CELLULAR AND MOLECULAR HOST-PATHOGEN INTERACTIONS DURING *CHLAMYDIA PNEUMONIAE* INFECTION

By

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CELLULAR AND MOLECULAR HOST-PATHOGEN INTERACTIONS DURING *CHLAMYDIA PNEUMONIAE* INFECTION

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To Megan, Ryan
Linda and Ken

With all my love

ABSTRACT

Chlamydia pneumoniae is a Gram-negative bacterial pathogen that has evolved to survive completely within the intracellular environment of a host cell. The obligate intracellular lifestyle of this bacterium necessitates an efficient invasion strategy, exemplified by a broad host cell tropism with little propensity for any single cell type and the ability to replicate within both professional phagocytic cells and cells with low phagocytic capacity. After cellular invasion, *C. pneumoniae* actively evades host immune defenses, establishes a parasitic relationship with the host cell and forms a microcolony by dividing within a non-fusogenic cytoplasmic vacuole called an inclusion. The varied spectrum of host cell pathways modified by *C. pneumoniae* suggests a complex interaction between the bacteria and the host cell.

Identifying the requisite host-pathogen interactions contributing to *C. pneumoniae* virulence is a major research goal. While the morphological features of the chlamydial developmental cycle have been described in detail, our understanding of the cellular and molecular events underlying *C. pneumoniae* invasion and potential mechanisms of disease pathogenesis are only preliminary. The work presented in this thesis examines cellular and molecular interactions between *C. pneumoniae* and various types of host cells during invasion and intracellular growth of chlamydiae. Some of the research questions addressed herein are framed within the context of atherosclerosis and coronary artery disease, with the *a posteriori* reasoning that a potential microbiologic contribution to human atherosclerosis, specifically due to *C. pneumoniae* infection, is suggested by several converging lines of investigation.

We found that *C. pneumoniae* invasion of human epithelial cells requires bacterial-induced remodeling of the host actin cytoskeleton and the activation of at least two host cell signal transduction pathways. These host modifications are required for *C. pneumoniae* uptake but not cellular attachment, suggesting that the *C. pneumoniae* invasion sequence is biphasic, whereby initial attachment is rapidly followed by activation of host cell signaling and actin polymerization to facilitate uptake. Secondly, we used cDNA array technology to study the endothelial cell transcriptional response to *C. pneumoniae* infection and found a prominent transactivation of several cytokines, chemokines and smooth muscle cell growth factor genes during early times after infection. Furthermore, using cell culture-based experiments and an established rabbit model of *C. pneumoniae*-induced atherosclerosis, we showed that *C. pneumoniae* infection is associated with paracrine activation of smooth muscle cell proliferation and aortic intimal thickening, potentially mediated through platelet-derived growth factor.

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In research...things have more of a chance of going wrong than right.
But when things go right, there is nothing more exciting.
-Dr. Michael Smith, Nobel Laureate (Chemistry 1993)

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LIST OF ABBREVIATIONS AND TERMS

CAD	-coronary artery disease
CAM	-chloramphenicol; antibiotic, disrupts prokaryotic protein translation
CMV	-cytomegalovirus
cOMC	-chlamydia outer membrane complex
CPM	-counts per minute
DNA	-deoxyribonucleic acid
dNTP	-deoxynucleoside triphosphate
EB	-elementary body (bodies), extracellular infectious chlamydia particle
EDTA	-ethylenediaminetetra-acetic acid
ERK	-extracellular signal regulated kinase
FAK	-focal adhesion kinase
FBS	-fetal bovine serum
FITC	-fluorescein isothyocyanate
HEp2 cells	-human laryngeal epithelial cells; transformed; for *C. pneumoniae* propagation
HMEC-1	-human microvascular endothelial cells; transformed
hpi	-hours post-infection
HRP	-horseradish peroxidase
HSP	-heat shock protein
HUVEC	-human umbilical vein endothelial cells, non-transformed
IB	-immunoblot
IFU	-inclusion forming unit, definition of one infectious EB
IP	-immunoprecipitation
IL-8	-interleukin 8; chemokine
LPS	-lipopolysaccharide
mAb	-monoclonal antibody
MCP-1	-monocyte chemotactic protein-1; chemokine
MIT	-maximal intimal thickening
MOI	-multiplicity of infection
N-terminus	-amino terminus
NTP	-nucleoside triphosphate
NZW	-New Zealand White, outbred rabbit strain
PAGE	-polyacrylamide gel electrophoresis
PCR	-polymerase chain reaction
PDGF	-platelet-derived growth factor
PI 3-kinase	-phosphoinositide 3-kinase
PMSF	-phenylmethylsulfonyl fluoride ($C_7H_7O_2SF$); serine protease inhibitor
POMP	-polymorphic outer membrane protein
RB	-reticulate body (bodies), intracellular vegetative chlamydia particle
RNA	-ribonucleic acid

RT	-reverse transcriptase
SEM	-standard error of the mean; or scanning electron microscopy
SDS	-sodium dodecyl sulfate, anionic detergent
SF	-serum-free
SI	-stimulation index
SMC	-smooth muscle cells
SPG	-sucrose-phosphate-glutamic acid buffer
TEM	-transmission electron microscopy
VR1310	-respiratory isolate of *C. pneumoniae*

CHAPTER ONE

INTRODUCTION

THE GENUS CHLAMYDIAE

General biology and host range

Chlamydiae are a diversified group of obligate intracellular Gram-negative bacteria capable of infecting a wide range of both warm and cold-blooded animals. These pathogens propagate by first invading a susceptible host cell and then commandeering host metabolic pathways for their own replication needs. During evolution, chlamydiae appear to have acquired sophisticated mechanisms of invasion and intracellular survival, exemplified by their ability to grow inside professional phagocytic cells and non-phagocytic cells alike.

All chlamydiae possess a unique biphasic developmental cycle that shuttles between a stable infectious elementary body (EB) that is highly adapted for extracellular survival and a non-infectious, metabolically active intracellular reticulate body (RB). This unique developmental cycle differentiates chlamydiae from all other microorganisms (Moulder, 1991) and is the basis for their taxonomic classification into a separate biological order, *Chlamydiales*. RB divide by binary fission within a sequestered microcolony in the cytoplasm of host cells enclosed within a membrane bound vacuole called an inclusion. The bacterial membrane and inclusion membrane are decorated with specialized transporter machinery used to acquire from the host cell metabolic intermediates and lipids for macromolecular synthesis (Tjaden et al., 1999; Kubo and Stephens, 2001). Recently, a third morphological form called the persistent body (PB) has been recognized for chlamydiae (Beatty et al., 1994). PB are enlarged, non-dividing yet viable intracellular bacterial particles that develop in response to adverse environmental conditions such as glucose withdrawal, amino acid catabolism, heat shock, or in response to antibiotic treatment. The ability

of chlamydiae to contribute to chronic disease may be predicated on specialized mechanisms of intracellular survival and the unique ability of PB to chronically infect host cells (Ward, 1999).

Members of the genus and spectrum of disease

Currently, the *Chlamydia* genus is composed of four recognized species, including *C. trachomatis*, *C. pneumoniae*, *C. psittaci* and *C. pecorum*. *C. trachomatis* and *C. pneumoniae* are primarily human pathogens, with *C. trachomatis* causing trachoma and blindness, conjunctivitis, lymphogranuloma venereum, genital tract diseases, tubal infertility, infantile pneumonia and reactive arthritis. *C. psittaci* causes a zoonotic infection contracted by humans from infected avian species and can cause pneumonia or systemic infections producing endocarditis. *C. pecorum* is a recently described species whose biological distribution is not yet known and whose pathogenic role is not clear. What is clear is that human chlamydial infections are highly pervasive. *C. pneumoniae* seroprevalence rates exceed 70% after the age of 50 world wide and annual incidence rates of *C. trachomatis* infections are estimated at 90 million, with 6 million cases of preventable blindness in trachoma-endemic areas and 4 million new cases of sexually transmitted infection in the United States alone (World Health Organization, 1996). With these rates of infection, chlamydiae are arguably among the most ubiquitous of the human pathogens. The ability of the different chlamydial species to cause a varied spectrum of human diseases in light of their remarkable structural and functional similarities is biological testimony of the evolutionary fitness of these bacteria. Currently there are no approved vaccines for any of the chlamydia species.

Clarification of chlamydia taxonomy

The order *Chlamydiales* is made up of *Chlamydia* species that display the unique developmental cycle signature for which this order was named. Recent analysis of 16S and 23S ribosomal RNA (rRNA) gene sequences has indicated that a new taxonomic classification may be warranted (Everett et al., 1999). This new classification would divide the existing family *Chlamydiaceae* into two genera, (i) *Chlamydia* (to include *C. trachomatis, C. muridarum,* and *C. suis)* and (ii) *Chlamydophila* (to include *C. pneumoniae*, *C. pecorum, C. abortus, C. caviae* and *C. felis*). However, since these proposed taxonomic changes are currently being debated (Schachter et al., 2001) all reference to *C. pneumoniae* in this document implies *Chlamydia pneumoniae.*

CHLAMYDIA PNEUMONIAE: A DESCRIPTION OF THE SPECIES

General description of *C. pneumoniae* biology and genomics

C. pneumoniae is a highly evolved pathogen capable of infecting a variety of cell types ranging from eukaryotic cells to the soil protist Acanthamoeba. Like all chlamydiae, *C. pneumoniae* are nonmotile, Gram-negative cocci with an intracellular lifestyle, replicating exclusively within a non-fusogenic intracellular inclusion. The limiting membrane of the inclusion affords the bacteria a sequestered niche in which to replicate and allows for careful control of the luminal contents through various bacterial-derived inclusion membrane proteins (Wyllie et al., 1999) and bacterial surface proteins (Kubo and Stephens, 2000, 2001) that function as specialized porins. Intensive genetic analysis has deciphered the *C. pneumoniae* genome (Kalman et al., 1999; Read et al., 2000), which is a 1,230,230 base pair chromosome comprising 1054 open reading frames and 214 unique protein-coding sequences not found in *C. trachomatis*

and most without homologues to other known sequences deposited in public genomic databases (National Center for Biotechnology Information Database; *C. pneumoniae* genome: www.ncbi.nlm.nih.gov/cgi-bin/Entrez/framik?db=Genome&gi=140). When analyzed against other sequenced chlamydia strains, prominent comparative findings in *C. pneumoniae* include expansion of a novel family of 21 sequence-variant outer-membrane proteins called Pmps (polymorphic membrane proteins), conservation of a type-III secretion virulence system, three eukaryotic-type serine/threonine protein kinases, additional purine and biotin biosynthetic capability and the loss of genes for tryptophan biosynthesis. In addition, *C. trachomatis* bears an extrachromosomal plasmid that is lacking in *C. pneumoniae.* These genomic differences may be implicated in the unique properties that differentiate *C. pneumoniae* and *C. trachomatis* with respect to tissue tropism and disease spectrum.

Spectrum of human diseases

First characterized in 1986 by Grayston and colleagues (1986), *C. pneumoniae* is now a recognized etiologic agent of upper and lower respiratory tract infections (Grayston, 2000). Seroepidemiologic studies suggest that infections are frequently acquired during adolescence and early adulthood resulting in seroprevalences up to 70% by the fifth decade of life (Kuo et al., 1995). Given that antibodies raised following primary infection are lost in 3-5 years (Patnode et al., 1990), continually rising seroprevalence rates out to the eighth decade of life suggest that re-infections and / or reactivation of persistent infections occur. *C. pneumoniae* is an etiologic agent in approximately 10-15% of community-acquired bacterial pneumonia cases with an estimated annual incidence rate in the United States of ~300,000 (Guthrie, 2001). Other manifestations of acute infection include pharyngitis, bronchitis and sinusitis. Very often however, acute infections

are asymptomatic or produce symptoms so benign that they are often not treated medically. During the last decade, intensive scientific investigation has focused on a new emerging disease association linked to *C. pneumoniae* infection – that being atherosclerosis, a chronic inflammatory disease state underlying coronary and carotid artery disease.

Atherosclerosis as an emerging disease association

Atherosclerosis develops from a chronic inflammatory response at the vessel wall characterized by cellular hyperplasia, lipid deposition and intimal thickening leading to narrowing of the vessel lumen (Ross, 1993, 1999). Based on several lines of investigation, there is now an accepted association between *C. pneumoniae* and atherosclerotic disease in the coronary- and carotid arteries (Grayston, 2000). The first association between *C. pneumoniae* and atherosclerosis was reported in a 1988 serologic study by Saikku and colleagues in Finland (Saikku et al., 1988) who demonstrated that patients with established coronary artery disease were significantly more likely to have anti-*C. pneumoniae* antibodies when compared with matched control patients. This initial finding has been reproduced several dozen times in various geographical locations and populations, however the acknowledged lack of consistent serologic criteria, inherent problems with performance of the serologic methods and the lack of a reliable surrogate marker for chronic or persistent *C. pneumoniae* infection have hampered the interpretation of some of these studies. To compensate, investigators are now turning to improved diagnostic technology in an attempt to examine the pervasiveness of *C. pneumoniae* in atheromatous tissue and to uncover potential mechanisms that may be implicated in the disease process.

Pathological studies have provided stronger evidence for an association between *C. pneumoniae* and coronary artery disease. A direct examination of human tissue has identified the organism collectively in over 50% of all diseased atherosclerotic tissues tested by several methods including polymerase chain reaction (PCR), *in situ* hybridization, immunohistochemical staining, electron microscopy and culture (Kuo et al., 2000; Grayston, 2000; Ouchi et al., 2000; Vink et al., 2001). In these same studies, the detection rate in samples from non-atheromatous arterial specimens was less than 5%. Comprehensive pathological studies have also suggested a predilection of *C. pneumoniae* for atheromatous tissue over other anatomical sites (Ouchi et al., 2000; Jackson et al., 2000). In 1996, *C. pneumoniae* was cultured for the first time from a human atheroma (Ramirez et al., 1996), which has since been reproduced (Jackson et al., 1997; Apfalter et al., 2000) albeit at a low frequency. That viable organism is found in the vascular tree suggests a unique virulence determinant allowing these bacteria to invade and survive within blood leukocytes and thereby gain access to the cellular components of the vessel wall. This notion is supported by experimental evidence showing that detection of *C. pneumoniae* DNA in peripheral blood mononuclear cells is predictive of vascular infection in humans (Blasi et al., 1999). Chronic infection with *C. pneumoniae* may be facilitated by the ability of this organism to persist *in vivo* despite appropriate antibiotic therapy (Hammerschlag et al., 1992; Gieffers et al., 2001) and it has been suggested that the ability to persist and evade host immune mechanisms while chronically stimulating the immune system with bacterial antigens may be important biological properties of *C. pneumoniae* contributing to the chronic inflammatory process of atherosclerosis (Mahony and Coombes, 2001). The advent of reproducible and robust animal models together with cellular and molecular studies *in vitro* has been helpful in experimentally probing these complicated issues.

CELLULAR MICROBIOLOGY

Some organisms gain entry into their hosts by entering and surviving inside eukaryotic cells and macrophages, thereby functioning as intracellular parasites. Therefore, research into the biochemistry of how these pathogens adhere, enter, survive, replicate within and exit their hosts must take place within the cellular milieu of the host cell. The burgeoning field of 'cellular microbiology' attempts to define determinants of bacterial virulence in the context of the host cell, such that the lessons learned will offer a more rational approach to therapeutic intervention. Techniques forged out with other models of intracellular bacteria are beginning to be applied to the study of chlamydia. However, the fastidious growth requirements of obligate intracellular bacteria such as *C. pneumoniae* is further hampered by the inability to stably introduce recombinant DNA into this organism, thus precluding our ability to generate genetic mutants. For these reasons, elucidation of host cell receptors for chlamydiae and their cognate bacterial ligands and virulence factors presents a special research challenge that has not currently been overcome. Nevertheless, progress into the interactions that occur between chlamydia and host cells, including the molecular mechanisms of host-bacteria interactions has been made possible by employing well-defined cell culture models of infection together with novel cell- and animal models. Such studies have allowed the examination of an expanded host range and of different biological states of the organism that may be clinically relevant such as persistent infections (see section entitled 'Persistent chlamydia and chronicity of disease' below).

Cell culture models of acute *C. pneumoniae* infection

In the laboratory, strains of *C. pneumoniae* can infect and replicate within epithelial cells, endothelial cells, smooth muscle cells (SMC) and macrophages (Gaydos et al., 1996; Coombes and Mahony, 1999, 2001). Recent reports of the infection of T lymphocytes (Haranaga et al., 2001) raises the intriguing possibility that *C. pneumoniae* may be able to infect virtually any mammalian cell. Survival of the bacteria within mammalian cells is largely attributed to the incomplete fusion of the inclusion with pre-lysosomal and lysosomal compartments, resulting in attenuated luminal acidification of the inclusion (Schramm et al., 1996). The molecular mechanism by which the inclusion confers resistance to lysosomal killing remains unresolved. Almost all of what is known about the chlamydial developmental cycle, cellular invasion and the ensuing host response to invasion has been garnered from *in vitro* cell culture model systems that attempt to imitate an actively replicating acute infection. These models are indispensable for the study of chlamydial biology.

Developmental cycle

All species of chlamydiae share a unique biphasic developmental cycle consisting of an infectious EB exhibiting little to no metabolic activity and a metabolically active RB that divides by binary fission inside host cells. EB (diameter ~300 nm) attach to a susceptible host cell via an unknown cellular receptor(s) and bacterial ligand(s) (refer to Appendix A, Figure A.1). The outer membrane structure of an EB consists of a framework of disulfide bonds, conferring to the EB particle extreme structural rigidity and osmotic stability (Hatch, 1996). This unique structure is constructed from several cysteine-rich outer membrane proteins, the most notable being OmcB (M_r 60,000; ~7% cysteine residues) and OmcA (M_r 12,000; ~21% cysteine residues) that,

together with OmpA, form the supramolecular outer membrane structure of the EB. It is now clear that inter- and intramolecular disulfide bonding within the OMC is responsible for the rigid EB structure.

C. pneumoniae remains inside a vacuolar inclusion during the intracellular stage of its developmental cycle, with the inclusion being the only environment known to support the growth of this organism. The inclusion membrane is derived from the host cell plasma membrane during uptake and through an unknown process requiring bacterial protein synthesis (van Ooij et al., 1998), inclusions do not mature into late endosomes or fuse with lysosomes. Instead, the inclusion is rapidly diverted to a perinuclear region where it intercepts sphingomyelin-containing exocytotic vesicles trafficking from the trans-Golgi network to the plasma membrane (Wolf and Hackstadt, 2001). Intracellular EB reorganize into less condensed RB (diameter ~ 0.6-1 μm) through a process involving reduction of the disulfide-bonded outer membrane complex (refer to Appendix A, Figure A.2 for detail of EB and RB morphology). Reduced RB then initiate a replication phase characterized by RNA and DNA synthesis, protein translation and division by binary fission. The replication phase of RB lasts for approximately 48-72 hours as assessed in cell culture models and is followed by asynchronous reorganization of RB back into infectious EB. The signals that control this process and the bacterial proteins required for morphological transformations are poorly understood but involve, in part, the formation of inter- and intra-protein disulfide bonds between the cysteine-rich outer membrane proteins to regenerate the rigid and osmotically stable EB that is adapted for extracellular survival. Concomitantly, metabolic activity ceases in the bacterial progeny and EB are lytically released from host cells to initiate a new developmental cycle in neighbouring cells.

Invasion of non-phagocytic cells

Primary chlamydia infections are initiated in the mucosal epithelium by invasion into non-professional phagocytic epithelial cells. A highly efficient cellular invasion stratagem is necessitated by the intracellular lifestyle of these bacteria and accordingly, it is clear that *C. pneumoniae* possess the required machinery to infect both professional phagocytes and non-professional phagocytic cells (Gaydos et al., 1996; Coombes and Mahony, 1999). Intensive efforts to understand how *Chlamydia* spp. attach to susceptible host cells have yet to reveal specific host cell receptors and to date, no adhesin has been shown unequivocally to mediate binding and invasion of chlamydiae into cells. Several surface molecules have been purported as bacterial adhesins, including the major outer membrane protein (Su et al., 1996), OmcB (Ting et al., 1995), heat shock protein 70 (Raulston et al., 1993) and heparin sulfate-like proteoglycans (HSPG) (Zhang and Stephens, 1992). In the current model of chlamydial attachment to cells, sulfated carbohydrate molecules either on the bacteria (Stephens et al., 2001) or on the host cell surface (Taraktchoglou et al., 2001; Wupperman et al., 2001) appear to be important in mediating non-specific adhesion of both *C. pneumoniae* and some biovariants of *C. trachomatis* to cells. Other bacteria, most notably *Neisseria gonorrhoeae*, have been shown to utilize cell surface HSPG for binding and / or invasion yet these bacteria also require other receptors for internalization, suggesting that other undefined chlamydial receptors may exist. Of the two predominant types of invasion sequences described for intracellular bacteria – the so-called trigger and zipper mechanisms (Finlay and Falkow, 1997; Dramsi and Cossart, 1998)– scanning electron microscopic studies reveal that *C. pneumoniae* invasion better resembles the zipper-type process (Wolf et al., 2000), where the host cell membrane is in close juxtaposition around EB being internalized. The molecular determinants of chlamydial attachment are believed to reside

within the bacterial outer membrane complex, since purified outer membrane envelopes can reproduce membrane binding events to fibroblasts (Levy and Moulder, 1982), and *C. psittaci* Omp2 and MOMP, two bona fide outer membrane proteins, can directly bind HeLa cell surfaces in a trypsin-sensitive manner (Ting et al., 1995). The putative chlamydial receptor(s) is believed to be proteinaceous, since it is exquisitely sensitive to proteolysis. Mild trypsin proteolysis of host cell surfaces renders cells refractive to infection with chlamydiae and at least four hours of incubation is required to recover susceptibility to infection, which can be inhibited by the protein translation inhibitor, cycloheximide (Byrne and Moulder, 1978). Indeed, resistance of EB to release by proteases has been used as an indicator of internalized EB (Byrne and Moulder, 1978) and was the basis for an invasion assay developed for the work described in chapter 2 of this thesis.

By comparing the biology of intracellular parasitism gleaned from other published studies, one can appreciate that while several viruses and intracellular bacteria use HSPG as non-specific adhesin receptors, most, if not all binding and internalization events have been shown to be multifaceted, requiring at least one other specific secondary receptor to facilitate invasion. A similar bipartite mechanism of invasion may also exist for chlamydiae, whereby initial attachment to the cell surface is followed by receptor activation, or interaction with a secondary receptor leading to uptake. Such a mechanism is supported by experimental evidence describing the ability to block chlamydial internalization without affecting binding to the cell surface. Ting and colleagues (1995) showed a contrast between the apparent narrow range of heat treatment required to attenuate chlamydia infectivity and the much broader range required to reduce bacterial attachment. More recently, using a panel of wild type and mutant Chinese hamster ovary cell lines Carabeo and Hackstadt (2001) reported data that showed reversible binding of *C.*

trachomatis to cell surface glycosaminoglycans (GAG) and irreversible attachment to an unknown cell surface component subsequent to GAG binding, supporting the notion that attachment and internalization are two discrete events. Further, experiments described herein show that inhibition of specific host cell signal transduction cascades or actin polymerization can abrogate *C. pneumoniae* uptake without blocking attachment to the surface of HEp2 cells, suggestive of additional steps in the invasion process subsequent to initial attachment that are required for internalization. Based on the currently available data with regard to chlamydia binding mechanisms and intrinsic catalytic signaling activity ascribed to HSPG, I hypothesize that for infection of epithelial cells, *C. pneumoniae* use HSPG but also interact with a secondary, as of yet unidentified receptor leading to activation of signaling pathways in the host to promote bacterial entry. It is anticipated that genomics and proteomics-based approaches will see new progress in the identification of potential bacterial ligands involved in chlamydial invasion. Indeed, such studies are already garnering valuable data (Vandahl et al., 2001; Montigiani et al., 2002).

Host cell response to chlamydial invasion

In this section I describe the intimate relationship between chlamydiae and the host cell with the intention of illustrating the extent to which chlamydial infection pervades host cell biology.

(i) Activation of host cell signaling pathways- Intracellular pathogens rely completely on interactions with host cells for all aspects of their survival, from the initial attachment to host cell membranes, to cellular invasion, acquisition of host cell metabolites and intracellular replication.

Invasion by pathogenic bacteria often relies on manipulation of host cell signaling pathways in order to commandeer the cytoskeleton for uptake or motility or for full virulence *in vivo* (Finlay and Cossart, 1997). The molecular mediators to activate and deactivate specific cellular signaling cascades are frequently delivered in the form of bacterial Type III secretion effector proteins during invasion at the cell surface. These proteins are exported or translocated from the bacterial cytoplasm into the host cell cytosol via a conserved and highly specialized protein secretion apparatus called Type III (Hueck, 1998). Type III secretion is used exclusively by pathogenic bacteria for delivery of virulence factors to their hosts prior to, and during infection.

Mammalian signal transduction pathways are comprised of a collection of kinases, phosphatases and adaptor molecules that collectively regulate diverse cellular processes such as cell proliferation and differentiation, survival, apoptosis, cytoskeletal rearrangements and regulated gene expression. Modular domains contained in signaling proteins allow a sequential interaction of signaling molecules required for the specificity of the desired cellular response. Intracellular bacteria have evolved mechanisms to selectively activate and deactivate cellular signaling cascades in order to manipulate the intracellular environment in a way that is compatible with invasion, growth and intracellular residency (Ireton and Cossart, 1998). Chlamydiae are no exception to this doctrine. Previous studies have identified tyrosine phosphorylation of host cell proteins migrating at 140, 97, and 64-68 kDa in HeLa cells infected with *C. trachomatis* L2 (Birkelund et al., 1994). In this same study actin bundles and phosphotyrosine proteins were observed in close proximity to invading EB, leading the authors to suggest that *C. trachomatis*-induced tyrosine phosphorylation was associated with microfilament-dependent uptake. In a similar study, Fawaz and coworkers (1997) identified tyrosine phosphorylated proteins of ~100 kDa and 75/85 kDa in *C. trachomatis*- infected cells. These

same authors demonstrated the co-localization of phosphotyrosine proteins with the actin-binding protein cortactin and using subcellular fractionation techniques suggested that the 75/85 kDa phosphotyrosine complex was associated with the host cell cytoskeleton. From these limited studies on *C. trachomatis* infection of epithelial cells, the general implication is that chlamydiae induce the selective activation of signal transduction cascades in the host cell leading to rearrangements in the actin cytoskeleton in ways that are compatible with bacterial entry. Currently, there is a paucity of similar studies examining cellular invasion mechanisms of *C. pneumoniae*. Krüll et al. (1999) demonstrated tyrosine phosphorylation of host p42/p44 mitogen activated protein kinase in endothelial cells infected with *C. pneumoniae* and a second study demonstrated a similar finding in infected SMC (Sasu et al., 2001). An important caveat to both studies however relates to the infection methodology, in which the inoculum was prepared in serum-containing medium. Since components of serum, including growth factors and cytokines can activate various mammalian signal transduction cascades (Whitmarsh et al., 1995; Gineitis et al., 2001), the results of these earlier studies must be interpreted with caution.

(ii) Host transcriptional response to infection – The availability of complete genome sequences for both microbial pathogens and host cells forms of the basis of microarray technology to study host-pathogen interactions. Using approaches such as hybrid selection on high-density arrays of oligonucleotide and cDNA probes, the simultaneous characterization of mRNA expression levels of a large number of genes can be accomplished (Cummings and Relman, 2001; Kato-Maeda et al., 2001). This approach is amenable to the complementary analysis of both host- and bacterial responses to infection and has been used to probe the transcriptional response of host cells to several bacterial pathogens including *H. pylori*, *Shigella*

flexneri, *Streptococcus pneumoniae*, *Salmonella* and *Listeria monocytogenes* (reviewed by Kato-Maeda et al., 2001). Using more conventional approaches to analyze mRNA expression such as reverse transcriptase (RT)-PCR, several investigators have reported upregulation of cytokine and adhesion molecule genes such as MCP-1, IL-8, intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 in chlamydiae-infected cells (Kol et al., 1999; Molestina et al., 1999, Jahn et al., 2000). To initiate a more pervasive investigation into *C. pneumoniae*-inducible genes, we used a cDNA filter array to characterize the mRNA expression profiles of 268 human genes following infection with *C. pneumoniae* (Coombes and Mahony, 2001). The results of this study are described in detail in chapter three of this thesis.

(iii) Unification of host cell metabolism with chlamydial parasitism- The obligate intracellular lifestyle of chlamydiae obviates their need to encode complex biochemical pathways in their genome. As such, chlamydiae have deleted several genes encoding biosynthetic intermediates and coenzymes during their evolution, thereby becoming auxotrophic for the end products and requiring transport systems to acquire these metabolites from the host cell. This is true for most of the amino acid biosynthetic operons, pyridine nucleotides (NAD/NADH), coenzyme A, and enzymes of both the *de novo* and salvage pathways of nucleotide metabolism. The complete genome sequences of both *C. trachomatis* (Stephens et al., 1998) and *C. pneumoniae* (Kalman et al., 1999) have now been published, identifying several biochemical pathways that may contribute to metabolic activities not historically associated with chlamydiae. These include peptidoglycan biosynthesis, genes for nucleotide conversion, ribonucleotide reductase, tricarboxylic acid cycle enzymes, and most surprisingly, enzymes and cofactors of the electron transport chain to generate a putative ATPase complex capable of oxidative

phosphorylation (Kalman et al., 1999). A cautionary note should be made that these putative metabolic activities are based on information deduced from genomics and the functionality of these pathways in chlamydiae requires biochemical confirmation.

The mechanisms by which chlamydiae acquire metabolites from the host cell are being elucidated. Tjaden and colleagues (1999) have identified a functional ATP-ADP chlamydial translocase and a second nucleoside triphosphate transporter that imports all four ribonucleoside triphosphates (NTPs) thus providing a means to acquire not only energy, but also the net uptake of NTPs essential for anabolic reactions. Kubo and Stephens (2001) have recently identified a dicarboxylate-specific porin on the chlamydial surface that may import 2-oxoglutarate to feed the incomplete bacterial tricarboxylic acid cycle. Furthermore, that chlamydiae intercept sphingomyelin-containing vesicles from the host Golgi apparatus (van Ooji et al., 2000; Wolf and Hackstadt, 2001) and eventually develop a phospholipid composition that mimics that of the host cell in which it is grown (Hatch and McClarty, 1998) is compelling evidence for the interaction of the bacteria with host lipid metabolism.

ANIMAL MODELS OF *C. PNEUMONAIE* INFECTION: TOWARDS UNDERSTANDING PERSISTENCE AND CHRONIC INFECTION

An historical view of chlamydia animal models

Animal models of *C. pneumoniae* infection were first developed in non-human primates (Bell et al., 1989), mice (Yang et al., 1993) and rabbits (Moazed et al, 1996) to study respiratory diseases induced by infection. The mouse and rabbit models have since been refined largely to investigate the apparent role of this organism in atherosclerosis, suggested from earlier

epidemiologic and pathologic investigations. These models offer a controlled environment in which to discriminate between disease caused by chronic / persistent infections and acute re-infections. Secondarily, these models have allowed for a more investigative look into chlamydial persistence and in understanding bacterial dissemination in a host animal.

Persistent chlamydia and chronicity of disease

Chlamydial persistence describes a long-term association between chlamydiae and their host cell and is meant to describe a condition discrete from acute re-infections. Persistent chlamydiae remain in a viable yet culture negative state without overt chlamydial growth. This cryptic state is akin to viral latency where the normal progression of the microorganism through the developmental cycle is interrupted. This concept is introduced here in order to provide adequate background for further discussion of chlamydial persistence alluded to in subsequent chapters. Persistent chlamydial infections can readily be induced in cell culture following treatment of infected cells with antibiotics, IFN-γ, or removal of essential amino acids (Beatty et al., 1994). These experiments have identified at least two potential molecular mechanisms of chlamydial persistence. From the infected host cell perspective, induction of persistence can involve the activation of indoleamine-2,3-dioxygenase-mediated tryptophan catabolism (Mehta et al., 1998) and from the bacterial perspective, a persistent phenotype appears to correlate with a downregulation of *ftsK* and *ftsW* genes, whose gene products are required for cytokinesis (Gerard et al., 2001). Yet key questions remain; are persistent chlamydiae a real clinical entity and what role might these infections play in the development of chronic disease?

The concept that chlamydiae have the potential to produce chronic infections was made evident in earlier studies of human *C. trachomatis* infection showing that long-term carriage of

apparently non-productive bacteria in the eye conjunctiva could potentiate the development of scarring trachoma (Mabey et al., 1987). The ability of clandestine chlamydial infections to cause chronic disease is further supported by indirect reports documenting individuals who emigrated out of trachoma-endemic areas and had not experienced active *C. trachomatis* infection since childhood but who nonetheless developed acute trachoma decades later (Schachter, 1978). Similar studies looking at *C. pneumoniae* infections in humans have shown persistence of *C. pneumoniae* infection with prolonged respiratory symptoms for up to 6 years despite routine antibiotic therapy (Hammerschlag et al., 1992). The complex chlamydial life cycle is likely responsible for this phenomenon. Metabolically inert EB are not affected by antibiotics and may persist in the body for an indeterminate amount of time and intracellular RB, although susceptible to antibiotics, are somewhat protected in an intracellular niche with three membranes (RB membrane, inclusion membrane and host cell membrane) separating the bacteria from the extracellular environment. Furthermore, there is evidence that the persistent body, shown in cell culture to be induced following antibiotic treatment, may be refractory to further antimicrobial activity (Gieffers et al., 2001).

Murine models of *C. pneumoniae* infection

Murine models of atherosclerosis using inbred mouse strains harboring genetic deficiencies in either apolipoprotein E (ApoE) or the low-density lipoprotein receptor (LDL-R) have corroborated the ability of *C. pneumoniae* to exacerbate atherosclerosis (Hu et al., 1999; Moazed et al., 1999; Burnett et al., 2001; Rothstein et al., 2001), yet other studies using similar murine models have suggested that infectious agents are not necessary for the development of atherosclerosis (Wright et al., 2000; Caligiuri et al., 2001). Data from these latter studies

however does not rule out an involvement of *C. pneumoniae* in the progression of atherogenesis in wild type mice not genetically predisposed to the disease. A principal limitation of animal models using genetically modified and wild type inbred mice is that they measure outcomes within a genetically restricted homogeneous population. For this reason, outbred animal models representing a genetically heterogeneous population may be more relevant when extrapolating results to the human disease counterpart.

Rabbit models of *C. pneumoniae* infection

An animal model using outbred New Zealand White (NZW) rabbits to study the potential of *C. pneumoniae* to induce atherosclerosis was first developed through the research collaboration of our laboratory and Dr. I. Fong at the University of Toronto (Fong et al., 1997). Since then, our model has been reproduced several times (Laitinen et al., 1997; Muhulestein et al., 1997; Fong et al., 1999a), demonstrating *de novo* aortic atherosclerotic disease following intranasal administration of *C. pneumoniae.* Studies in rabbits have identified a synergistic interaction between this organism and other defined risk factors for human coronary artery disease such as high blood cholesterol levels. Rabbit models have also been useful for understanding how this organism disseminates in a susceptible host animal, identifying *C. pneumoniae* within peripheral blood mononuclear cells, in secondary lymphoid organs such as spleen and in the cardiovascular tree, thereby confirming hematogenous dissemination of the bacteria following primary infection of lung epithelium. Our laboratory has shown that antibiotics administered up to 5 days after inoculation, but not after 14 days of infection can attenuate the development of atherosclerotic lesions in rabbits infected via the respiratory tract (Fong et al., 1999b), suggesting that after

resolution of acute infection, the pro-atherogenic effects of *C. pneumoniae* infection persist, perhaps due to persistence of the organism in tissues inaccessible to the antibiotic.

One important finding garnered from rabbit models of *C. pneumoniae*-induced atherosclerosis is that unlike wild type mice, exacerbation of atherosclerosis in rabbits can take place without the addition of a high fat diet (Fong et al., 1997, 1999a). Our research has demonstrated that approximately 40% of normocholesterolemic rabbits infected with *C. pneumoniae* reproducibly develop atherosclerotic disease by three months post-infection. This has proven to be a useful model in which to delineate the contribution of *C. pneumoniae* infection per se without the confounding background of an atherogenic high cholesterol diet. Accordingly, the normocholesterolemic rabbit model was used for the investigations described in chapter five of this thesis.

Limitations of current animal models

A brief introduction to the limitations of these animal models seems warranted in order to construct a framework on which to adjudicate these and other animal experiments. It is unlikely that any animal model used to investigate the relationship between *C. pneumoniae* and atherosclerosis will fulfill all the criteria of Koch's postulates, which historically were developed for the identification of an acute infectious disease with a single etiology; one infection, one disease. With chronic diseases, the time delay between inoculation and disease manifestation present issues that are difficult to reconcile with the preconceived definition of an acute infectious disease. Furthermore, the impact of aggregate infectious burden (ie co-infection with multiple pathogens) on the disease process is a recently identified phenomenon (Espinola-Klein et al., 2002) that complicates the issue further in human populations. Our interpretation of data from

animal models is currently limited by the inability to recover viable, cultivable *C. pneumoniae* from atheromatous tissue and by the lack of surrogate markers for persistent infections. Consolidated data from multiple animal models focusing on potential mechanisms of the disease process and improved identification and recovery of persistent bacteria should be helpful in elucidating the putative role of *C. pneumoniae* in this disease process.

OBJECTIVES AND THESIS ORGANIZATION

C. pneumoniae is a highly adapted pathogen that infects a large number of individuals and whose clinical spectrum of disease has recently expanded to include atherosclerosis and chronic coronary artery disease. Characterizing the molecular and cellular interactions between this obligate intracellular bacteria and it's host is an important first step in (i) understanding how bacterial invasion is initiated (ii) for identifying mechanisms of disease pathogenesis and (iii) for identifying new therapeutic targets to combat chlamydial infections.

The direction of this research project may best be described as a 'creative evolution', guided by the mutual research interests of Dr. Mahony and myself and by important knowledge gaps in the body of chlamydia literature that we felt needed to be addressed. While the specific research objectives are defined in each of the research chapters 2 through 5, the comprehensive objectives of this research project were to explore the basic molecular mechanisms of cellular invasion by *C. pneumoniae* and to identify potential molecular mechanisms by which *C. pneumoniae* could contribute to the process of atherosclerosis. This line of investigation has been approached using both cell culture-based experimentation together with an established *in vivo* rabbit model of *C. pneumoniae* infection. It is hoped that this information will foster additional

research investigation into the biology of *C. pneumoniae* with a focus on the molecular and cellular determinants dictating invasion and disease pathogenesis.

This thesis is organized into four major research chapters (chapters 2 through 5) comprising three published research articles and one manuscript submitted for publication, together with introductory and concluding chapters. In this way, it represents a "sandwich thesis", or a collection of my published works that address the research questions put forth during the course of this project. I was the principal author of all these papers and conducted the experiments described herein unless otherwise stated. Chapter two examines the requirement of specific host cell signal transduction pathways and the actin cytoskeleton for *C. pneumoniae* attachment to and uptake by epithelial cells and presents data on the characterization of early molecular events associated with the *C. pneumoniae* invasion sequence. This work has been submitted to *Cellular Microbiology* and is presented in manuscript format as specified by the Journal. To characterize the molecular host cell response to infection, we used cDNA array technology to profile the transcriptional response of endothelial cells once *C. pneumoniae* infection had been initiated. These data have been published in *Infection and Immunity* and are presented in Chapter three in manuscript format as specified by the Journal. Chapter four describes cellular proliferation assays that demonstrate that *C. pneumoniae* infection of human endothelial cells induces the production of biologically active endothelial-derived SMC growth factors. This work is presented in manuscript format and has been published in *Infection and Immunity*. In Chapter five, we used a rabbit model of *C. pneumoniae*-induced atherosclerosis to extend our *in vitro* findings of SMC proliferation. To this end, we examined the ability of *C. pneumoniae* infection to cause cellular and molecular changes consistent with atherogenesis in the vessel wall. Dr. Brian Chiu, formerly at the University of Toronto and now at the University

of Alberta in Edmonton performed the preliminary immunocytochemical staining of aortic tissue sections used for this study and was instrumental in teaching me the technique of microscopic measurement of intimal thickening. Dr. Ignatius Fong at the University of Toronto and St. Michael's Hospital has been a long-standing collaborator on our animal work with *C. pneumoniae* infection and atherosclerotic disease. The pathologic expertise of Dr. Fong and the technical support of his research team made these animal studies possible. Dr. Fong has been listed as a co-author on this manuscript and the technical support of his research team has been acknowledged. This manuscript has been accepted for publication in *The Journal of Infectious Diseases* and is presented in manuscript format as outlined by the Journal. Dr. Mahony provided valuable comments on all manuscript drafts prior to submission to the respective Journals and in doing so, helped me improve the final versions presented here.

