our study confirmed that IL-1β expression increased after *C. trachomatis* infection. Moreover, this enhanced secretion was subsequently down-regulated. IL-1β down-regulation may be explained by the differential regulation of IL-1β with its natural inhibitor, IL-1 receptor antagonist (IL-1RA). It has been found that the

differentiation of monocytes to macrophages leads to down- regulation of IL-1β, but to up-regulation of IL-1RA [32]. In airway epithelial cells, TNF-α can influence the levels of IL-1β and IL-1RA [40]. Also, considering the importance of IL-1β in immune response initiation, it is possible that *C. trachomatis* by itself down-regulates IL-1β expression. Viral compounds, for example, can inhibit the enzyme named ICE, converting pro-IL-1β to active IL-1β [25]. Thus, to understand how down-regulation occurs, it will be interesting to measure the concomitant expression of IL-1β, IL-1RA and ICE activity over the time.

TNF-α, IL-10 and IL-12p70 production in infected co-cultured HeLa/THP-1 cell is virtually similar to that seen in the 2 kinds of cells separately. It was very surprising to find that the increased expression of TNF-α was not sustained. TNF-α, with IL-1, may play a role in the pathogenesis of rheumatoid arthritis, for which *C. trachomatis* is one of the etiologic agents [23].

By secreting IL-10 and IL-12p70, HeLa cells may be implicated in the regulation of antichlamydial-specific immune responses. The balance between IL-10 and IL-12 is actually deciding in the induction of specific adaptive responses. IL-12 favours the Th1 response that is required to resolve infection, whereas IL-10 induces the ineffective Th2 response [38]. However, in the 3 kinds of cellular systems examined in this study, no preferential or differential expression of any of these 2 regulatory proteins was noticed. Thus, if *C. trachomatis* modulates IL-10 or IL-12 expression, this regulation may occur very late during in vitro infection.

Some studies have revealed that antimicrobial compounds, such as IFN-γ or ciprofloxacin, evoke persistent chlamydial infection [1, 11]. Our work showed that bactericidal Dox treatment induced also persistent *C. trachomatis* L2 infection. It is important to know if the transition between active and persistent infection elicited changes in cytokine expression. The present study disclosed that Dox treatment leads to TNF-α, IL-8, IL-1β and IL-6 synthesis inhibition. Although IL-10 and IL-12p70 expression is not affected, it is not sustained. The cytokine synthesis inhibition was not

due to the anti-inflammatory effect of Dox, but was caused by chlamydial persistence, as stated before. Our experiment revealed that when *C. trachomatis* remained in a persistent state, the amount of pro-inflammatory cytokines was reduced. This finding is in accordance with the correlation between chlamydial growth and cytokine level [24]. The reduction of pro-inflammatory cytokine synthesis may influence the intensity of inflammation that may become insufficient to support adaptive responses to resolve infection. However, over time this diminished inflammation may lead to tissue damage.

In conclusion, we found that *C. trachomatis* serovar L2 provoked lasting IL-6 and IL-8 expression in a co-culture model of HeLa/THP-1 cells. When increased IL-1β expression became down-regulated with time, TNF-β, IL-10 and IL-12p70 expression was not sustained. In addition, we found that in persistent infection induced by Dox treatment, IL-1β, IL-6, IL-8 and TNF-α secretion was inhibited, whereas IL-10 and IL-12p70 synthesis remained unchanged, but not sustained. Understanding the role of IL-6 and IL-8 in acute and chronic infection may help to elucidate chlamydial immunopathogenesis.

5.7 ACKNOWLEDGEMENTS

This work was supported in part by "Agence Canadienne de la Francophonie" and by the "Programme Canadien des Bourses de la Francophonie" (scholarship to M.P.). We are grateful to Robert Boileau and Michal Abrahamowicz for their valuable assistance with statistical analysis. The editorial work of Ovid Da Silva is acknowledged.

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5.9 FIGURES

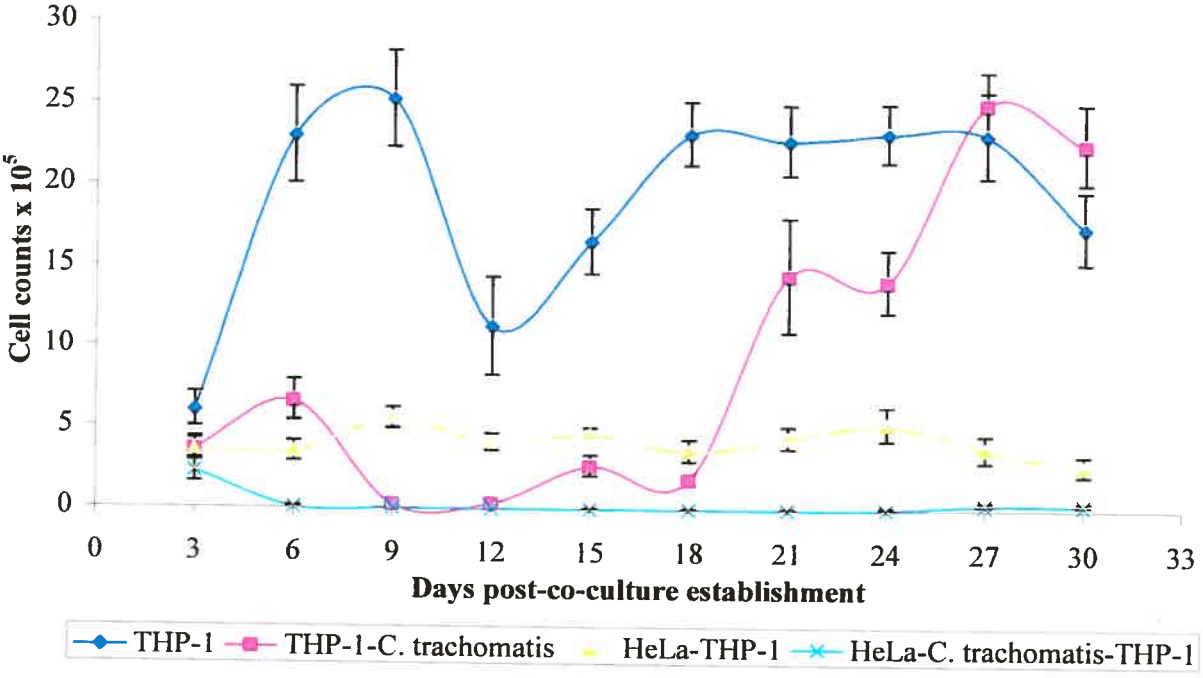
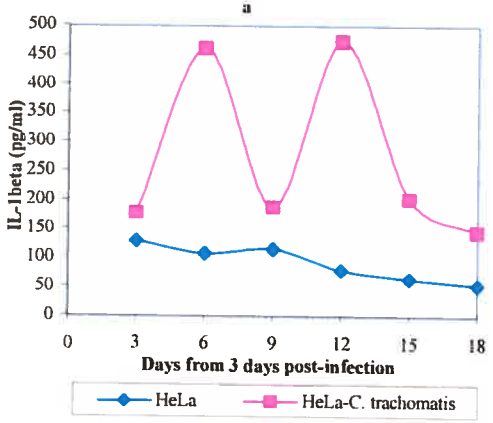
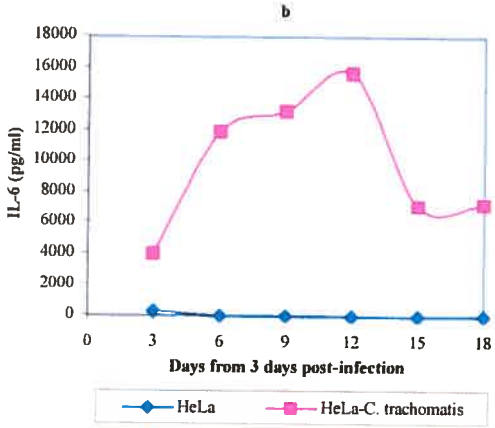