CHAPTER TWO

AUTHOR'S PREFACE TO CHAPTER 2

In this chapter, I describe novel molecular and ultrastructural host cell events associated with attachment and uptake of *C. pneumoniae* by epithelial cells. This work identifies the first requisite host cell signaling cascades utilized by *C. pneumoniae* during invasion of epithelial cells. The overall implication of these findings is that (i) *C. pneumoniae* activates dual host cell signaling cascades and actin polymerization that are required for bacterial invasion and (ii) the *C. pneumoniae* invasion sequence may be biphasic, whereby initial attachment is rapidly followed by activation of specific host cell signaling pathways and actin polymerization required to facilitate bacterial uptake. This was suggested by the identification of inhibitors of signaling cascades and actin polymerization that attenuated *C. pneumoniae* uptake without affecting attachment to the host cell surface.

The material presented in Chapter 2 has been accepted for publication in the peer-reviewed journal, *Cellular Microbiology*. The experiments described in this chapter and the final version of the published manuscript are my original contributions. Dr. James Mahony provided comments on the manuscript and minor changes were incorporated into the text prior to publication in the Journal. The accepted version of the manuscript is presented here. The references have been incorporated into the Reference list at the end of the thesis to avoid considerable redundancy with other chapters and have been reformatted to Harvard style for consistency throughout this thesis.

The full citation is:

Brian K. Coombes, and James B. Mahony. (2002) Identification of MEK- and phosphoinositide 3-kinase-dependent signaling as essential events during *Chlamydia pneumoniae* invasion of epithelial cells. Cellular Microbiology. In press.

Portions of this work will be presented as an oral presentation at the 10th International Symposium on Human Chlamydial Infections in Antalya, Turkey, June 16-21, 2002.

IDENTIFICATION OF MEK- AND PHOSPHOINOSITIDE 3-KINASE-DEPENDENT SIGNALING AS ESSENTIAL EVENTS DURING *CHLAMYDIA PNEUMONIAE* INVASION OF HEP2 CELLS

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Running Title: Host cell signaling during *C. pneumoniae* invasion

Key Words: *Chlamydia pneumoniae*, host-response, invasion, signal transduction, ERK1/2, PI 3-kinase

SUMMARY

The ability of *Chlamydia pneumoniae* to survive and cause disease is predicated on efficient invasion of cellular hosts. While it is recognized that chlamydial determinants are important for mediating attachment and uptake into non-phagocytic cells, little is known about the bacterial ligands and cellular receptors that facilitate invasion or host cell signal transduction pathways implicated in this process. We used transmission and scanning electron microscopy to demonstrate that attachment of bacteria to host cells induced the appearance of microvilli on host cell membranes. Invasion occurred 30 min to 120 min after cell contact with the subsequent loss of membrane microvilli. Using an epithelial cell infection model, *C. pneumoniae* invasion caused a rapid and sustained increase in MEK-dependent phosphorylation and activation of ERK1/2, followed by PI 3-kinase-dependent phosphorylation and activation of Akt. Tyrosine phosphorylation of focal adhesion kinase (FAK) preceded its appearance in a complex with the p85 subunit of PI 3-kinase during chlamydial invasion and isoform-specific tyrosine phosphorylation of the docking protein Shc also occurred at the time of attachment and entry of bacteria. Chlamydia entry but not attachment could be abrogated with specific inhibitors of MEK, PI 3-kinase and actin polymerization, demonstrating the importance of these signaling pathways and an intact actin cytoskeleton for *C. pneumoniae* invasion. These results suggest that activation of specific cell signaling pathways is an essential strategy used by *C. pneumoniae* to invade HEp2 cells.

INTRODUCTION

Chlamydiae are a diversified group of intracellular Gram-negative bacteria capable of infecting a wide range of both warm and cold-blooded animals. These pathogens survive by first invading a susceptible host cell and then commandeering host pathways for their own metabolic- and energy requirements. During evolution, *Chlamydia pneumoniae* have acquired sophisticated mechanisms of cellular invasion and intracellular survival, exemplified by their ability to survive within professional phagocytes and non-phagocytic cells. *C. pneumoniae* initiates primary infections in the mucosal epithelium of the upper and lower respiratory tract, causing pneumonia, bronchitis and sinusitis (Kuo et al., 1995). The appearance of *C. pneumoniae* within atherosclerotic lesions subsequent to primary infection may occur as a result of hematogenous carriage and dissemination by infected monocytes (reviewed by Grayston, 2000).

As an obligate intracellular pathogen, *C. pneumoniae* interacts with host cells during its entire developmental cycle (Wyrick, 2000). Invasion of mammalian cells is initiated when the infectious extracelluar elementary body (EB) attaches to the host cell surface. Once internalized, bacteria reside exclusively inside a membrane-bound vacuole that separates from the endosomal pathway, avoiding fusion with mature lysosomes and joining an exocytic-like pathway, fusing with Golgi-derived vacuoles containing sphingomyelin (Wolf and Hackstadt, 2001). Inside the vacuole EB rapidly undergo morphological transformation into reticulate bodies (RB) that divide by binary fission for 60-80 hours in cell culture models followed by differentiation back into infectious EB prior to lytic release from host cells. The complete genome sequences of both *C. trachomatis* (Stephens et al., 1998) and *C. pneumoniae* (Kalman et al., 1999) have revealed a molecular complexity to this bacteria, notably with regard to the outer membrane complex, that

may harbor molecules important for binding and entry into multiple cells types. Indeed, the ability of *C. pneumoniae* strains to infect and replicate within epithelial cells, endothelial cells, smooth muscle cells, and macrophages (Gaydos et al., 1996; Coombes and Mahony, 1999) may implicate multiple routes of bacterial entry. The current model of chlamydial attachment suggests that sulfated glycosaminoglycan (GAG) molecules either on the bacteria (Stephens et al., 2000, 2001) or the host cell surface (Taraktchoglou et al., 2001; Wuppermann et al., 2001) are important in mediating attachment of both *C. pneumoniae* and some biovars of *C. trachomatis* to mammalian cells. Although these findings have identified potential modes of chlamydia attachment to host cell surfaces, the mechanisms used by this bacterium to facilitate post-attachment uptake remain obscure.

Intracellular bacteria have evolved sophisticated mechanisms to selectively activate and deactivate signal transduction pathways in mammalian cells in order to manipulate the cellular environment for invasion, growth and development within host cells (reviewed by Finlay and Cossart, 1997; Ireton and Cossart, 1998). Previous studies have identified host protein tyrosine phosphorylation (Birkelund et al., 1994; Fawaz et al., 1997; Krüll et al., 1999), nuclear translocation of NFkB (Dechend et al., 1999), host transcriptional activation (Coombes and Mahony, 2001) and perturbation of the host apoptotic program (Fan et al., 1998; Geng et al., 2000; Dean and Powers, 2001; Stenner-Liewen et al., 2002) in chlamydiae-infected cells. The wide spectrum of host cell signaling pathways modified by chlamydiae suggests a complex interaction between the bacteria and the host cell. Despite the requirement of cellular invasion for *C. pneumoniae* survival, specific host cell signaling pathways activated during invasion of epithelial cells have not been previously identified.

The aims of this study were to (i) establish early kinetics of binding and invasion of *C. pneumoniae* into HEp2 cells under static conditions, (ii) identify specific mammalian signal transduction pathways that become activated in response to *C. pneumoniae* invasion and (iii) determine the requirement of these pathways for bacterial invasion. We demonstrate here that successful invasion of epithelial cells by *C. pneumoniae* requires host cell participation through activation of specific signal transduction cascades that manifest as cytoskeletal rearrangements necessary for bacterial uptake. These data suggest that in addition to chlamydial-derived factors involved in the attachment phase of invasion, host cell factors are also required for intracellular changes leading to chlamydial uptake.

RESULTS

Invasion of HEp2 cells induces host cell membrane microvilli – We used electron microscopy to establish invasion kinetics under static culture conditions and to define the morphological features of initial cell surface interactions between *C. pneumoniae* and host cells. At a multiplicity of infection (MOI) of ~50, *C. pneumoniae* EB associated with the host-cell surface within 15 minutes as assessed by transmission electron microscopic (TEM) examination of fixed cells (Fig. 1, panel a). In some cases EB were associated with membrane projections presumed to be microvilli, but were also observed to interact directly with the host cell surface. At these early time points after exposure to bacteria, scanning EM (SEM) confirmed the presence of membrane projections (shown in Fig 1, panel b) on host cells, but these projections were largely absent on uninfected cells (Fig 1, panel h). By 30 minutes after infection, EB could be seen intimately associated with the host cell surface as revealed by both TEM (Fig 1, panel c) and SEM (Fig 1, panel d). After 2 hours of infection, it was difficult to locate EB associated with the host cell surface. Between 60-120 min after infection we observed by TEM bacteria contained within membrane-bound vacuoles inside cells (Fig. 1 panel e) and a reduction in the amount of surface bound EB by SEM (Fig 1. panel f). At 60-120 minutes post-infection, SEM also revealed the relative absence of membrane extensions on infected cells, indicating that these projections developed transiently in response to extracellular bacteria. To verify that infection of HEp2 cells in the absence of centrifugation and cycloheximide lead to a productive infection, infected cells were fixed and stained at 48-72 h post infection for the presence of intracellular bacterial inclusions. Fig 1, panel g demonstrates the presence of multiple intracellular chlamydial inclusions in HEp2 cell monolayers as determined in Nomarski-immunofluorescent overlays.

Additional experiments revealed that the bacterial progeny produced under these infection conditions were viable (data not shown). In subsequent SEM experiments, the formation of membrane microvilli was chlamydia dose-dependent since the appearance of microvilli was delayed in cells infected with decreasing concentrations of EB (B.K. Coombes, Appendix D). Collectively, these experiments demonstrated that exposure of HEp2 cells to *C. pneumoniae* under the described inoculation conditions resulted in (i) extensive binding of the bacteria to the host cell surface and microvilli formation during the first 15 min of infection, (ii) invasion of cells between 30 to 120 min, followed by (iii) the growth and development of intracellular inclusions containing viable bacterial progeny.

Inhibitors of MEK, PI 3-kinase and actin polymerization block *C. pneumoniae* uptake – Previous studies have revealed tyrosine phosphorylation of several host cell proteins in response to chlamydial infection (Birkelund et al., 1994; Fawaz et al., 1997; Krüll et al., 1999). To examine the possibility that *C. pneumoniae* invasion requires participation of the host cell via activated signal transduction cascades and cytoskeletal rearrangements, we infected HEp2 cells in the presence of specific inhibitors of MEK, PI 3-kinase, and actin polymerization – signaling pathways known to be important for the uptake of other bacteria. Cell invasion experiments were designed to discriminate between *C. pneumoniae* attachment and invasion by exploiting the fact that the putative chlamydia receptor is a surface-exposed, proteinaceous antigen, as even mild trypsin proteolysis of host cell monolayers renders the cells refractory to infection (Byrne and Moulder, 1978). Thus, treatment of infected cells with trypsin releases attached, non-internalized bacteria from the cell surface while intracellular bacteria are resistant to trypsin treatment. As shown in Fig. 2, treatment of HEp2 cells with the MEK inhibitor, U0126 inhibited *C. pneumoniae*

invasion without affecting binding of the bacteria to the cell surface. U0126 is a specific inhibitor of MEK1/2 but not MEK3. At 1 μM and 10 μM U0126, uptake of *C. pneumoniae* was inhibited 60% and 69% respectively from 4 independent experiments. HEp2 cells treated with 10 μM and 50 μM LY294004, a specific inhibitor of PI 3-kinase also prevented invasion by *C. pneumoniae* by 64% and 77%, respectively, again without affecting bacterial binding to host cells. We then performed experiments in the presence of the actin-depolymerizing agent, cytochalasin D (1.5 μg/ml), since actin polymerization has been shown to be important in the uptake of certain biovars of *C. trachomatis*. Treatment of HEp2 cell with cytochalasin D inhibited invasion by 87% compared to control cells, which was similar to inhibition in the presence of both LY294002 (50μM) and U0126 (10 μM) (87%). None of the drugs were cytotoxic to either HEp2 cells or *C. pneumoniae* under the described assay conditions. Trypan blue staining of HEp2 cells treated with drugs or vehicle did not reveal significant differences in the number of viable cells (>95% of cells were viable). Chlamydiae pre-treated with inhibitors and then released into fresh medium produced inclusions in HEp2 cells that were not significantly different in number compared with bacteria treated with vehicle (data not shown).

***C. pneumoniae* invasion induces rapid and sustained MEK-dependent activation of ERK1/2**– In order to identify early signalling cascades involved in *C. pneumoniae* binding and invasion, serum-starved HEp2 cells were cultured with *C. pneumoniae* for times ranging from 5 minutes to 2 hours. At the indicated times, the status of ERK1/2 phosphorylation in cell lysates was analyzed by immunoblotting with specific antibodies recognizing either anti-phospho-ERK1/2 or total ERK1/2. As shown in Figure 3 A, *C. pneumoniae* induced a rapid

phosphorylation of ERK1/2 that was sustained until at least 120 minutes after infection. Since ERK1/2 is a specific substrate of MEK1/2, we tested whether MEK was required for *C. pneumoniae*-induced ERK1/2-phosphorylation. HEp2 cells were treated with the MEK-specific inhibitor U0126 (Favata et al., 1998) or DMSO for 30 minutes and then infected with *C. pneumoniae* for 20 minutes in the presence of inhibitor. ERK1/2 $^{Thr202/Tyr204}$ phosphorylation was inhibited in a dose-dependent fashion in cells pretreated with 1-25 μM U0126 then infected with *C. pneumoniae* (Fig. 3 B). Phosphorylation of ERK1/2 in response to the agonist EGF was also abrogated in epithelial cells pretreated with the MEK inhibitor at a concentration of 25 μM. In contrast, cells treated with DMSO vehicle were able to phosphorylate ERK1/2 in response to *C. pneumoniae* to a similar level achieved in previous time course experiments. These data indicate that *C. pneumoniae*-induced phosphorylation of ERK1/2 requires the upstream activation of MEK1/2. To confirm the activation of ERK1/2, we measured the enzymatic activity of immunoprecipitated ERK by assaying its ability to transfer γ^{32}-phosphate from ATP to myelin basic protein (MPB). As predicted by the pattern of ERK phosphorylation in response to chlamydia, ERK kinase activity was rapidly activated within 10 minutes after exposure of cells to bacteria (Fig. 4) and remained elevated at 2 hours post infection. In support of previous experiments implicating MEK involvement in ERK activation, ERK kinase activity in response to chlamydia was completely abrogated by U0126 (Fig. 4, lane 6). Together, these data demonstrate that chlamydia induces rapid and sustained MEK-dependent activation of ERK, which correlates with the phosphorylation kinetics of ERK from previous experiments.

Akt phosphorylation occurs subsequent to ERK1/2 during *C. pneumoniae* invasion- There is precedent for the usage of PI 3-kinase-dependent signaling pathways by at least three other

intracellular bacteria (Ireton et al., 1996; Reddy et al., 2000; Steele-Mortimer et al., 2000) and *C. pneumoniae* may also use a similar strategy. Since Akt is a 'downstream' molecular target of PI 3-kinase signaling, the phosphorylation status of Akt was investigated during *C. pneumoniae* invasion. Serum-starved HEp2 cells were infected with *C. pneumoniae* for 10 min to 2 h, and total Akt was immunoprecipitated from soluble whole cell lysates. Immunoprecipitated Akt was first probed with an anti-phospho Akt polyclonal antibody recognizing Ser-473 phosphorylation, followed by an antibody recognizing total Akt. As shown in Fig 5 A, Akt Ser-473 phosphorylation was induced beginning at 20 min after addition of bacteria to cells and reached maximal levels at 40 min. Akt phosphorylation remained elevated above basal levels for the duration of the experiment (2h). In repeat experiments, we were able to detect Ser-473 phosphorylation of Akt directly in immunoblots from 100 μg of whole cell lysate from chlamydia-infected cells without concentrating the sample by immunoprecipitation (see Fig 5 B). The kinetics of Akt phosphorylation in these repeat experiments was similar to the data shown in Fig 5 A, with maximal Akt phosphorylation occurring at ~40 min after addition of bacteria to HEp2 cells.

Akt phosphorylation requires PI 3-kinase activity – Phosphorylation of Akt and activation of its effector functions has been shown to be PI 3-kinase-dependent. To determine whether *C. pneumoniae*-induced phosphorylation of Akt required PI 3-kinase activity, HEp2 cells were pretreated with either LY294002 (Vlahos et al., 1994) or wortmannin (Wymann et al., 1996), two structurally unrelated specific inhibitors of PI 3-kinase, followed by chlamydia infection and immunoblotting for Akt phosphorylation at Ser-473. Control cell cultures were treated with an appropriate concentration of DMSO. Treatment of HEp2 cells with the lowest concentration of

LY294002 (10μM) or wortmannin (5 nM) completely inhibited Akt Ser-473 phosphorylation at 40 min after infection (Fig 5, B). In control experiments, concentrations of 40 μM LY294002 or 50 nM wortmannin also completely blocked Akt Ser-473 phosphorylation in response to the PI 3-kinase agonist, EGF (Fig. 5, B). Having defined the pattern of Akt phosphorylation in response to chlamydia, we next determined whether the kinase activity of Akt was also increased. HEp2 cells were infected with chlamydia and Akt was recovered from whole cell lysates by immunoprecipitation with an anti-Akt polyclonal antibody. Immune complexes were tested for their ability to transfer radiolabeled phosphate to GSK-3 fusion protein, a known substrate of Akt. Infection with chlamydia caused a marked increase in Akt kinase activity (Fig 6) that correlated with its phosphorylation status at 40 min as determined in previous time course experiments (Fig 5). Akt activity was completely blocked by LY294002, verifying that Akt activation is PI 3-kinase-dependent as suggested from previous phosphorylation experiments. These results demonstrate that Akt is activated after *C. pneumoniae* infection, however this activity follows a strikingly different pattern compared to that of ERK, with Akt activation appearing 30 to 45 minutes after ERK activation.

***C. pneumoniae* induces interaction between p85 PI 3-kinase and tyrosine-phosphorylated proteins** – The p85 regulatory subunit of PI 3-kinase is known to interact with tyrosine phosphorylated proteins in response to certain agonists and our previous experiments demonstrated PI 3-kinase activation during chlamydial invasion. In order to identify protein interactions with p85, cell lysates were immunoprecipitated with anti-phosphotyrosine (anti-Tyr(P)) antibodies, and then probed with an anti-p85 antibody. Infection with chlamydia induced the appearance of p85 in anti-Tyr(P) immunoprecipitates starting at 5 min after exposure to

bacteria, and remained elevated at 75 min (Fig. 7, A). Similarly, treatment of HEp2 cells with chlamydiae induced the appearance of several bands representing tyrosine-phosphorylated proteins in anti-p85 immunoprecipitates with similar kinetics, beginning 5 min after exposure to bacteria (Fig. 7 B, top panel). The presence of p85 in anti-Tyr(P) immunoprecipitates and the phosphotyrosine protein profile that co-immunoprecipitated with p85 in response to EGF are shown in the right-hand panels in Fig. 7A and 7B, respectively. These experiments suggest that activation of PI 3-kinase signaling results in the tyrosine phosphorylation of several additional proteins during chlamydiae invasion of cells.

Chlamydia invasion induces phosphorylation of FAK and its interaction with p85 – Co-immunoprecipitation experiments with an anti-p85 antibody showed the appearance of a tyrosine-phosphorylated protein with an apparent molecular mass of ~125-kDa (Fig. 7A, arrow). Work by others has demonstrated that the p85 subunit of PI 3-kinase can interact with activated protein tyrosine kinases through its SH2 domain and that one PI 3-kinase binding partner is FAK, a 125-kDa non-receptor tyrosine kinase that is important in assembly of signaling complexes that mediate local changes in the actin cytoskeleton in response to specific agonists (Chen and Guan, 1994). Exposure of HEp2 cells to *C. pneumoniae* induced tyrosine phosphorylation of FAK that peaked at 5 min post-exposure and remained slightly elevated until 45 min after infection (Fig. 8, top panel). Infection also increased the amount of p85 found in anti-FAK immunoprecipitates (Fig. 8, middle panel) that was maximal at 10 min post-infection and remained elevated until 45 min. The kinetics of p85-FAK interaction observed in reciprocal immunoprecipitation experiments was identical to that observed in anti-FAK IP/anti-p85 blots (compare Fig. 7 B, middle panel with Fig. 8, middle panel). Taken together, these experiments demonstrate that

FAK is tyrosine-phosphorylated above basal levels by 5 min after exposure to *C. pneumoniae* and that tyrosine-phosphorylated FAK interacts with the p85 regulatory subunit of PI 3-kinase beginning at 10 minutes after exposure to bacteria.

Chlamydia invasion involves isoform-specific tyrosine phosphorylation of the adaptor protein Shc – The Shc docking protein binds phosphotyrosine residues on activated receptor tyrosine kinases through SH2 and PTB phosphotyrosine binding domains and exists as three isoforms (of 46, 52, and 66-kDa) in mammalian cells, derived from differential initiation of translation or alternative mRNA splicing (Migliaccio et al., 1997). We tested whether Shc becomes tyrosine-phosphorylated during *C. pneumoniae* invasion and whether this protein interacts with the p85 regulatory subunit of PI 3-kinase. Shc proteins were immunoprecipitated from infected HEp2 cells using an antibody that recognizes all three Shc isoforms. Tyrosine phosphorylation of Shc was then assessed by immunoblot analysis using an anti-Tyr(P) antibody. Chlamydia infection induced tyrosine phosphorylation of Shc within 5 min of infection, which was sustained for 75 min when the experiment was stopped (Fig. 9, top panel). Interestingly, only the 52-kDa isoform of Shc was tyrosine-phosphorylated above basal levels in response to chlamydiae, with tyrosine-phosphorylation of the 46- and 66-kDa isoform remaining at similar levels to that in uninfected control cells. (Fig. 9, top panel). To verify the repertoire of Shc proteins in HEp2 cells, tyrosine phosphorylation of Shc immunoprecipitated from EGF-stimulated cells was determined by immunoblot analysis. Tyrosine phosphorylation of all three isoforms of Shc was detected in EGF-stimulated HEp2 cells thus confirming that these cells are indeed capable of producing these three polypeptides (Fig. 9, right panel). This pattern of Shc

tyrosine phosphorylation was verified in reciprocal experiments where anti-Tyr(P) immunoprecipitates were blotted with anti-Shc antibodies (data not shown).

DISCUSSION

Pathogenic bacteria manipulate host cell signaling pathways in order to commandeer the cytoskeleton for invasion, intracellular motility or for full virulence *in vivo* (Finlay and Cossart, 1997; Ireton and Cossart, 1998). For *C. pneumoniae*, efficient cellular invasion is mandatory for their survival and is exemplified by a unique ability to invade both phagocytes and non-professional phagocytic mammalian cells. Our results highlight a host cell signaling program utilizing at least two signaling pathways and actin polymerization to facilitate invasion of epithelial cells by *C. pneumoniae.*

Based on our scanning EM studies, bacterial attachment to the cell surface followed by reorganization of host cell membranes to form microvilli was temporally associated with ERK activation. ERK1/2 is activated after phosphorylation by MEK at Thr and Tyr residues in response to a variety of extracellular stimuli including growth factor receptor tyrosine kinases and integrin ligation to the extracellular matrix (Chang and Karin, 2001). The disappearance of microvilli and the appearance of intracellular bacteria within membrane-bound vacuoles temporally coincided (40 min after infection) with the activation of Akt, a mammalian kinase involved in a number of tightly regulated cellular processes such as proliferation and differentiation, cell survival and regulation of apoptosis. In the current model, inactive Akt resides in the cytoplasm and is recruited to the lipid membrane by phosphoinositide (PI) 3-kinase inositol phospholipids, PtdIns(3,4)P2 and PtdIns(3,4,5)P3 through the N-terminal Akt pleckstrin homology (PH) domain (Vanhaesebroeck and Alessi, 2001). Akt is activated by phosphorylation on Thr-308 and Ser-473 located within the activation loop of the kinase domain, and the hydrophobic C-terminal regulatory motif, respectively by PDK1 and possibly integrin linked

kinase (ILK) (Persad et al., 2001). The temporal separation of ERK and Akt activation might reflect the sequential activation of host cell pathways during invasion and modification of the intracellular environment. The activity of MEK and PI 3-kinase did not appear to be fully redundant for invasion since inhibitors of either pathway could block bacterial entry but the combined action of both signaling pathways was necessary for full invasiveness.

How might PI 3-kinase and ERK signaling lead to bacterial uptake? We speculate that *C. pneumoniae* invasion is a biphasic event whereby initial attachment is rapidly followed by activation of a cell surface receptor(s) leading to two distinct forward signals involving PI 3-kinase and MEK-ERK (and perhaps other pathways). That PI 3-kinase is an upstream and downstream effector of several members of the Ras superfamily of small guanosine triphosphatases involved in actin polymerization (Nobes and Hall, 1995; Keely et al., 1997; Hall, 1998) raises the possibility that one outcome of PI 3-kinase activation by chlamydia might be to mediate actin polymerization required for bacterial uptake. This hypothesis is supported by invasion experiments with PI 3-kinase antagonists that reduced *C. pneumoniae* uptake by similar levels to that achieved with cytochalasin D. The reversibility of this inhibitory effect following release from drug inhibition after a 2 h infection is difficult to quantitatively assess under our assay conditions since prolonged exposure of extracellular EB to physiological temperatures has been shown to significantly compromise bacterial viability (Kuo and Grayston, 1998) and consequently their ability to invade cells. For this reason, quantitative assessment of reversibility will require additional empirical studies using different culture conditions. In preliminary experiments we found that pharmacological inhibition of MEK and PI 3-kinase could antagonize microvilli formation induced by *C. pneumoniae* and that inhibition of microvilli can also be

achieved by blocking actin polymerization (B.K. Coombes, unpublished data). Actin polymerization has been previously shown to be essential for the uptake of certain biovars and serovars of *C. trachomatis* (Majeed and Kihlstrom, 1991; Schramm and Wyrick, 1995; Boleti et al., 1999. However, experimental and biological variation in the requirement for actin polymerization during *C. trachomatis* invasion is suggested from these studies. Namely, while it has been demonstrated that *C. trachomatis* serovar E of the trachoma biovar appears to require host actin for invasion of epithelial cells (Majeed and Kihlstrom, 1991; Schramm and Wyrick, 1995), the lymphogranuloma biovar, serovar L2 appears to require actin for epithelial cell invasion in some studies (Boleti *et al.,* 1999; Majeed and Kihlstrom, 1991) but not in others (Schramm and Wyrick, 1995). Complex biological differences between *C. trachomatis* biovars and serovars may account for this apparent variation.

Our data support the notion that early host cell signaling cascades may operate, at least in part, to facilitate cytoskeletal rearrangements leading to efficient bacterial uptake, perhaps through activated FAK. FAK is a non-receptor tyrosine kinase that when phosphorylated, presents docking sites for SH2 domains on other signaling molecules (Chen and Guan, 1994; Schaller, 2001). In this way, FAK is important in transducing growth factor and integrin signals to downstream pathways such as PI 3-kinase and ERK1/2. The temporal appearance of FAK-p85 complexes during *C. pneumoniae* invasion was consistent with a model whereby prior tyrosine phosphorylation of FAK was required to render it a competent binding partner for the SH2 domain of p85. Rapid FAK phosphorylation following exposure to chlamydia and its interaction with p85 may indicate a bifurcation of early PI 3-kinase signaling at the lipid membrane leading to MEK-ERK signaling, recruitment of specific adapter molecules and activation of Akt, similar to what has been described in lysophosphatidic acid-treated Cos7 cells (Bondeva et al., 1998).

Recent studies have demonstrated the translocation of peripheral ERK to newly forming focal adhesions (Finchman et al., 2000) and evidence exists for the ability of cytochalasin D to abolish tyrosine phosphorylated FAK targeting to membranes (Schaller, 2001). Together with the ability of PI 3-kinase inhibitors to block microvilli during infection (unpublished data), these findings are consistent with a possible role for PI 3-kinase- and ERK-dependent regulation of actin polymerization required for *C. pneumoniae* uptake.

We found a preferential phosphorylation of the 52-kDa Shc isoform in response to chlamydia infection despite the fact that cells in our model system expressed all three Shc isoforms. Shc lacks detectable intrinsic catalytic activity but serves as a molecular adaptor linking activated receptor tyrosine kinases with downstream signaling molecules via an N-terminus PTB domain and C-terminus SH2 domain (Chang and Karin, 2001). Previous work by others has shown that certain signaling pathways can recruit Shc in an isoform-specific manner (Okada et al., 1995; Sato et al., 2000), suggesting that the specificity of diverse signaling pathways utilizing Shc as a ubiquitous docking protein are afforded, in part, by specific Shc isoforms that recruit different adaptors molecules and/or kinases for the desired cellular response. In two other reports citing phosphorylation of Shc in response to intracellular bacteria, this response was isoform-nonspecific in the case of Listeria (Ireton et al., 1999), and appeared to be specific for the 46- and 52-kDa isoforms during EPEC infection of T84 human colonic cells (Czerucka et al., 2001). However, in the latter study, the 66-kDa Shc isoform was not apparent in EGF-treated cells suggesting that T84 colonic cells do not produce the higher molecular weight Shc. To our knowledge, our study is the first report showing single Shc isoform-specificity during invasion by an intracellular bacterium. Given that a primary role of Shc is to bind activated receptor tyrosine kinase molecules in response to extracellular agonists, it is possible

that elucidation of Shc binding partners in response to chlamydia may offer clues to the identity of the putative chlamydia receptor, which could be a receptor tyrosine kinase (RTK) or other membrane protein that associates with an RTK.

Similar signaling molecule dynamics have been described during entry of other intracellular bacteria and protozoan parasites into various mammalian cells. Using a Listeria model, Ireton and colleagues showed PI 3-kinase activation by the bacterial protein InlB (Ireton et al., 1999) – an agonist of the hepatocyte growth factor (HGF)/Met receptor tyrosine kinase (Shen et al., 2000). *Salmonella enterica* serovar Typhimurium (Steele-Mortimer et al., 2000), *Escherichia coli* (Reddy et al., 2000) and the protozoan parasite *Trypanosoma cruzi* (Chuenkova et al., 2001; Wilkowsky et al., 2001) also activate PI 3-kinase signaling and localized cytoskeletal changes involved in bacterial uptake. For chlamydial invasion, it is possible that type III-secreted proteins play a role in activation of host signaling molecules and/or invasion since others have furnished evidence arguing for the existence of chlamydial type III effector molecules (Fields and Hackstadt, 2001). Other groups have reported host tyrosine phosphorylation in response to *C. trachomatis* infection of epithelial cells (Birkelund et al., 1994; Fawaz et al., 1997) and *C. pneumoniae* infection of endothelial cells (Krüll et al., 1999), however these previous experiments were conducted in the presence of serum (containing various growth factors), making the interpretation of the data more difficult. Birkelund and colleagues (1994) first reported the tyrosine phosphorylation of 64, 66, 68, 100 and 140 kDa proteins in HeLa cells infected with *C. trachomatis* but the identity of these proteins and their role in infection were not determined. *C. trachomatis* infection of HeLa cells (Fawaz et al., 1997) has also been shown to induce co-localization of cortactin – a 75 kDa to 85 kDa activator of actin assembly nucleated by the Arp2/3 complex (Schafer, 2002)– with host tyrosine phosphoproteins and the chlamydial

vacuole. Since actin and its associated proteins interact with components of the endocytic machinery, they could help coordinate signaling events and actin assembly involved in chlamydial uptake. These data are consistent with our hypothesis that *C. pneumoniae* activates a specific set of host signaling pathways that manifest, in part, as cytoskeletal alterations and bacterial uptake.

What binding events at the cell surface might be involved in activation of these signaling pathways and internalization of chlamydia? To date, no specific host cell receptor or chlamydia adhesin has been shown unequivocally to mediate invasion into host cells, yet there is some evidence that heparan sulfate glycosaminoglycans (HSPG) facilitate chlamydia attachment to cell surfaces (Stephens et al., 2000; 2001; Taraktchoglou et al., 2001; Wuppermann et al., 2001). Based on the data currently available with regard to chlamydia binding mechanisms and intrinsic catalytic signaling activity ascribed to HSPG, we hypothesize that *C. pneumoniae* utilize a bipartite invasion sequence whereby initial attachment to HSPG facilitates tyrosine phosphorylation of a secondary, as of yet unidentified receptor to initiate PI 3-kinase and MEK-ERK activity in the host to promote bacterial entry. This model is supported by experiments showing that inhibition of chlamydial uptake can take place independent of attachment to the host cell surface. Ting and colleagues (1995) demonstrated that a narrow range of heat treatment could attenuate chlamydial infectivity, yet a much broader range was needed to reduce bacterial attachment. More recently, using a panel of wild type and mutant Chinese hamster ovary cell lines, Carabeo and Hackstadt (2001) reported data that showed reversible binding of *C. trachomatis* to cell surface glycosaminoglycans (GAG) and irreversible attachment to an unknown cell surface component subsequent to GAG binding, thus supporting the notion that attachment and internalization are two discrete molecular events. Further, experiments described

here show that inhibition of specific host cell signaling pathways and actin polymerization can reduce *C. pneumoniae* uptake independent of surface attachment, suggestive of additional steps in the invasion sequence subsequent to initial attachment that are required for internalization.

In summary, our data identify a dual signaling mechanism that provides novel insight into the host cell machinery required for uptake of *C. pneumoniae* into HEp2 cells. Further experiments with different chlamydial species and host cell types will be required to extend the generalizability of these data. The activation of two separate host cell signaling pathways with known involvement in cytoskeletal rearrangements may offer chlamydia and perhaps other obligate intracellular bacteria access to a wider range of cellular hosts. Activation of survival pathways by chlamydiae such as those involving Akt (Datta et al., 1999) may transcend invasion to facilitate a long-term association with host cells during chronic infections.

EXPERIMENTAL PROCEDURES

Antibodies and other reagents - Polyclonal phospho-Akt Ser 473 rabbit antiserum was purchased from New England Biolabs (Mississauga, ON). Phospho-independent Akt rabbit antiserum was also from New England Biolabs and recognizes both phosphorylated and non-phosphorylated AKT. Polyclonal anti-ERK1/2 Thr202/Tyr204 antibody, and a phospho-independent anti-ERK1/2 polyclonal antibody were from New England Biolabs. Anti-phosphotyrosine monoclonal antibody (TyrP, clone 4G10) and polyclonal antiserum raised against the p85 subunit of rat PI3 kinase was purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal anti-Shc antibody was from Transduction Laboratories (Mississauga ON) and rabbit polyclonal anti-FAK antibody (c20) was from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated secondary antibodies raised in goat against either mouse or rabbit were purchased from Sigma (St. Louis, MD). Protein A-agarose beads were purchased from Roche Molecular Biochemicals (Laval, QC). Epidermal growth factor (EGF; Sigma) was prepared as a 100 μg/ml stock solution and stored at -20°C. LY294002 (Sigma) was reconstituted in dimethylsulfoxide (DMSO) at 10 or 50 μM and stored at -80°C. Wortmannin was diluted in DMSO and stored as a 2 mM stock solution at –20°C. The MEK1/2 inhibitor, U0126 (New England Biolabs) was diluted in DMSO, stored as a 10 or 50 mM stock at -80°C and used within 1 week. Cytochalasin D (Sigma) was reconstituted in DMSO and stored at -20°C. Fresh aliquots of inhibitors were diluted to the desired working concentration as needed for individual experiments. All other fine chemicals were obtained from Sigma unless otherwise stated.

Cell Culture, bacterial strain and Chlamydia propagation - HEp2 epithelial cells (ATCC CCL-23) were cultured in minimal essential medium (MEM; Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 2 mM L-glutamine. HEp2 cells were subcultured into 6- or 12-well culture dishes and allowed to adhere for 24 h prior to infection with chlamydiae. At all times, cells were grown at 37°C, in 5% CO_2 humidified air. *C. pneumoniae* strain VR1310 is a respiratory isolate of chlamydia and was propagated in cycloheximide-treated HEp2 cells with centrifugation-assisted inoculation as described previously (Coombes and Mahony, 2001) During the last passage of bacteria prior to final harvest, HEp2 cells were infected under serum-free conditions and maintained in serum-free (SF)-MEM throughout the course of infection. Bacteria were purified on discontinuous density gradients as described by Caldwell et al. for *C. trachomatis*, (Caldwell et al., 1981) except Renografin was replaced with Gastrografin (Braco Diagnostics, Princeton, NJ, USA). Purified bacteria were stored in a 50% sucrose-phosphate-glutamate buffer and 50% serum-free MEM mixture at -80°C and titers were determined in HEp2 cells using immunofluorescent staining with a genus-specific fluorescein isothyocyanate-labeled monoclonal antibody (Kallestad, Chaska, MN). Stained cells were visualized with either an Olympus BH-2 epifluorescent microscope, or a Zeiss LSM 510 confocal laser-scanning microscope. Bacterial titers were expressed as inclusion forming units (IFU) per ml.

Metabolic labeling of Chlamydia with ^{35}S- Chlamydia EB were metabolically labeled with [^{35}S]- methionine and cysteine. HEp2 cells were cultured in 75 cm2 culture flasks and inoculated with *C. pneumoniae* VR1310. Infected cells were centrifuged for 45 min at $1250 \times g$ then cultured at 37°C for 1h. The culture medium was replaced with MEM containing 1 μg/ml

cycloheximide and plates were further incubated for ~24 h. At 24 h post-infection, the medium was replaced with MEM containing 30 μg/ml cold methionine and 30 μg/ml cold cysteine and supplemented with 10% dialyzed FBS, 2 mM L-glutamine, 2.5 μg/ml cycloheximide and 100 μCi/ml of ExpreS35S protein labeling mix (Perkin Elmer Life Sciences, Boston, MA) containing 77% [^{35}S]-methionine and 17% [^{35}S]-cysteine. At 72- h post-infection, cells were detached from the culture flasks with sterile 3mm glass beads, sonicated, and then centrifuged at 1500 × *g* to remove cellular debris. Radiolabeled EB from the supernatant fraction were purified by centrifugation through discontinuous Gastrografin gradients as described above. Radiolabeled bacteria were stored in 50% MEM and 50% SPG buffer in aliquots and typically yielded ~1.3 CPM per IFU.

Bacterial infection of HEp2 cells - HEp2 cells were cultured in 6-well dishes for ~ 20 h at 37°C, 5% CO_2. Cells were washed three times with serum-free (SF) MEM, and then cultured for 3-4 h in SF-MEM prior to stimulation or infection. For infection with *C. pneumoniae*, host cells were overlaid with a suspension of *C. pneumoniae* EB prepared in SF-MEM at a multiplicity of infection (MOI) of ~50 IFU per host cell without centrifugation and in the absence of cycloheximide. Cells were incubated at 37°C, 5% CO_2 for the specified time interval. After infection, cell monolayers were washed with ice-cold PBS and lysed immediately in solubilization buffer composed of 50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM glycerophosphate, 1 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride (PMSF). In experiments containing the PI3 kinase inhibitors LY294002 (Vlahos et al., 1994) and wortmannin

(Wymann et al., 1996) or the MEK1/2 inhibitor U0126 (30), inhibitors were diluted in SF-MEM to the desired working concentration and pre-incubated with HEp2 cells for 30 minutes prior to infection. Inhibitors were maintained in the culture medium for the duration of the infection period at the concentration given in individual figure legends. Control cells were cultured in 0.1% DMSO vehicle. At the concentrations and durations used in this study, these inhibitors did not affect cell viability as determined by trypan blue staining and did not cause cells to detach from the culture dishes.

Electron microscopy – For transmission electron microscopy (TEM), preformed monolayers of HEp2 cells grown in 4.5 cm2 plastic dishes were overlaid with a suspension of chlamydia at a multiplicity of ~50 for times ranging from 15 min to 2 h. After the indicated time, cells were washed with PBS and fixed with 2% glutaraldehyde in sodium cacodylate buffer. Fixed cells were post-fixed in 1% osmium tetroxide in buffer, dehydrated in a graded ethanol series, and then treated with propylene oxide. Cells were detached from culture dishes, embedded in Spurrs resin and thin sections (80 nm) were stained with uranyl acetate and lead citrate. Sections were viewed with a Phillips EM 300 electron microscope. For scanning electron microscopy (SEM), HEp2 cells were cultured in 8-well chamber slides (0.64 cm^2 growth area per well) and infected with *C. pneumoniae* as described above for TEM. Cells were fixed in 2% glutaraldehyde, post-fixed in osmium tetroxide and dehydrated with 100% ethanol in situ. Cells were critical point dried, sputter-coated with gold and viewed with a Jeol JSM-840 scanning electron microscope.

Immunoprecipitations – ~80% confluent monolayers of HEp2 cells grown in 6-well dishes were cultured for 4 h in SF-MEM prior to stimulation or infection. Infected cells were lysed in 0.5 ml

of ice-cold solubilization buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% (v/v) Triton X-100, 1 mM EDTA, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM glycerophosphate and supplemented with 1 μg/ml leupeptin and 1 mM PMSF. Separate control cells were mock infected or stimulated with EGF at 100 ng/ml for 2 min. Lysates were centrifuged at 20,000 × *g* for 5 minutes at 4°C and the protein content in the supernatant was determined by the Bradford assay. Lysate containing 0.5 to 0.7 mg of protein was used for immunoprecipitations. Lysates were incubated with the appropriate antibody (typically 4-6 μg per sample) overnight at 4°C on a rocking platform. Then, 50 μl of Protein A-agarose beads were added and incubated with gentle rocking for 2-4 h at 4°C. Beads were collected by centrifugation and washed four times with ice-cold solubilization buffer. For immunoblotting, the beads were resuspended in 50 μl 2× SDS-PAGE sample buffer, boiled, and clarified samples were used for SDS-PAGE. For in vitro kinase assays, immunocomplexes were washed an additional two times in 1× kinase buffer and used immediately.

Immunoblotting – As a positive control for all immunoblotting experiments, the agonist EGF was suspended in SF-MEM at a final concentration of 100 ng/ml and applied to serum-starved HEp2 cells for 2 min. After EGF-treatment or chlamydia infection, cells were washed with ice-cold TBS and then lysed in 0.5 ml of ice-cold solubilization buffer (for composition, see above). Cells were then scraped into 1.5 ml microfuge tubes and incubated on ice for 15 minutes. Lysates were centrifuged at 20,000 × *g* for 15 minutes at 4°C and the protein content of the detergent-soluble fraction was determined by the Bradford method (see "Supplemental Materials and Methods", Appendix B). Equal amounts of protein samples were mixed with an equal volume of

2× SDS-PAGE sample buffer (100 mM Tris-HCl, pH 6.8, 20% glycerol, 4% sodium dodecyl sulfate (SDS), 0.002% bromophenol blue and 200 mM dithiothreitol) and heated at 100°C for 5 minutes. Protein samples were separated using 7.5%- or 10% SDS-polyacrylamide gels followed by transfer to nitrocellulose membranes. Membranes were blocked for 1-3 h in 1× TBS, 0.1% Tween-20 (TBS-T), plus 3% bovine serum albumin (BSA) for anti-phosphotyrosine blots or TBS-T with 5% (w/v) non-fat milk for all other antibody detections, then incubated overnight at 4°C with a 1:1000 dilution of primary antibody in TBS-T containing the appropriate blocking agent. Membranes were washed and incubated with either a secondary goat anti-rabbit IgG (1:2000), or goat anti-mouse IgG (1:2000) antibody conjugated to horseradish peroxidase for 1 h at room temperature. Antibody complexes were detected using enhanced chemiluminescence (Amersham Pharmacia Biotech, Baie d'Urfé, QC). In some experiments, blots were stripped after detection in 62.5 mM Tris-HCl, pH 7.2, 2% SDS, 100 mM 2-mercaptoethanol at 57°C then reused for a second immunodetection.

In vitro kinase assays – HEp2 cells were serum-starved for 5 hours prior to infection or stimulation with EGF. When inhibitors were present, cell were pretreated with either U0126 or LY294002 for 30 minutes and inhibitors were present throughout the infection period at the concentrations given in individual figure legends. Cells were incubated for the indicated times with either chlamydia or EGF, washed with ice-cold PBS, then solubilized in lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM glycerophosphate and supplemented with 1 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride (PMSF). Samples were centrifuged for 10 min at 20,000 × *g* and the supernatants were incubated with 4-5 μg of either

anti-Akt or anti-phospho-ERK antibodies overnight on a rocking platform. 50 μl of protein A-agarose beads were used to precipitate immune complexes and beads were washed twice in lysis buffer and twice in kinase buffer containing 25 mM Tris-HCl, pH 7.2, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1mM DTT, 0.1 mM Na_3VO_4 and 5 mM glycerophosphate. Beads were suspended in 21 μl kinase buffer and supplemented with 9 μl of ATP/substrate mix in kinase buffer, such that the final concentration of cold ATP was 50 μM (for ERK assays) or 100 μM cold ATP (for Akt assays) and 5 μCi [γ^{32}P] ATP per reaction. As a substrate for Akt, 2 μg of a fusion protein containing the Ser 21/9 residues of GSK-3α/β was added to the reaction and incubated at 30°C for 30 minutes. For ERK kinase assays, myelin basic protein (MBP) was used as a substrate at a final concentration of 1 mg per ml and incubated at 30°C for 30 minutes. Reactions were terminated by addition of 2× SDS sample buffer, vortexed and boiled for 5 min. Reactions were subjected to 13% SDS-PAGE, transferred to nitrocellulose membranes and autoradiographs were developed to detect phosphorylated substrate. Phosphorylated substrates were then excised from the nitrocellulose filter and radioactivity was counted using liquid scintillation counting (Packard 1900 CA Tri-Carb Liquid Scintillation Analyzer, Meriden, CT).

Cell binding and invasion experiments – To measure chlamydia binding and entry into HEp2 cells we used an invasion assay modified from others (Byrne and Moulder, 1978; Stephens et al., 2000). HEp2 cells were seeded into 24-well culture plates and allowed to adhere for 24 h. Prior to infection, cell monolayers were washed with fresh medium, and then pre-incubated for 30 minutes in the presence of various concentrations of LY294002, U0126, or the actin-depolymerizing agent, cytochalasin D (Sigma) at a final concentration of 1.5 μg per ml.

Inhibitors were diluted in fresh SF-MEM just before experimentation and control cells were cultured in an appropriate concentration of DMSO diluted in SF-MEM. Following pre-incubation, cell monolayers were inoculated with 35S-radiolabeled *C. pneumoniae* at an MOI of ~1 or 10 in the presence of inhibitors and incubated at 37°C for 2h to allow binding and invasion to take place. Centrifugation-assisted inoculation was not employed in this assay. The medium was discarded and cells were washed twice with PBS to remove unbound bacteria, which was also discarded. Then, cells were trypsinized from the culture dishes to release attached, but not internalized bacteria. HEp2 cells were collected by centrifugation at 1200 × *g* and the supernatant containing trypsin-sensitive (attached) bacteria was counted by liquid scintillation counting. The cell pellet containing trypsin-resistant (internalized) bacteria was washed with PBS, then lysed with 2% SDS. Radioactivity contained in the cell lysate was quantified by liquid scintillation counting. In control experiments, binding and invasion assays were performed at 4°C, conditions that have previously shown to allow binding but not internalization of chlamydia. Data were expressed as the number of internalized and attached bacteria as a percent of the control cells.

Statistical Analysis- Statistical analysis of the data was performed using the Prism GraphPad software package (San Diego, CA). Data from the binding and invasion experiments were analyzed using one-way analysis of variance with the Neuman-Keuls post-test. P- values <0.05 were considered significant.

Footnotes

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Figure 2.1 - Binding and invasion of HEp2 cells by *C. pneumoniae*

HEp2 cells were inoculated with *C. pneumoniae* for 15 to 120 min without centrifugation and in the absence of cycloheximide and visualized by TEM (*panels a, c, e*), SEM (*panels b, d, f*) and immunofluorescent microscopy (*panel g*). Binding of Chlamydia EBs to the host cell surface occurs within 15 min after addition of bacterial to cells (*a* and *b*). TEM revealed EB interaction with membrane processes (shown in *panel a*) although the direct interaction between EB and the cell surface was also observed. Under similar magnification, p*anel b* shows an SEM photomicrograph of infected cells at 15 minutes, highlighting membrane projections (*inset*), that were not present in uninfected cells (*panel h*). At 30 minutes post infection (*panels c and d*), EB begin invasion of cells as revealed by TEM (panel c) and SEM (*panel d*). Incubation of cells with bacteria for 120 min lead to the appearance of intracellular bacteria contained within membrane-bound vacuoles (*panel e*) and the disappearance of both EB bound to the host cell surface and of extended membrane microvilli (*panel f*). *Panel g* shows a Nomarski differential interference contrast image merged with the FITC green channel from infected cells stained with an anti-LPS antibody conjugated to FITC to visualize intracellular inclusions at 48 hours post-infection. Bright spots indicate green fluorescence of intracellular bacterial inclusions and demonstrate productive intracellular replication of the bacteria. *Panel h* is a scanning electron micrograph showing the morphology of uninfected HEp2 cells, with very few membrane extensions and relatively smooth morphology. Bars indicate 1 μm in all panels except for *panel g*, where the bar represents 50 μm.

Figure 2.1

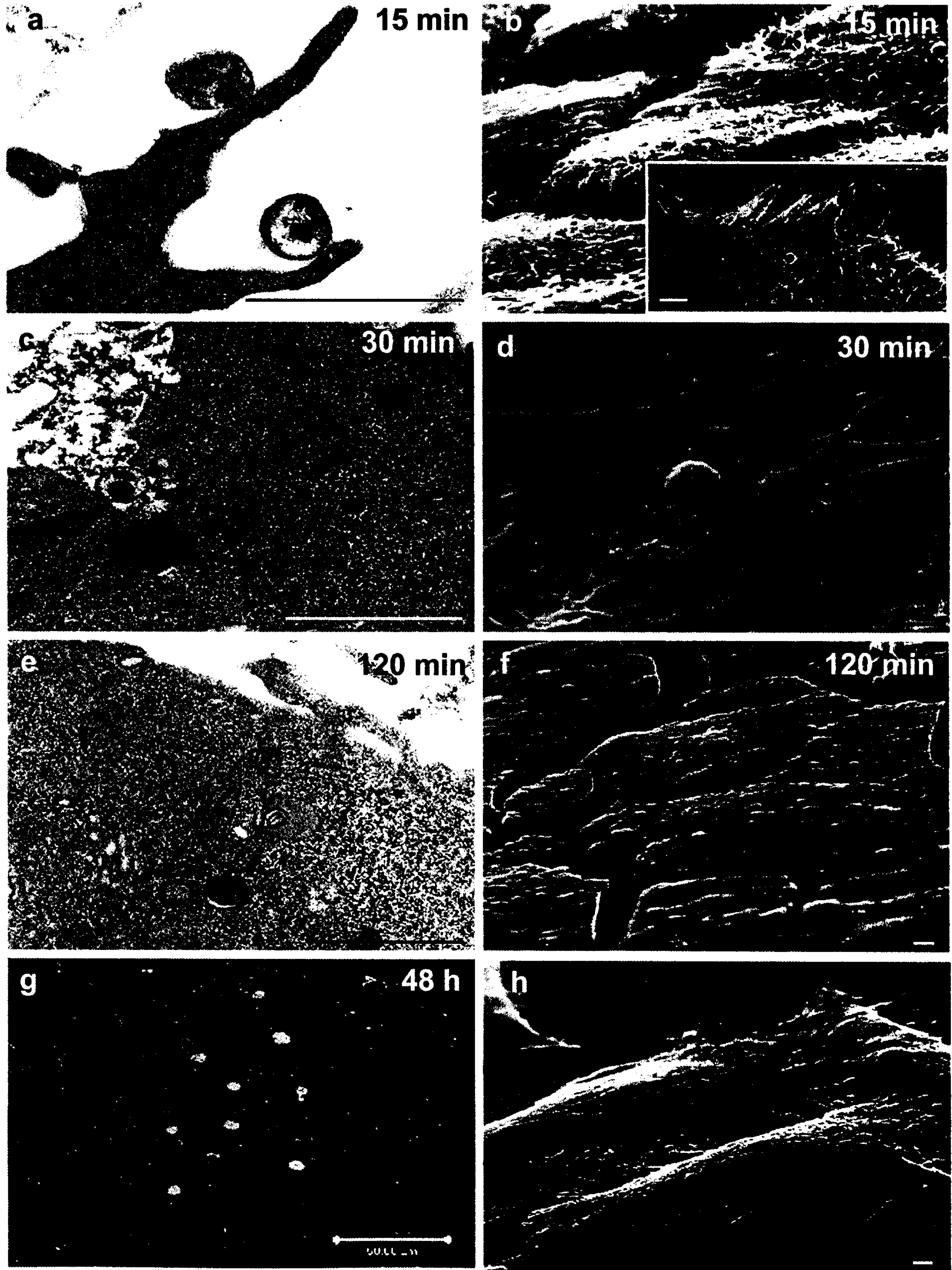


Figure 2.2 - Inhibitors of MEK, PI 3-kinase and actin polymerization block *C. pneumoniae* invasion but not binding

HEp2 cells were cultured in 24-well plates, then infected with ^{35}S-labeled *C. pneumoniae* for 2h in the presence or absence of inhibitors of MEK (U0126), PI 3-kinase (LY294002) or actin polymerization (cytochalasin D) at the concentrations given in the figure. After 2h of infection, the inoculum and two cell washes were discarded to remove unbound bacteria and monolayers were trypsinized to detach cells from the dishes and to release attached, but not internalized bacteria. HEp2 cells were collected by centrifugation and the supernatant containing the trypsin-sensitive (attached) bacteria was removed to a separate tube. The cell pellet containing trypsin-resistant (internalized) bacteria was washed with PBS, lysed with SDS and all samples were measured by liquid scintillation counting. Radioactivity contained in the cell fraction (black bars) represents internalized bacteria and radioactivity in the supernatant fraction (dotted bars) represents attached, but not internalized bacteria. Data presented is the mean with standard errors from 4 independent binding and invasion experiments each performed in duplicate. *, P<0.001 compared to control cells treated with DMSO.

Figure 2.2

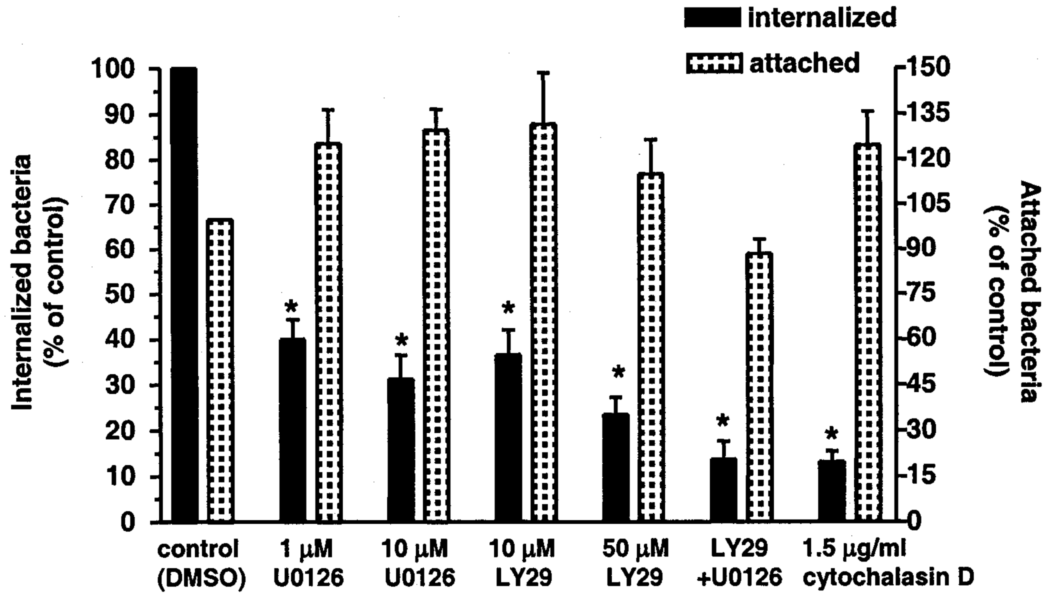
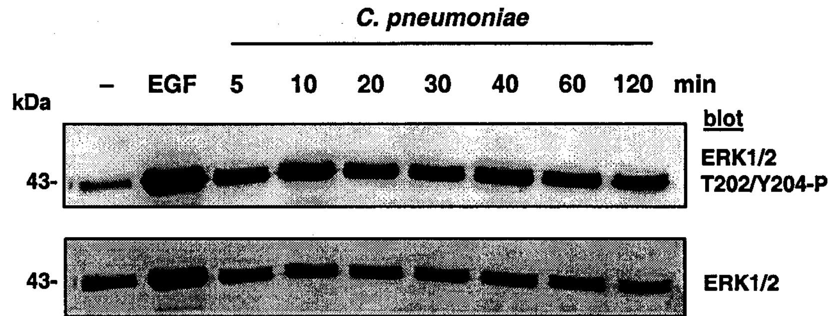


Figure 2.3 - *C. pneumoniae* infection induces rapid MEK-dependent phosphorylation of ERK1/2 in epithelial cells

(A) Infection time course with immunoblot detection of phosphorylated ERK1/2 in response to *C. pneumoniae*. Serum-starved HEp2 cells were stimulated with 100 ng/ml EGF for 2 min or cultured in the presence of *C. pneumoniae* for the times indicated in the figure. Cells were then solubilized and lysates were separated by SDS-PAGE and transferred to membranes. Phosphorylation of ERK1/2 was analyzed by probing blots with an anti-phospho T202/Y204 ERK1/2 antibody (upper panel). Blots were then stripped and probed with a phospho-independent anti-ERK1/2 antibody that recognizes total ERK1/2 (lower panel). (-), uninfected, unstimulated control cells. Positions of molecular mass standards are indicated in kilodaltons. Similar results were obtained in 2 other experiments. **(B)** Dose-dependent inhibition of ERK1/2 phosphorylation with the MEK-inhibitor, U0126. HEp2 cells were preincubated with the MEK1/2 inhibitor, U0126 or 0.1% DMSO in culture medium for 30 minutes then stimulated with EGF (100 ng/ml) for 2 minutes, or infected with *C. pneumoniae* for 20 minutes. Cells were lysed and immunoblotted for phosphorylated ERK1/2 ^{Ser473} (upper panel). Membranes were stripped and re-probed for total ERK1/2 protein (lower panel). Concentrations of U0126 are in μM. (–), untreated, uninfected HEp2 cells. Similar data was obtained in 2 other experiments.

Figure 2.3

A.



B

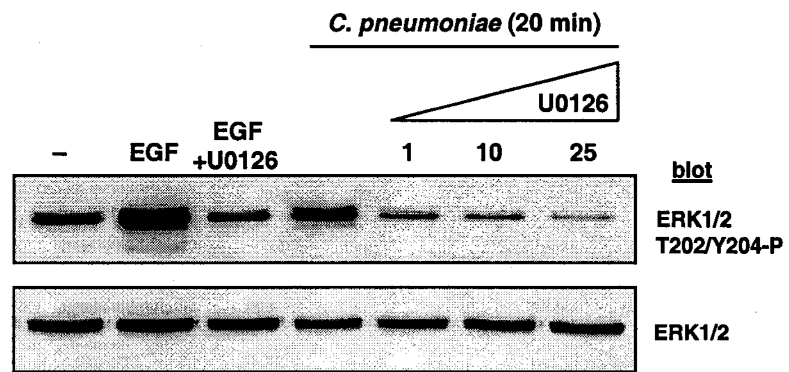


Figure 2.4 - ERK1/2 kinase activity is increased in chlamydia-infected cultures

(A) Cells were infected for the indicated times in the presence or absence of U0126 (25 μM), or left uninfected, or stimulated with EGF as described. Solubilized cell lysates were immunoprecipitated with an anti-ERK antibody and the ability of ERK to transfer γ^{32}-phosphate to MBP was measured by *in vitro* kinase assays. Data shown is a representative autoradiogram developed from nitrocellulose filters containing kinase reaction products separated by 13% SDS-PAGE. Bands indicate radiolabeled MBP migrating at approximately 20,000 molecular weight. **(B)** Radioactivity incorporated into MBP from each reaction was then measured by liquid scintillation counting and normalized to total protein content. The results are shown as counts per minute (CPM) of radioactivity incorporated into MBP for each condition.

Figure 2.4

A

B

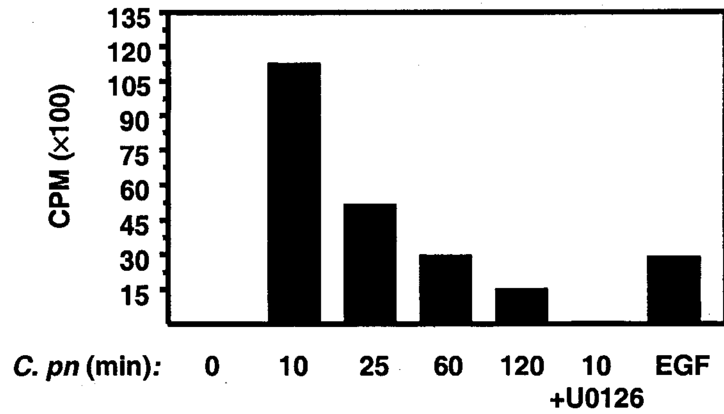
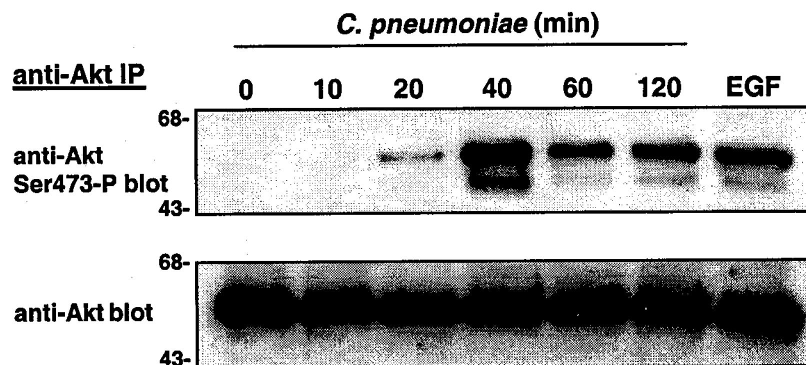


Figure 2.5 - *C. pneumoniae* stimulates a delayed PI 3-kinase-dependent phosphorylation of Akt in epithelial cells

(A) Immunoblot analysis of Akt phosphorylation in response to chlamydia. Serum-starved HEp2 cells were stimulated with 100 ng/ml EGF for 2 min or cultured in the presence of *C. pneumoniae* for the times indicated in the figure. The status of AKT phosphorylation was analyzed by probing AKT immunoprecipitates with an anti-phospho Akt ^{Ser473} antibody. (upper panel). Blots were stripped and re-probed with an anti-total Akt antibody (lower panel). (0), uninfected, unstimulated control cells. Positions of molecular mass standards are indicated in kilodaltons. **(B)** PI 3-kinase inhibitors abrogate Chlamydia-induced Akt phosphorylation. HEp2 cells were preincubated with the PI3 kinase inhibitors, LY294002 or wortmannin diluted in culture medium for 30 minutes then stimulated with EGF (100 ng/ml) for 2 minutes, or infected with *C. pneumoniae* for 40 minutes. Control cells were preincubated with 0.1% DMSO in place of specific inhibitors. Cells were lysed and immunoblotted for phosphorylated Akt^{Ser473} (upper panel). Membranes were then stripped and re-probed for total Akt protein (lower panel). Concentrations of LY294002 (LY) are in µM and wortmannin (WM) concentrations are in nM. (–), untreated, uninfected HEp2 cells. Similar results were obtained in two other experiments.

Figure 2.5 A



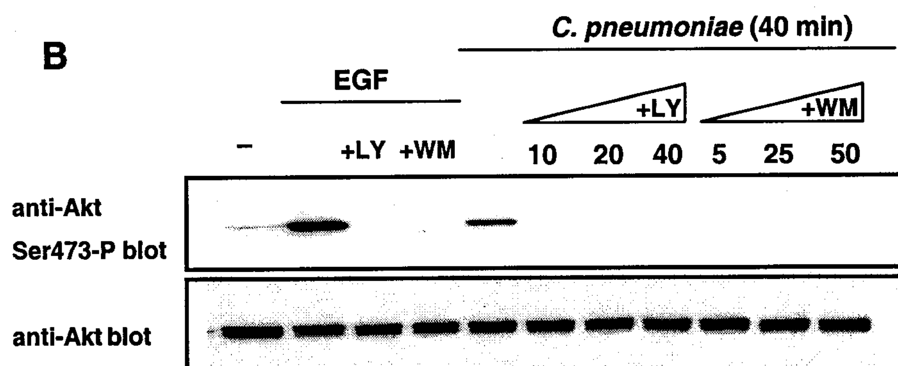
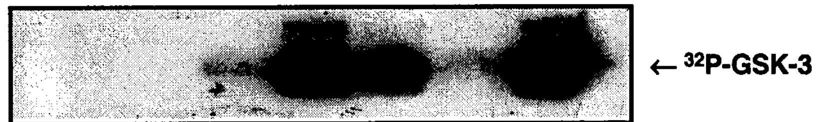


Figure 2.6 - Increased Akt activity is reflective of the pattern of Akt phosphorylation following infection

(A) Akt kinase activity was measured by incubating immunoprecipitated Akt with [γ^{32}P]ATP and a GSK-3 fusion protein containing phosphate acceptor sites as a specific substrate of Akt. Bands indicate radiolabeled GSK-3 from a representative autoradiogram developed from nitrocellulose filters containing separated kinase reaction products. The GSK-3 fusion protein migrates at ~30,000 molecular weight. **(B)** Radioactivity incorporated into GSK-3 from each reaction was analyzed by liquid scintillation counting and normalized to total protein content. Data presented are counts per minute (CPM) of radioactivity incorporated into GSK-3.

Figure 2.6

A



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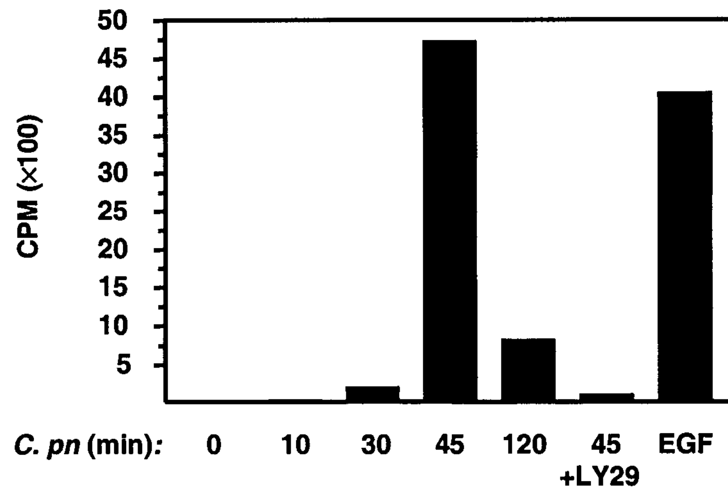
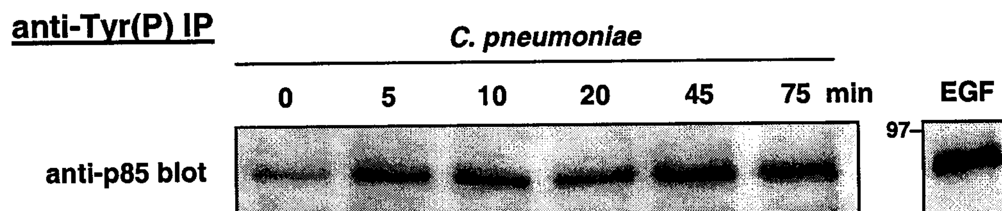


Figure 2.7 - *C. pneumoniae* induces interaction between p85 and FAK and other tyrosine-phosphorylated proteins

The association of p85 with tyrosine-phosphorylated proteins was assessed by immunoblotting anti-Tyr(P) immunoprecipitates with an anti-p85 antibody (*A*) and also by immunoblotting anti-p85 immunoprecipitates with anti-Tyr(P) antibodies (*B*, top panel). **(A)** HEp2 cells were incubated with *C. pneumoniae* for 5 to 75 min followed by solubilization and immunoprecipitation with an anti-Tyr(P) antibody. The presence of p85 in Tyr(P) immunoprecipitates was analyzed by immunoblotting with an anti-p85 antibody. The *top* panel and *right* panel in *A* are from the same SDS-polyacrylamide gel. **(B)** HEp2 cells were incubated with *C. pneumoniae* for 5 to 75 min followed by solubilization and immunoprecipitation of p85. Anti-p85 immunoprecipitates were probed with an anti-Tyr(P) antibody (top panel). The arrowhead in *A* indicates the ~125 kDa tyrosine-phosphorylated protein that co-immunoprecipitated with p85 and is believed to be FAK. The identity of the ~160-170 phosphoprotein in *B, (top panel)* that is phosphorylated above basal levels in response to Chlamydia and EGF-stimulation is unknown. The appearance of a phosphoprotein at ~85 kDa was not obvious in anti-p85 immunoprecipitates in this and repeats of this experiment, suggesting that p85 itself is not detectably phosphorylated under these conditions. In the middle panel in *B*, the anti-Tyr(P) blot was stripped and immunoblotted for FAK. In the bottom panel in *B,* the membrane was stripped a second time and probed with an anti-p85 antibody to verify equivalent amounts of p85 were present in the immunoprecipitates. Positions of molecular weight standards are indicated in kilodaltons. These results are representative of three experiments.

Figure 2.7

A



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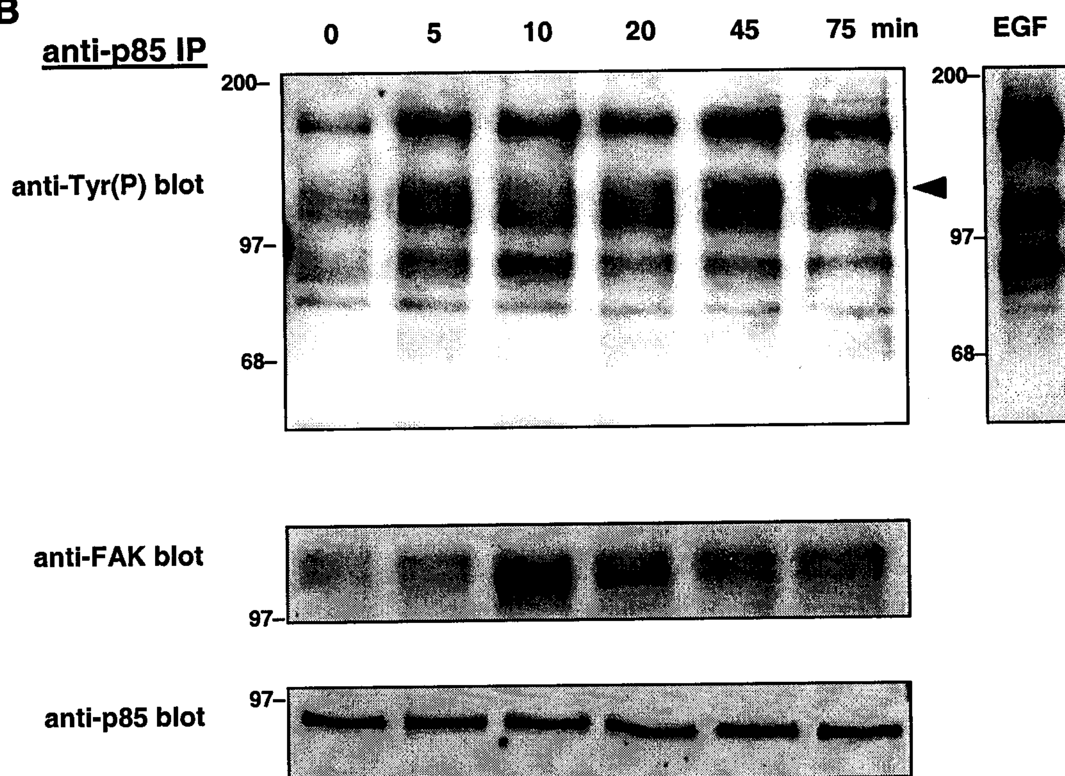


Figure 2.8 - Chlamydia infection results in tyrosine-phosphorylation of FAK and association with the p85 subunit of PI 3-kinase

HEp2 cells were left uninfected ('0' lane), or infected with *C. pneumoniae* for 5 to 75 min. Solubilized lysates were immunoprecipitated with an anti-FAK antibody and probed with an anti-Tyr(P) antibody (top panel). Membranes were stripped and re-probed with an anti-p85 antibody (middle panel), followed by one more round of membrane stripping and a final immunoblot with anti-FAK antibody to ensure equivalent amounts of FAK in the immunoprecipitates (lower panel). Locations of molecular mass standards are given on the left in kilodaltons.

Figure 2.8

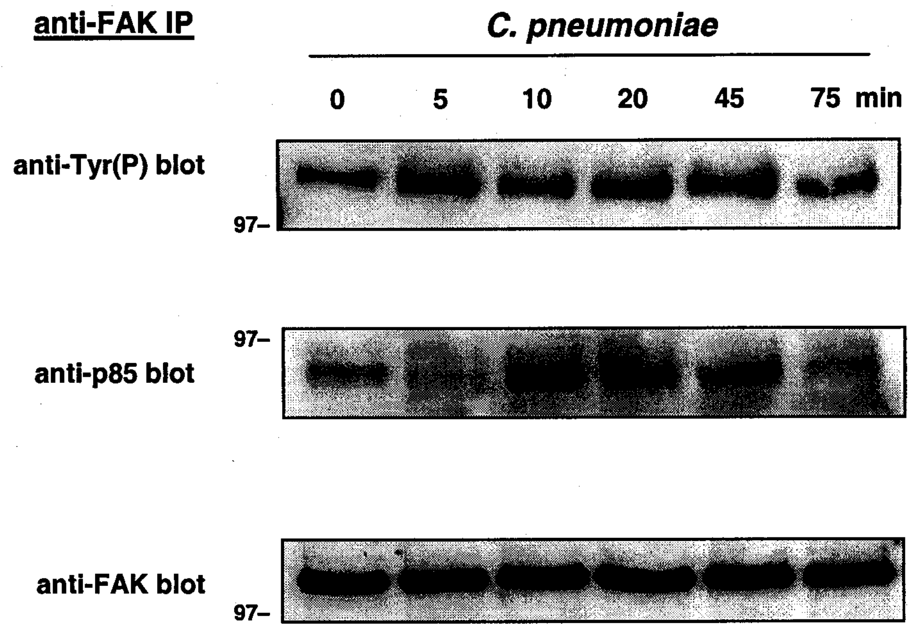
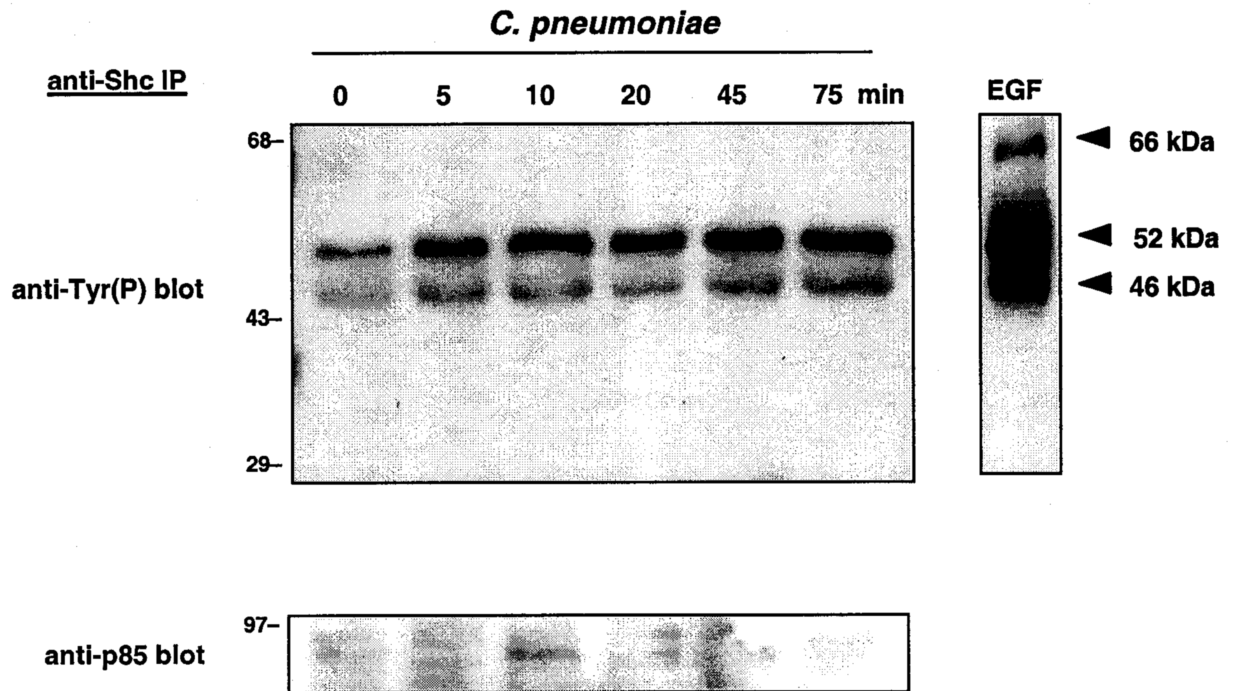


Figure 2.9 - Isoform-specific phosphorylation of Shc in response to chlamydia invasion

HEp2 cells were left uninfected (0 min), or exposed to chlamydia for 5 to 75 min then solubilized and subjected to immunoprecipitation. Tyrosine-phosphorylation of Shc protein was analyzed by immunoblotting Shc immunoprecipitates with an anti-Tyr(P) antibody (top panel). As a positive control, cells were treated with 100 ng/ml EGF for 2 min, then subjected to the same solubilization, immunoprecipitation and immunoblotting procedure as for Chlamydia-treated cells (right panel). Membranes were stripped and re-probed with an anti-p85 antibody (bottom panel). Positions of molecular weight standards are indicated on the left and arrowheads indicate the ~46, 52 and 66 kDa isoforms of Shc. Membranes were stripped a second time and probed with anti-Shc antibodies, but these blots generally gave low signals after two rounds of membrane stripping. Control experiments in which anti-Shc immunoprecipitates were directly probed with anti-Shc antibodies verified that equivalent amounts of Shc were immunoprecipitated from each condition. The top panel and right panel are from the same SDS-polyacrylamide gel. These results are representative of 2 independent experiments.