30
25
20
15
10
5
0
Cell counts x 10^5
0
3
6
9
12
15
18
21
24
27
30
33
Days post-co-culture establishment
THP-1
THP-1-C. trachomatis
HeLa-THP-1
HeLa-C. trachomatis-THP-1

Figure 1: Influence of *C. trachomatis* serovar L2 infection on THP-1 cells alone or in co-culture with HeLa cells. 10^5 HeLa cells or 1.5 x 10^5 THP-1 cells were infected with 10^4 chlamydial EBs. Co-culture was established 3 days post-infection with addition of 1.5 x 10^5 fresh THP-1 cells to infected or non-infected HeLa cell culture. Viable THP-1 cells in culture were counted every 3 days by the trypan blue dye exclusion method. Data are means ± standard deviations (error bars) of 3 experiments.

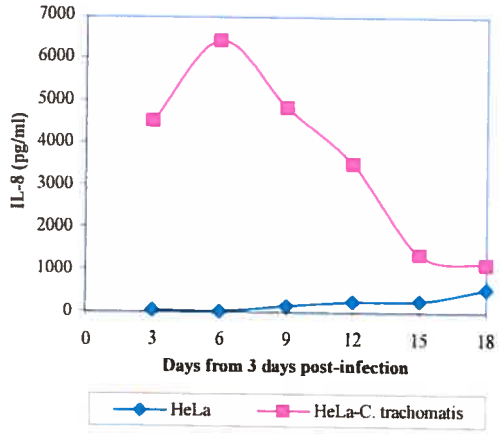
a


IL-1beta (pg/ml)
500
450
400
350
300
250
200
150
100
50
0
0 3 6 9 12 15 18
Days from 3 days post-infection
HeLa
HeLa-C. trachomatis

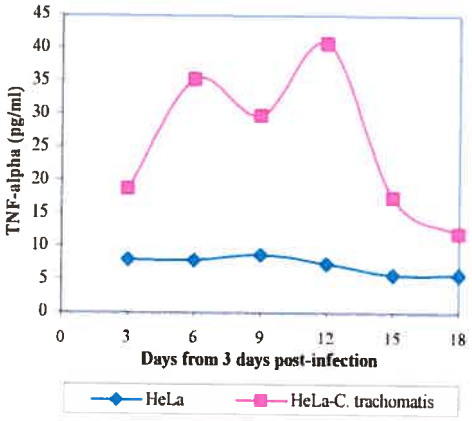
b


IL-6 (pg/ml)
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
0 3 6 9 12 15 18
Days from 3 days post-infection
HeLa
HeLa-C. trachomatis

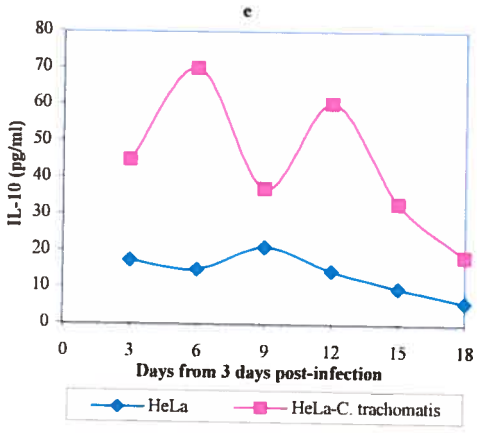
c


IL-8 (pg/ml)
7000
6000
5000
4000
3000
2000
1000
0
0 3 6 9 12 15 18
Days from 3 days post-infection
HeLa
HeLa-C. trachomatis

d


TNF-alpha (pg/ml)
45
40
35
30
25
20
15
10
5
0
0 3 6 9 12 15 18
Days from 3 days post-infection
HeLa
HeLa-C. trachomatis

e


IL-10 (pg/ml)
80
70
60
50
40
30
20
10
0
0 3 6 9 12 15 18
Days from 3 days post-infection
HeLa
HeLa-C. trachomatis

f

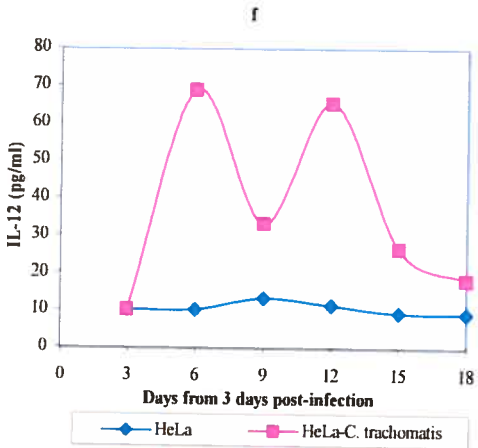
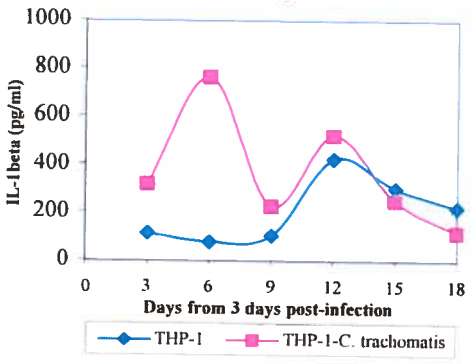
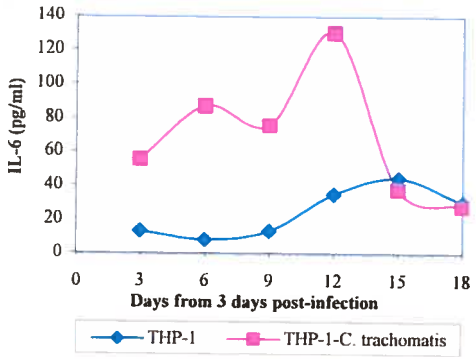

IL-12 (pg/ml)
80
70
60
50
40
30
20
10
0
0 3 6 9 12 15 18
Days from 3 days post-infection
HeLa
HeLa-C. trachomatis

Figure 2: IL-1β (A), IL-6 (B), IL-8 (C), TNF-α (D), IL-10 (E) and IL-12p70 (F) synthesis by *C. trachomatis* serovar L2-infected HeLa cells. 10^5 HeLa cells were infected with 10^4 chlamydial infectious particles. 3 days post-infection, once infection was really established, cycloheximide was removed from the culture media. From this day on, supernatants were collected every 3 days and stored for further pro-inflammatory cytokine identification by microarray bead assay.

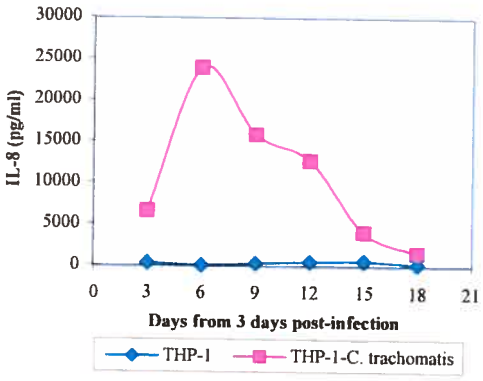
a


IL-1beta (pg/ml)
1000
800
600
400
200
0
0 3 6 9 12 15 18
Days from 3 days post-infection
THP-1
THP-1-C. trachomatis

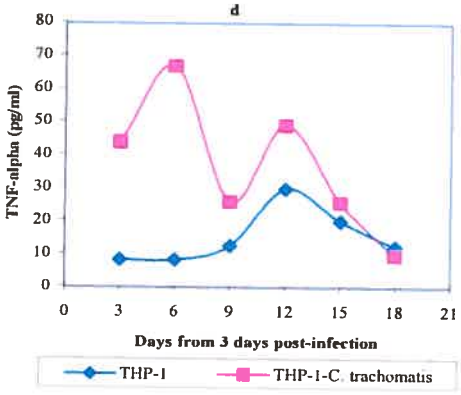
b


IL-6 (pg/ml)
140
120
100
80
60
40
20
0
0 3 6 9 12 15 18
Days from 3 days post-infection
THP-1
THP-1-C. trachomatis

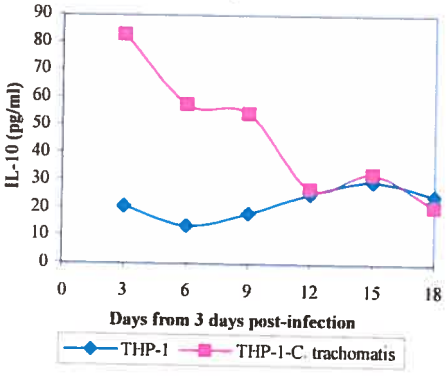
c


IL-8 (pg/ml)
30000
25000
20000
15000
10000
5000
0
0 3 6 9 12 15 18 21
Days from 3 days post-infection
THP-1
THP-1-C. trachomatis

d


TNF-alpha (pg/ml)
80
70
60
50
40
30
20
10
0
0 3 6 9 12 15 18 21
Days from 3 days post-infection
THP-1
THP-1-C. trachomatis

e


IL-10 (pg/ml)
90
80
70
60
50
40
30
20
10
0
0 3 6 9 12 15 18
Days from 3 days post-infection
THP-1
THP-1-C. trachomatis