Figure 2.9



CHAPTER THREE

AUTHOR'S PREFACE TO CHAPTER 3

In Chapter 3, I describe an investigation into the molecular host cell response to *C. pneumoniae* at the level of gene expression. Using molecular techniques to measure transcriptional activation of specific host cell genes in response to infection, we identified a host transcriptional program including genes for cytokines, chemokines, growth factors and surface receptor molecules. Approximately 85% of the induced genes identified in our array experiments represent novel contributions, while the remaining observations corroborated previously published data.

The material presented in Chapter 3 has been published in the peer-reviewed journal *Infection and Immunity.* The experiments conducted for this work and the final version of the published manuscript are my original contributions. Dr. James Mahony provided comments on the manuscript and minor changes were incorporated into the text prior to publication in the Journal. The manuscript is presented in its published form but the references have been reformatted to Harvard style for consistency throughout this thesis and have been incorporated into the thesis Reference list to avoid redundancy with other chapters.

The full citation is:

Brian K. Coombes, and James B. Mahony. (2001) cDNA array analysis of altered gene expression in human endothelial cells in response to *Chlamydia pneumoniae* infection. Infect Immun. 69(3): 1420-1427.

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Part of the work described in chapter 3 were presented at the IV European Chlamydia Congress annual meeting in Helsinki, Finland, August 20-23, 2000 and have been published as a contribution to a book entitled Proceedings of the European Society for Chlamydia Research, p169, 2000. A Lois Bond Travel Award was awarded to B.K.C. to support traveling to this meeting.

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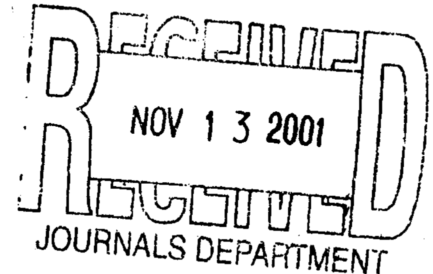

November 2, 2001

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> **Coombes, B.K.**, and J.B. Mahony. 2001. cDNA array analysis of altered gene expression in human endothelial cells in response to *Chlamydia pneumoniae* infection. Infect Immun. 2001 Mar;69(3):1420-1427.

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cDNA ARRAY ANALYSIS OF ALTERED GENE EXPRESSION IN HUMAN ENDOTHELIAL CELLS IN RESPONSE TO *CHLAMYDIA PNEUMONIAE* INFECTION

Brian K. Coombes [1] and James B. Mahony [1,2]

Graduate Programme in Medical Sciences, McMaster University, Father Sean O'Sullivan Research Centre, St. Joseph's Hospital [1], and the Department of Pathology and Molecular Medicine [2], McMaster University, Hamilton, CANADA. L8N 4A6.

Running title: Endothelial cell mRNA responses to *C. pneumoniae* infection

ABSTRACT

Strong epidemiological and pathological evidence supports a role for *Chlamydia pneumoniae* infection in atherosclerosis and human coronary heart disease. Animal models have shown that *C. pneumoniae* disseminates hematogenously in infected monocytes and macrophages while *in vitro* data suggests that infected macrophages can transmit *C. pneumoniae* infection directly to endothelial cells. Endothelial cells may be key *in vivo* targets for *C. pneumoniae* infection and that these cells are important in regulating the dynamics of the vessel wall, we used cDNA microarrays to study the transcriptional response of endothelial cells to infection with *C. pneumoniae*. cDNA arrays were used to characterize the mRNA expression profile for 268 human genes following infection with *C. pneumoniae* and were compared to mRNA profiles of uninfected cells. Selected genes of interest were further investigated by reverse transcription polymerase chain reaction throughout a 24 h period of infection. *C. pneumoniae* infection upregulated mRNA expression for approximately 20 genes (8%) of the genes studied. Genes coding for cytokines (interleukin-1), chemokines (monocyte chemotactic protein-1, interleukin-8), and cellular growth factors (heparin-binding epidermal-like growth factor, basic fibroblast growth factor, platelet-derived growth factor B-chain) were the most prominently upregulated. In addition to these families of genes, increases in mRNA levels for intracellular kinases and cell surface receptors with signal transduction activities were also observed. Time-course experiments showed that mRNA levels were upregulated within 2 h following infection. These results expand our knowledge of the response of endothelial cells to *C. pneumoniae* by further defining the repertoire of *C. pneumoniae*-inducible genes, and provide new insight into potential mechanisms of atherogenesis. In addition, the use of cDNA microarrays may prove useful for the

study of host cell responses to *C. pneumoniae* infection during latent and replicative stages of infection and related pathology.

INTRODUCTION

C. pneumoniae is an obligate intracellular pathogen of humans and causes acute respiratory illnesses such as pneumonia, sinusitis, bronchitis and pharyngitis (Grayston et al., 1989). An association of this organism with chronic diseases such as atherosclerosis and coronary heart disease has been established based on several seroepidemiological and pathological studies. Pathological studies have identified the organism in diseased atherosclerotic tissue by a variety of techniques including polymerase chain reaction, immunocytochemistry, electron microscopy and culture (recently reviewed by Kuo et al., 1993a; Grayston, 2000). Chronic infection of cells with *C. pneumoniae* may be facilitated by the ability of this organism to persist within host cells in an aberrant, non-dividing morphological form (Beatty et al., 1994). Furthermore, infected cells shedding chlamydial envelope antigens have been shown to promote a sustained inflammatory response *in vitro* (Wyrick et al., 1999). Given that atherosclerosis is a chronic inflammatory response at the vessel wall (Ross, 1993, 1999) interaction of *C. pneumoniae* with host cells and the subsequent host cell response to infection may be important in the pathogenesis of atherosclerosis (Gibbs et al., 1998; Gupta, 1999).

Studies attempting to identify mechanisms by which *C. pneumoniae* may alter the hemodynamic properties of the vessel wall are ongoing. Data emerging from these *in vitro* experiments focus on the host cell response to infection and have identified several important pathways that are activated in atherogenesis. For example *in vitro, C. pneumoniae* lipopolysaccharide (LPS) has been shown to enhance foam cell formation in macrophages exposed to oxidized low density lipoprotein (oxLDL) (Kalayoglu and Byrne, 1998). Another *C. pneumoniae* component, heat shock protein-60 has been shown to promote the oxidation of LDL

to its proatherogenic form (Kalayoglu et al., 1999) and to stimulate the synthesis of matrix metalloproteinases (MMP) in macrophages (Kol et al., 1998).

One of the hallmark features of atherosclerosis is the migration and proliferation of medial smooth muscle cells into the arterial intima (Ross and Glomset, 1973; Ross, 1993; Navab et al., 1995). Studies from our laboratory have shown that infection of human umbilical vein endothelial cells (HUVEC) resulted in the production of endothelial cell-derived soluble factor(s) that stimulated DNA synthesis in SMC and increased SMC proliferation (Coombes and Mahony, 1999). Cellular proliferation and induction of various genes are tightly controlled by intercellular cytokine, chemokine and growth factor networks, which may be affected by *C. pneumoniae* infection. Evidence for this is suggested by the *in vitro* finding that *C. pneumoniae* activates several host cell signaling pathways whose downstream effector proteins are transcription factors capable of transactivation of several genes with important immunological and regulatory functions. A recent report shows that signal transduction cascades involving several host cell protein tyrosine kinases are induced within 5 minutes of *C. pneumoniae* binding to host endothelial cells (Krüll et al., 1999) and activation of the transcription factor NFκB has been shown to translocate to the nucleus of *C. pneumoniae*-infected endothelial cells within 15 minutes following infection (Krüll et al., 1999), potentially affecting the transcriptional regulation of various host cell genes.

Transcriptional activity of endothelial cells following infection with *C. pneumoniae* has been reported. Induction of various molecules with immunological and procoagulant activity has been observed, including monocyte chemotactic protein-1 (MCP-1) and interleukin 8 (IL-8) (Kaukoranta-Tolvanen et al., 1996; Molestina et al., 2000). These findings are consistent with a role of *C. pneumoniae* in the pathogenesis of atherosclerosis. These reports however, focus only

on a small number of genes encoding for immunoregulatory proteins and may represent a small subset of inducible genes that are activated in endothelial cells following infection with *C. pneumoniae.*

Microarray technology is now readily available and allows characterization of the mRNA levels for a large number of genes simultaneously, thus providing a useful tool to identify broad-spectrum changes in gene expression in cells in response to a given stimulus (Der et al., 1998; Duggan et al., 1999; van Hal et al., 2000). cDNA arrays have been used to analyze transcription in host cells in response to several intracellular pathogens including *Salmonella* (Eckmann et al., 2000) and *Staphlococus aureus* (Wang et al., 2000), yet this approach has not been applied to chlamydia-infected cells. In an effort to expand the repertoire of human host cell genes that are upregulated by *C. pneumoniae,* we have used cDNA microarrays to analyze mRNA expression for a large number of genes in human microvascular endothelial cells following infection with *C. pneumoniae.*

MATERIALS AND METHODS

Cell Culture. HEp2 cells (ATCC CCL-23) were grown in 75-cm2 culture flasks with minimal essential medium (MEM; Gibco BRL, Gaithersburg, MD) containing Earle's salts and supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL) and 2 mM L-glutamine. HEp2 cells were subcultured into 25 cm^2 flasks or shell vials containing glass coverslips prior to infection with *C. pneumoniae.* Human microvascular endothelial cells (HMEC-1) were obtained from E. Ades (Centres for Disease Control and Prevention, Atlanta, GA). HMEC-1 were cultured in MCDB-131 medium (Gibco BRL) supplemented with 10% heat-inactivated FBS, 10 ng/ml epidermal growth factor (Sigma, St. Louis, MO) and 1 μg/ml hydrocortisone (Sigma) at 37°C and 5% CO_2. Prior to infection, cells were seeded into 25 cm^2 flasks (for cDNA array experiments) or into 6-well plates (for RT-PCR experiments) at a density of 1.7×10^5 cells/cm^2 without supplements and allowed to adhere for 24 h. Human umbilical vein endothelial cells (HUVEC; ATCC 1730-CRL) were maintained in Ham's F12K medium (Gibco BRL) supplemented with 10% FBS, 30 μg of endothelial cell growth supplements (Sigma) per ml and 10 units of heparin (Sigma) per ml. Cells were maintained at 37°C and 5% CO_2 in gelatin-coated culture flasks. Prior to infection, cells were seeded into gelatin-coated 6-well plates and allowed to adhere for 24 h in the absence of supplements.

***Chlamydia pneumoniae* propagation**. *C. pneumoniae* VR-1310 (ATCC 1310-VR) was propagated in HEp2 cells as described by Roblin et al. (Roblin et al., 1992) with slight modifications. *C. pneumoniae* was inoculated onto confluent monolayers of HEp2 cells,

centrifuged at 1,000 × *g* for 60 min at 25°C, then incubated at 37°C for 1 h. The inoculum was removed and replaced with growth medium consisting of MEM containing 1 μg/ml cycloheximide and incubated 72 h at 37°C and 5% CO_2. *C. pneumoniae* was harvested by disruption of HEp2 cells with glass beads followed by sonication and centrifugation at 500 × *g* to remove cellular debris. Supernatants containing *C. pneumoniae* were centrifuged at 30,000 × *g* for 30 min at 4°C to pellet *C. pneumoniae* elementary bodies (EB). EB pellets were suspended in sucrose-phosphate-glutamate buffer (SPG), aliquoted and stored at -70°C. *C. pneumoniae* titrations were performed on frozen stocks using immunofluorescent staining with a genus-specific fluorescein isothiocyanate (FITC)-labeled monoclonal antibody (Kallestad, Chaska, Minn). *C. pneumoniae* titres were expressed as inclusion-forming units (IFU) per ml.

HMEC-1 infection protocol. HMEC-1 cells were infected as described above at a multiplicity of infection (MOI) of 1. Following centrifugation at 1000 × *g* and incubation at 37° C for 1h, the inoculum was removed and cells were washed twice with Hanks balanced salt solution and cultured in MCDB-131 medium containing 0.1% FBS but lacking growth supplements and cycloheximide. Host cell RNA was isolated at various times as indicated in the figure legends for individual experiments. Intracellular inclusions could be seen in *C. pneumoniae*-infected cultures under these growth conditions after 48-72 h, but the viability of the bacterial progeny was not examined.

Analysis of mRNA expression using cDNA arrays. Infected and uninfected HMEC-1 cells were trypsinized and collected by centrifugation. Total cellular RNA was isolated by lysis of

cells in 4.0 M guanidinium thiocyantate followed by a series of phenol-chloroform extractions. The final aqueous phase containing total RNA was treated with RNase-free DNase to remove genomic DNA, re-extracted with phenol-chloroform-isoamyl alcohol and RNA was isolated by precipitation with 2.5 volumes of absolute ethanol and 0.1 volume of 2 M sodium acetate (pH 4.5). Total RNA was collected by centrifugation and washed with ice-cold 75% ethanol. The integrity of RNA transcripts was verified by electrophoresis through denaturing agarose-formaldehyde gels followed by ethidium bromide staining according to standard protocols (Sambrook et al., 1989). Subsequently, poly A^+ RNA was purified from total RNA using Oligotex polystyrene-latex resin (Qiagen, Mississauga, ON) according to the manufacturer's instructions. Analysis of mRNA expression was performed by hybridization of radioactively labeled cDNA to membrane-bound cDNAs corresponding to various genes. The array used in this study was the Atlas Cytokine/Receptor cDNA Expression Array from Clontech Laboratories (Palo Alto, CA). Preparation of radiolabeled cDNAs and hybridizations were performed as outlined by the manufacturer. Briefly, 1 μg of poly A^+ RNA was reverse transcribed using Moloney murine leukemia virus (MMLV) reverse transcriptase in the presence of 35 μCi of [α-^{32}P]dATP and 268 gene-specific primers. cDNA was purified by passage through a CHROMA SPIN-200 column (Clontech) and column fractions were analyzed by scintillation counting for incorporation of radioactive label. Each cDNA probe pool was adjusted to 10^6 cpm/ml and hybridized to separate nylon Atlas arrays at 68°C overnight in ExpressHyb™ hybridization solution (Clontech). Membranes were washed three times in 2× SSC, 1% SDS for 30 min at 68° and twice in 0.1× SSC, 0.5% SDS for 30 min at 68°C. Membranes were exposed to x-ray film with intensifying screens at -70°C for 1-3 days and mRNA expression levels were analyzed by

scanning densitometry of autoradiographs using an Image Master VDS v. 2.0 (Amersham Pharmacia Biotech). Analysis of differential patterns of gene expression was assessed by preparing cDNA probe pools from both uninfected HMEC-1 controls and *C. pneumoniae*-infected HMEC-1 and hybridizing these cDNAs in parallel to pairs of identical cDNA arrays. Array data are expressed as relative changes in mRNA expression following normalization of gene signals to levels of β-actin mRNA to ensure analysis of equivalent amounts of RNA.

Analysis of mRNA expression using RT-PCR. Total RNA was isolated from uninfected and *C. pneumoniae*-infected HMEC-1 cells at various times after infection using RNeasy columns (Qiagen) according to the manufacturer's instructions. Total RNA was treated with RNase-free DNase and further purified by salted alcohol precipitation as described above. For cDNA preparation, 1.5 μg total RNA was reverse transcribed with MMLV-reverse transcriptase in the presence of 0.5 μg oligo $(dT)_{12\text{-}18}$. Primer sequences for RT-PCR were as follows; MCP-1 (forward) 5'-CAAACTGAAGCTCGCACTCTCGCC-3', MCP-1 (reverse) 5'-ATTCTTGGGTTGTGGAGTGAGTGTTCA-3' (Lin et al., 1998). IL-8 (forward) 5'-ATGACTTCCAAGCTGGCCGTCGCT-3', IL-8 (reverse) 5'-TCTCAGCCCTCTTCAAAAACTTCTC-3' (Ehrlich et al., 1998), and β-actin (forward) 5'-CCAACCGCGAGAAGATGACC-3', β-actin (reverse) 5'-GATCTTCATGAGGTAGTCAGT-3' (Jobin et al., 1997). Sequences for PCR primers specific for other individual genes of interest were obtained from CLONTECH and primers were synthesized by the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University, Hamilton, ON. 2 μl of cDNA was used as template for individual polymerase chain reactions with pairs of gene-specific

primers. Each PCR reaction contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.5 µM each primer, and 1.5 units of AmpliTaq Gold DNA polymerase (Perkin Elmer). Thermal cycling programs consisted of 10 min denaturation at 95°C followed by 1 min at 94°C, 1 min at 56°C, and 2 min at 72°C for 23 cycles and a final extension of 5 min at 72°C. PCR products were analyzed by electrophoresis through 2% agarose gels and visualized by ethidium bromide staining. RT-PCR data was analyzed by scanning densitometry of gel bands and normalized to β-actin signals obtained from the same time point. The normalized data was expressed as relative changes in mRNA levels between *C. pneumoniae*-infected HMEC-1 cells and uninfected controls. The numerical data was analyzed using a two-tailed Student's t-test. A P value of <0.05 was considered significant.

RESULTS

Analysis of endothelial cell mRNA expression by cDNA microarrays in response to *C. pneumoniae* infection. To study changes in mRNA expression in endothelial cells in response to infection with *C. pneumoniae*, we employed a cDNA microarray approach using a commercially available membrane-based array (Atlas Human Cytokine/Receptor cDNA Microarray, CLONTECH). This array represents 268 different human genes including cytokines and other immunological regulatory genes such as chemokines, growth factors and cellular receptors. Each gene is represented on the array as duplicate spots containing immobilized cDNA fragments, to which experimentally prepared cDNAs are hybridized. mRNA was isolated from uninfected control HMEC-1 cells and *C. pneumoniae*-infected cells at 18 h post infection and converted to radioactively labeled cDNAs by reverse transcription using a single gene-specific primer for each gene represented on the array. cDNA pools from uninfected cells and *C. pneumoniae*-infected cells were hybridized in parallel to identical pairs of cDNA array membranes under identical hybridization conditions. Subsequent wash steps, generation of autoradiographs and densitometric analysis of data was performed in parallel. This approach facilitates the direct comparison of mRNA levels between infected and uninfected cells. mRNA for approximately 84 out of 268 arrayed genes (31%) was expressed in uninfected HMEC-1 cells. In *C. pneumoniae*-infected cells, 99 out of 268 genes (37%) were expressed (Figure 1). Upregulation of 20 genes (7.5%) of the total arrayed genes was identified using the cDNA microarrays, where 15 genes were induced from undetectable levels and 5 genes were upregulated. Therefore, the majority of genes expressed by HMEC-1 under these experimental conditions were not affected by *C. pneumoniae* infection indicating that the endothelial cell

mRNA responses to *C. pneumoniae* infection was relatively narrow and specific. Table 1 identifies the *C. pneumoniae*-induced genes in HMEC-1 cells at 18 h after infection.

Analysis of mRNA expression using RT-PCR. In order to confirm the data obtained using the cDNA arrays and to further characterize the mRNA expression profile for selected genes of interest, we chose a panel of genes representing various chemokines and cellular growth factors whose expression levels were increased in infected HMEC-1 cells. We also included one gene (CD40) that was not expressed by control HMEC-1 cells or cells infected with *C. pneumoniae* to verify the specificity of the cDNA array for unexpressed transcripts. The mRNA expression levels of these selected genes were then analyzed by RT-PCR at various time points after *C. pneumoniae* infection ranging from 0 to 24 h. RNA from infected and uninfected HMEC-1 cells was harvested and processed in parallel under identical conditions for each time point. In some cases, primer sequences for selected genes of interest were obtained from CLONTECH. RT-PCR was performed for a minimum number of cycles (18-23 cycles) previously determined to be within the linear range of amplification (data not shown). As shown in Figure 2 for the genes chosen for further analysis, RT-PCR confirmed most of the data obtained using the cDNA arrays. As summarized in Table 2, for seven genes chosen for further analysis whose mRNA levels were increased in the cDNA array, RT-PCR confirmed upregulation for five of these genes. IL-8, MCP-1, heparin-binding epidermal-like growth factor (HB-EGF), basic fibroblast growth factor (bFGF) and platelet-derived growth factor-B chain were all upregulated at least 2-fold. Table 2 lists the maximum fold-induction of these genes in *C. pneumoniae*-infected HMEC-1 cells compared to uninfected control cells, which in most cases occurred between 2 to 4 h post-infection. Also listed in Table 2 are the expression levels of these genes at 12 h post infection,

demonstrating that expression levels were lower as the time of infection increased. Insulin-like growth factor binding protein-4 (IGFBP4) and thrombin receptor however, showed no differences in the mRNA expression levels using RT-PCR. Figure 3 shows the level of mRNA for the seven genes selected for further study at various times after infection. Five genes (IL-8, MCP-1, PDGF B-chain, bFGF and HBEGF) were upregulated as early as 2 h post infection. Levels of mRNA for these genes declined from 2 to 4 h, reaching basal levels by 12 to 24 h in most cases. Using the more sensitive technique of RT-PCR, the finding that CD40 was not expressed by HMEC-1 under the conditions used in this study confirmed the cDNA array result and verified the ability of the cDNA array to provide a true negative result for a non-expressed gene.

Confirmation of mRNA responses in another endothelial cell culture model. In order to extend the results obtained using HMEC-1 cells, we used the well-characterized human umbilical vein endothelial cells (HUVEC) as a secondary cell culture model system to study the mRNA responses for the two genes with the highest increases in mRNA expression as measured by RT-PCR. HUVEC were infected with *C. pneumoniae* as outlined for HMEC-1 cells and mRNA for IL-8 and MCP-1 was assessed by RT-PCR at 2 h after infection. As shown in Figure 4, MCP-1 and IL-8 mRNA was upregulated in HUVEC 11-fold and 21-fold respectively. This was similar to the 8.3- and 17.4-fold induction levels seen in HMEC-1 cells (Table 2).

DISCUSSION

C. pneumoniae infection has been associated with atherosclerosis in many seroepidemiological studies and has been demonstrated in coronary, carotid or popliteal arteries in over 40 studies using a variety of techniques including PCR, immunohistochemistry and culture (recently reviewed by Grayston, 2000). *C. pneumoniae* may enter pre-formed or forming atheromas from infected peripheral blood mononuclear cells or via endothelial cells that become infected indirectly from infected mononuclear cells. Studies aimed at characterizing the host response to *C. pneumoniae* infection are necessary in order to discern how infection with this organism contributes to the pathophysiology of atherosclerosis. In the present study, we have shown that approximately 20 genes are upregulated in human vascular endothelial cells in response to *C. pneumoniae* infection. These genes include cytokines, chemokines, growth factors and cellular receptors, all of which are involved in inflammation and may include a pathophysiological role for *C. pneumoniae* in atherogenesis.

In this study, we used cDNA microarray technology to characterize gene expression in human endothelial cells infected with *C. pneumoniae*. Since atherosclerosis is a chronic inflammatory process involving several cell types within the vessel wall (*viz.* endothelial cells, SMC and macrophages), we chose an array containing 268 cDNA probes representing known human genes whose functions included cytokines, cellular receptors and other secreted growth-regulatory molecules (CLONTECH, Atlas Cytokine/Receptor cDNA Microarray). These arrays are composed of gene-specific cDNA probes immobilized on a solid phase nylon membrane. mRNA pools from cultured cells are detected by their ability to hybridize to a given cDNA probe on the array. Upregulation of specific genes with cDNA microarrays was confirmed using a

semi-quantitative RT-PCR to measure fold increases in mRNA between *C. pneumoniae*-infected HMEC-1 cells and uninfected control cells.

Previous studies from our laboratory have shown that infection of human endothelial cells with *C. pneumoniae* leads to the production of soluble factors with mitogenic and proliferative activity towards SMC (Coombes and Mahony, 1999). SMC proliferation in the neointima is a hallmark feature of atherosclerosis and is controlled, in large part, by paracrine growth factors secreted by neighbouring cells (Campbell et al., 1989; Ross, 1993). Intercellular communication between arterial cells mediated by soluble molecules and cellular receptors likely plays an important role in the progression of the chronic inflammatory atherosclerotic lesion since these molecules control tightly regulated cellular and molecular events. It has been suggested that perturbation of these networks due to intracellular infection with *C. pneumoniae* may contribute to the cellular dysfunction associated with atherogenesis (Mahony and Coombes, submitted for publication).

Our approach using a cDNA microarray demonstrated that infection of HMEC-1 cells with *C. pneumoniae* induced expression of a relatively small number of genes (20 out of 268), and suggested that the endothelial mRNA response to infection is not a generalized response. Of these responses, some of the findings confirmed previous studies, while others represent novel findings. MCP-1 and IL-8 proteins were detected in the supernatants of *C. pneumoniae*-infected endothelial cell cultures by Molestina et al., suggesting transcriptional induction of mRNA for these proteins (Molestina et al., 1998). Of interest in this study was the novel finding that mRNA coding for several growth factors including basic fibroblast growth factor (bFGF), platelet-derived growth factor-B chain (PDGF-B) and heparin-binding epidermal-like growth factor (HBEGF) were induced by *C. pneumoniae* infection of endothelial cells. Recently, Rödel et al.

(Rödel et al., 2000) reported the accumulation of bFGF mRNA in SMC infected with *C. pneumoniae*, indicating a possible common induction cascade in endothelial cells and SMC.

The production of growth factors by endothelial cells that may induce proliferation of SMC supports our previous finding showing SMC proliferation in response to culture supernatants from *C. pneumoniae*-infected endothelial cells (Coombes and Mahony, 1999) and could represent a significant new mechanism of *C. pneumoniae* involvement in atherogenesis. For example, HBEGF has been shown to be a potent mitogen with apparent specificity for SMC (Higashiyama et al., 1991) and has been implicated in a variety of pathological processes including SMC hyperplasia and atherosclerosis (Raab and Klagstrum, 1997). Similarly, homodimers of PDGF-B with growth promoting activity for SMC have also been associated with neointimal proliferation of SMC (Ross, 1993). PDGF-associated protein, a growth factor accessory molecule that modulates the activity of specific growth factors was also upregulated by *C. pneumoniae*. Together HBEGF and PDGF-BB could initiate or regulate the migration and proliferation of medial-derived SMC in the intima of a progressing atherosclerotic lesion. However, we do not yet know whether one or more of these specific growth factors were responsible for SMC proliferation in our previous study.

The finding of increased mRNA expression for activin A or erythroid differentiation protein was also a novel finding in our study. Activin A is a member of the transforming growth factor β (TGFβ) superfamily and is functionally composed of a homodimer of βA chains of the inhibin/activin group (Yu and Dolter, 1997). This molecule has recently been shown to modulate monocyte/macrophage functions including immunological activation of monocytes (Eramaa et al., 1992) and induction of matrix metalloprotease 2 (MMP-2) (Ogawa et al., 2000). Association of activin A during atherogenesis has also been reported by others. Using cDNA microarrays,

deWaard et al. reported induction of activin A mRNA from human endothelial cells exposed to conditioned medium from monocytes exposed to oxidized LDL (de Waard et al., 1999). Upregulation of activin A may be important since this molecule has been demonstrated in atherosclerotic lesions of both humans (M.A. Engelse, et al.; unpublished data) and in animal models (Inoue et al., 1994). Its role in lesion progression may be to influence phenotypic changes in SMC (Engelse et al., 1999) or act as a paracrine or autocrine mediator of macrophage activation as described above.

Time course analysis of gene expression in *C. pneumoniae*-infected endothelial cells by RT-PCR revealed a tightly controlled temporal regulation of gene induction. For the genes studied in *C. pneumoniae*-infected cells, mRNA was maximal between 2 to 6 h post-infection and declined thereafter, reaching a steady state at 24 h in most cases. This was a consistent finding for all the genes studied. These findings, at least for MCP-1, differ somewhat from those reported by Molestina et al. (2000). Although an early MCP-1 mRNA response was noted following *C. pneumoniae* infection in their study, this response was not maximal until 12 h post infection and remained significantly elevated at 24 h post infection. These differences may be due to cell-specific variations as the endothelial cells used in their study were derived from human umbilical vein, while HMEC-1 cells are derived from the human microvasculature.

However, our data on early activation of mRNA responses are consistent with signal transduction pathways activated early in endothelial cells following chlamydial infection (Fawaz et al., 1997; Krüll et al., 1999). A recent report shows that signal transduction cascades involving several host cell protein tyrosine kinases including p42/p44 mitogen-activated protein kinase (MAPK) are induced within 5 minutes of *C.* pneumoniae binding to host endothelial cells (Krüll et al., 1999). In addition, the ubiquitous transcription factor, NF-κB which controls inducible

transcriptional activation of several immunological genes has been shown by several investigators to be activated and nucleus-associated within 10 to 15 minutes following *C. pneumoniae* interaction with host cells. (Krüll et al., 1999; Dechend et al., 1999; Molestina et al., 2000). This early transcription factor activation is reduced to basal levels by 24 h post infection, indicating that the transcriptional response of cells to *C. pneumoniae* infection is elicited at an early time point after infection. An early transcriptional response in less than 2 h would be consistent with signal transduction events following contact of EBs with specific membrane molecules leading to activation of host cell signaling and nuclear relocation of transcription factors, such as NF-κB. Consistent with this chronology, is the finding that maximal increases of E-selectin, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) mRNA in endothelial cells occurs at 2 h post infection with *C. pneumoniae*, which return to basal levels by 24 h post infection (Krüll et al., 1999). These genes all contain consensus NF-κB binding sequences within their promoter regions and are known to be inducible following activation of NF-κB (May and Ghosh, 1998).

In addition to these signal transduction pathways, our data suggest the activation of other signal transduction cascades in *C. pneumoniae*-infected endothelial cells. The upregulation of mRNA corresponding to TKT tyrosine kinase, a member of a cell adhesion kinase receptor family (Karn et al., 1993) was observed, along with insulin-like growth factor receptor 1, which is a transmembrane tyrosine kinase linked to the Ras-Raf MAPK cascade. Other intracellular gene products for which mRNA was upregulated included the interferon γ-responsive transcription factor, interferon regulatory factor 1 (IRF-1). This transcription factor binds to regulatory DNA binding sequences upstream from IFN-inducible genes and controls their transactivation. This finding further suggests that endothelial cells may play an important role in controlling initial

immunological responses to *C. pneumoniae* infection at the vessel wall and may play an important role in the production of inflammatory mediators in the atherosclerotic plaque.

Despite these early initial responses of endothelial cells to infection with *C. pneumoniae in vitro*, sustained activation of these molecules may occur *in vivo*, during different stages of infection. For example, infection of various cells by *C. pneumoniae* would not be synchronized, so it may be possible that specific gene products accumulate in high levels in tissues as new cells become infected during an ongoing chlamydial infection. Alternatively, the apparent ability of *chlamydiae* to enter into a persistent stage of infection where the organism aborts its normal developmental cycle and appears to reside in viable, non-replicating form may provide a sustained antigenic stimulation of both immune and non-immune cells which contributes to a chronic activated state of cells present in atheromatous lesions. This idea of chronic cell activation is supported by the *in vitro* demonstration of sustained activation of endothelial cells in response to persistent chlamydia envelope antigens following antibiotic treatment of infected cell cultures (Wyrick et al., 1999).

Technical issues relating to the use of cDNA arrays for the study of differential gene expression following a given stimulus were noted in our study. For example, in some cases for genes whose expression levels were found to be upregulated by the cDNA arrays, induction could not be confirmed RT-PCR analysis. This was the case for 2 genes out of 7 chosen for further study, insulin-like growth factor binding protein 4, and thrombin receptor. In these cases, the confirmatory approach revealed mRNA expression for these genes, yet their levels were not significantly upregulated in infected cells compared to the uninfected controls. This issue underscores the importance of confirmatory testing of cDNA microarray results using a second technology. A similar conclusion has been reached in other studies using oligonucleotide arrays

for analysis of differential gene expression in cells (Eckmann et al., 2000). It is likely that the discrepancies noted above relate to the different sensitivities between the cDNA arrays and in this case, RT-PCR. Where RT-PCR can provide exquisitely better sensitivity owing to amplification of starting products, thereby improving the detection of mRNA in low abundance, the cDNA microarray may not reach this level of sensitivity. In this case, small increases in mRNA abundance for a given gene in response to infection may become visible on the array, but the low-level expression from the uninfected samples may be below the detection threshold. During densitometric analysis of these signals, changes may be overestimated, since a weak signal from the infected array is being compared to an absent signal from the uninfected array. In these cases, RT-PCR may be a better indicator of actual differences in the mRNA populations between the two samples.

The use of cDNA microarrays for the study of host-pathogen interactions has proved to be a valuable tool for extending and characterizing the repertoire of host cellular responses to *C. pneumoniae* infection. An understanding of these responses at a molecular level will be necessary to evaluate the biological role infection may play in the development or progression of certain diseases.

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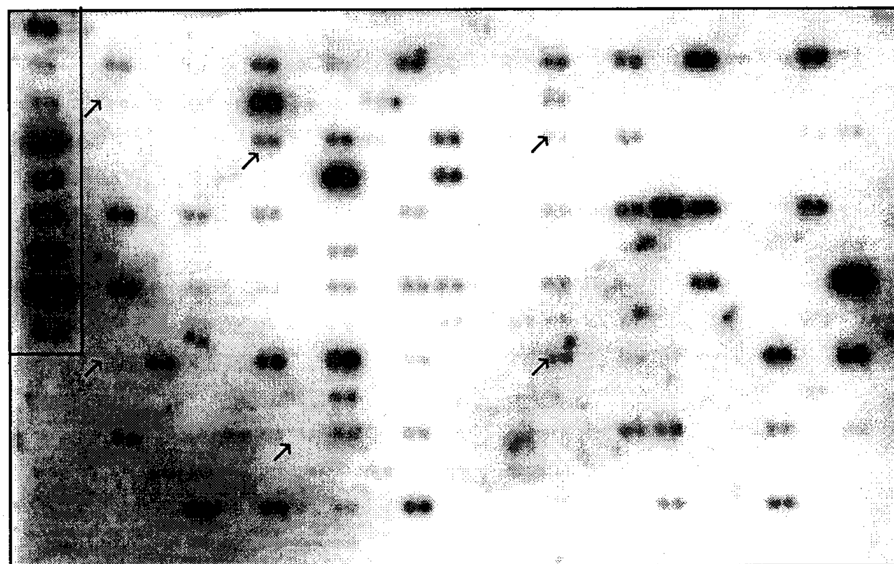
Figure 3.1 - cDNA microarray analysis of gene expression in response to *C. pneumoniae* infection

Radioactively labeled cDNA probes generated from polyA+ mRNA from uninfected HMEC-1 (A) and HMEC-1 infected with *C. pneumoniae* for 18 h (B) were hybridized in parallel to pairs of identical cDNA arrays. Hybridization patterns were assessed by autoradiography for 24 to 72 h and analyzed by scanning densitometry. The relative expression levels for specific genes was normalized using housekeeping gene controls (boxed area). Arrows indicate the location of several representative genes whose expression levels increased in response to *C. pneumoniae* infection.

Figure 3.1

A



B

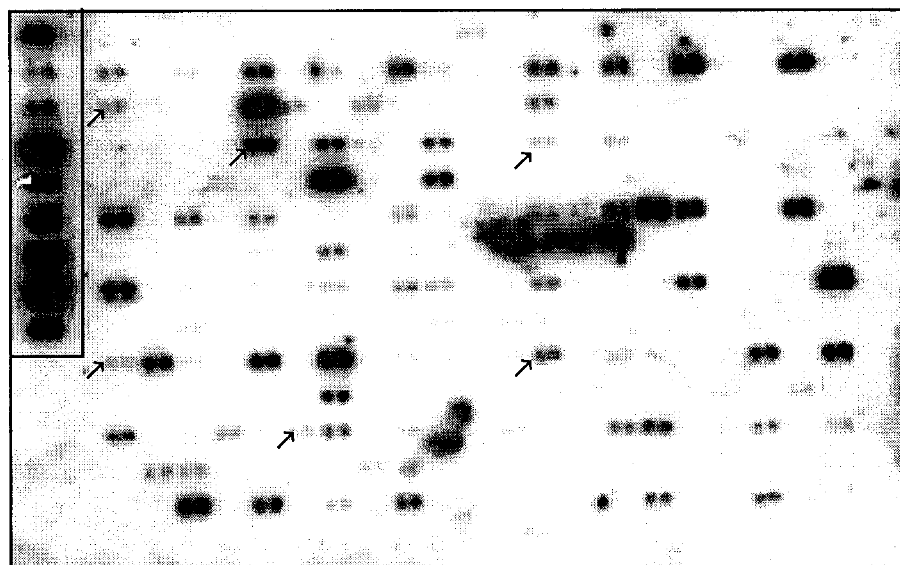


Figure 3.2 - RT-PCR analysis of mRNA expression for various genes in uninfected HMEC-1 cells and HMEC-1 cells infected with *C. pneumoniae*

Total RNA was harvested at 0, 2, 4, 8, 12, and 24 h and levels of specific mRNA were determined by RT-PCR as described in Materials and Methods section. RT-PCR products were analyzed by scanning densitometry for relative changes in mRNA expression. (–) no RNA control; bp, size of amplified cDNA in base pairs.

Figure 3.2

uninfected

C. pneumoniae-infected

– 0 2 4 8 12 24 0 2 4 8 12 24

bp

MCP-1 354

IL-8 289

HB-EGF 241

FGFβ 262

PDGFβ 462

IGFBP4 310

Thrombin R 400

CD40 205

β-actin 236

Figure 3.3 - RT-PCR analysis of mRNA expression in *C. pneumoniae*-infected HMEC-1

Levels of specific mRNA following infection of HMEC-1 with *C. pneumoniae* were assessed by RT-PCR at various time points as described under Materials and Methods. RT-PCR products were first measured by plotting the densitometric [gene]/[β-actin] RT-PCR product ratios for both infected and uninfected HMEC-1. Normalized data for infected cells was then converted to fold-induction by expressing the densitometric data as a ratio of uninfected controls (O.D. ratio $_{\text{infected cells}}$/O.D. ratio $_{\text{uninfected controls}}$). Genes are given in the legends for individual panels. Data points represent the means ± standard errors from two separate experiments. *, $P<0.03$.