f

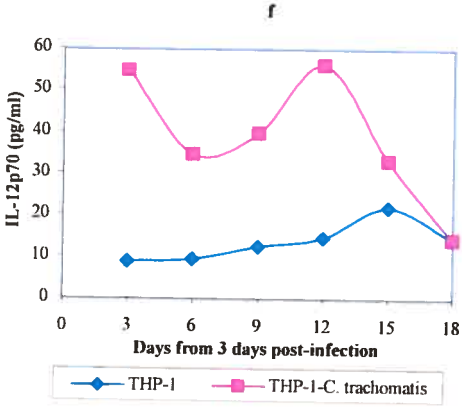
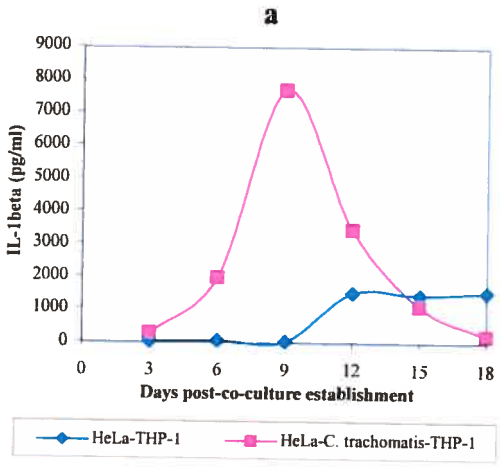
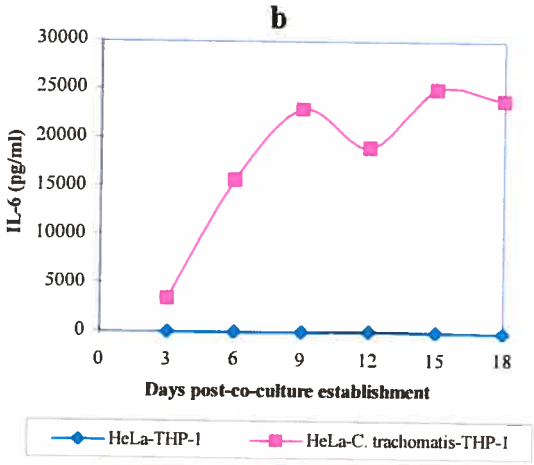
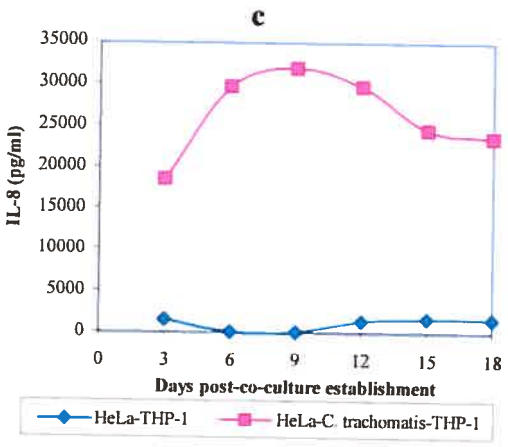
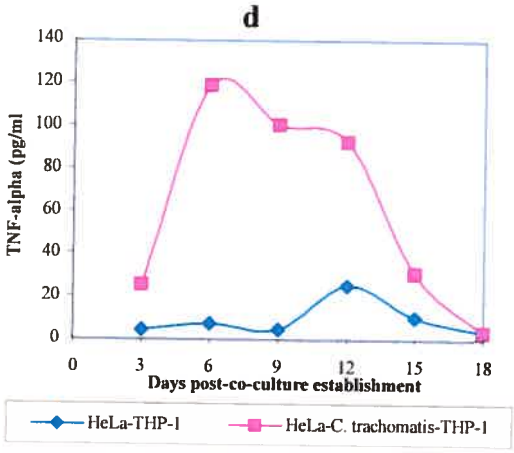

IL-12p70 (pg/ml)
60
50
40
30
20
10
0
0 3 6 9 12 15 18
Days from 3 days post-infection
THP-1
THP-1-C. trachomatis

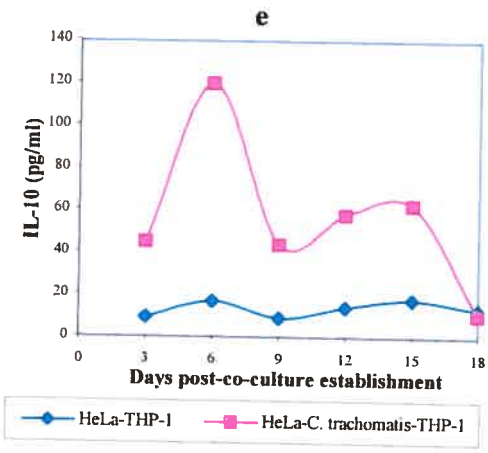
Figure 3: IL-1β (A), IL-6 (B), IL-8 (C), TNF-α (D), IL-10 (E) and IL-12p70 (F) synthesis by *C. trachomatis* serovar L2-infected THP-1 cells. 1.5 x 10^5 THP-1 cells were infected with 10^4 chlamydial infectious particles. 3 days post-infection, once infection was really established, cycloheximide was removed from the culture media. From this day on, the culture media were collected every 3 days and stored for further pro-inflammatory cytokine identification.


a
IL-1beta (pg/ml)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0 3 6 9 12 15 18
Days post-co-culture establishment
HeLa-THP-1
HeLa-C. trachomatis-THP-1


b
IL-6 (pg/ml)
30000
25000
20000
15000
10000
5000
0
0 3 6 9 12 15 18
Days post-co-culture establishment
HeLa-THP-1
HeLa-C. trachomatis-THP-1


c
IL-8 (pg/ml)
35000
30000
25000
20000
15000
10000
5000
0
0 3 6 9 12 15 18
Days post-co-culture establishment
HeLa-THP-1
HeLa-C. trachomatis-THP-1


d
TNF-alpha (pg/ml
140
120
100
80
60
40
20
0
0 3 6 9 12 15 18
Days post-co-culture establishment
HeLa-THP-1
HeLa-C. trachomatis-THP-1


e
IL-10 (pg/ml)
140
120
100
80
60
40
20
0
0 3 6 9 12 15 18
Days post-co-culture establishment
HeLa-THP-1
HeLa-C. trachomatis-THP-1

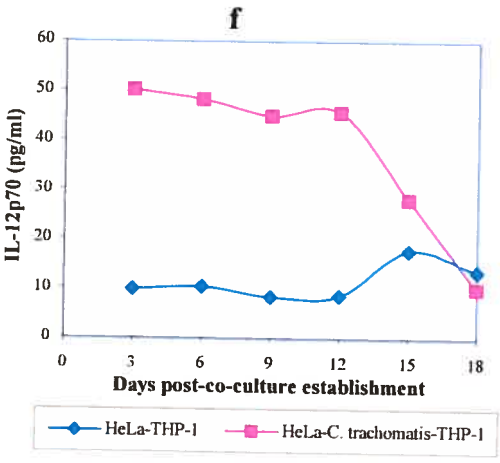

f
IL-12p70 (pg/ml)
60
50
40
30
20
10
0
0 3 6 9 12 15 18
Days post-co-culture establishment
HeLa-THP-1
HeLa-C. trachomatis-THP-1

Figure 4: IL-1β (A), IL-6 (B), IL-8 (C), TNF-α (D), IL-10 (E) and IL-12p70 (F) synthesis by *C. trachomatis* serovar L2-infected co-cultured HeLa/THP-1 cells. 10^5 HeLa cells were infected with 10^4 chlamydial infectious particles. To establish co-culture, 3 days post-infection, once infection was really established, 1.5 x 10^5 fresh THP-1 cells in culture media without cycloheximide were added on the infected HeLa monolayer. Then, supernatants were collected every 3 days and stored for further pro-inflammatory cytokine identification.