Figure 3.3

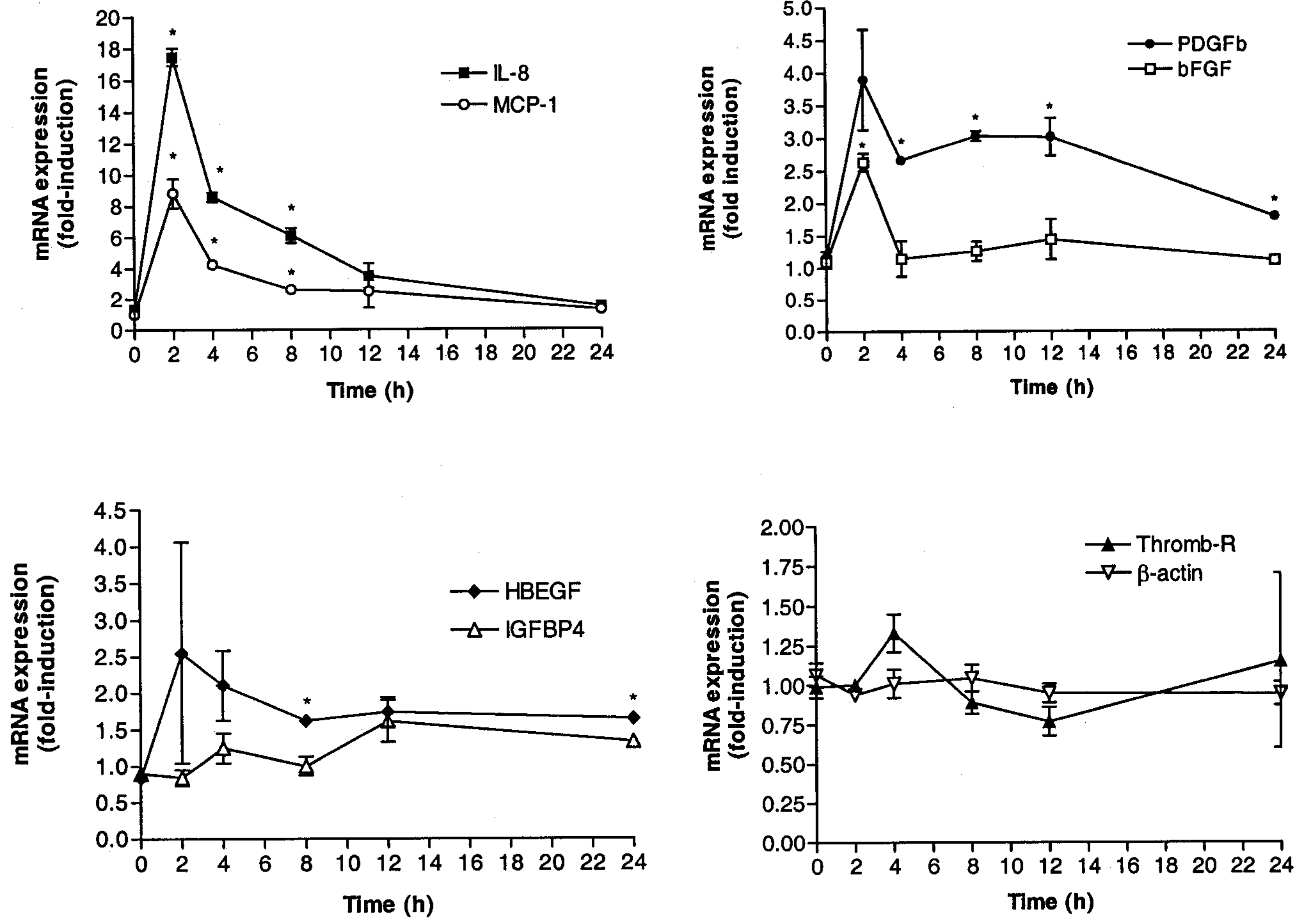


Figure 3.4 - Analysis of gene expression in HUVEC by RT-PCR

HUVEC were infected with *C. pneumoniae* or left uninfected for 2 h, then processed for RT-PCR as described under Materials and Methods for IL-8, MCP-1 and β-actin. Top panels represent RT-PCR products resolved by agarose gel electrophoresis. Bottom figure is a numerical representation of the RT-PCR data as determined by densitometric analysis of gel bands. Fold induction is calculated as the maximum optical density (O.D.) of *C. pneumoniae*-infected samples / O.D. of uninfected control samples, normalized to β-actin. NC, negative control; U, uninfected HMEC-1; I, *C. pneumoniae*-infected HMEC-1.

Figure 3.4

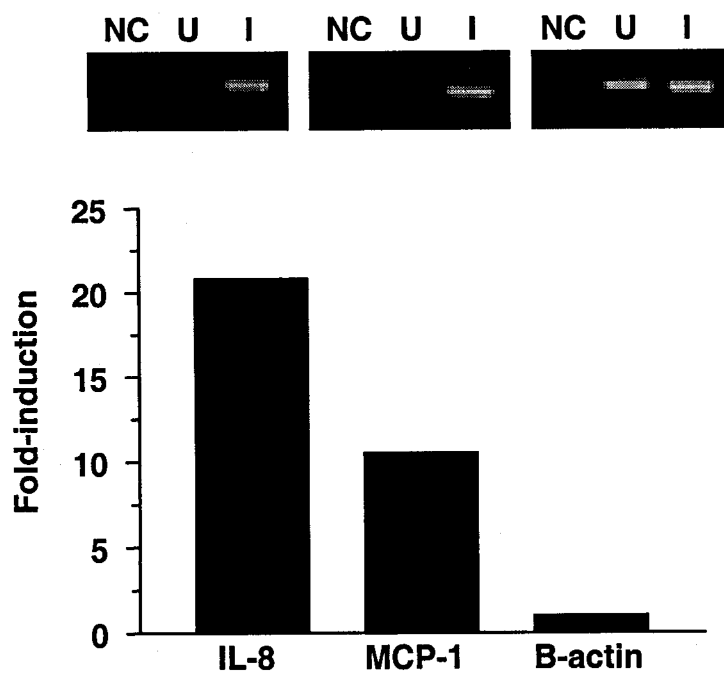


Table 3.1 – Identification of *C. pneumoniae*-induced genes in HMEC-1 cells by cDNA array

Footnote to Table 1:

cDNA microarrays were analyzed by scanning densitometry as described under Materials and Methods and gene signals were normalized to the β-actin housekeeping gene.

[a] cDNA probe accession numbers correspond to gene sequences from GenBank

[b] Relative mRNA expression is a ratio of the normalized optical density gene signal from *C. pneumoniae*-infected cells / normalized optical density gene signal from uninfected control cells. mRNA expression was assessed at 18 h post infection.

TABLE 3.1 Identification of *C. pneumoniae*-induced genes in HMEC-1 by cDNA array

Gene	cDNA probe accession [a]	Major function(s)	Relative mRNA expression ratio [b]
-Heparin-binding EGF-like growth factor	M60278	Blastocyst implantation, would healing, tumor growth, SMC hyperplasia, atherosclerosis, membrane-bound and soluble forms; soluble form is mitogenic and chemotactic for SMC and fibroblasts	17.3
-Insulin-like growth factor protein-4	M62403	Transport of IGFs in circulation; modulation of IGF binding to receptors and growth-promoting actions	15
-Stromal-derived factor 1A	L36034	CXC chemokine; B-lymphophoiesis, cardiogenesis	13
-Growth-assoc. protein-43	M25667	Neuronal growth; intracellular signaling involved in cytoskeletal reorganization	10
-Interferon α receptor	J03171	Jak tyrosine kinase-associated; STAT transcription factor activation	8
-Insulin-like growth factor I receptor	X04434	Signal transduction molecule; transmembrane tyrosine kinase linked to the ras/raf MAPK cascade; antiapoptotic effects in vivo and in vitro	7.5
-Interferon regulatory factor-1	X14454	Interferon-inducible transcription factor, regulates the activation of IFN gene and genes involved in cell cycle progression and apoptosis	5.8
-PDGF-α receptor	M21574	Member of the protein tyrosine kinase family of receptors	4.1
-MCP-1	M24545	CC chemokine; monocyte chemoattractant	3.5
-Brain-derived neurotrophic factor	M61176	Involved in neuron integrity; stimulates synaptic strength and survival of neurons	3.0
-IL-8	Y00787	Chemotactic for neutrophils; neutrophil activation	2.7
-IL-1β	K02770	T-cell activation; macrophage activation	2.5
-Platelet-derived growth factor associated protein	U41745	Binds PDGF with low affinity and enhances the mitogenic effect of PDGF-A, but not PDGF-B	2.5
-PDGF-B chain	X02811	Mitogenic for connective tissue cells, chemokine for smooth muscle cells, fibroblasts; BB homodimers biologically active	2.5
-Erythroid differentiation protein	J03634	Also called Activin A; member of the TGF-β superfamily; acts as a paracrine and autocrine mediator of host defenses	2.4
-FMLP-related receptor	M76673	Seven-transmembrane receptor, linked to G-protein-coupled receptors to activate chemotaxis and exocytosis	2.3
-Basic FGF	M27968	Widespread mitogenic and neurotrophic activity, activates receptor tyrosine kinases	2.3
-TNF-inducible hyaluronate-binding protein (TSG-6)	M31165	Cytokine-inducible secreted glycoprotein; anti-inflammatory activity, plasmin inhibitor, possibly involved in SMC growth	2.1
-Coagulation factor II receptor	M62424	Seven transmembrane, G protein-coupled receptor for thrombin	2.0

Table 3. 2 – RT-PCR analysis of mRNA expression in HMEC-1 cells following *C. pneumoniae* infection

Footnote to Table 2:

[a] cDNA probe accession numbers correspond to gene sequences deposited in GenBank.

[b] Fold-induction was calculated as normalized optical density signals obtained from *C. pneumoniae*-infected cells / normalized optical density signals obtained from uninfected HMEC-1 controls.

[c] Maximum fold-induction is the largest increase in mRNA expression at a single time point between 0-24 h post-infection.

Table 3.2 RT-PCR analysis of mRNA expression in HMEC-1 cells following *C. pneumoniae* infection

Gene	cDNA probe accession number [a]	Fold-induction [b]	
		Maximum fold-induction [c]	*12 h post-infection*
IL-8	Y00787	17.45 ± 0.54	3.52 ± 0.80
MCP-1	M24545	8.33 ± 0.93	2.52 ± 1.08
PDGF-B chain	X02811	3.90 ± 0.76	3.02 ± 0.29
Basic FGF	M27968	2.63 ± 0.13	1.44 ± 0.31
HBEGF	M60278	2.55± 1.51	1.74 ± 0.20
IGFBP4	M62403	1.62 ± 0.29	1.62 ± 0.29
Thrombin Receptor	M62424	1.66 ± 0.05	0.77 ± 0.09

[a] cDNA probe accession numbers correspond to gene sequences deposited in GenBank.

[b] Fold-induction was calculated as normalized optical density signals obtained from *C. pneumoniae*-infected cells / normalized optical density signals obtained from uninfected HMEC-1 controls.

[c] Maximum fold-induction is the largest increase in mRNA expression at a single time point between 0-24 h post-infection

CHAPTER FOUR

AUTHOR'S PREFACE TO CHAPTER 4

In this chapter, I identify a novel mitogenic activity that can be transferred in conditioned medium from *C. pneumoniae*-infected endothelial cells to SMC cultures. Together with the identification of SMC growth factors in the cDNA array experiments, this work suggested that chlamydia-induced growth factors have biological activity towards SMC and that SMC proliferation induced by this bacterium may be relevant to its documented association with atherosclerotic disease.

The material presented in Chapter 4 has been published in the peer-reviewed journal *Infection and Immunity*. The experiments described in this chapter and the final version of the published manuscript are my original contributions. Dr. James Mahony provided comments on the manuscript and his changes were incorporated prior to publication in the Journal. The manuscript is presented in its published format. The references have been converted to Harvard style for consistency throughout this thesis and have been incorporated into the thesis Reference list to avoid redundancy.

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Preliminary data pertaining to this work was presented in part at the American Society for Microbiology 99th General Meeting in Chicago, IL, May 30-Jun 3, 1999. Subsequent data pertaining to this work was presented in part at the Canadian Association for Clinical Microbiology and Infectious Diseases conference in Ottawa, ON, November 5-9, 2000. The latter poster presentation was awarded the Canadian College of Microbiologists Award for outstanding student presentation. A McMaster Health Sciences Centennial Travel Bursary Award was awarded to B.K.C to provide financial assistance while traveling to the latter meeting.

CHLAMYDIA PNEUMONIAE INFECTION OF HUMAN ENDOTHELIAL CELLS INDUCES PROLIFERATION OF SMOOTH MUSCLE CELLS VIA ENDOTHELIAL CELL-DERIVED SOLUBLE FACTOR(S).

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Running title: Chlamydia-induced production of SMC proliferative factors.

ABSTRACT

An association of *Chlamydia pneumoniae* with atherosclerosis and coronary heart disease has been determined epidemiologically and by the detection of *C. pneumoniae* organisms in atherosclerotic lesions in both humans and animal models of atherosclerosis. Previously, it has been shown that *C. pneumoniae* is capable of replicating in cell types found within atheromatous lesions, *viz.* endothelial cells, smooth muscle cells (SMC) and macrophages, yet the role *C. pneumoniae* in the pathogenesis of atherosclerosis has not been determined. Since intimal thickening is a hallmark of atherosclerosis, we investigated whether *C. pneumoniae* infection of human umbilical vein endothelial cells (HUVEC) could induce the expression of soluble factor(s) with mitogenic potential for SMC using [^{3}H]-thymidine incorporation and direct cell counting. Conditioned medium harvested from HUVEC infected with *C. pneumoniae* stimulated SMC replication in a time- and dose-dependent fashion. Infection studies using various multiplicities of infection (MOI) ranging from 0.001 to 1 demonstrated a dose-dependent production of the soluble factor(s). At a MOI of 1, SMC stimulation indices were 8.4 ($P<0.01$) and 12.2 ($P<0.01$) for conditioned media harvested at 24 and 48 hours respectively. To determine whether viable *C. pneumoniae* was required for production of the soluble factor(s), HUVEC were infected with either heat inactivated *C. pneumoniae*, or cells were infected in the presence of chloramphenicol. Both treatments produced stimulation indices similar to those for live *C. pneumoniae* in the absence of chloramphenicol ($P>0.05$), indicating that the factor(s) were produced by HUVEC cells and not by *C. pneumoniae*, and that signal transduction events following chlamydia endocytosis may be important in the production of soluble factor(s). The ability of *C.*

pneumoniae to elicit endothelial cell-derived soluble factor(s) that stimulate SMC proliferation may be important in the pathogenesis of atherosclerosis.

INTRODUCTION

Chlamydia pneumoniae is a common cause of acute respiratory conditions such as pneumonia, sinusitis, bronchitis and pharyngitis (Grayston et al., 1989; Kuo et al., 1995). More recently, *C. pneumoniae* has been implicated as a possible etiologic agent of coronary artery disease and atherosclerosis (Linnanmaki et al., 1993; Melnick et al., 1993; Gibbs et al., 1998). Saikku and colleagues reported the first evidence for such a relationship by demonstrating increasing titres of *C. pneumoniae* antibodies in men with coronary heart disease and acute myocardial infarction (Saikku, 1993). Since then, new evidence has emerged which support a role for *C. pneumoniae* in the pathogenesis of atherosclerosis. *C. pneumoniae* has been detected in atherosclerotic arteries by several techniques (Kuo et al., 1993a; Kuo et al., 1993b; Campbell et al., 1995) and the organism has been isolated from both coronary (Ramirez et al., 1996) and carotid (Jackson et al., 1997) atheromas. Recent animal models have suggested that *C. pneumoniae* is capable of inducing atherosclerosis in both rabbit (Fong et al., 1997; Muhlestein et al., 1997) and mouse (Moazed et al., 1997) models of atherosclerosis. Furthermore, human clinical treatment studies have been initiated which examined the use of antichlamydial macrolide antibiotics in patients with coronary atherosclerosis. In these placebo-controlled studies, a 5-10% reduction in secondary adverse cardiovascular events was observed in patients receiving either azithromycin (Gupta et al, 1997) or roxithromycin (Gurfinkel et al., 1997) compared to age- and sex-matched controls.

Atherosclerosis is thought to occur following an inflammatory response to injury of the vessel wall (Ross, 1993; Berliner et al., 1995; Navab et al., 1995). During this process, peripheral leukocytes such as monocytes and T-lymphocytes bind to upregulated receptors on activated

endothelium via vascular cell adhesion molecule-1 (VCAM-1) and leukocyte function antigen-1 (LFA-1) and transmigrate through the endothelium into the subendothelial space. Here, macrophages accumulate oxidized LDL, forming foam cells and together with media-derived smooth muscle cells (SMC), form the initial fatty streak. Grade 2 and 3 lesions then ensue which contain increased numbers of foam cells surrounding a central core of lipid and cell debris, ultimately forming the end-stage fibrous plaque (Ross, 1993; Navab et al., 1995).

Several groups have demonstrated the ability of *C. pneumoniae* to infect and replicate in cell types found within the atherosclerotic lesion including endothelial cells, SMC and macrophages (Godzik et al., 1995; Gaydos et al., 1996). Infection of these cell types has been shown to result in the production of pro-inflammatory cytokines, which may be involved in atherogenesis. The ability of chlamydiae to persist within host cells and produce antigens in the absence of replication may provide sustained immunogenic stimulation necessary for the development of chronic inflammatory diseases such as atherosclerosis. *C. pneumoniae* infection of endothelial cells has been shown to upregulate the expression of endothelial adhesion molecules (Kaukoranta-Tolvanen et al., 1996) and several inflammatory mediators including MCP-1, IL-8 (Molestina et al., 1998) and IL-1β (Heinemann et al., 1996). During atherosclerosis, these cytokines are also upregulated (Ross and Glomset, 1973) and may potentiate the development of atheromatous lesions. *C. pneumoniae* LPS has also been shown to promote the formation of macrophage foam cells *in vitro* (Kalayoglu and Bryne, 1998).

Central to the pathology of atherosclerosis is the proliferation of SMC in the arterial intima (Stary, 1990; Ishida et al., 1997). Endothelial cells have been shown to secrete several soluble factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1) (Ross, 1993), which are important in SMC migration and

proliferation during atherogenesis. The production of these factors during chlamydial infection however, has not been studied. To better understand the pathophysiology of atherosclerosis and the role of *C. pneumoniae* in atherogenesis, we examined whether *C. pneumoniae* infection of endothelial cells could induce the production of soluble factors that stimulate SMC replication.

MATERIALS AND METHODS

Cell Culture. HEp2 cells (ATCC CCL-23) were maintained in minimal essential medium (MEM) (Gibco BRL, Gaithersburg, MD) containing Earles salts and supplemented with 10% heat inactivated fetal bovine serum (Gibco BRL) and 2 mM L-glutamine (Gibco BRL). Cells were maintained at 37°C and 5% CO_2 in 75 cm^2 culture flasks and subcultured into either 25 cm^2 flasks or shell vials containing glass cover slips prior to infection with *C. pneumoniae.*

Human umbilical vein endothelial cells (HUVEC; ATCC 1730-CRL) were maintained in Ham's F12K medium (Gibco BRL) supplemented with 10% fetal bovine serum, 30 μg/ml endothelial cell growth supplement (Sigma, St. Louis, Mo.) and 10 U/ml heparin (Sigma). Cells were maintained at 37°C and 5% CO_2 in gelatin-coated 75 cm^2 culture flasks. Prior to infection, cells were seeded into gelatin-coated 24-well plates at a density of 2×10^5 cells/well and allowed to adhere for 24 hours in the absence of endothelial cell growth supplement or heparin.

Primary smooth muscle cells were established from bronchial explants by a method described previously (Zhang and Cox, 1996), and were provided at passage two by Dr. G. Cox (Dept. of Medicine, McMaster University). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, BRL) supplemented with 10% fetal bovine serum, penicillin (50 units/ml) and streptomycin (50 μg/ml) (Gibco BRL). Cells were maintained at 37°C and 5% CO_2 in 75 cm^2 culture flasks and subcultured at ~90% confluency. SMC showed typical hill and valley morphology and the expression of α-actin was confirmed by immunofluorescence. Cells were suspended in DMEM at a density of 5×10^4 cells/ml and transferred into 24-well plates (5×10^4 cells/well). SMC were used at the third and fourth passage for all experiments.

***Chlamydia pneumoniae* Propagation.** *C. pneumoniae* VR-1310 (ATCC 1310-VR) was propagated in HEp2 cells as described by Roblin et al. (Roblin et al., 1992) with slight modifications. *C. pneumoniae* was inoculated onto preformed monolayers of HEp2 cells in 25 cm^2 flasks, centrifuged at 1000 × *g*. for 60 minutes at 25° C and incubated at 37°C for 1 hour. The inoculum was removed and replaced with growth medium consisting of minimal essential media containing 1 μg/ml cycloheximide. Infected cultures were incubated for 48 to 72 h at 37°C and 5% CO_2. *C. pneumoniae* were harvested by disrupting HEp2 cells with glass beads followed by sonication and centrifugation at 250 × *g* to remove cellular debris. Supernatants containing *C. pneumoniae* were aliquoted and frozen at -70° C. For some experiments, supernatants containing *C. pneumoniae* were further centrifuged at 20,000 × *g* for 20 minutes to pellet EBs. EB pellets were then suspended in sucrose-phosphate-glutamate buffer (SPG), aliquoted, and stored at -70°C. HEp2 monolayers grown in shell vials were used to monitor the course of infection by immunofluorescent (IF) staining using a genus-specific fluorescein isothiocyanate-labeled monoclonal antibody (Kallestad, Chaska, Minn.). *C. pneumoniae* titrations were performed on frozen stocks and were expressed as inclusion forming units (IFU) per ml.

Preparation of HUVEC culture conditioned medium. HUVEC were infected in 24-well plates as described above at various multiplicities of infection (MOI). Following centrifugation at 1000 × *g* for 60 min and incubation at 37°C for 1 h the inoculum was removed and cells were washed twice with Hanks balanced salt solution and cultured in 1 ml of serum-free Hams F12K media lacking growth supplements and cycloheximide. HEp2 cell lysates were prepared according to the same procedure used for *C. pneumoniae* isolation and used for control mock infection of

HUVEC. Conditioned medium from infected or uninfected HUVEC cell cultures was harvested at 24 or 48 h, clarified by centrifugation at 20,000 × *g* for 30 minutes and assessed immediately for the ability to stimulate SMC using a [^{3}H]-thymidine incorporation assay. In some experiments, chlamydiae were heat inactivated at 56°C for 30 minutes, or cultured in the presence of chloramphenicol (80 μg/ml; Sigma). Both treatments inhibited the growth of *C. pneumoniae* as determined by IF staining for chlamydial inclusions.

SMC DNA Synthesis. DNA synthesis in SMC was assessed by the incorporation of [^{3}H]-thymidine as described previously (Ishida et al., 1997) with slight modifications. SMC were suspended in DMEM, 10% FBS and seeded into 24-well plates at 5×10^4 cells/well and cultured for 48 h. to ensure logarithmic growth. At 48 h. cells were washed 3 times in serum-free (SF) DMEM and cultured an additional 48 h. in SF-DMEM containing 0.1% BSA (Sigma) in order to induce cells into G_o quiescence. Quiescent SMC were washed and conditioned assay medium from uninfected, mock infected, or *C. pneumoniae*-infected HUVEC was applied to quiescent SMC cultures for 24 h. 1μCi/ml [^{3}H]-thymidine (methyl-^{3}H; 6.7 Ci/mmol, 37 MBq/ml; NEN, Boston, MA) was added after 16 h. for an 8-hour pulse. Each assay condition was assessed in triplicate. DMEM containing 10% FBS was used as a positive control for [^{3}H]-thymidine incorporation. Following the 24 h. incubation period, medium was discarded and cells were washed 3 times with ice cold phosphate buffered saline, then lysed in 0.25 ml of 0.2N NaOH for 20 minutes at room temperature. DNA was precipitated by addition of 1.25 ml of 10% trichloroacetic acid (Sigma) for 1 h at 4°C. The TCA insoluble fraction was filtered onto GF/F glass fibre disks (Whatman, Clifton, NJ) washed and air-dried. Filters were placed into 5 ml of

scintillation fluid (BCA-NA; Amersham) and radioactivity was determined using a liquid scintillation β-counter (LS5801, Beckman Instruments) and expressed as counts per minute (CPM). Stimulation indices were calculated as follows: [^{3}H]-thymidine CPM for conditioned medium ÷ mean [^{3}H]-thymidine CPM for uninfected medium.

SMC Proliferation assay. Proliferation of SMC was determined by cell counting. Subconfluent (5×10^4) SMC were induced into quiescence for 48 h in SF-DMEM, 0.1% BSA as described above. SMC were cultured in the presence of conditioned assay medium from uninfected, mock infected, or *C. pneumoniae*-infected HUVEC for 7 days. Cells were counted every other day by removing the medium, treating cells with 0.3 ml of 0.05% trypsin-EDTA, resuspending in medium, and staining cells with 0.4% trypan blue. Cell counts were done in triplicate in a Neubauer chamber and expressed as mean counts.

Statistical Analysis. Statistical comparisons were made using one-way analysis of variance (ANOVA) with the Dunnett's multiple comparison post-test. *P* values of <0.05 were considered to be significant.

RESULTS

Growth of *C. pneumoniae* in HUVEC. An initial experiment was performed to confirm the earlier observation that HUVEC cells support the growth of *C. pneumoniae*. HUVEC supported the replication of *C. pneumoniae* VR-1310 as demonstrated by visualizing intracellular inclusions at 48 h post infection. Figure 1 shows an infected HUVEC cell with one typical inclusion body. The number of chlamydial inclusions per high-powered field increased with increasing titres of *C. pneumoniae* (data not shown). Infection studies using heat inactivated *C. pneumoniae* failed to give rise to productive infections in HUVEC as evidenced by the absence of any visible inclusions at either 48 or 72 h post infection (data not shown). The chlamydial developmental cycle was also blocked when HUVEC were infected in the presence of chloramphenicol, which inhibits prokaryotic translation, but does not effect eukaryotic translation at the concentrations used in these studies.

***C. pneumoniae* infection of HUVEC results in the production of soluble factor(s) with mitogenic activity for SMC.** To study whether *C. pneumoniae* infection of HUVEC could produce soluble factors that stimulate SMC replication, conditioned medium harvested from infected cell culture was assayed for the ability to stimulate [^{3}H]-thymidine incorporation into SMC. Conditioned medium was clarified by high-speed centrifugation to remove HUVEC cell debris and any *C. pneumoniae* elementary bodies. The clarified conditioned medium failed to give rise to chlamydial inclusions following inoculation onto HEp2 cell monolayers verifying that *C. pneumoniae* EBs were absent from the medium (data not shown). Initially, the production of soluble factors by HUVEC cells in response to *C. pneumoniae* infection was determined at

various MOIs ranging from 0.001 to 1. Conditioned medium from these infected cultures stimulated DNA synthesis in SMC cultures in a dose-dependent fashion. The production of HUVEC-derived soluble factor(s) was dependent on the initial dose of *C. pneumoniae* used to infect cultures, with the largest stimulation index (SI), (7.4 ± 0.9; $p<0.01$) seen at an MOI of 1 and the smallest SI (3.4 ± 0.3; $p<0.05$) at an MOI of 0.001, as shown in Fig. 2.

The SMC mitogenic activity of conditioned medium increases with the time of HUVEC infection. The kinetics of the production of soluble factor(s) from HUVEC infected with *C. pneumoniae* was investigated by using various MOI and by comparing the ability of 24- or 48-h post infection conditioned medium to stimulate DNA synthesis. As shown in Fig. 3, by 24 h post infection (MOI 0.1), SI were 6.65 ± 0.57 ($p<0.01$), and increased to 9.64 ± 0.56 by 48 h ($p<0.01$), compared to uninfected controls. In contrast, mock infection of HUVEC with HEp-2 cell lysates (SI =1.99 ± 0.13) gave SI similar to that of uninfected cell controls (SI=1.0 ±0.13; $p>0.05$) at 24 and 48 hours (48 h. shown). Using a MOI of 1, SI reached 9.62 ± 1.3 ($p<0.01$) at 24 h. post infection and increased to 12.2 ± 0.61 ($P<0.01$) at 48 h post infection. Figure 4 shows the results of a separate experiment to confirm the effect of soluble factor(s) on SMC proliferation using direct cell counting. Conditioned medium from infected HUVEC stimulated the proliferation of SMC during a 7-day observation period, whereas medium from mock-infected or uninfected HUVEC cultures did not result in an increase in cell number. By day 7 cell numbers had increased by 86.6% ($p<0.05$) for medium from HUVEC infected with *C. pneumoniae* at an MOI of 0.1 and 87.5% ($p<0.01$) for HUVEC infected at a MOI of 1; these increases in cell numbers were significantly higher than either mock-infected HUVEC, 11.7% or uninfected HUVEC, 9.5%

(Fig. 4B). During the same observation period, control SMC cultures overlaid with uninfected HUVEC medium but containing 10% FBS showed an increase in cell number of 168% at day 7.

***C. pneumoniae* endocytosis followed by signal transduction events appears to be important in the induction of mitogenic activity.** To determine whether viable *C. pneumoniae* was required for the induction of mitogenic factor(s) from HUVEC cells, experiments were carried out using heat-inactivated *C. pneumoniae* or viable organisms in the presence of chloramphenicol. Figure 5 demonstrates that both heat inactivated *C. pneumoniae* and *C. pneumoniae* grown in the presence of chloramphenicol were able to induce the synthesis of soluble factor(s) that stimulated SMC replication. Although the stimulation index obtained from HUVEC cells infected with either viable, heat inactivated, or chloramphenicol treated *C. pneumoniae* were significantly different than uninfected or mock infected control groups ($p<0.01$), there was no significant difference between the infected groups ($p>0.05$), indicating that heat treated EBs and EBs grown in the presence of chloramphenicol were similar to viable organisms in their ability to induce HUVEC-derived factors. These results suggest that chlamydial endocytosis or cell surface contact alone is sufficient for the production of soluble factor(s).

DISCUSSION

Our results demonstrate that HUVEC infected with *C. pneumoniae* produce soluble factor(s) that are mitogenic for SMC. Induction of this SMC growth factor activity by HUVEC cells was dependent on the time of infection, as medium harvested at 48 hours post infection had greater mitogenic activity than that of medium harvested at 24 hours. In addition, the HUVEC response to *C. pneumoniae* was dose-dependent, as HUVEC infected with higher MOIs of *C. pneumoniae* produced conditioned medium with increased mitogenic potential for SMC, compared with lower MOI. This is presumably due to the increased proportion of HUVEC cells infected at higher MOI and more cells producing the soluble factor. Interestingly, HUVEC infected with extremely low doses of *C. pneumoniae* (MOI $=10^{-3}$), were also able to generate SMC stimulatory activity indicating that the HUVEC response to *C. pneumoniae* is sensitive and may involve the production of soluble cytokines/chemokines by a signal transduction phenomenon and/or an autocrine feedback mechanism, where cytokines released from HUVEC could stimulate the production of mitogenic factors and thereby amplify the response at low *C. pneumoniae* doses.

Experiments using chloramphenicol and heat-inactivated *C. pneumoniae* were performed to investigate whether newly synthesized chlamydial cell components were required for the production of SMC growth factor(s). In the presence of chloramphenicol, chlamydiae can bind to host cells and become phagocytosed, but their developmental cycle is blocked. Likewise, heat inactivated chlamydiae can bind to host cells, albeit at a lower efficiency than native, viable organisms (Byrne and Moulder, 1978) and become endocytosed. From a signal transduction perspective, the vesicular trafficking events following uptake are similar in both cases. Heat

inactivated organisms as well as chloramphenicol treated organisms do not have the ability to actively prevent phagolysosomal fusion since this event in *C. trachomatis* has been shown to be dependent on early de novo chlamydial protein synthesis (Scidmore et al, 1996). The ability of heat-inactivated and chloramphenicol treated organisms to activate HUVEC in a similar manner to viable ones suggests that the SMC growth factor(s) is a bona fide HUVEC product, since both types of treated organisms do not have the potential to synthesize bacterial proteins. Furthermore, our results demonstrate that viable *C. pneumoniae* is not required for HUVEC stimulation, suggesting that cell surface binding and/or phagosomal entry events are important in transducing the signals involved in upregulating cytokine/chemokine gene expression in HUVEC. Such early events capable of inducing host cell signal transduction pathways via host protein tyrosine phosphorylation have been described for *C. trachomatis* (Fawaz et al., 1993, 1997) and are activated also by heat- or UV-treated *C. trachomatis*. A common signal transduction pathway may be activated in *C. pneumoniae*-infected HUVEC cells. Preliminary experiments with *C. trachomatis* have indicated that this species also stimulates the production of SMC growth factors in a similar fashion to that of *C. pneumoniae,* however, the existence of *C. trachomatis* in atherosclerotic lesions has not been demonstrated, making this observation less relevant to atherogenesis.

Existing evidence linking *C. pneumoniae* to atherosclerosis is expanding (Saikku et al., 1988; Linnanmaki et al., 1993; Melnick et al., 1993; Kuo et al., 1993b), and different pathophysiological mechanisms have been suggested (Leinonen, 1993; Danesh et al., 1997; Libby et al., 1997; Kalayoglu and Byrne, 1998). Implicit in the progression of atherosclerotic lesions is the crucial role played by SMC. Intimal SMC proliferation has been described as a hallmark of disease progression (Ross and Glomset, 1973) and is under complex regulation in the artery

(Ross, 1993) by way of growth factors and cytokine agonists and antagonists. For example, several growth factors have been detected in atherosclerotic lesion, such as PDGF, FGF, EGF and IGF-1. It is thought that these factors induce not only proliferation of vascular SMC, but also in some cases, the migration of SMC into the intima. The dual chemotactic/proliferative nature of these growth factors is thought to be largely responsible for the focal accumulation of media-derived SMC during advanced lesions. As a result of these growth factors, SMC growth antagonists are also at work in the artery. For example, IL-1, TNF-α and TGF-β are all factors that can inhibit cell proliferation, and attenuate the action of growth factors (Ross, 1993) as a regulatory mechanism to control the response of SMC to growth factors. In addition to these traditional SMC growth factors, evidence is emerging to suggest that other chemokines may be more promiscuous in their actions than previously thought. For example, the β-chemokine interferon-inducible protein-10 (IP-10), originally described as a chemokine for activated T-lymphocytes, may include SMC chemotactic and mitogenic capabilities in its repertoire (Wang et al., 1996). Interestingly, monocyte chemotactic protein-1 (MCP-1), which has been found to be upregulated in the atherosclerotic artery and during *C. pneumoniae* infection of HUVEC cells (Molestina et al., 1998), has been shown to be mitogenic for rat vascular smooth muscle cells (Porreca et al, 1997).

Pathological mechanisms resulting in excessive SMC proliferation in the artery have been described, including the ability of mildly oxidized LDL to activate SMC mitogen-activated protein kinase (MAPK), and transduce S-phase entry signals for vascular SMC (Auge et al., 1998). Since arterial hyperlipidemia is an established risk factor for atherosclerosis, this phenomenon may play a significant role in SMC growth regulation in vivo. Our results are consistent with a role of *C. pneumoniae* in atherogenesis where *C. pneumoniae* infected vascular

endothelial cells release soluble growth factors that stimulate SMC replication and contribute to intimal thickening and fibrous plaque formation. Of interest, is the identification of the endothelial factor(s) that stimulate SMC, and the bacterial component that triggers the signal transduction pathways resulting in production of the growth factor(s). There are several candidate factors that could be produced by HUVEC cells in response to *C. pneumoniae* infection, which are mitogenic for SMC. For example, many classical SMC growth factors are endothelial cell-derived, such as PDGF and FGF and may play a role in SMC proliferation during the progression of atherosclerosis due to the regional proximity between endothelial cells and intimal SMC. Other factors may also play a role in this scenario. Endothelial cells produce both IP-10 and MCP-1 and evidence suggests that these inflammatory mediators may have proliferative actions on SMC. Experiments using neutralizing monoclonal antibodies to specific cytokines or growth factors are underway to identify the factor(s) generated by HUVEC in response to *C. pneumoniae* infection and to characterize the molecular mechanism responsible for activation of the host cells. Possible candidates responsible for the induction of these factors in HUVEC include chlamydial lipopolysaccharide (LPS), components of the bacterial outer membrane complex, or heat shock protein-60 (HSP-60), which are known to be highly immunogenic. HSP-60 has recently been shown to induce cytokine and adhesion molecule expression in HUVEC cells (Galdiero et al., 1997), and has been found to co-localize with human HSP-60 in lesions of atherosclerosis (Kol et al., 1998). The induction of SMC growth factor(s) by *C. pneumoniae*-infected endothelial cells represents a novel mechanism by which this bacterium may contribute to the immunopathogenesis of atherosclerosis.

ACKNOWLEDGEMENTS

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Figure 4.1 - *C. pneumoniae* infection of HUVEC cells

HUVEC cells were infected with *C. pneumoniae* and at 48 h. post infection, monolayers were stained with an FITC-conjugated anti-LPS specific monoclonal antibody. HUVEC cells containing typical intracellular inclusions are shown. Neighboring uninfected cells do not contain typical inclusion bodies. (original magnification, 400×; epifluorescent microscopy).

Figure 4.1

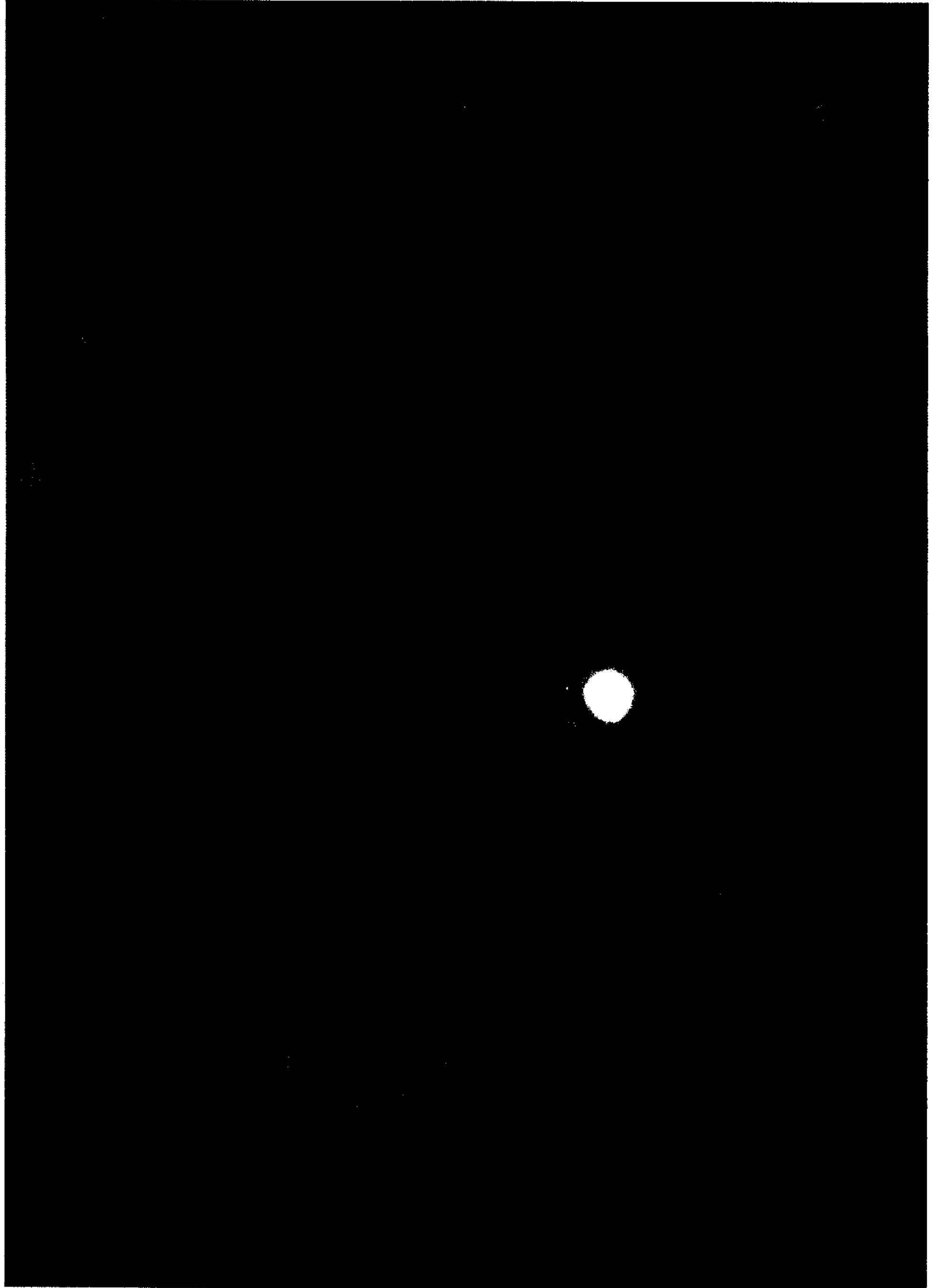


Figure 4.2 - [^{3}H]-thymidine incorporation by SMC treated with conditioned medium from *C. pneumoniae*- infected HUVEC at various doses for 48 hours

HUVEC cultures were infected with *C. pneumoniae* at various MOIs and conditioned medium was harvested at 48 h. and applied to SMC cultures for 24 h. [^{3}H]-thymidine was added for the last 8 h. and incorporation was determined as described in "Materials and Methods". Stimulation index data are expressed as the mean ± S.E. of one representative experiment performed in triplicate. * $P<0.05$, ** $P<0.01$, compared to uninfected control.

Figure 4.2

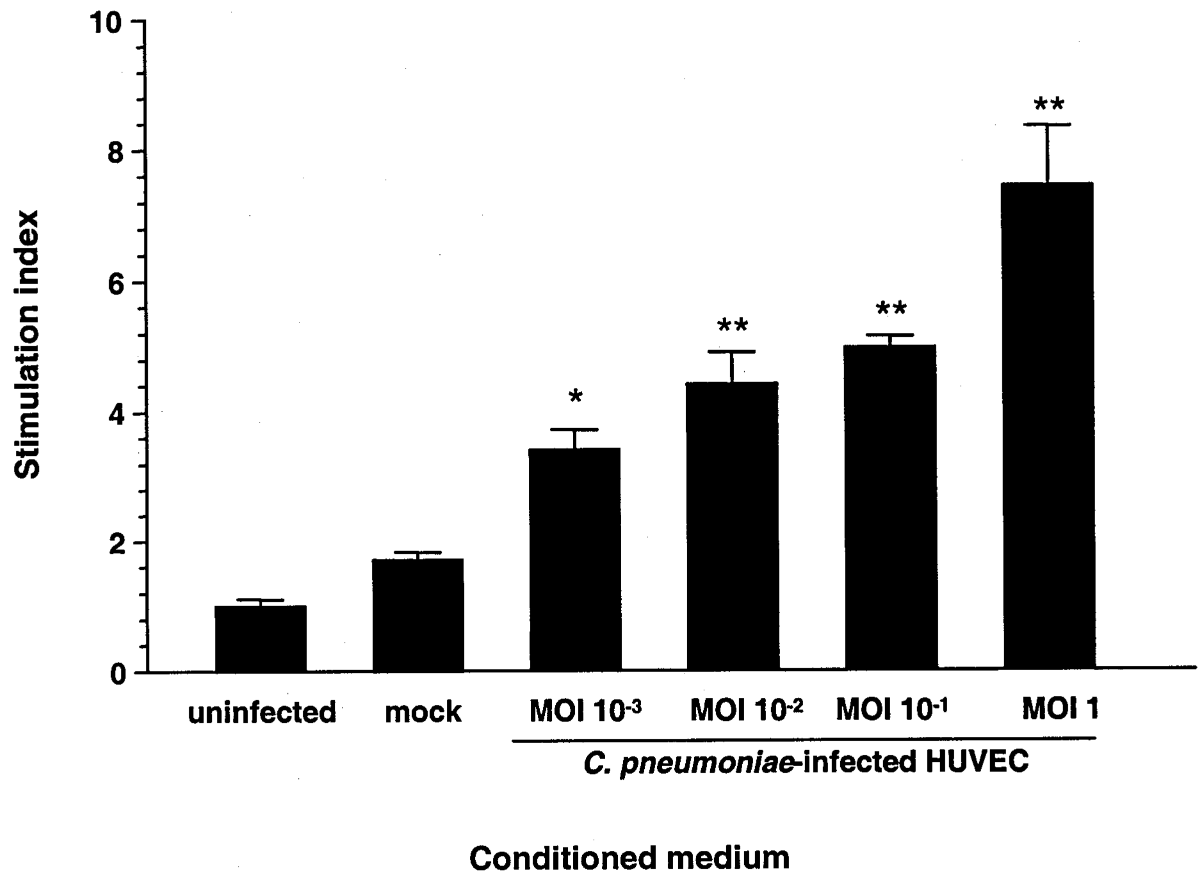


Figure 4.3 - [^{3}H]-thymidine uptake by SMC in response to conditioned medium from HUVEC cultures infected with *C. pneumoniae* for 24 and 48 h

HUVEC cultures were either uninfected, mock infected, or infected with *C. pneumoniae* at an MOI of 0.1 (striped bars) or MOI 1 (solid bars). Conditioned medium from these cultures was harvested at either 24 or 48 h. and applied to SMC cultures for 24 h. as described in Fig. 2. Data illustrated are the means ± S.E. of triplicate determinations of one representative experiment. **P<0.01, compared to uninfected control.

Figure 4.3

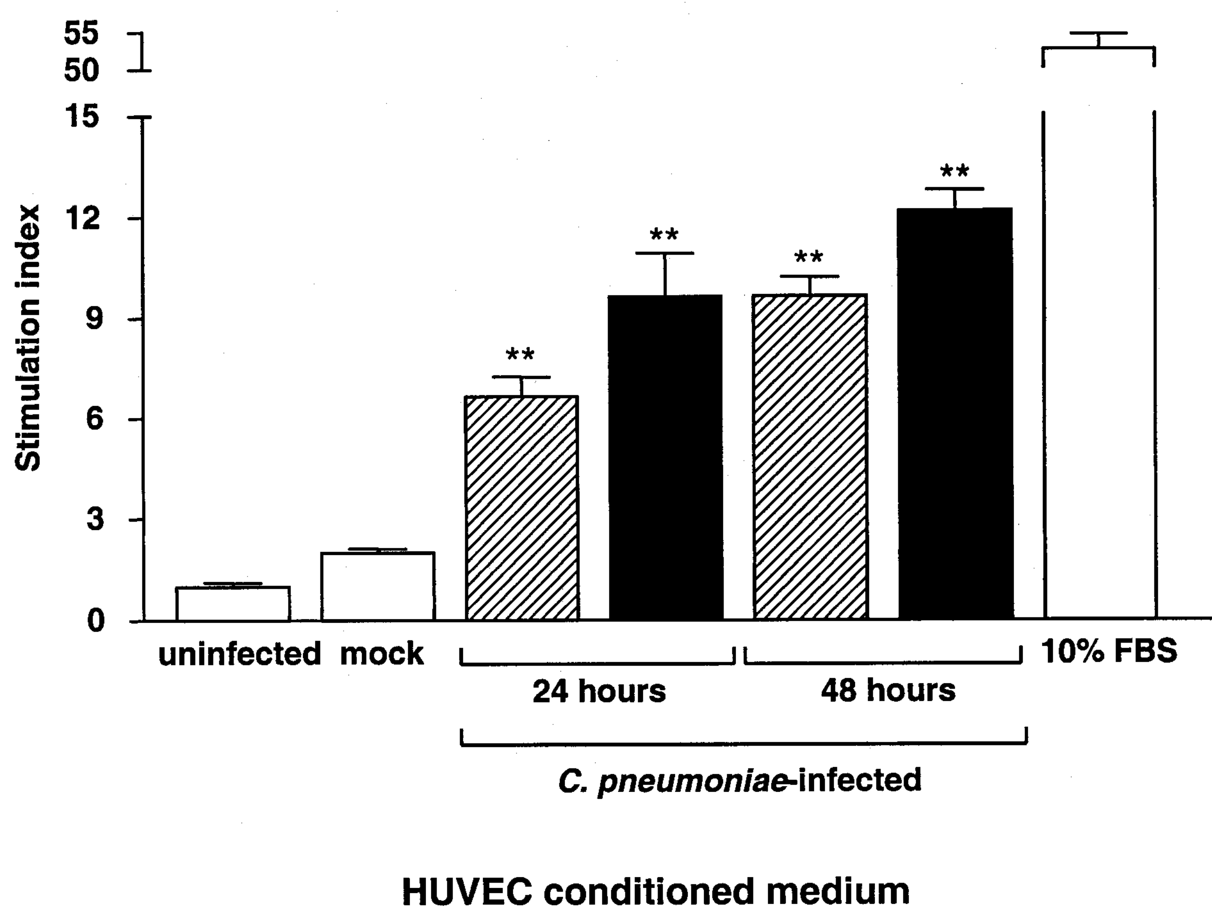


Figure 4.4 - SMC replication in response to conditioned medium from *C. pneumoniae*-infected HUVEC cultures

SMC were grown in 24-well plates and overlaid with the indicated conditioned medium from either uninfected or mock infected HUVEC, or HUVEC that were infected with *C. pneumoniae* for 48 h. On days 1, 3, 5 and 7, cells were harvested and counted as described in "Materials and Methods". **(A)** Increase in SMC numbers as measured by direct cell counting and **(B)** increase in SMC cell number on day 7 expressed as a percentage of cell number on day 1. The data are illustrated as mean ± S.E. of one experiment performed in triplicate. * $P<0.05$, ** $P<0.01$, compared to uninfected control.

Figure 4.4

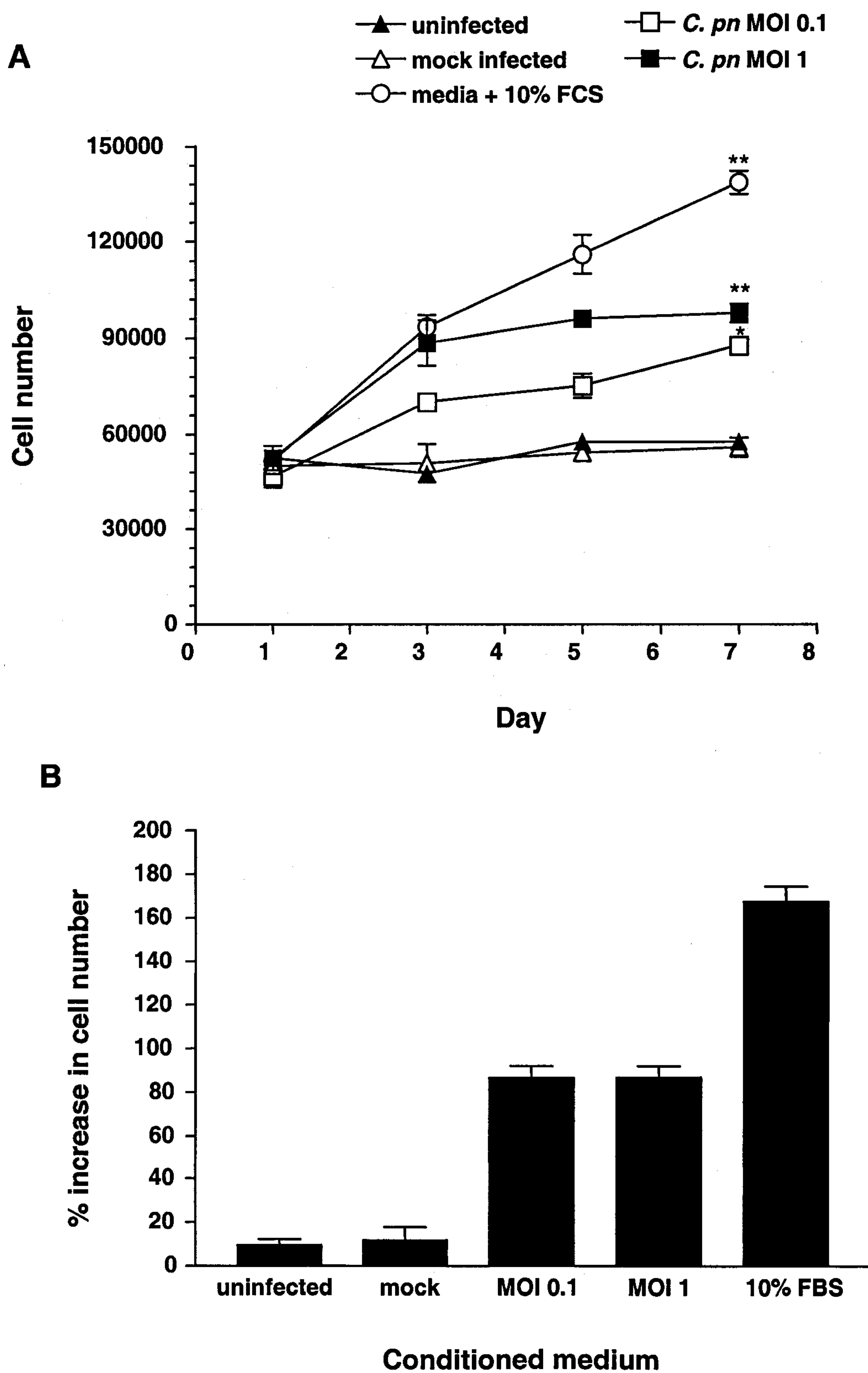
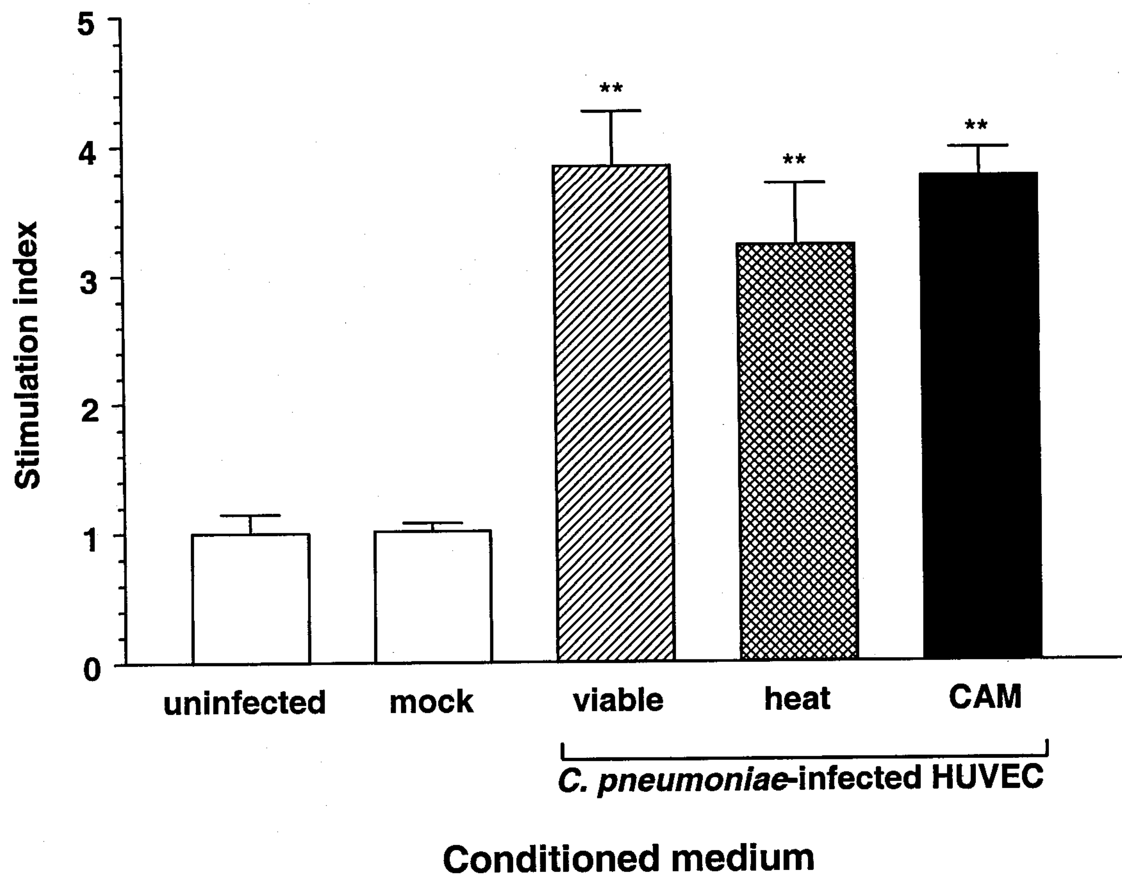


Figure 4.5 - Effect of heat treatment and chloramphenicol on the production of soluble factor(s) by HUVEC cell cultures infected with *C. pneumoniae*

HUVEC cultures were infected with viable (striped bar), or heat-treated (thatched bar) *C. pneumoniae*, or with viable *C. pneumoniae* in the presence of chloramphenicol (solid bar). Conditioned medium was harvested at 48 h. and applied to SMC for 24 h. as described in Fig. 2. Data are given as the mean ± S.E. of triplicate determinations of one representative experiment. $^{**}P<0.01$, compared to uninfected control

Figure 4.5



CHAPTER FIVE

AUTHOR'S PREFACE TO CHAPTER 5

The work described in this chapter utilized an animal model to demonstrate that *C. pneumoniae* infection of normocholesterolemic rabbits induces intimal thickening in the aorta at three months post-infection. A positive correlation was also shown for both *C. pneumoniae* antigen and platelet-derived growth factor-B with aortic intimal thickening in this rabbit model. The implication of these findings is that infection of the vessel wall with *C. pneumoniae* can potentiate the localized production of PDGF-B and intimal thickening, which are features consistent with the pathogenesis of atherogenesis.

The material presented in Chapter 5 has been published in the peer-reviewed journal, *Journal of Infectious Diseases*. The tissue specimens used in this study were obtained from the laboratory of Dr. Ignatius W. Fong (St. Michael's Hospital, Toronto, ON). Dr. Brian Chiu (St. Michael's Hospital) provided expertise with pathological grading of atherosclerotic specimens and performed the immunocytochemical staining referred to in this study. My original contributions include (i) the *in vitro* experimentation, (ii) the microscopic measurements of intimal thickness of the rabbit aorta specimens, (iii) the analysis of the data with the assistance of Dr. Marek Smieja and (iv) the writing of the research paper. Dr. James Mahony provided comments on the manuscript, which were incorporated into the text prior to publication in the Journal. The references were converted to Harvard style for consistency within the thesis prior to incorporation into the thesis Reference list.

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February 26, 2002

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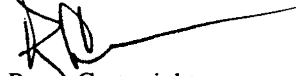

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CHLAMYDIA PNEUMONIAE INFECTION OF ENDOTHELIAL CELLS INDUCES TRANSCRIPTIONAL ACTIVATION OF PDGF-B: A POTENTIAL LINK TO INTIMAL THICKENING IN A RABBIT MODEL OF ATHEROSCLEROSIS

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Running head:Chlamydia linked to intimal thickening through PDGF

Footnotes

All animal protocols were approved by the Animal Care Committee at St. Michael's Hospital, Toronto, Ontario, Canada and animal care was in accordance with published institutional guidelines.

This work was supported in part by a University-Industry Operating Grant from the Canadian Institutes for Health Research (formerly Medical Research Council of Canada) to I.W.F. and J.B.M.

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ABSTRACT

Smooth muscle cell (SMC) proliferation and intimal thickening are hallmark features of atherosclerotic disease and *Chlamydia pneumoniae* may contribute to atherogenesis by imparting biological effects on SMC. An in vitro endothelial cell (EC) model of *C. pneumoniae* infection and a normocholesterolemic rabbit model of *C. pneumoniae*-induced atherosclerosis were used to test the hypothesis that respiratory tract infection with *C. pneumoniae* results in platelet-derived growth factor (PDGF)-B production, SMC proliferation and aortic intimal thickening in the absence of high cholesterol. Using RT-PCR, it was demonstrated that *C. pneumoniae* infection of EC induced PDGF-B mRNA expression. In *C. pneumoniae*-infected rabbits, maximal intimal thickening (MIT) was significantly greater than in sham-infected animals ($P<0.0001$). MIT correlated with the presence of both *C. pneumoniae* antigen ($P=0.043$) and PDGF-B ($P=0.002$) in aortic tissues and *C. pneumoniae* antigen was independently correlated with PDGF-B in aortic tissues ($P=0.009$). These results suggest that *C. pneumoniae*-induced SMC proliferation and intimal thickening may be mediated through PDGF-B and offer a molecular mechanism by which *C. pneumoniae* infection could contribute to atherosclerotic disease.

INTRODUCTION

C. pneumoniae is an obligate intracellular pathogen and an etiological agent of acute respiratory illnesses (Kuo et al., 1995). An association with chronic atherosclerotic disease in the coronary- and carotid arteries has now been established, with pathological studies collectively identifying the organism in over 50% of all diseased atherosclerotic tissues tested by several methods including polymerase chain reaction, immunocytochemistry and culture (Kuo et al., 2000; Grayston, 2000; Ouchi et al., 2000; Vink et al., 2001). Chronic infection with *C. pneumoniae* may be facilitated by the ability of this organism to persist in vivo despite appropriate antibiotic therapy (Hammerschlag et al., 1992). Furthermore, persistent chlamydial outer membrane antigens promote a sustained inflammatory response in vitro (Wyrick et al., 1999), resulting in increased expression of cellular adhesion molecules and cytokine production by endothelial cells exposed to chlamydial membranes. The ability to persist and evade host immune mechanisms while chronically stimulating the immune system with bacterial antigens may be important biological properties of *C. pneumoniae* contributing to the chronic inflammatory process of atherosclerosis (Mahony and Coombes, 2001).

In humans, the recovery of viable *C. pneumoniae* from atherosclerotic lesions in coronary and carotid arteries (Jackson et al., 1997; Ramirez et al., 1996) suggests a unique virulence determinant allowing these bacteria to invade and survive within blood leukocytes and thereby gain access to various cell types within vessel walls. This notion is supported by experimental evidence showing that detection of *C. pneumoniae* DNA in peripheral blood mononuclear cells is predictive of vascular infection (Blasi et al., 1999). Outbred animal models (Fong et al., 1997, 1999a; Laitinen et al., 1997; Muhlestein et al., 1997) have demonstrated hematogenous

dissemination of *C. pneumoniae* and *de novo* atherosclerotic disease following intranasal inoculation and have established a synergistic interaction between this organism and other defined risk factors for human coronary artery disease such as high blood cholesterol levels. Our laboratory has shown that antibiotics administered up to 5 days after inoculation, but not after 14 days of infection can attenuate the development of atherosclerotic lesions in rabbits infected via the respiratory tract (Fong et al., 1999b), suggesting that after resolution of acute infection, the pro-atherogenic effects of *C. pneumoniae* infection persist, perhaps due to persistence of the organism in tissues inaccessible to the antibiotic. Murine models of atherosclerosis using inbred mouse strains harboring genetic deficiencies in either apolipoprotein E (ApoE) or the low-density lipoprotein receptor (LDL-R) have corroborated the ability of *C. pneumoniae* to exacerbate atherosclerosis (Hu et al., 1999; Moazed et al., 1999; Burnett et al., 2001; Rothstein et al., 2001), while other studies using similar murine models have suggested that infectious agents are not necessary for the development of atherosclerosis (Wright et al., 2000; Caligiuri et al., 2001). Data from these latter studies however do not rule out an involvement of *C. pneumoniae* in the progression of atherogenesis in wild-type mice not genetically predisposed to the disease.

Infection of endothelial cells with *C. pneumoniae* elicits several host cell changes, including increased adherence and transmigration of monocytes and neutrophils (Molestina et al., 1999), increased procoagulant activity (Fryer et al., 1997; Dechend et al., 1999) and the expression of several proinflammatory cytokines, chemokines and growth factors known to play a role in the progression of atherosclerosis (Coombes and Mahony, 1999, 2001). Cellular cross communication in the vessel wall may alter the dynamics of atherosclerotic lesion progression, including migration and proliferation of smooth muscle cells (SMC) (Mahony and Coombes, 2001). For example, molecules mitogenic for SMC such as platelet-derived growth factor

(PDGF), fibroblast growth factor (FGF) and insulin-like growth factor (IGF) are produced by endothelial cells (Ross, 1993; Ross and Glomset, 1973) and may be overexpressed in *C. pneumoniae* infected cells.

One growth factor associated with atherosclerosis is PDGF, a protein composed of two chains, A and B, held together in a homodimeric or heterodimeric configuration by interchain disulfide bonds. PDGF is a potent mitogen and chemoattractant for cells of mesenchymal origin, with considerable evidence showing that neointimal thickening of arterial walls with atherosclerosis is, in large part, due to the chemotactic and mitogenic activity of PDGF on SMC (Ross and Glomset, 1973). PDGF-B mRNA and protein have been detected in human atherosclerotic lesions (Ross et al., 1990; Ueda et al., 1996) and in human coronary arteries following percutaneous transluminal angioplasty (Ueda et al., 1996). Furthermore, infusion of recombinant PDGF-BB stimulates SMC migration, proliferation and intimal thickening in a rat model of carotid artery balloon angioplasty (Jawien et al., 1992). As intimal thickening has been shown to predict the presence of atherosclerotic disease in humans (Zureik et al., 2000), these data support a potential pathologic role for PDGF in SMC proliferation during atherosclerosis, contributing to intimal thickening and narrowing of the vessel lumen.

To further explore a possible role of *C. pneumoniae* in atherosclerosis, we examined whether *C. pneumoniae* infection of endothelial cells could induce the transcriptional activation of PDGF-B in vitro, and whether Chlamydial antigens and/or PDGF-B production was a feature of aortic intimal thickening in a rabbit model of *C. pneumoniae*-induced atherosclerosis.

MATERIALS AND METHODS

Endothelial cell culture. HEp2 cells (ATCC CCL-23) were grown in 75 cm^2 culture flasks with minimal essential medium (MEM) (Life Technologies, Gaithersburg, MD) containing Earles salts and supplemented with 10% heat inactivated FBS (Life Technologies) and 2 mM L-glutamine. Human microvascular endothelial cells (HMEC-1) were obtained from Dr. E. Ades (Centers for Disease Control and Prevention, Atlanta, Ga.). HMEC-1 were cultured in MCDB-131 medium (Life Technologies) supplemented with 10% FBS, 2 mM L-glutamine, 10 ng/ml epidermal growth factor (EGF; Sigma) and 1 µg/ml hydrocortisone (Sigma). All cells were incubated at 37°C with a 5% CO_2 atmosphere.

***Chlamydia pneumoniae* propagation.** The respiratory isolate, *C. pneumoniae* VR-1310 (ATCC 1310-VR) was propagated in cycloheximide-treated HEp2 cells as described previously (Coombes and Mahony, 2001). *C. pneumoniae* were harvested at 72h by disrupting HEp2 cells with glass beads followed by sonication and differential centrifugation. Elementary bodies (EB) were pelleted, washed and suspended in sucrose-phosphate-glutamate buffer (SPG), and stored in aliquots at -70°C. For determination of bacterial titres, HEp2 monolayers grown on glass coverslips were infected and stained with a genus-specific mouse IgG-FITC conjugated monoclonal antibody directed against chlamydial LPS (Bio-Rad, Hercules, CA). *C. pneumoniae* titres were expressed as inclusion forming units (IFU) per ml. HEp2 cells and *C. pneumoniae* VR1310 were confirmed as Mycoplasma negative by a genus-specific PCR.

Analysis of mRNA expression using RT-PCR. HMEC-1 were infected in 6-well plates as described above at a multiplicity of infection (MOI) of ~8 with either viable EB, EB inactivated by heat at 70°C for 30 min, or EB in the presence of 80 μg/ml chloramphenicol. Following centrifugation at 1000 × *g* for 60 min and incubation at 37°C for 1 h, the inoculum was removed and cells were cultured in medium containing 0.5% serum, lacking growth supplements and cycloheximide. HEp2 cell lysates were prepared according to the same procedure used for *C. pneumoniae* isolation and used for mock infection of HMEC-1. Total RNA was isolated from uninfected and *C. pneumoniae*-infected HMEC-1 at various times after infection by lysing cells directly in culture dishes and extracting total RNA using RNeasy columns (Qiagen, Mississauga, On.) according to the manufacturer's instructions, followed by treatment with RNase-free DNase. The purity and yield of total RNA was assessed spectrophotometrically prior to cDNA synthesis. cDNA was synthesized in 20 μl reactions containing 5 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1.5 μg of total RNA, 0.5 μg of oligo$(dT)_{12-18}$ and 2 pmol of PDGF-B reverse primer, 0.6 mM each dNTP and 200 units of Moloney murine leukemia virus-reverse transcriptase (Life Technologies). Reactions were first heated to 70°C in the absence of enzyme, chilled on ice and then incubated for 60 min at 37°C in the presence of reverse transcriptase. 2μl of cDNA was used as template in PDGF-B and β-actin PCR reactions containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.5 μM each primer and 1.5 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer). Primer sequences for PDGF-B mRNA were purchased from Clontech Laboratories (Palo Alto, Calif.) and primer sequences for β-actin were: (forward) 5'-CCAACCGCGAGAAGATGACC-3' and (reverse) 5'-GATCTTCATGAGGTAGTCAGT-3' (Jobin et al., 1997). Primers were synthesized by the

Central Facility of the Institute for Molecular Biology and Biotechnology (McMaster University, Hamilton, Ontario). Thermal cycling programs consisted of 5 min of denaturation at 95°C, following by 30 sec at 95°C, 60 sec at 56°C and 90 sec at 72°C for 25 cycles and a final incubation of 5 min at 72°C. PCR products were separated by 2% agarose gel electrophoresis with ethidium bromide staining. RT-PCR products were analyzed by scanning densitometry of gel bands using the Un-Scan It-gel software package (Silk Scientific, Orem, Utah) or ImageJ analysis software obtained from the National Institutes of Health. PDGF-B RT-PCR products were normalized to actin signals obtained from each time point and the normalized data were expressed as fold increase in PDGF-B mRNA compared to uninfected controls at the same time point. The numerical data were analyzed using a two-tailed Student's *t* test with *P* values of <0.05 being considered significant.

Validation of RT-PCR linearity. To validate the semi-quantitative assessment of mRNA levels at both low concentrations and high concentrations of PDGF-B mRNA, we verified that the RT-PCR was linear with respect to cDNA input and PCR cycle number for both PDGF-B and actin targets. Various amounts of cDNA (0.5 μl to 3 μl) corresponding to low PDGF-B mRNA (from uninfected cells) and high PDGF-B mRNA (from infected cells) levels were used for PDGF-B and actin PCR using 25 cycles of amplification. PCR products were separated by agarose gel electrophoresis and the intensity of the gel bands was quantified by densitometry. Optical density values were plotted for each cDNA template concentration and linear regression analysis was applied to the raw data. For PCR cycle number validation experiments, 2 μl of template cDNA corresponding to low level PDGF-B-containing samples (from uninfected cells) and high level

PDGF-B mRNA samples (from infected cells) was amplified by PCR for PDGF-B and actin for various cycle numbers ranging from 19-31. PCR products were resolved by agarose gel electrophoresis and the intensity of the gel bands was quantified by densitometry. Optical density values were plotted for various PCR cycle numbers and linear regression analysis was used to assess linearity.

Experimental animals. All animal protocols were approved by the Animal Care Committee of St. Michael's Hospital and animal care was in accordance with published institutional guidelines. Animals used in this study were part of ongoing studies reported on previously by our laboratory (Fong et al., 1997, 1999a). One-month-old pathogen-free New Zealand White rabbits (NZW) were fed cholesterol-free, standard chow diets. Two groups of rabbits were studied; (i) 24 rabbits were inoculated with 1×10^7 IFU of *C. pneumoniae* via the nasopharynx using a catheter as described (Fong et al., 1997, 1999a). Rabbits received three separate inoculations, two weeks apart for 6 weeks and were sacrificed 3 months after the last inoculation; (ii) 24 rabbits were mock-infected with HEp2-cell lysates processed as described for *C. pneumoniae* and sacrificed after 3 months.

Histological examination and measurement of maximal intimal thickness. Descending thoracic aorta sections from *C. pneumoniae*-infected and mock-infected animals were fixed in 10% buffered formalin, processed and paraffin embedded. Four micron transverse sections from each segment were prepared and stained with Movat's pentachrome stain or Miller's elastic stain and examined microscopically with the observer blinded to the animal infection status. Maximal intimal thickness (MIT) was assessed by quantitative measurement using a Leitz Laborlux

microscope equipped with an eyepiece micrometer. MIT was defined as the maximum radial distance from the luminal surface to the internal elastic lamina of the vessel wall (one measurement per tissue section) and expressed in millimeters.

Immunohistochemical staining. Immunohistochemical staining was performed on paraffin-embedded aortic sections by the modified streptavidin-biotin-peroxidase method (Cartun et al., 1989). Chlamydial antigens were detected by reacting tissue sections with a *Chlamydia* genus-specific mouse monoclonal antibody directed against chlamydial lipopolysaccharide (CF2; Washington Research Foundation), using CF2-conjugated to biotin at 1:600 dilution. Two *C. pneumoniae* species-specific monoclonal IgG antibodies were also used; RR402 (Washington Research Foundation) at a dilution of 1:300 and Chlamydia CelPn (Cellab, Bellvue, Queensland, Australia) at 1:150 dilution. Tissue sections were reacted with normal mouse ascitic fluid as negative controls and paraffin-embedded HEp2 cells infected with *C. pneumoniae* were used as positive controls. Tissue sections containing at least one focus with ≥ 5 positively stained cells were scored as positive. A subset of tissue sections was also stained with anti-human PDGF-B (Pharmingen, San Diego, Calif.) at a 1:25 dilution. This antibody was shown to cross-react with rabbit PDGF-B in a pilot study conducted in our laboratory.

Statistical analysis. Statistical analysis of the data was performed using GraphPad Prism (San Diego, CA) and SPSS version 10.1 software. For RT-PCR linearity validation experiments, linear regression analysis was applied to the raw data and the correlation of determination (r^2) was calculated. Analysis of residuals was performed to test whether a linear equation fit the data and the slope of the linear equation was analyzed using an F-test to test whether the slope

significantly deviated from zero. For MIT measurements, animal groups were compared using a Wilcoxon Rank Sum test (Mann-Whitney Test). For 2 ×2-table analyses, the Fisher's exact test was used. P values of < 0.05 were considered significant for all tests.

RESULTS

***C. pneumoniae* infection induces SMC proliferation and intimal thickening in a normocholesterolemic rabbit model of *C. pneumoniae*-induced atherosclerosis.** Since intimal thickening is due primarily to SMC proliferation in the intima (Igarashi et al., 1997; Komukai et al., 1999; Hirata et al., 2000) we used intimal thickness as a surrogate marker of SMC proliferation in the vessel wall. Twenty-four rabbits in each experimental group were studied and maximal intimal thickness (MIT) from 3-6 aortic sections from each animal was measured. Averaged data was plotted as the mean MIT with standard errors. Figure 1 shows the MIT results for each group. MIT from the infected animals (group mean; 0.0405 ± 0.0064 mm) was significantly greater than MIT from uninfected rabbits (group mean; 0.0097 ± 0.0012 mm. $P<$ 0.0001). Upon histological examination, intimal thickness of uninfected rabbits was uniform and largely acellular, whereas MIT in infected animals was more variable. A photomicrograph showing representative intimal morphology from an uninfected rabbit is shown in Figure 2A, and demonstrates a uniformly acellular intimal layer. In contrast, the intimal region of infected rabbits showed varying degrees of increased cellularity (red/black nuclei), increased deposition of extracellular matrix and ground substance (blue/green) and degradation of the internal elastic lamina. Figure 2 (B-D) shows representative aortic sections from rabbits with mild MIT (Fig. 2B), moderate MIT (Fig. 2C) and a raised atherosclerotic lesion with severe MIT (Fig. 2D).

***C. pneumoniae* infection of EC induces transcription of PDGF-B mRNA.** HMEC-1 cells were left uninfected or infected for various times with viable *C. pneumoniae*, heat-inactivated *C. pneumoniae* or viable *C. pneumoniae* in the presence of chloramphenicol and total RNA from

these cultures was assessed for PDGF-B mRNA levels by RT-PCR. As shown in Figure 3 A and B, infection of HMEC-1 with viable *C. pneumoniae* induced transcription of PDGF-B mRNA beginning at 2 hours post-infection, which remained elevated throughout the 24 h of infection. In cultures inoculated with heat-inactivated bacteria, there was no induction of PDGF-B mRNA above basal levels throughout the 24 h observation period, while in cultures infected with *C. pneumoniae* in the presence of chloramphenicol, PDGF-B mRNA was induced at 2-4 h post infection, albeit to reduced levels compared to viable bacteria. In chloramphenicol-treated cultures, PDGF-B mRNA returned to similar levels seen in uninfected cultures by 24 h. To validate that the RT-PCR was quantitative under the conditions used, we verified that RNA input levels and PCR cycle numbers chosen were within the linear range of amplification at both low concentrations of PDGF-B mRNA and high concentrations of PDGF-B mRNA. The linearity of the RT-PCR assay was verified using various PCR cycle numbers (Figure 4 A) and different concentrations of cDNA template (Figure 4 B). Densitometric analysis of the RT-PCR products followed by linear regression analysis confirmed that RT-PCR amplification was linear with 2μl cDNA template at 25 cycles of amplification for all samples tested including those with low- and high concentrations of PDGF-B mRNA.

Intimal thickening is positively correlated with both *C. pneumoniae* antigen and PDGF-B in aortic tissue in infected animals. Aortic sections from infected and uninfected animals were stained for PDGF-B and *C. pneumoniae* antigens and then correlated with intimal thickening measurements. As shown in Figure 5, the mean maximal intimal thickness of the *C. pneumoniae* antigen-positive group was 0.044 mm ± 0.010 mm compared with 0.021 mm ± 0.003 mm for the *C. pneumoniae* antigen-negative group (P=0.043) and 0.010 mm ± 0.001 mm for the uninfected

group (*P*=0.002 compared to *C. pneumoniae*-infected, antigen-negative group). The mean MIT for the PDGF-B-positive group (n=7 infected animals) was 0.055 mm ± 0.008 mm compared with 0.016 mm ± 0.003 mm for the PDGF-negative group (n=17 infected animals; *P*=0.002 for PDGF (+) group compared to PDGF (-) group) (Figure 6).

The presence of *C. pneumoniae* in aortic sections predicts the presence of PDGF-B. Since both *C. pneumoniae* antigen and PDGF-B correlated with intimal thickening, we tested the covariation between *C. pneumoniae* and PDGF-B in aortic tissue sections from infected animals. A 2 × 2 contingency table analysis of the staining results indicated a positive correlation between the presence of *C. pneumoniae* antigen and PDGF in aortic tissue from infected animals (OR=18.8; 95% CI 1.5-388.5, *P*=0.009) (Table 1). These data indicate a correlation between *C. pneumoniae* antigen and PDGF-B in aortic tissues and that the presence of either *C. pneumoniae* antigen or PDGF-B predicts the occurrence of intimal thickening in infected rabbits.

DISCUSSION

The combination of *in vitro* and *in vivo* findings from our study provides insight into a potential molecular mechanism for a role of *C. pneumoniae* in atherogenesis by demonstrating a positive correlation between the degree of intimal thickening and the presence of *C. pneumoniae* antigen in aortic tissues. Further, we show that intimal thickening in infected rabbits may be mediated through PDGF-B, whose presence is predicted by *C. pneumoniae* antigen in the same aortic tissue.

Intercellular communication between infected cells and neighbouring cells may play an important role in the dynamics of vessel wall remodeling following local *C. pneumoniae* infection of the vasculature possibly through the action of secreted growth factors. In an effort to determine whether viable bacteria were required for the transcriptional activation of PDGF in EC, we inhibited prokaryotic translation using chloramphenicol (CAM), or rendered bacteria non-infectious by heat treatment prior to infection of HMEC-1. Chlamydiae treated with CAM are able to bind and infect host cells, but because inhibition of endosome-lysosome fusion requires de novo bacterial protein synthesis, CAM-treated bacteria are eventually degraded in lysosomes. The ability of CAM-treated bacteria but not heat-inactivated bacteria to induce PDGF-B mRNA expression is consistent with the idea that events associated with bacterial entry, and /or intracellular growth are involved in the transcriptional response of the host cell because heat treated bacteria have been shown to be severely attenuated in uptake by cells, whereas CAM-treated bacteria can still invade cells, albeit to slightly lower levels than untreated bacteria. Since cells infected with viable bacteria sustained the transcriptional response until at least 24h, this also suggests that the intracellular presence of the bacteria may be responsible for this activity. A

candidate host cell molecule that may be important in inducible gene expression in infected cells is the transcription factor NFκB, which has recently been shown to translocate to the nucleus of *C. pneumoniae*-infected cells within 15-30 minutes following infection (Dechend et al., 1999; Jahn et al., 2000). Given that the PDGF-B gene promoter contains a DNA binding element recognized by NFκB (Khachigian et al., 1995), it is possible that inducible PDGF-B expression in *C. pneumoniae*-infected EC may be controlled, at least in part, by this transcriptional activator.