6.10 TABLES

Table 1 *C. trachomatis* serovar L2 growth [a]

Days	Inclusions/well x 10^4
2	2.45 ± 0.62
4	3.18 ± 0.69
6	2.48 ± 0.25
8	2.99 ± 0.47
10	4.32 ± 0.88
12	4.06 ± 0.77
14	0.60 ± 0.51
16	0.38 ± 0.26
18	0.09 ± 0.18
20	0.08 ± 0.16
22	0.15 ± 0.29
24	0.17 ± 0.35

[a] 10^5 HeLa cells, seeded in 24-well plates, were infected with 10^4 *C. trachomatis* serovar L2 IFUs. From the third day of infection the cells were exposed to May-Grünwald-Giemsa stain, every 2 days. Inclusions were then enumerated by light microscopy. The data are mean numbers of inclusions/well in 4 experiments.

Table 2 Effect of Dox on cytokine secretion by co-cultured HeLa/THP-1 cells infected with *C. trachomatis* serovar L2[a]

Days post-treatment	Quantities of cytokine produced (ng/ml)								
	IL-1β			IL-6			IL-8		
	- Dox	+ Dox	Ratio(-/+)	- Dox	+ Dox	Ratio(-/+)	- Dox	+ Dox	Ratio(-/+)
3	0.30	0.47	0.63	3.46	1.68	2.06	18.64	13.97	1.33
6	1.99	0.37	5.37	15.72	3.20	4.9	29.80	24.00	1.24
9	7.70	0.42	18.41	23.00	1.45	15.87	32.04	22.18	1.44
12	3.46	0.32	10.43	19.06	0.27	69.12	29.79	11.09	2.68
15	1.11	0.12	0.93	25.00	0.05	469.04	24.59	2.74	8.98
18	2.28	0.03	7.33	23.89	0.02	1380.95	23.65	0.78	30.09

[a] HeLa cells were infected with *C. trachomatis* serovar L2 at MOI of 0.1. Three days later, 1.5 x 10^5 monocytic THP-1 cells were added to the medium with or without 1μg/ml Dox. From that moment on, culture supernatants were collected every 3 days and stored for further quantification of pro-inflammatory cytokines by cytometric bead assay

PARTIE III:
DISCUSSION GÉNÉRALE, CONCLUSION ET PERSPECTIVES

CHAPITRE VI: DISCUSSION ET CONCLUSION

6.1 DISCUSSION

Les maladies causées par *C. trachomatis*, d'abord aiguës, évoluent souvent en maladies chroniques avec de séquelles graves parmi lesquelles: les grossesses ectopiques et l'infertilité tubaire chez les femmes sexuellement atteintes, la cécité chez des personnes infectées au niveau oculaire. Les maladies chlamydiennes chroniques avec de séquelles graves ne s'installent qu'à la suite de multiples re-infections ou d'infections initiales non résolues. En réalité, les infections chlamydiennes aiguës peuvent être spontanément résolues par le système de défense de l'hôte uniquement, chez certains individus. L'antibiothérapie est également pratiquée. Depuis des lustres, la doxycycline, la tétracycline, l'érythromycine, et beaucoup plus récemment l'ofloxacin, l'azithromycine ont été introduites dans la thérapie des infections chlamydiennes. Cependant, malgré la disponibilité de la thérapie, les infections à *C. trachomatis* demeurent parfois exacerbantes. Les raisons du développement d'infections exacerbantes chroniques sont multiples, parmi elles, la persistance est évoquée. La persistance, état de quiescence chlamydienne, se développerait durant les conditions altérant la croissance. Au vu de tout cela, l'hypothèse selon laquelle les antibiotiques requis dans le traitement des infections chlamydiennes pourraient induire la persistance chlamydienne à la place de résoudre l'infection a été posée.

Pour vérifier cette hypothèse, un modèle de culture *in vitro* a été adopté. Tout d'abord, les concentrations minimales bactéricides (CMBs) de la Dox, de la Tet et de l'Ery ont été déterminées par la méthode conventionnelle (Committee for investigation methods, 1992; Suchland et *al.*, 2003). Les CMBs de la Dox, Tet et l'Ery déterminées sont respectivement de 1.0, 3.0, et 2.0µg/ml. Ces valeurs sont en accord avec celles rapportées, soit 0.125-1.0, 2.4, 2.0-4.0µg/ml pour la Dox, la Tet et l'Ery respectivement (Bowie et Lee, 1978; Mourad et *al.*, 1980; Bailey et *al.*, 1984). La CMB est la plus faible concentration de l'antibiotique qui ne permet pas la croissance de *Chlamydia* après deux générations, c'est-à-dire la concentration létale.

Les cellules épithéliales HeLa ont été infectées et traitées durant 24 jours avec la Tet, Dox ou l'Ery aux concentrations minimales bactéricides. Les résultats révèlent que ces traitements ne résolvent pas l'infection. Même si après 10 jours de traitements, ni les particules infectieuses, ni les inclusions typiques ne sont observées dans le milieu de culture, *C. trachomatis* viable

continue d'y demeurer. En effet, les ARNr 16S chlamydiens non matures sont continuellement exprimés même après 24 jours de traitement, suggérant inéquivoquement la présence de particules chlamydiennes viables, mais non infectieuses. De telles particules rappellent la forme persistante de *C. trachomatis* observée dans différentes conditions non propices à la croissance, contenant par exemple l'IFN - γ, la pénicilline, ou déficientes en nutriments importants comme le tryptophane (Beatty et *al.*, 1994; Harper et *al.*, 2000; Wyrick et *al.*, 2004). Cette étude montre que le traitement bactéricide de *C. trachomatis* en culture dans les cellules HeLa ne tue pas le *C. trachomatis*, mais favorise la forme persistante du pathogène. Nos résultats sont en accord avec la littérature. Suchland et *al.* (2003) ont montré que l'azithromycine, l'Ery, l'ofloxacin et la Tet, aux concentrations 100 fois supérieures à la CMI, ne tuaient pas *C. trachomatis* en culture. Dresses-Werringloer et *al.* (2000) ont également constaté que l'ofloxacin et la ciprofloxacin ne résolvaient pas l'infection à *C. trachomatis in vitro*. L'agent pathogène demeurait dans le milieu de culture viable, mais non infectieuse. Ainsi la persistance chlamydienne pourrait survenir malgré le traitement thérapeutique approprié. Ceci pourrait expliquer en partie les échecs thérapeutiques rapportés, après les traitements appropriés à la Dox, à la Tet ou à l'azithromycine (Dean et *al.*, 2000; Bragina et *al.*, 2001; Smith et *al.*, 2001). Également, l'étude de Blythe et *al.* (1992) révèle les infections récurrentes chez les jeunes adolescentes ayant été traitées convenablement. Dans une cohorte de 177 patientes, 38.4% ont eu de récurrences. En général, ces récurrences étaient dues par le même serovar, ce qui suggérait des réinfections par les partenaires non traités. La présente étude suggère que la persistance chlamydienne pourrait être aussi une des causes des échecs thérapeutiques rapportés.

Le mécanisme par lequel la persistance se forme demeure indéterminé. La particule chlamydienne persistante serait un CR atypique devenu très large, répliquant continuellement son ADN, mais dont la croissance est stoppée avec l'inhibition de scission binaire (Beatty et *al.*, 1994; Belland et *al.*, 2003). On pourrait également penser que les CEs intracellulaires et les particules intermédiaires entre les CEs et les CRs, qui exhibent déjà une résistance naturelle due à leur métabolisme moindre puissent persister ou requérir de quantités d'antibiotiques importantes pour être détruits. En fait, les CMI et les CMB déterminés par la méthode standard pourraient ne pas être appropriées. Utilisant la RT-PCR de l'ARNr 16S, Misyurina et *al.* (2002) ont déterminées les CMBs très élevées de la Dox et de l'Ery supérieures à 2.56 et à 12.8 µg/ml

respectivement. Les différences de sensibilité aux antibiotiques des particules chlamydiennes pourraient bien expliquer la survie chlamydienne hétérotypique suggérée par Suchland et *al.* (2003).

C. trachomatis serovar L2 se développe activement dans les cellules monocytiques THP-1, mais une portion parmi ces cellules échappent à l'infection productive et deviennent infectées de manière persistante. Par contre, les cellules monocytiques, U-937, sont uniquement infectées de manière persistante, c'est-à-dire non productive. L'infection persistante chlamydienne des cellules monocytiques THP-1 et U-937 est résolue par la Dox après 30 jours de traitement alors que les cellules HeLa demeurent continuellement infectées. Cette résolution dans les monocytes pourrait s'expliquer par les effets combinés des antibiotiques et des réactions de défense générées beaucoup plus efficaces dans les cellules inflammatoires monocytiques que dans les cellules HeLa. La fusion lysosomale requise pour la destruction lytique des particules chlamydiennes est bloquée dans les cellules HeLa, alors qu'elle surviendrait dans les monocytes (Ojcius et *al.*, 1997; Scidmore et *al.*, 2003). Ce résultat montre que l'infection persistante est remédiable, grâce à une action concertée de la defense de l'hôte et des anti-chlamydiens. Si les cellules HeLa demeurent infectées de manière persistante, elles pourraient constituer un réservoir de particules chlamydiennes capables d'infecter continuellement les monocytes. Ce résultat pourrait expliquer pourquoi les infections persistantes ne surviendraient que chez certains individus, probablement ceux produisant une réponse immunitaire anti-chlamydienne insuffisante.

Afin de comprendre comment une infection persistante puisse engendrer une infection productive typique, les antibiotiques ont été enlevés du milieu de culture à différents moments. Le développement de l'infection productive ne se produit que si le traitement antimicrobien ne dure au plus que 10 jours. Au delà de 10 jours, l'infection demeure dans un état inapparent, même plus de 10 jours après la suppression de l'antibiotique. Ce résultat suggère que l'infection persistante ne se convertit pas en infection productive, ce qui est en désaccord avec des études antérieures. Ce désaccord pourrait s'expliquer par les différences de concentrations d'agents inducteurs utilisés. Beatty et *al.*(1994) ont constaté la réversion des particules persistantes en particule normales si cette persistance avait été induite par l'IFN-γ 0.2 ng/ml et non si la quantité

de l'IFN-γ inductrice de persistance était de 2.0 ng/ml. L'utilisation des concentrations sub-bactéricides n'exclut pas définitivement la possibilité que les CEs et les CRs typiques ne soient pas complètement tués. Koehler et *al.* (1997) ont constaté que les particules persistantes dans les monocytes U-937 ne se convertissent pas en particules normales malgré la disponibilité du tryptophane dont la pénurie expliquerait la persistance. Dans la présente étude, nous avons constaté que seulement les cellules monocytiques ayant acquis la persistance à la suite de leur interaction avec les cellules HeLa infectées préalablement étaient capables de libérer les particules infectieuses. Ceci suggère que l'interaction préalable entre les monocytes et les cellules HeLa confère un signal permettant l'émission de particules infectieuses à partir des monocytes infectés de manière persistante.

Les maladies chlamydiennes sont souvent caractérisées par une inflammation chronique pouvant être, avec les autres réponses immunitaires enclenchées, responsables de sérieuses séquelles résultantes. *C. trachomatis* persistant dans les cellules constitue une source antigénique importante pour maintenir une inflammation prolongée et délétère. Les cytokines pro-inflammatoires, parmi lesquelles IL-1β, IL-6, IL-8, IL-12 et le TNF-α, sont requises pour la modulation et l'entretien de la réponse inflammatoire. Le 2nd objet de ce projet de recherche était de déterminer le profil différentiel des cytokines pro-inflammatoires selon que l'infection est productive ou persistante. Pour ce faire, les différentes cytokines induites par *C. trachomatis* dans 3 modèles de cultures *in vitro*, cellules HeLa, les cellules THP-1 et les cellules THP-1 et HeLa en co-culture, ont été mesurées. Les résultats montrent une expression accrue de IL-1β, IL-6, IL-8, IL-12p70, TNF-α et l'IL-10. Cette synthèse accrue finit par décliner probablement dû à la dégradation de la monocouche de cellules HeLa. Ces résultats sont en accord avec la littérature en ce sens que l'infection chlamydienne accroît la synthèse des cytokines (Bianchi et *al.*, 1997; Rasmussen et *al.*, 1997). Il semble que l'infection des cellules épithéliales par les particules chlamydiennes ne suffit pas à déclencher la réponse immunitaire. Les cytokines pro-inflammatoires induites, libérées au niveau de cellules épithéliales, site initial de l'infection, seraient requises dans l'initiation de l'inflammation et donc de la réponse spécifique (Rasmussen et *al.*, 1997). La synthèse de IL-1β, IL-6, IL-8, IL-12p70 et le TNF-α et l'IL-10 dans les cellules monocytique THP-1 est aussi accrue, mais décline par la suite. Les cytokines synthétisées par les monocytes seraient impliquées dans l'amplification des réponses immunitaires déjà induites.