This study demonstrated an increase in SMC proliferation in aortas of *C. pneumoniae*-infected rabbits as measured by histological staining and microscopic measurement of intimal thickness. Although intimal thickening in response to *C. pneumoniae* infection has been reported previously, to our knowledge, this report is the first to measure intimal thickening in normocholesterolemic animals infected with *C. pneumoniae*. SMC migration from the media to the intima and neointimal proliferation of SMC is an important feature of atherogenesis. This process is mediated, in part, by matrix metalloproteases that degrade portions of the extracellular matrix and internal elastic lamina allowing SMC access to the intimal space. Growth factor stimulation leads to SMC accumulation in the intima and increased secretion of stromal glycosaminoglycans and other extracellular matrix components by these cells, which accounts for up to 60% of the volume of areas of intimal thickening (Stary et al, 1992). The vast majority of cells in the neointima are SMC (Campbell and Campbell, 1989) and several studies using balloon catheterization animal models have demonstrated that intimal thickening can be prevented by pharmacological inhibition of SMC proliferation (Hirata et al., 2000; Wiernicki et al., 1996; Gallo et al., 1999; Schmidt et al., 2000). In our study the microscopic observation of intimal thickening in infected rabbit aortas occurred concomitantly with discontinuity of the internal elastic lamina, increased cellularity of the neointima and deposition of extracellular matrix, thus supporting data

from human studies attempting to define a role for *C. pneumoniae* in atherosclerotic lesions. In hypertensive men at risk for cardiovascular disease, Schmidt and colleagues demonstrated a correlation between elevated *C. pneumoniae* IgM, IgA and/or IgG and increased carotid intima-media thickness as assessed by B-mode ultrasound (Schmidt et al., 2000). Ericson and colleagues (Ericson et al., 2000) extended these findings by demonstrating that positive immunostaining for *C. pneumoniae* in human coronary arteries correlated with the severity of atherosclerosis when a microscopic measure of intimal thickening similar to that used in the present study was applied to autopsy specimens. Our study complements and extends these data by demonstrating spatial localization of *C. pneumoniae* antigen with increased intimal thickness and a known SMC growth factor in an animal model. One caveat of our data relates to the type of in vitro cell system used and the in vivo extrapolation of that data. Since our in vitro experiments were performed with cultured human endothelial cells and our in vivo experiments were performed in a rabbit model, it remains to be seen whether our data is generalizable to a natural *C. pneumoniae* infection in a human vessel.

Our demonstration of increased intimal thickness in *C. pneumoniae*-infected rabbits corroborates and extends the work of Muhlestein et al. (Muhlestein et al., 1997), with the important difference being that our model involves non-cholesterol supplemented rabbits with normal cholesterol profiles. In the study reported by Muhlestein, rabbits receiving a 0.25% cholesterol-supplemented diet had intimal thickening that was exacerbated by concurrent *C. pneumoniae* infection. The lower MIT measurements reported in the present study are most likely explained by the lack of elevated serum cholesterol concentrations in our animals (mean serum cholesterol concentrations 0.8 ± 0.3 mM). Indeed, observations from our rabbit model indicated that a 0.5% cholesterol diet produced a substantial and uniform increase in intimal

thickness in the absence of *C. pneumoniae* infection, while an intermediate cholesterol diet (0.15%) gave rise to mild, discontinuous or patchy intimal thickening similar to that observed in the normocholesterolemic infected rabbits (Fong et al., 1999a; B. Coombes, unpublished data).

Microscopic measurements demonstrated that intimal thickening was greater in infected animals than uninfected when analyzed as a group, but also that intimal thickening was non-uniform and not present in all infected rabbits. Similarly, positive immunocytochemical staining for *C. pneumoniae* antigen was not observed in all aortic sections tested from infected animals. Our in vitro data showing that *C. pneumoniae* infection could upregulate PDGF-B mRNA expression lead us to hypothesize that localized *C. pneumoniae* infection in the vessel wall could be contributing to PDGF production, SMC proliferation and focal intimal thickening. Upon further examination, it was revealed that aortic sections representing areas of intimal thickening were significantly more likely to be *C. pneumoniae* antigen positive and PDGF-B positive compared to areas without intimal thickening. These data are consistent with our *in vitro* finding of transcriptional upregulation of PDGF-B and support a model whereby infected cells secrete PDGF-B to affect local SMC proliferation leading to intimal thickening and remodeling of the vessel wall. Although our animal experiments did not reveal the cellular source of PDGF-B, it is quite possible that infected endothelial cells secreting PDGF are responsible, at least in part, for the observed intimal SMC proliferation. Also, since chlamydial antigens such as heat shock protein 60 can activate not only EC, but also SMC and macrophages (Kol et al., 1999), it may also be possible that infected SMC or macrophages secreting growth factors contribute to intimal thickening. While the majority of aortic tissue sections from infected rabbits were negative for both *C. pneumoniae* and PDGF antigens, this is not surprising in light of the fact that the bacterial load in a single clinical specimen from both humans and animals is low (Smieja et al., 2001; J.

Mahony, unpublished data). The lack of PDGF staining in tissue sections negative for *C. pneumoniae* antigen also supports our hypothesis that the presence of *C. pneumoniae* contributes to PDGF production. Lastly, it is of particular interest that cytomegalovirus (CMV), another pathogen associated with atherosclerosis and restenosis can affect PDGF-B homeostasis. CMV immediate early gene products have been shown to promote SMC migration, proliferation and expression of PDGF-B receptor in rat SMC (Zhou et al., 1999) and CMV infection in vivo can induce expression of PDGF mRNA (Lemstrom et al., 1994), smooth muscle proliferation and intimal thickening (Lemstrom et al., 1993) in rat aortic allografts.

Our results provide additional evidence for a role of *C. pneumoniae* in atherogenesis by demonstrating a temporal and spatial co-localization of *C. pneumoniae* antigen, PDGF and intimal thickening in a cholesterol-free rabbit model of atherosclerosis. It remains possible that a chronic infection in the vessel wall with persistent non-replicating *C. pneumoniae* releasing bacterial antigens may exacerbate an inflammatory cascade during atheroma development thereby contributing to atherogenesis (Mahony and Coombes, 2001; Kol and Libby, 1999). Molecular tests to identify persistent organisms in biological samples together with novel antimicrobials that are effective against non-dividing bacteria will be helpful to test the hypothesis that persistent *C. pneumoniae* in the vessel wall contribute to the chronic inflammatory process of atherogenesis.

ACKNOWLEDGEMENTS

We are grateful to E. Viira and S. Muffasar for excellent technical assistance in preparation of the histological specimens, Dr. M. Smieja for assistance with statistical analysis and Dr. A. Murdin for critical review of the manuscript.

Figure 5.1 - Maximal intimal thickness (MIT) of normocholesterolemic rabbit aortas

Rabbits were infected with *C. pneumoniae* (n=24) or mock infected with HEp2 cell lysates (n=24) and MIT of aortic sections were measured. Each data point represents the mean MIT of 3-6 aortic sections obtained for one animal. Horizontal lines indicate the group mean. $P<0.0001$ for infected animal group compared to uninfected controls.

Figure 5.1

MIT (mm)

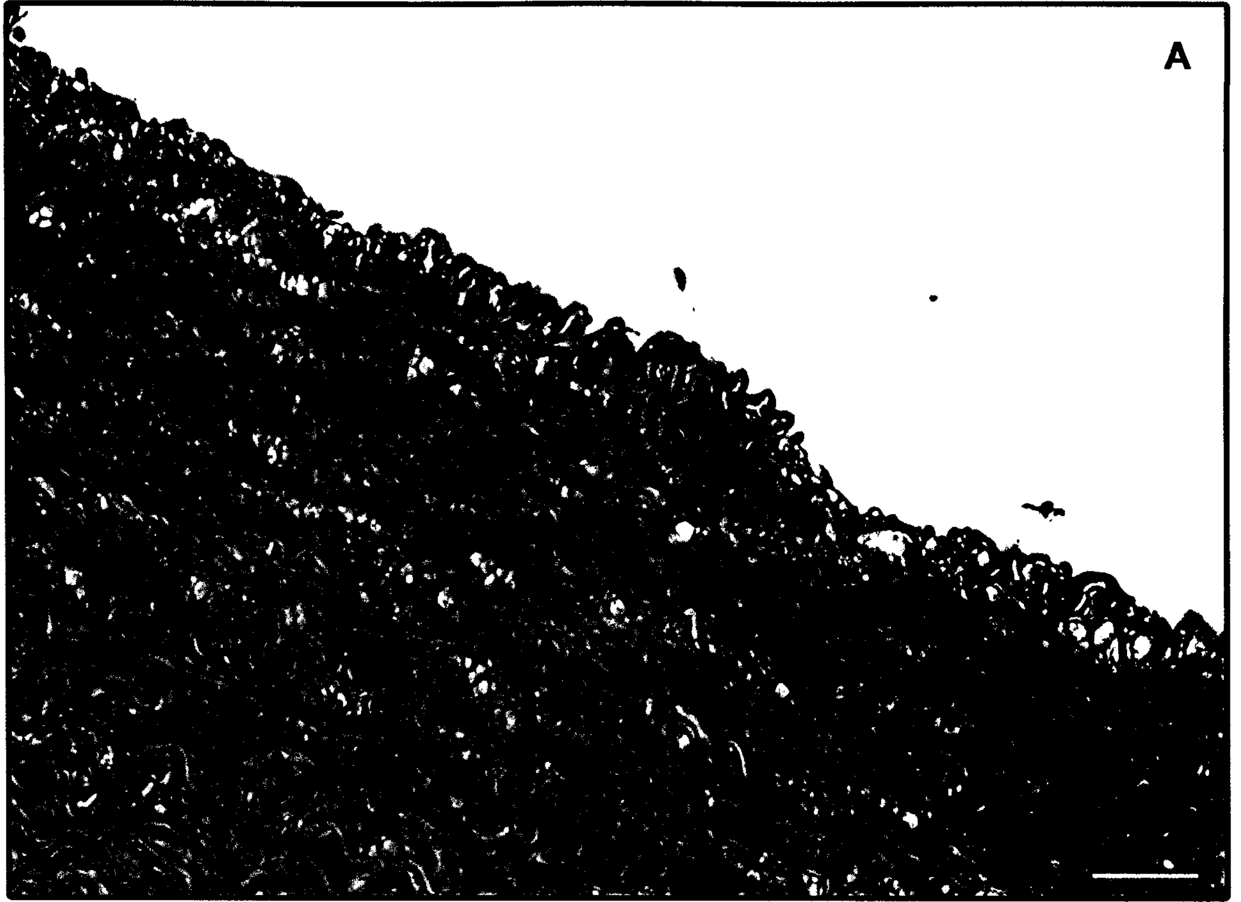
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00

infected
uninfected controls

Figure 5.2 - Photomicrographs of rabbit aorta tissue sections

Aortic sections were stained with Movat's pentachrome stain and visualized microscopically. Arrowheads indicate the internal elastic lamina in each field. **(A)** Typical vessel morphology from an uninfected animal showing an acellular intima. Original magnification, 400X **(B)** Mild intimal thickening from a *C. pneumoniae*-infected rabbit. The intima shows mild cellularity (red/black nuclei) and deposition of extracellular matrix and ground substance (blue/green). Original magnification, 400X **(C)** Moderate intimal thickening from an infected animal. The intimal layer shows increased cellularity and deposition of extracellular matrix with breaks in the internal elastic lamina (circles) not present in sections from uninfected animals. Original magnification, 400X. **(D)** A raised atherosclerotic lesion from an infected animal, showing marked intimal thickening from the internal elastic lamina (arrowheads), matrix deposition (blue) and cellular proliferation (dark nuclei). Original magnification, 100X. White bars in A-C represent 50 μm and the bar in D represents 200 μm.

Figure 5.2



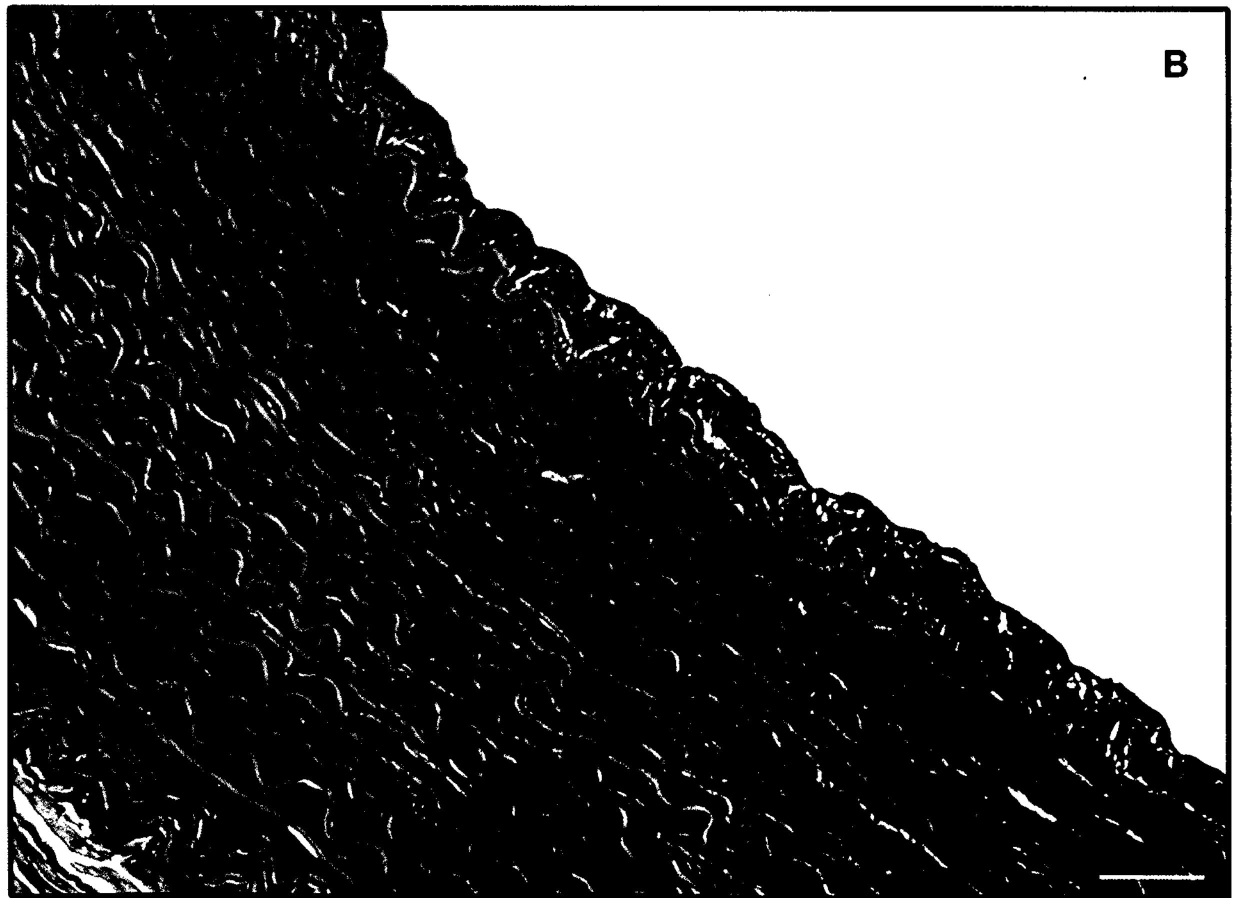


Figure 5.2

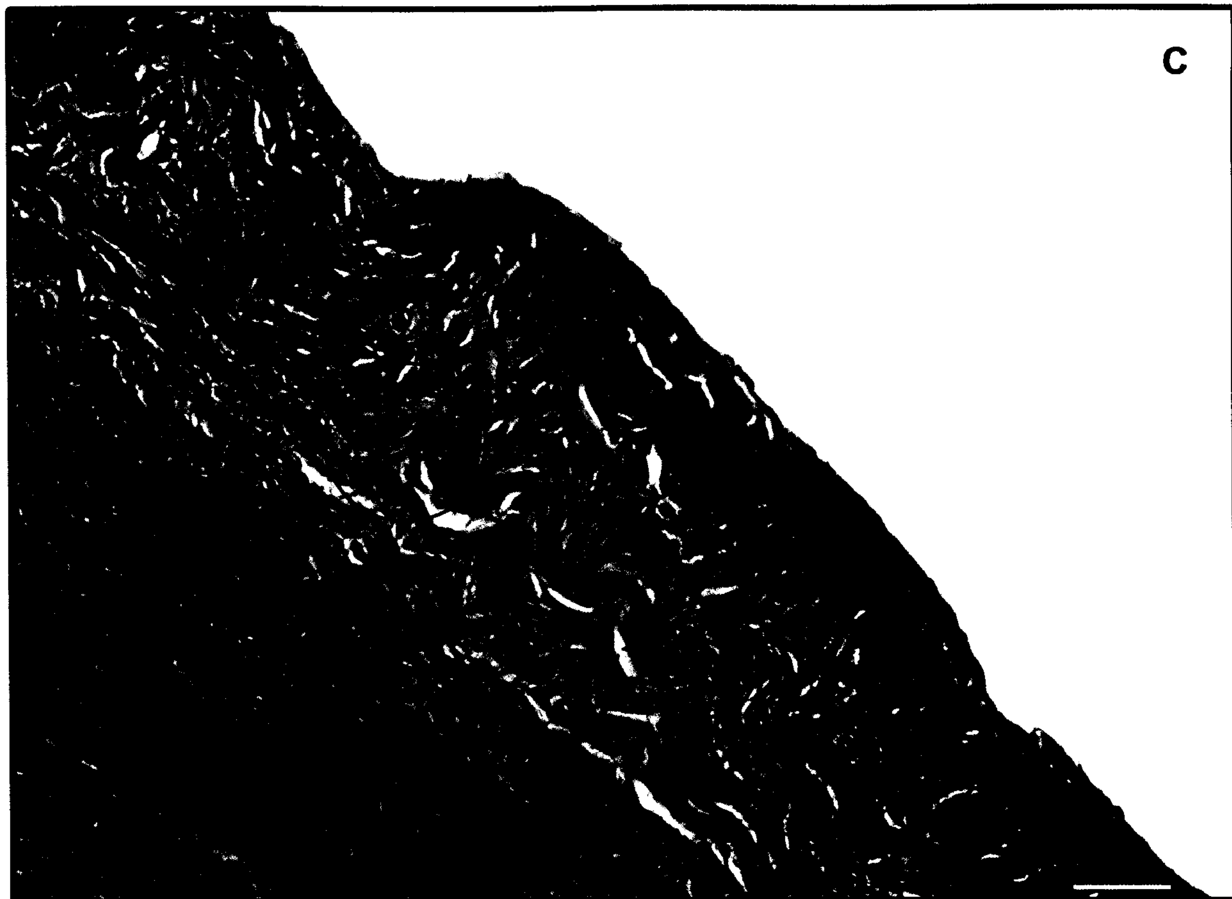


Figure 5.3 - *C. pneumoniae* induces transcriptional activation of PDGF-B

HMEC-1 cells were infected with *C. pneumoniae* as described in Material and Methods. RNA was harvested at the indicated times and tested for platelet-derived growth factor (PDGF)-B mRNA and actin mRNA by reverse-transcriptase polymerase chain reaction (RT-PCR). (A) Panels show representative photographs of ethidium bromide-stained agarose gels of RT-PCR products for PDGF-B (462 bp) and actin mRNA (213 bp). M, molecular weight markers; NT, no template control. (B) PDGF-B mRNA levels at various times post-infection. Signals from each band in Fig 5.3A were quantified by densitometry and expressed initially as optical density in arbitrary units. PDGF-B signals from both infected HMEC-1 cells and the uninfected controls were normalized using actin signals at each time point and mRNA expression was quantitated by expressing PDGF-B levels as a ratio (infected/uninfected) for each time point. Data represents the means with standard errors from two separate experiments. *, $P<0.05$; **, $P<0.01$.

Figure 5.3 A

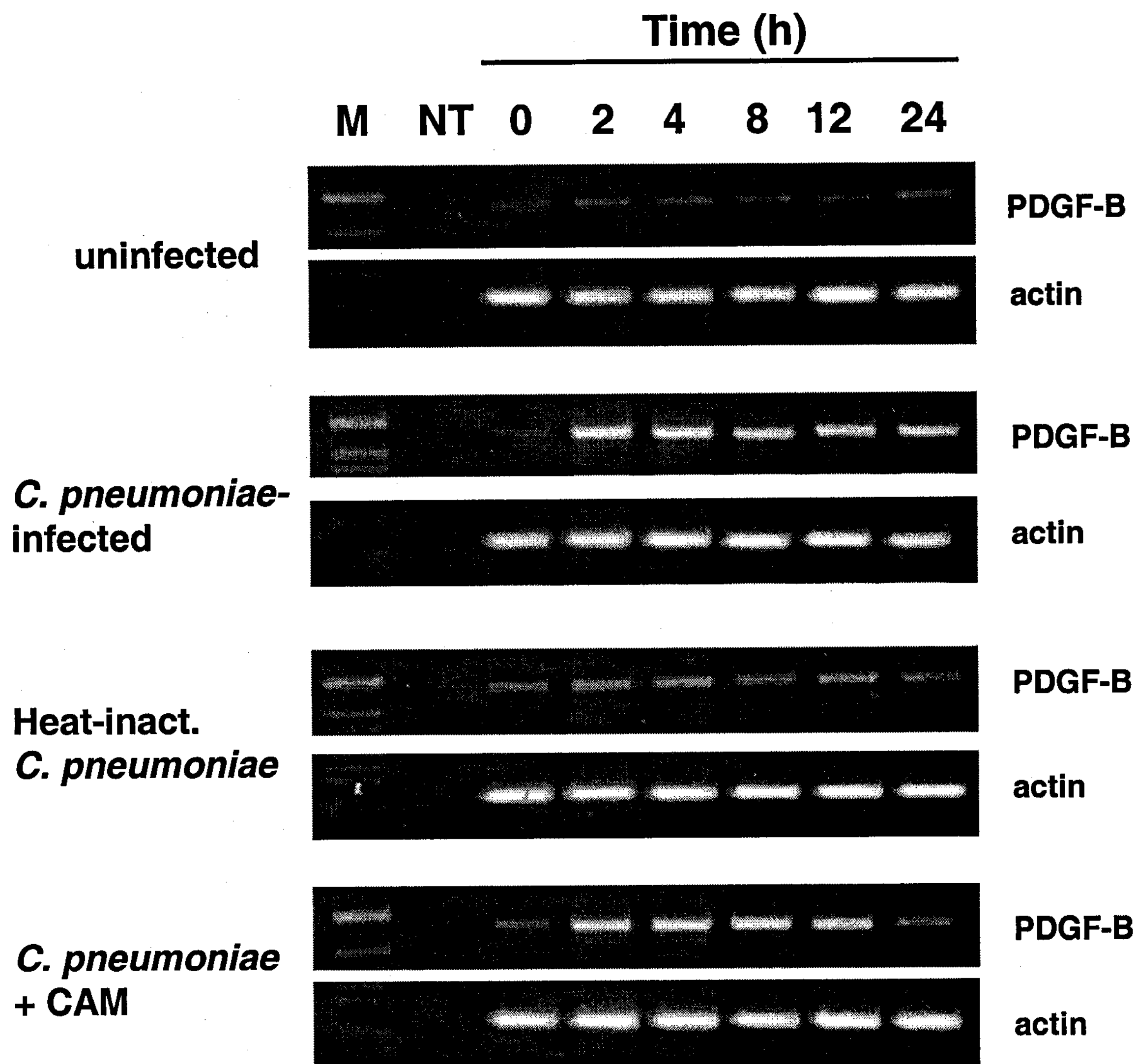


Figure 5.3 B

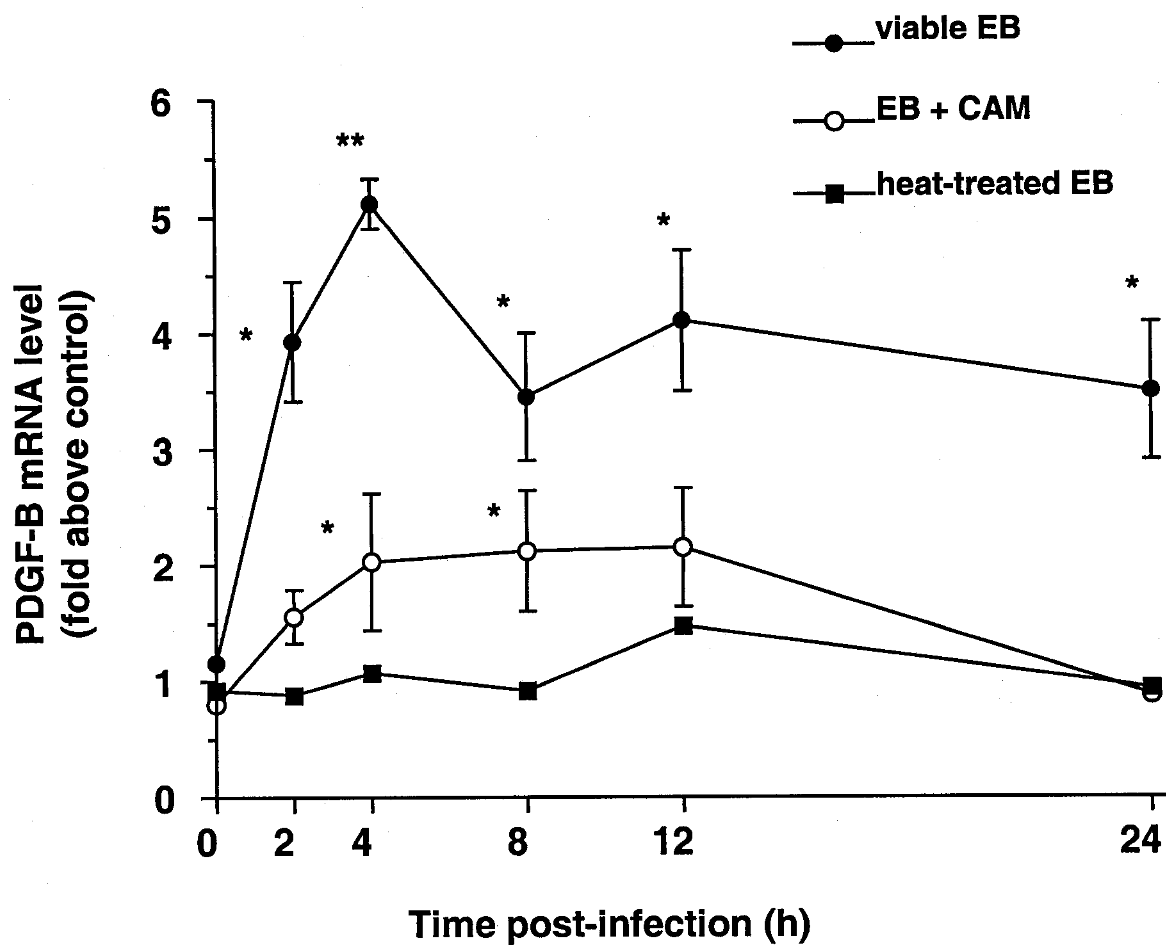
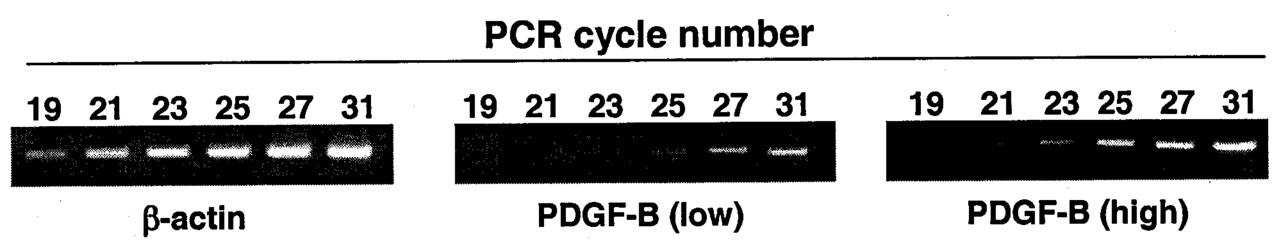
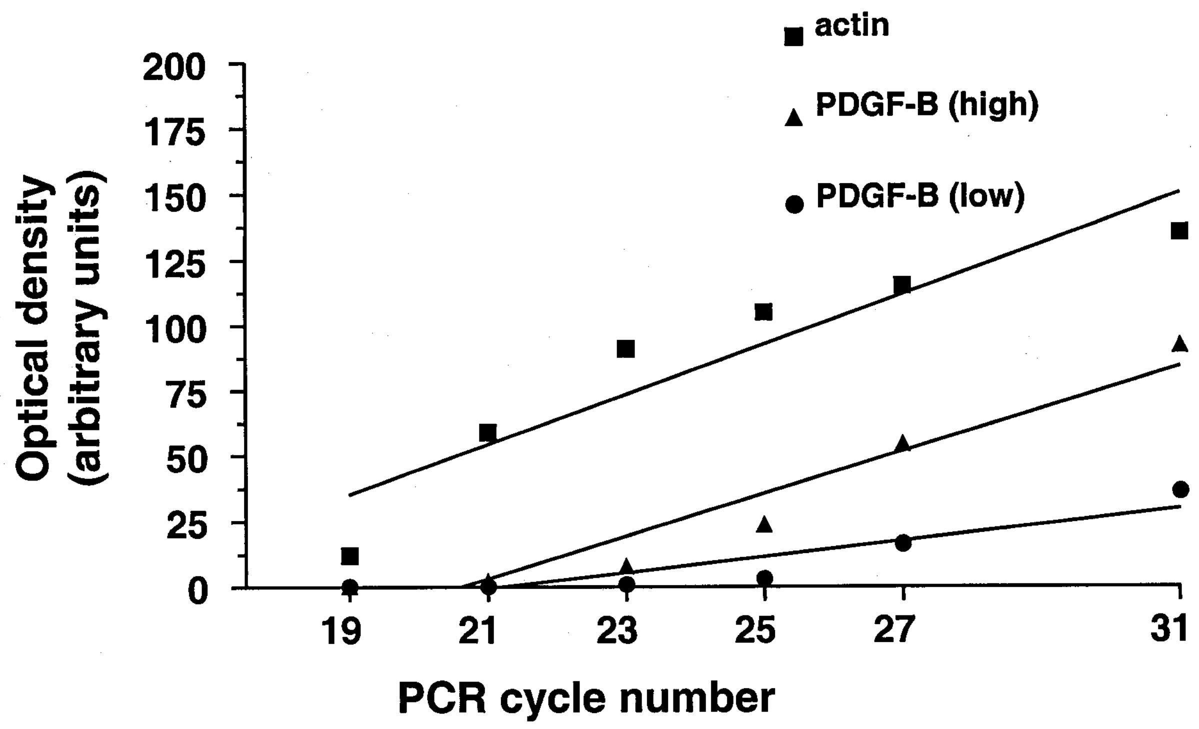


Figure 5.4 - Validation of RT-PCR linearity for cycle number (A) and cDNA input levels (B)

(A). Complementary DNA (cDNA) template was amplified by polymerase chain reaction (PCR) for various cycle numbers indicated in the figure. Top panels show representative photographs of RT-PCR products corresponding to actin (left), a sample with low concentration of platelet-derived growth factor (PDGF)-B mRNA (middle) and a sample with higher concentration of PDGF-B mRNA (right panel). Densitometric analysis was performed on each RT-PCR product and the data plotted as optical density in arbitrary units (graph). Linear regression analysis was performed as described in Materials and Methods to assess linearity. (B). Various amounts of cDNA template containing low or high concentrations of PDGF-B mRNA were used as input for PCR amplification. cDNA templates were amplified by PCR as described in Materials and Methods. Densitometric analysis was performed on each RT-PCR product and the data plotted as optical density in arbitrary units (graph). Linear regression analysis was performed as described in Materials and Methods.

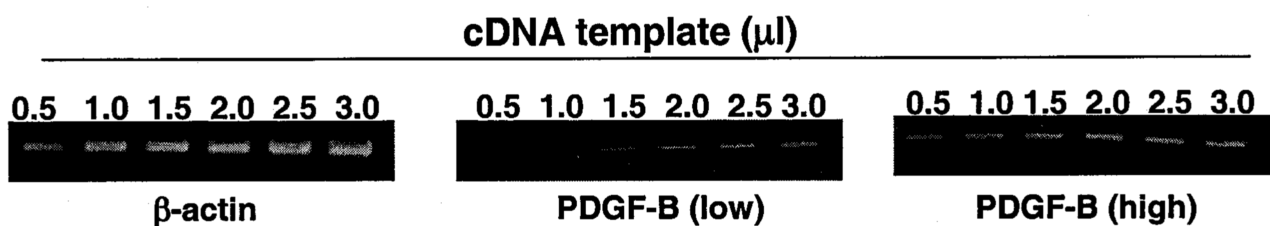
Figure 5.4 A

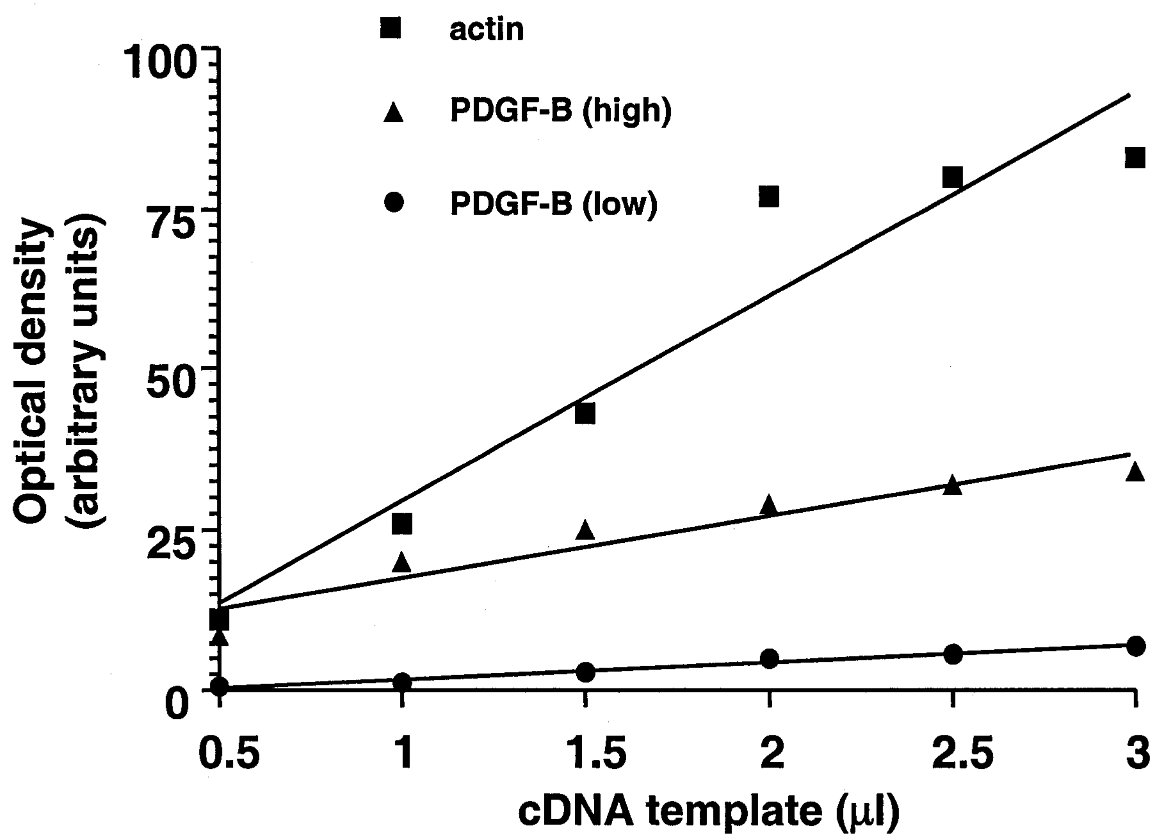




Actin: r^2=0.87, F-test: P=0.0064
PDGF-G (high): r^2=0.82, F-test, P=0.0025
PDGF-B (low): r^2=0.92, F-test: P=0.0127

Figure 5.4 B





Actin:	r^2=0.91, F-test: P=0.0035
PDGF-G (high):	r^2=0.93, F-test, P=0.0024
PDGF-B (low):	r^2=0.98, F-test: P=0.0002

Figure 5.5 - Maximal intimal thickness correlates with the detection of *C. pneumoniae* antigen in aortic sections from infected rabbits

Aortic tissue sections from infected rabbits were processed as described and *C. pneumoniae* antigens were detected by immunocytochemistry. Aortic sections were scored as either positive or negative for the presence of *C. pneumoniae* antigen as described in Material and Methods. For each data set, boxes extend from the 25th percentile to the 75th percentile, with horizontal lines indicating the 50th percentile (median). Bars indicate the range of the MIT data for each experimental group. *, P=0.043 for *C. pneumoniae* antigen (+) compared to *C. pneumoniae* antigen (-) for the infected group. **, P=0.002 for *C. pneumoniae* antigen (-) from the infected group compared to uninfected animals.

Figure 5.5

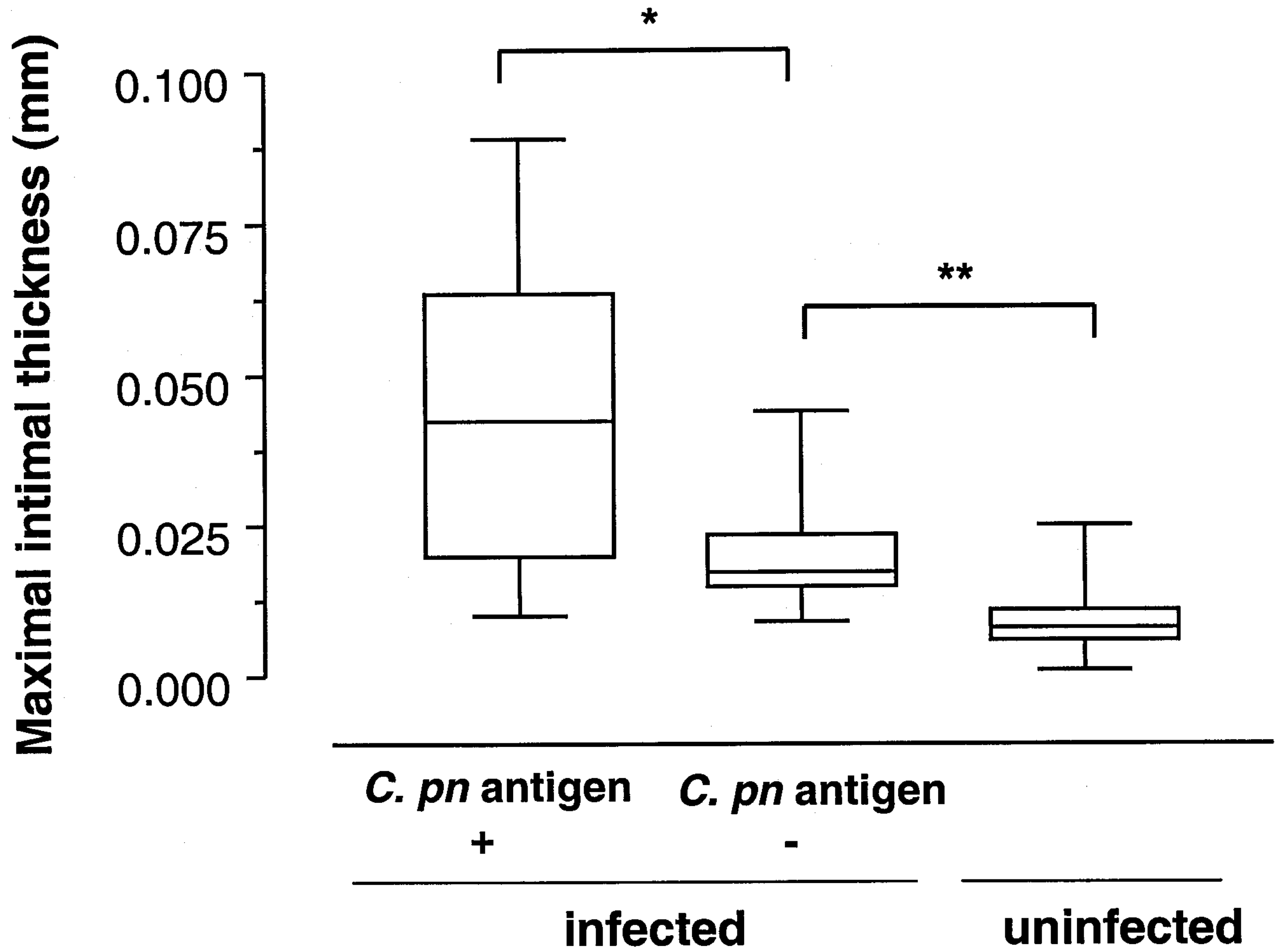


Figure 5.6 - Correlation between PDGF staining and MIT of rabbit aorta

Aortic tissue sections were processed for immunocytochemistry as described. Tissues were stained for the presence of platelet-derived growth factor (PDGF)-B and scored as positive or negative by a microscopist blinded to the animal treatment group. Tissue sections with one or more foci with ≥ 5 positive cells were scored PDGF-positive. **, *P*=0.002 for PDGF (+) group compared to PDGF (-) group. For each data set, boxes extend from the 25th percentile to the 75th percentile, with horizontal lines indicating the 50th percentile (median). Bars indicate the range of the MIT data for each experimental group.