La croissance de *C. trachomatis* dans le modèle de co-culture HeLa/THP-1 s'accompagne d'une induction intense et soutenue de l'IL-6 et IL-8. Au contraire, les quantités de l'IL-12p70, de l'IL-10 et du TNF-α produites ne sont pas significativement plus importantes que celles observées dans les cellules HeLa et THP-1 séparément. Ceci suggère que les quantités élevées et soutenues de l'IL-6 et de l'IL-8 ne résultent pas de simples effets additifs, mais d'une réelle up-regulation. Si différentes études ont révélé la synthèse initiale accrue de l'IL-8 et l'IL-6 dans différents types de cellules (Bianchi et *al.*, 1997; Rasmussen et *al.*, 1997), notre présente étude est originale puisqu'elle est la première qui montre l'expression soutenue de l'IL-8 et IL-6 durant l'infection chlamydienne. On ne sait pas exactement pourquoi ces deux cytokines spécifiquement demeurent exprimées de manière soutenue. L'IL-6 est une cytokine pléiotropique qui serait requise dans la défense initiale optimale (Williams et *al.*, 1998). Il semble que l'IL-6 interviendrait dans le processus immunitaire en général en bloquant l'activité suppressive des cellules T régulatrices CD4+CD25+ afin de permettre une réponse immunitaire adaptive appropriée au pathogène (Pasare et Medzhitov, 2003). Dans le cas des infections chlamydiennes, la réponse Th1 serait requise dans la résolution de l'infection. Il est possible que l'accomplissement de cette fonction de l'IL-6 ainsi que la fonction chemoattractive de l'IL-8 puisse requérir leur expression soutenue, mais pas brève.

Cependant, la production continue de l'IL-8 et de l'IL-6 pourrait s'avérer pathologique. Par exemple, les neutrophiles qui repondent au chimiotactisme exercé par l'IL-8 renferment dans leurs granules primaires et secondaires différentes enzymes lytiques. De plus, une fois actives, les neutrophiles permettent la production des dérivés d'oxygène et d'azote réactifs. La libération continue de ces différents composés cytotoxiques peut contribuer aux lésions tissulaires que l'on pourrait constater dans les infections chlamydiennes chroniques. Également, l'IL-6 et l'IL-8 seraient impliquées dans le développement et la progression de différents types de cancer dont le mélanome et le cancer de l'ovaire (Singh et *al.*, 1999; Ivarsson et *al.*, 1998). Il est rapporté que *C. trachomatis* puisse jouer un rôle dans le développement du cancer cervical causé par papillomavirus (Smith et *al.*, 2002; Silins et *al.*, 2005). *C. trachomatis* participerait-elle au développement du cancer du col utérin à travers sa capacité de promouvoir la synthèse de l'IL-6 et de l'IL-8? Le niveau élevé de l'IL-8 et de l'IL-6 a été, en effet, constaté dans les secrétions cervicales des patients souffrant du cancer cervical (Tjiong et *al.*, 1999).

Étant donné que les infections à *C. trachomatis* sont parfois caractésées par une inflammation chronique avec des séquelles graves, il est plus que primordial d'identifier les protéines chlamydiennes qui regulent la synthèse de cytokines. La protéine du choc thermique de 60 kDa (Hsp60) semble être un candidat crédible compte tenu de ses activités pro-inflammatoires constatées. Hsp60 chlamydien recombinant induit l'activation de NF-κB et l'activité du promoteur de l'IL-8 dans les cellules endothéliales humaines (Bulut et *al.*, 2002). En effet, NF-κB est requis dans l'activation des gènes de cytokines pro-inflammatoires. Les composés resultant du metabolisme des phospholipides membranaires, la prostaglandine E2, et une des enzymes requises dans ce matabolisme, la cychlooxygenase 2, seraient des inducteurs de l'IL-8 (Fukuda et *al.*, 2005). La synthèse continue de l'IL-8 durant l'infection productive pourrait donc s'expliquer par la libération continue de ces agents inducteurs.

Le profil d'expression de l'IL-1β dans les cellules HeLa et THP-1 en co-culture et infectées par *C. trachomatis* serovar L2 présente la forme d'une cloche, soit une synthèse initiale croissante pour atteindre un pic, suivie d'une baisse drastique. Il est stipulé que la pénétration de *C. trachomatis* au sein de son hôte se passerait inaperçue par le système de défense de l'hôte, et que la libération de l'IL-1β par des cellules endommagées à la suite de la croissance chlamydienne induirait la synthèse des autres cytokines pro-inflammatoires et ainsi permettrait le déclenchement de la défense de l'hôte (Rasmussen et *al.*, 1997). L'IL-β est en général préformée sous forme pro-IL-1β et converti en IL-1β mature par l'enzyme convertissant l'IL-1β (ICE). On ne sait pas si la baisse drastique de l'IL-1β s'explique par l'expression plus accrue de l'inhibiteur naturel de l'IL-1β, l'antagoniste du récepteur de l'IL-1 (IL-1RA) pouvant se produire lors de la différentiation des monocytes en macrophages (Scotton et *al.*, 2005). La mesure concomitante de l'IL-1β, de l'IL-1RA et de l'ICE aurait permis de comprendre le profil d'expression de l'IL-1β.

La synthèse de l'IL-10 ou l'IL-12p70, avec un profil variable, est augmentée dès le départ, mais cette synthèse finit par s'estomper avec le temps. L'IL-10 favoriserait la réponse Th2 inefficace dans la résolution des infections chlamydiennes, alors que l'IL-12 avantagerait la reponse Th1 requise pour remédier l'infection. La synthèse accrue de l'IL-10 serait associée aux pathologies chroniques (Yang, 2001). On ne sait pas exactement si *C. trachomatis* module la reponse de

l'hôte de façon à favoriser une reponse donnée. Cette étude ne montre pas une synthèse préférentielle de l'une de ces deux cytokines.

Il est surprenant que la synthèse du TNF-α, augmentée par rapport à la production contrôle, ne soit pas soutenue. Le TNF-α infligerait les dommages aux tissus, contribuant ainsi aux séquelles caractérisant les maladies chroniques chlamydiennes. Les quantités élevées du TNF-α ont été constatées dans les larmes des patients atteints de *C. trachomatis* oculaire (Conway et *al.*, 1997). Bien que la synthèse accrue du TNF-α ne soit pas soutenue dans cette étude, cela n'exclut pas son possible rôle dommageable lors des infections chlamydiennes *in vivo*. Une légère augmentation physiologique du TNF-α pourrait s'avérer suffisante pour exercer des effets dommageables.

Le traitement des cellules HeLa infectées par *C. trachomatis* serovar L2 avec la Dox à la CMB ne résout pas l'infection chlamydienne mais aboutit à l'installation de l'infection persistante. La question se pose de savoir si le passage de l'infection productive en infection persistante s'accompagne d'un changement de profil de cytokines pro-inflammatoires exprimées. Les résultats obtenus révèlent une inhibition de l'expression de l'IL-1β, l'IL-6, l'IL-8, le TNF-α alors que la production de l'IL-10 et de l'IL-12p70 demeure inchangée. Malgré la persistance chlamydienne, aucune de ces cytokines n'est exprimée de manière continue. L'inhibition de l'expression de ces cytokines n'est pas due aux effets anti-inflammatoires de la Dox. Les études antérieures ont montré que la Dox inhibe la synthèse des cytokines à une concentration d'au moins 0.01 mM (Krakauer et *al.*, 2003). Pour cette étude, la Dox a été utilisée à une concentration de 0.002 mM. Les études antérieures corrèlent la diminution de la synthèse de cytokines en fonction du nombre de particules chlamydiennes présentes dans le milieu. Plus la croissance de *Chlamydia* est forte, plus grande est la production des cytokines (Rasmussen et *al.*, 1998). Kutlin et *al.* (2002) ont également constaté une synthèse réduite de cytokines durant l'infection persistante de *C. pneumoniae*. La signification biologique de l'inhibition de la synthèse de cytokines durant l'infection persistante n'est pas claire. Étant donné que les cytokines pro-inflammatoires modulent et promouvoient l'inflammation, la réduction de leur synthèse peut atténuer l'intensité de la réponse inflammatoire générée. Une inflammation insuffisante s'avérerait inefficace à résoudre l'infection persistante de cellules HeLa. Cependant,

bien que cette inflammation soit de faible intensité, à long terme, elle pourrait s'avérer dommageable pour les cellules.

6.2 CONCLUSION ET PERSPECTIVE

Bien que l'antibiothérapie des maladies chlamydiennes soit pratiquée, les infections exacerbantes sont rapportées et pourraient se développer en des infections chroniques avec des séquelles graves caractéristiques. La présente étude démontre que les traitements appropriés des infections de cellules HeLa à *C. trachomatis* avec de quantités minimales bactéricides de Dox, Tet et Ery, antibiotiques habituellement utilisés dans la thérapie des infections chlamydiennes, ne résolvent pas nécessairement l'infection. Au contraire, il se forme de particules persistantes viables non infectieuses uniquement détectables par la RT-PCR de différents transcripts dont l'ARNr 16S non mature, mais non par la méthode conventionnelle de culture. Par contre dans les cellules monocytiques THP-1 et U-937, les particules chlamydiennes s'y développant sont résolues grâce à l'action concomitante de la Dox et probablement de réactions de défense généralement générées dans les cellules inflammatoires. Ces résultats montrent que les cellules épithéliales, non les cellules monocytiques, pourraient constituer un réservoir de particules chlamydiennes persistantes.

Cette étude révèle que l'infection de cellules HeLa et THP-1 par *C. trachomatis* serovar L2 s'accompagne d'une synthèse accrue de différentes cytokines IL-1β, IL-6, IL-8, IL-10, IL-12p70 et le TNF-α. Dans le modèle de co-culture *in vitro* de cellules HeLa et THP-1, mimant l'interaction entre les cellules épithéliales et monocytes, les cytokines pro-inflammatoires IL-6 et IL-8 sont synthétisées de manière intense et soutenue durant toute la durée de l'infection productive, alors que lorsque l'infection devient persistante à la suite du traitement à la Dox, la synthèse de IL-1β, IL-6, IL-8 et du TNF-α est inhibée. Nos données suggèrent la possibilité de la reduction de la réponse inflammatoire durant l'infection persistante.

Les résultats obtenus dans la présente étude montrent que l'infection chlamydienne persistante est remédiable dans les monocytes. Il serait intéressant d'étudier l'infection chlamydienne de monocytes et de mener les investigations afin de déterminer la réponse de défense générée dans ces cellules, dans le but de comprendre le processus de résolution intervenant.

Le mécanisme de persistance chlamydienne n'est pas encore bien défini. Cependant, il est soupçonné que les différentes particules chlamydiennes n'aient pas la même sensibilité aux

antibiotiques et donc pas la même capacité de survie. Il serait interessant de méner une exploration aux microscope électronique à transmission afin d'identifier les effets de différents antichlamydiens sur la structure et la viabilité de différentes particules chlamydiennes, précisement les CEs, les particules chlamydiennes en différenciation, les CRs.

La présente étude a été faite dans les modèles *in vitro* utilisant les lignées de cellules déjà établies (cellules HeLa et THP-1), les résultats obtenus ne reflètent fidèlement l'histoire naturelle de *C. trachomatis*. Il est nécessaire d'évaluer la production de ces différentes cytokines (IL-1β, IL-6, IL-8, le TNF-α, IL-10 et IL-12p70) durant l'infection chlamydienne de cellules épithéliales ou moncytaires primaires. Finalement, étant donné que la réduction de la synthèse de cytokines inflammatoires a été constatée dans la présente étude, il serait intéressant de comparer l'inflammation durant l'infection productive par rapport à celle persistante. Ceci pourrait permettre de comprendre les événements immunologiques déterminants dans la destruction des tissus.

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CHAPITRE VII:
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