Figure 5.6

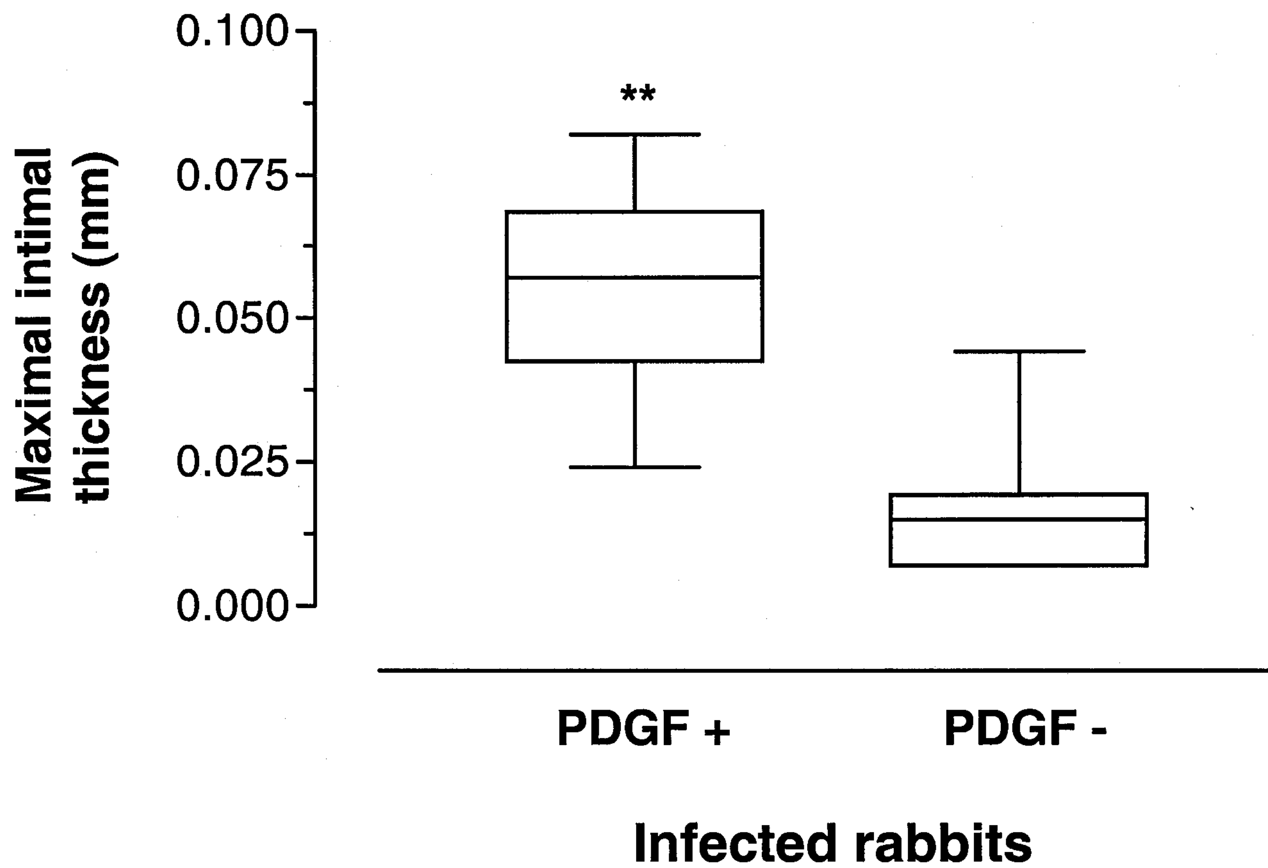


Table 5. 1- Correlation of *C. pneumoniae* antigen and PDGF-B in aortic sections from *C. pneumoniae*-infected rabbits

Table 5.1

Correlation of *C. pneumoniae* antigen and PDGF-B in aortic sections from *C. pneumoniae*-infected rabbits [a]

		C. pneumoniae antigen	
		+	-
PDGF	+	5	2
	-	2	15

[a] Data in table represents the number of individual animals positive for *C. pneumoniae* antigen or PDGF-B by immunohistochemical staining performed as described in Materials and Methods. OR=18.8 (1.5-388.5) [95% CI]; *P* = 0.009

CHAPTER SIX

DISCUSSION

Interference with host cell processes and the ensuing host response to infection govern the way in which bacteria take command of a cell. These interactions often involve activation of specific cell signaling cascades to facilitate bacterial entry and to prime the intracellular environment, making it compatible with bacterial growth, survival and evasion of host immune responses. Pasquelle Cossart and colleagues in 1996 (Cossart et al, 1996) first coined the term 'cellular microbiology' to describe a new scientific discipline bridging fundamental cell biology and microbiology. This came from the expanding appreciation that pathogenic bacteria often behaved differently when exposed to mammalian host cells than when grown in pure broth cultures. By necessity, chlamydiologists had already been studying cellular microbiology for over 40 years, since the host cell is the only machine capable of replicating chlamydia. Cellular microbiology seeks to provide a deeper understanding of the evolution of pathogenicity and to understand how pathogenic bacteria live habitually within animal cells as inquilines and in turn, cause disease in the host animal.

I have studied cellular microbiology of *C. pneumoniae* by investigating the molecular mechanisms contributing to invasion of prototypical epithelial cells and by examining potential molecular and cellular mechanisms of pathogenesis using an operational model of *C. pneumoniae*-induced disease. To this end, I have employed novel cell culture models of *C. pneumoniae* infection together with a rabbit model of *C. pneumoniae*-induced atherosclerosis to experimentally address several questions.

In this chapter, I discuss the potential interrelationship between the data described in chapters 2 through 5. I have drawn on relevant findings from the chlamydial literature and have

attempted to extract relevant findings from complementary studies using other invasive pathogens in order to explore the overall implications of our data. Furthermore, I consider these foregoing studies in aggregate to put forth a provocative discussion toward an expanded view of chlamydial cell biology and host interactions.

CELL CULTURE SYSTEMS: RATIONALE

The work described herein utilized different cell types and thus the rationale for using these models deserves some consideration. First, I found that examining different biological responses necessitated the use of different cell types. Our invasion experiments aimed at identifying host cell signaling and cytoskeleton involvement were designed with the *a priori* reasoning that the invasion process should be efficient, somewhat synchronized, able to infect a large percentage of the cells in culture and amenable to the identification of *C. pneumoniae*-induced responses. For this reason, we required a cell type that was (i) highly permissive for *C. pneumoniae* infection, (ii) would allow us to measure samples very early after inoculation of cells, (iii) could tolerate large doses of *C. pneumoniae* without overt cytotoxicity, (iv) did not require centrifugation-assisted inoculation, which would likely artifactually perturb the host cell membrane and thus the cytoskeleton and (v) did not require exogenous growth factors that might conceal responses evoked by *C. pneumoniae* per se. For these experiments, we chose the well-characterized cell line HEp2, a human epithelial cell line derived from the larynx. HEp2 cells are more susceptible to *C. pneumoniae* infection than other common epithelial cell lines used for *C. pneumoniae* propagation, including HeLa 229, McCoy and HL cells (Roblin et al., 1994). In our own studies, *C. pneumoniae* infection of other cell types such as SMC and endothelial cells typically yields lower inclusion-forming units per milliliter compared with HEp2 cells (B.

Coombes, unpublished data). Our preliminary experiments also showed that successful infection of HEp2 cells with *C. pneumoniae* could be achieved in the absence of cycloheximide and without centrifugation-assisted inoculation, two common manipulations used to achieve high infectivity rates for *C. pneumoniae* in cell culture.

Secondly, our studies on the host transcriptional response to infection were initially framed within the context of atherosclerosis. As such, we wanted to use a cell type that might present a natural target for infection in the vessel wall. Studies by other investigators previously demonstrated that monocytes infected with *C. pneumoniae* can transmit infection directly to endothelial cells via cell contact (Quinn and Gaydos, 1999). With this in mind, we hypothesized that hematogenous dissemination within leukocytes during a natural *C. pneumoniae* infection may permit inoculation of endothelial cells during transmigration from circulation to the extravascular space. Pilot experiments performed with the well-characterized umbilical vein endothelial cells showed that while these cells were susceptible to infection, obtaining and maintaining the large quantities of HUVEC cells needed for RNA extraction, spectrophotometric quantification, electrophoresis and polyA+ mRNA purification was laborious and prohibitive. Primary vascular endothelial cells also seemed to pose a similar problem (Dr. James Summersgill, personal communication). To overcome these issues, we obtained from the Centers for Disease Control and Prevention in Atlanta, HMEC-1 cells (Ades et al., 1992), an immortalized microvascular endothelial cell line that could be propagated with relative ease and which were susceptible to *C. pneumoniae* infection (Coombes and Mahony, 2001). HMEC-1 cells had recently been used in other *C. pneumoniae* studies (Lin et al., 2000) and offered a useful alternative model system to study comparative transcriptional responses to *C. pneumoniae* infection.

HMEC-1 cells however did not appear to be adequate for studying the secretion of SMC growth factors following infection. In experiments related to those described in chapter four using HUVEC cells, we found that conditioned medium from uninfected HMEC-1 cells stimulated SMC DNA synthesis to similar levels achieved with conditioned medium from infected cells (B. Coombes, unpublished data). A hypothetical explanation for this finding is that the transformed phenotype of HMEC-1 cells results in increased basal level secretion of a growth factor(s) that masks the mitogenicity of the medium due to *C. pneumoniae* infection per se. The finding that conditioned medium from uninfected HMEC-1 cells stimulated SMC DNA synthesis to greater levels than conditioned medium from uninfected HUVEC (B. Coombes, unpublished data) is consistent with this hypothesis. So while HMEC-1 cells were useful for gene expression profiling and comparison on an individual gene level (cDNA array experiments and RT-PCR experiments), they presented limitations for use in a different experimental system in which specific gene products were not directly measured.

Cell culture systems are inherently simplified and we acknowledge this limitation in our experiments. While cell culture models offer a controlled and manipulatable system, many factors such as availability, simplicity and overall relevance are considered when choosing the cell type for different experimental models. These limitations are also kept in mind when interpreting the output data. In terms of extrapolating the generalizability of our cell culture models to other cell types, work by other investigators has shown the production of growth factors from SMC infected with *C. pneumoniae* (Rödel et al., 2000). In addition, a similar panel of cytokines and chemokines to that reported here appears to be induced in *C. pneumoniae*-infected epithelial cells (Jahn et al., 2000). These complementary studies expand the generalizability of our data insofar as representing a common host signaling cascade in response

to *C. pneumoniae* infection that converges on the nucleus to transactivate a similar gene set. Elucidation of such a putative pathway will require further experimentation but may involve, in part, activation of the ubiquitous transcription factor NFκB as discussed in chapters 3, 5 and below.

POTENTIAL MECHANISMS OF BACTERIAL SIGNALING TO THE HOST CELL

Surface events and early signaling

Pathogenic bacteria first engage a host cell at the outer membrane surface, where adhesive interactions assist in bringing the bacterium and the host target into close contact. As introduced previously, the host cell receptor(s) and bacterial ligand(s) that mediate this interaction during *C. pneumoniae* infection of mammalian cells remain unknown, largely because there is no reproducible genetic transformation system to introduce selected mutations into putative chlamydial adhesins. Even if such a system did exist, the lack of a cell-free growth system for chlamydiae presents an additional problem, since disruption of an essential surface ligand that mediates invasion into cells will be a terminal event, not allowing cloning of the mutants. These limitations have significantly disadvantaged the molecular characterization of chlamydiae and have allowed the bacteria to remain somewhat enigmatic. For these reasons, our approach was to focus on the host cell during *C. pneumoniae* invasion with the intent of characterizing the signal transduction pathways required for invasion. Identifying essential signaling pathways may secondarily furnish clues into the molecular surface events leading to bacterial attachment and cellular invasion. Based on the experiments described in chapter two, we found that *C. pneumoniae* invasion of epithelial cells involved the requisite activation of host cell MEK-ERK signaling together with PI 3-kinase activation, leading to the phosphorylation and activation of at

least one downstream target of PI 3-kinase, Akt. The invasion sequence also involved phosphorylation of focal adhesion kinase (FAK) and the adaptor protein Shc. A working model of host cell signaling in response to *C. pneumoniae* invasion is presented in Figure 6.1 below.

FAK is a non-receptor tyrosine kinase whose subcellular distribution is largely localized to regions of the cell that attach to the extracellular matrix (Schaller, 2001). FAK contains 6 tyrosine residues that are sites of phosphorylation, with Tyr397 being the major site of autophosphoryation. When phosphorylated, this residue presents a docking site for SH2 phosphotyrosine binding domains on other signaling molecules including PI 3-kinase (Chen and Guan, 1994). FAK is also important in integrating growth factor and integrin signals to downstream pathways such as PI 3-kinase and ERK1/2 (Schaller et al., 2001). We found that FAK was tyrosine phosphorylated above basal levels within 5 minutes after exposure to *C. pneumoniae* and was sustained until ~45 minutes. Since FAK phosphorylation preceded its interaction with the p85 subunit of PI 3-kinase, it is possible that tyrosine phosphorylation of FAK induced by *C. pneumoniae* allows PI 3-kinase interaction via the SH2 phosphotyrosine-binding motif on the p85 subunit. Consequently, FAK-p85 interaction could generate the cascading forward signals that are involved in bacterial uptake.

Extracellular signal-regulated kinase (ERK)-1/2 (formally p42/p44 mitogen activated protein kinase) is a member of the mitogen-activated protein kinase (MAPK) family, with roles in inducible gene expression, cell proliferation, survival and cytoskeletal rearrangements (Chang and Karin, 2001). ERK1/2 is activated in response to a variety of extracellular stimuli including growth factor receptor tyrosine kinases and integrin ligation to the extracellular matrix. Phosphorylation at a threonine and a tyrosine residue by the upstream activator MEK (formally

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MAPK kinase) activates ERK1/2 kinase activity and is required for all known biological functions ascribed to this kinase. In our experiments, phosphorylation and activation of ERK1/2 occurred within 5-10 minutes after addition of chlamydia to HEp2 cells and required upstream MEK activation as determined by infection experiments performed in the presence of MEK inhibition. Based on our scanning EM studies, the kinetics of ERK and PI 3-kinase activation temporally correlated with binding of the bacteria at the cell surface. Binding events and ERK / PI 3-kinase activity were accompanied by reorganization of host cell membranes and the appearance of membrane microvilli that in some cases were seen by SEM and TEM to bind chlamydia EB. Of interest, the invasive bacterium *Neisseria gonorrhoeae* was recently shown to activate membrane microvilli on host cells (Griffiss et al., 1999) that were similar in appearance to chlamydia-induced microvilli. Griffiss and colleagues reported that microvilli appeared to be involved in the engulfment of bacterial microcolonies attached to the surface, albeit microvilli structures were observed no earlier than one hour following incubation of host cells with bacteria, which is markedly different than the kinetics of microvilli formation in response to *C. pneumoniae*. In related invasion studies, we demonstrated that the formation of membrane microvilli during *C. pneumoniae* invasion was dependent on the multiplicity of infection of the bacterial inoculum (Appendix D) and could be inhibited by pre-administering to cells either MEK-, PI-3 kinase- or actin polymerization inhibitors (B. Coombes, unpublished data). Under these conditions of inhibition, *C. pneumoniae* EB could attach to cellular membranes but entry was markedly reduced, producing a redistribution of total cell-associated EB from the cell cytoplasm fraction to the host cell surface fraction (Chapter 2 and Appendix C). That the inhibitory effect on entry but not attachment with MEK and PI 3-kinase inhibitors could be reproduced by preventing actin polymerization alone is consistent with the idea that both MEK

and PI 3-kinase-mediated signaling plays a role in facilitating actin polymerization required for bacterial uptake. In addition, our data confirm the importance of microfilament rearrangement for chlamydial invasion, as has been described for *C. trachomatis* serovar E (Schramm and Wyrick, 1995; Boleti et al., 1999). Clausen and colleagues (1997) have also presented data showing that chlamydiae utilize the host cell microtubule network to redistribute inclusions to the trans-Golgi region shortly after invasion. Inhibition of dynein, (a microtubule-dependent motor protein known to be involved in directional vesicle transport along microtubules) in *C. trachomatis* E and L2-infected cells caused a pronounced defect in *C. trachomatis* intracellular survival (Boleti et al., 1999). The authors reported that inhibition of *C. trachomatis* development under conditions of microfilament inhibition manifested at the level of inclusion-lysosome fusion. A tentative conclusion drawn from these studies is that chlamydiae have evolved to exploit the host cell cytoskeleton in a biphasic manner, whereby actin microfilaments allow invasion and the establishment of the endocytic vacuole, followed by recruitment of the host microtubule network to deliver the bacterial inclusion to a sequestered location away from degradative lysosomes of the endocytic pathway. The bacterial molecule(s) governing inhibition of inclusion-lysosome fusion have been a long sought after target in chlamydial biology and are currently undefined.

Activation of PI 3-kinase may have additional advantageous outcomes for *C. pneumoniae* in addition to facilitating invasion. It is now well documented that PI 3-kinase-dependent Akt activation transmits survival signals in mammalian cells that are both necessary and sufficient for growth factor-induced suppression of apoptosis (Datta et al., 1999). Given that chlamydiae are obligate intracellular parasites that rely heavily on the survival and maintenance of host cell metabolism for their own survival, it is tempting to speculate that Akt activation following chlamydia infection may increase host cell survival to the benefit of the bacteria. This hypothesis

is supported by at least one line of indirect evidence demonstrating that anti-apoptotic mechanisms are employed by both *C. trachomatis* and *C. pneumoniae* during intracellular infection. Fan and colleagues first reported that HeLa 229 and U937 cells infected with *C. trachomatis* were resistant to the apoptotic stimulant staurosporine, the DNA-damaging agent etoposide, as well as immunological stimuli such as tumor necrosis factor (TNF) α, Fas antibody and granzyme B/perforin (Fan et al., 1998). The anti-apoptotic mechanism apparently involved, at least in part, inhibition of caspase-3, which was later confirmed by others for *C. trachomatis* (Dean and Powers, 2001), and for *C. pneumoniae*-infected cells (Geng et al., 2000). Of interest, the X-protein of hepatitis B virus was recently shown to activate a PI 3-kinase/Akt-dependent cell survival pathway in CHL cells by a mechanism involving inhibition of caspase-3 activity (Lee et al., 2001). In addition, expression of an anti-apoptotic protein, Bcl-2 is elevated in cells via $\alpha_v\beta_1$ or $\alpha_v\beta_3$ integrins in a Shc- and FAK-dependent manner, with subsequent involvement of the PI 3-kinase-Akt pathway (Matter and Ruoslahti, 2001). Mitogen-activated protein kinases such as MEK or ERK did not appear to be necessary for inhibition of apoptosis in this model.

Considering our data in light of the biology of chlamydial invasion and intracellular survival, it is plausible that the MEK-ERK and PI 3-kinase-Akt axes represent a bifurcation of early bacterial-induced signaling events leading to different functional consequences; the former involved in cytoskeletal rearrangements (Christerson et al., 1999; Fincham et al., 2000) necessary for invasion and the latter pathway involved in host cell survival and growth of the bacteria. Further work will be required to determine whether chlamydiae employ a PI 3-kinase/Akt-dependent pathway to promote inhibition of apoptosis and whether this mechanism might play a role in establishing persistence of the organism in chronic diseases.

Chlamydia receptor signaling: clues and novel views

As mentioned above, a secondary objective of examining the host cell signaling response to infection was to garner clues into the unknown binding events at the cell surface that precede *C. pneumoniae* invasion. Since cell biological studies have elucidated many of the complex signaling arrays originating from the cell surface (in response to such stimuli as growth factors and integrin ligation to the extracellular matrix), we speculated that characterizing host signals during *C. pneumoniae* invasion might allow us to make inference to their surface origin. The signaling events we observed were induced very early following inoculation of cells, suggesting that the putative cellular receptor for *C. pneumoniae* may contain intrinsic catalytic activity capable of generating the phosphotyrosine residues we observed. If this were the case, the most likely candidate family of host cell molecules might be receptor tyrosine kinases. There is precedent for the usage by bacterial pathogens of receptor tyrosine kinases for cellular invasion. *Salmonella typhimurium*, *Listeria monocytogenes* and *Neisseria meningitidis* engage the epidermal growth factor receptor (Galan et al., 1992), the Met receptor (hepatocyte growth factor receptor) (Shen et al., 2000) and the Erb2b receptor tyrosine kinase (Hoffmann et al., 2001), respectively to activate specific signaling pathways required for uptake into mammalian cells. The early activation of tyrosine phosphorylation and downstream signal transduction cascades such as ERK and PI 3-kinase during *C. pneumoniae* invasion are consistent with the activation of a receptor tyrosine kinase cytoplasmic domain, recruitment of adaptor molecules such as Shc to the inner membrane followed by movement of lipid kinases such as PI 3-kinase and Akt to a receptor-proximal location. Similar signaling molecule dynamics have been described during entry of *Listeria monocytogenes* (Ireton et al., 1996; 1999) and Enteropathogenic *E. coli* (Czerucka et al., 2001) into epithelial cells, whereby PI 3-kinase activation (in the case of *L.*

monocytogenes) and Shc phosphorylation (in both bacterial models) are involved in the invasion process. Our finding that only a single isoform of the adaptor protein Shc was phosphorylated in response to *C. pneumoniae* invasion is unique among all intracellular bacteria shown to recruit this adaptor molecule. As discussed in chapter two, this finding may also provide clues to a putative chlamydial receptor, since Shc has been shown in other studies to interact with different receptor tyrosine kinases in an isoform-specific fashion (Okada et al., 1995; Sato et al., 2000). It is likely that elucidation of Shc binding partners during *C. pneumoniae* invasion may bring us even closer to identifying a putative chlamydial receptor.

Alternatively, there are several examples of bacteria that engage cell surface integrins as a mechanism to gain entry into host cells (Finlay and Falkow, 1997). Integrins allow cells to interact with their environment by binding to extracellular matrix components, which then potentiate signaling cascades to the cell interior, coordinating processes such as inducible gene expression and anchorage-dependent cell growth and survival. While integrins do not appear to contain intrinsic catalytic activity, they recruit catalytic molecules to their intracellular domain following engagement of the extracellular matrix (Aplin et al., 2001). Many intracellular bacteria bind their cognate integrin receptor by mimicking extracellular matrix components – displaying the integrin binding tripeptide motif, arginine-glycine-aspartic acid (RGD) on one of their outer membrane proteins (Finlay and Cossart, 1997). In a recent genetic analysis of chlamydial outer membrane proteins, one novel protein in *C. pneumoniae* and two outer membrane proteins in *C. trachomatis* were found to contain the RGD signature (B. Coombes, unpublished data), raising the possibility that the chlamydial invasion sequence could also involve integrin binding. Supplementing this unpublished preliminary data is the signal transduction data presented in this work. The very early activation of ERK kinase activity is consistent with the engagement of

integrins at the cell surface, since anchorage-dependent ERK signaling via integrins is recognized as an important signaling pathway regulating cytoskeletal rearrangements and cell survival (Aplin et al., 2001; Howe et al., 2002).

It also remains possible that these two hypotheses – implicating receptor tyrosine kinases and integrins– are not mutually exclusive and that both could play a role in *C. pneumoniae* invasion of mammalian cells. Indeed, the ability of *C. pneumoniae* to infect nearly all mammalian cell types (at least *in vitro*) implicates multiple routes of bacterial entry. Our laboratory is actively pursuing the invasion mechanism of *C. pneumoniae* with emphasis on identifying putative chlamydial adhesins, cognate cellular ligands and Type III secretion effector molecules that might activate host cell signaling pathways. As introduced previously, analysis of the *C. pneumoniae* and *C. trachomatis* genomes revealed that chlamydiae possess genes encoding a Type III secretion apparatus – a highly conserved, contact-dependent secretion machinery involved in delivering virulence proteins to host cells subsequent to surface attachment. In other Gram-negative pathogens, type III secretion is used to translocate virulence proteins directly into the host cell cytoplasm, often in the form of kinase and phosphatase molecules (DeVinney et al., 2000) that interact directly with host cell signaling pathways, perturbing their normal function and rewiring them for the bacteria's own use. Recently, our laboratory has cloned and characterized the first *C. pneumoniae* serine/threonine protein kinase (annotated gene I.D. Cpn0148), designating it CpnPK1 (Johnson et al., 2002). Experiments are underway to identify potential targets of this and other chlamydial kinases, which may interact with host signaling pathways during invasion. In related work, recent studies have evaluated the possibility of a functional Type III secretion mechanism in chlamydia. Fields and Hackstadt (2001) demonstrated that CopN – a homologue to the secreted effector protein YopN of *Yersinia* spp.– is

a substrate for secretion by a heterololgous secretion apparatus in *Yersinia enterocolitica*. Subtil and colleagues (2001) also demonstrated that a heterololgous Type III secretion apparatus in *Shigella flexneri* secretes chlamydial proteins associated with the bacterial inclusion – the so-called Inc proteins– thus expanding the repertoire of putative chlamydial effector molecules and providing the rationale for our pursuit of chlamydial effector kinases. While the direct demonstration of functional Type III secretion in chlamydiae has yet to be shown, I suspect that the diversity of chlamydia interactions with host cell signaling components will become more complex as Type III effector proteins in these bacteria are identified.

THE HOST CELL RESPONSE TO INFECTION

Host cell signaling and inducible gene expression

We demonstrated that specific signaling pathways activated by *C. pneumoniae* were essential for bacterial invasion, which we speculate occurs at the level of the actin cytoskeleton. However, it is also possible that these same signaling pathways pervade deeper into the host cell response to infection at the level of inducible gene expression. This comes from the fact that many downstream substrates of the MEK-ERK pathway are transcription factors (Karin, 1996). Following activation, MEK phosphorylates its downstream target ERK1/2, which then dimerizes and undergoes nuclear translocation to phosphorylate specific nuclear transcription factors capable of modifying gene expression. The most widely studied transcription factor substrates of ERK1/2 are Elk1, ATF-2 and c-Jun, which collectively are involved in inducing transcription of several genes required for cell growth and survival and the induction of proinflammatory cytokines such as TNF-α, IFN-γ, IL-1β and IL-8 (Cobb and Goldsmith, 1995; Rao, 2001). Cutting-edge techniques using functional proteomics and mass spectrometry have revealed that

the effects of activated ERK1/2 may be even more pleiotropic than previously suspected. In a proteomics-based study, Lewis and colleagues (2000) identified cellular targets regulated by the MEK-ERK cascade. Remarkably, they identified twenty-five substrates of this activated cascade, of which only five were previously characterized as MEK-ERK effector molecules. Of the twenty novel targets, functional roles in nuclear transport, DNA repair, membrane trafficking and cytoskeletal rearrangements could be assigned, offering the view that functional outcomes of MEK-ERK signaling may be quite pleiotropic depending on the cellular context (that is, in different cell types and in response to different stimuli). In a study with the intracellular pathogen *Salmonella typhimurium*, the MAP kinase pathway was involved in the nuclear responses of epithelial cells following infection (Hobbie et al., 1997). *S. typhimurium* invasion was associated with Type III secretion-dependent nuclear translocation of ERK and activation and proinflammatory cytokines, thus demonstrating that invasion-associated signals at the cell surface can also be implicated as agonists of the nuclear response of the host.

A similar analogy can be made for the PI 3-kinase/Akt signaling pathway with relation to nuclear factor kappa B (NFκB)-mediated transcriptional activation. NFκB is a ubiquitous transcription factor sequestered in the cytoplasm by a resident inhibitor protein called IκB. NF-κB activation requires phosphorylation and degradation of IκB, thereby releasing NFκB for nuclear translocation. Nuclear NFκB plays an essential role in inducible expression of several proinflammatory cytokines, chemokines and growth factor genes (Barnes and Karin, 1997). Recent evidence suggests that in some anchorage-dependent cell types – such as endothelial cells and epithelial cells –Akt can activate NFκB to restrict apoptosis induced by certain stimuli. Of relevance, both viruses (Thomas et al., 2002) and intracellular bacteria (Mansell et al., 2001) have

been shown to exploit Akt-mediated NFκB activation to induce regulated gene transcription and to sustain the integrity of the host cell during intracellular residency. Data from the *Listeria monocytogenes* model system is intriguing, with several noteworthy similarities to the current discussion pertaining to *C. pneumoniae*. The *L. monocytogenes* surface protein Internalin B (InlB) functions as an invasin by binding directly to the hepatocyte growth factor/Met receptor tyrosine kinase (Shen et al., 2000). InlB-Met binding induces PI 3-kinase signaling in the host that is indispensable for bacterial uptake into epithelial cells (Ireton et al., 1999). It was later demonstrated that this same PI 3-kinase activity (involved in bacterial uptake) leads to Akt-dependent activation of NFκB and induction of IL-8 expression (Mansell et al., 2001). A similar bacterial-induced signaling pathway originating at the cell surface during invasion but also responsible for inducible gene expression has been demonstrated for the enteric pathogen *Yersinia enterocolitica* (Schulte et al., 2000). In this model, attachment of *Yersinia* to the epithelial cell surface via the outer membrane protein invasin triggered NFκB nuclear translocation and induction of IL-8 production by infected cells.

The duality of PI 3-kinase/Akt signaling –facilitating bacterial uptake and an inducible cytokine response by the host– may represent a dynamic interplay between host responses aimed at controlling infection and bacterial virulence mechanisms aimed at exploiting these same responses to gain entry into cellular hosts and colonize the host environment. In the case of *Y. enterocolitica* infection described above, IL-8-mediated recruitment of polymorphonuclear leukocytes (PMN) and local breaches in the mucosal epithelium during PMN extravasation is thought to be a virulence strategy allowing the bacteria access to the basolateral surface of the epithelium and for dissemination within host tissue (McCormick et al., 1997). Given their ability to infect and disseminate hematogenously within leukocytes, *C. pneumoniae*-activation of an

innate inflammatory response and recruitment of mononuclear cells to the site of infection may provide the bacteria with a secondary cellular reservoir in which to gain access to other anatomical sites. The idea that nuclear responses could be a downstream manifestation of the same events that lead to chlamydial invasion is only conjecture at this point. However, it may help establish a unified mechanism linking host signaling during *C. pneumoniae* entry with the subsequent induction of proinflammatory cytokines seen during *C. pneumoniae* infection *in vitro* (Coombes and Mahony, 2001) and *in vivo* (Ward, 1999).

Examining the host cell response to infection at the level of transcription was undertaken to further characterize putative mitogenic factors released by infected endothelial cells. Recall that in previous experiments, we demonstrated that conditioned medium from cultured endothelial cells infected with *C. pneumoniae* was mitogenic for quiescent SMC cultures, inducing SMC DNA replication and proliferation of the cells over a 7-day period. As a follow-up, we used a cDNA filter array to profile the steady-state level of 268 individual human genes in response to *C. pneumoniae* infection, followed by RT-PCR time course experiments for selected gene targets. While this study was modest in nature compared to high-density arrays containing thousands of host-cell genes, it provided the first, more pervasive exploration of host responses to *C. pneumoniae* infection at the level of gene expression. We found that specific proinflammatory cytokines (IL-1β), chemokines (IL-8, MCP-1) and SMC growth factors (FGFb, PDGF-B) were induced following *C. pneumoniae* infection. In more comprehensive time-course experiments we determined that this transcriptional response represented an early event during host cell infection, occurring between 1-6 hours after initial inoculation of cells. With reference to the bacterial invasion process, there was a temporal correlation between ERK1/2 and Akt activity and the kinetics of inducible gene expression. Both gene expression and kinase activity were observed to

overlap at 1-2 hours post-infection (albeit in different cell types –epithelial cells for invasion and intracellular signaling; endothelial cells for inducible gene expression). Work by others has repeatedly shown NFκB nuclear translocation within 15 minutes of *C. pneumoniae* infection in both epithelial (Jahn et al., 2000) and endothelial cells (Krüll et al., 1999), supporting the hypothesis that this transcription factor is involved in the inducible gene expression we observed and that upstream signaling cascades –activated during bacterial invasion– may be implicated in this transcriptional program. By using chemical and genetic inhibition of specific signaling pathways, it would seem possible to map out the host-signaling program responsible for inducible gene expression following *C. pneumoniae* infection.

A limitation of the foregoing studies is that they each measured host cell responses to bacteria undergoing a cyclic developmental cycle –akin to acute infection with actively replicating bacteria. Importantly, a comprehensive investigation of host cell transcription in persistently infected cells has not been undertaken but these experiments are highly justified, considering that chronicity is pathognomonic of chlamydial disease.

As an aside, data from chlamydial microarrays monitoring bacterial gene expression inside host cells are forthcoming and will undoubtedly offer valuable information about gene expression in a bacterial system that is notoriously difficult to work with. A molecular description of the bacterial transcriptional program in response to infection may allow one to (i) predict functions of unknown genes based on their temporal appearance in the bacterial developmental cycle, (ii) characterize the adaptive response of chlamydiae following environmental perturbation, (iii) define a global gene signature of persistent bodies, and (iv) to correlate the expression of putative virulence-associated genes with a given pathology. Investigation into some of these questions is currently underway in our laboratory.

HOST ANIMAL RESPONSE TO INFECTION: LINKS TO THE DISEASE PROCESS

Integrating molecular and cellular responses at the whole animal level

In order to integrate our cellular models with infection in a host animal, we used an established rabbit model of *C. pneumoniae* infection (Fong et al., 1997; 1999). Our rabbit model provided us with both the animal and the disease process, since we had earlier established that intranasal inoculation of rabbits with *C. pneumoniae* produces *de novo* atherosclerosis in the aorta. Links between atherogenesis and the molecular interactions described herein have already been suggested previously (refer to Discussion sections in chapters 4 and 5) but an expanded discussion will follow here.

SMC migration from the arterial media layer into the intima followed by intimal proliferation is an invariant feature of atherosclerosis. Accordingly, our studies focused on SMC proliferation as a potential point of involvement in chlamydia-enhanced atherosclerosis. SMC proliferation in the neointima and release of extracellular matrix components increases the volume of the intima, which encroaches into the vessel lumen resulting in narrowing and various degrees of occlusion. We hypothesized that host cell responses to *C. pneumoniae* infection may involve production of growth factors that could impart biological changes on SMC. Since atherosclerosis is considered an inflammatory-based disease involving endothelial cell injury, we further reasoned that the endothelial cell was a logical candidate to explore this hypothesis. Using *in vitro* techniques, we showed that *C. pneumoniae* infection of endothelial cells induced the production of SMC growth factors that could provoke a mitogenic response in SMC (Coombes and Mahony, 1999). We later identified several candidate SMC growth factors from a cDNA screening array (Coombes and Mahony, 2001) that were potential candidate mediators of the proliferative biological response. We chose to explore PDGF-B in further mechanistic studies

since previous reports implicated this growth factor in atherogenesis (Ross and Glomset, 1973; Ross et al., 1990). PDGF-B mRNA was upregulated in infected endothelial cells as accessed by both cDNA array and RT-PCR (Coombes and Mahony, 2001; Coombes et al., 2002). Furthermore, in a non-hypercholesterolemic rabbit model, there was a positive correlation between aortic intimal thickness and the presence of both *C. pneumoniae* antigen and PDGF-B (Coombes et al., 2002). Likewise, the presence of either *C. pneumoniae* antigen or PDGF-B could predict the presence of the other from the same aortic tissues. Given that *C. pneumoniae* antigen, PDGF-B and a hallmark pathologic feature of atherogenesis (intimal thickening) were co-localized in a temporal and spatial manner in the aorta, we suggested that *C. pneumoniae*-induced SMC proliferation and the resultant intimal thickening might be mediated through PDGF-B, thus offering a molecular mechanism by which *C. pneumoniae* infection could contribute to atherosclerotic disease.

This work is not without interpretive limitations. For example, while we can detect *C. pneumoniae* major outer membrane protein (OmpA), LPS and nucleic acid in aortic tissue, we currently cannot assess the degree of bacterial burden in the vessels. It would be useful to quantify the bacterial load in the tissue and correlate this with a pathological metric – in this case, intimal thickening. To this end, we have attempted quantitative culture to recover infectious EB from tissues. Regrettably, these attempts have been successful (with great difficulty) only in lung tissue shortly after acute infection of rabbits (Dr. J. Mahony, unpublished data). This may be due to existence of the bacteria in a viable, non-cultivable state of persistence that current culture techniques cannot overcome. Perhaps more concerning for the accurate correlation between bacterial burden and disease severity is that even newer molecular-based technology such as nucleic acid amplification tests have shown widely discordant results when applied to clinical

specimens. While *C. pneumoniae* DNA detection in peripheral blood mononuclear cells was – in some cases – predictive of vascular infection (Blasi et al., 1999), others have reported that *C. pneumoniae* copy number does not correlate with atherosclerotic disease severity (Berger et al., 2000). In a recent nine-membered multicenter comparison trial of PCR methods for detecting *C. pneumoniae* DNA in endarterectomy specimens, the (maximum) concordance rate for any single specimen was only 25% (Apfalter et al., 2001). Furthermore, positivity rates for (positive control) atheroma samples varied between 0 and 100% when the identical samples are analyzed by different centers. Currently there are no standardized nucleic acid amplification tests for *C. pneumoniae* and all reported clinical data are based on 'in-house' assays using different primers, a range of reaction conditions and various methods to detect the amplified products. Therefore, since discrepant results might be due to methodological issues, the application of PCR to clinical specimens must be interpreted with caution, especially when sampling error due to low bacterial copy number is suspected.

A problem with toxicity of rabbit tissue homogenates on human epithelial cell cultures was also been noted in our studies, thus precluding quantitative recovery of the organism in culture (Dr. A. Murdin, unpublished data). Furthermore, the aforementioned animal studies did not allow us to uncover the cellular source(s) of *C. pneumoniae* antigen or PDGF-B. While we (Fong et al, 1997) and others (Shor, 2001) have observed *C. pneumoniae* antigen within intimal SMC from rabbit and humans, respectively, this does not preclude other cell types as reservoirs of infection in the vessel wall that could contribute to either *C. pneumoniae* replication and/or PDGF-B production.

Pathogen burden and SMC proliferation

With reference to cytomegalovirus (CMV), another pathogen linked to atherosclerosis, it may be cautiously suggested that SMC proliferation could be an emerging paradigm of pathogen involvement in atherosclerosis. Although CMV is more strongly associated with restenosis following coronary angioplasty and accelerated graft vasculopathy in heart transplant recipients rather than native atherosclerosis, the pathogenesis and cellular involvement are similar. Previous work has demonstrated that CMV induces proliferation and migration of SMC (Zhou et al., 1999). Recently, this same group demonstrated that co-infection with *C. pneumoniae* induced transactivation of the major immediate early promoter of CMV (Wanishsawad et al., 2000), suggesting that if CMV and *C. pneumoniae* do indeed play a role in atherosclerosis, the summation of their individual host cell responses to infection may synergistically contribute to the disease process. These data speak to the concept that cumulative pathogen burden confers an increased risk to coronary artery disease. A recent analysis of human subjects determined that increasing pathogen burden was significantly associated with increasing CAD – with CAD prevalence being 48%, 69%, and 85% in individuals with antibodies to two or more pathogens, to three or four pathogens, and to five pathogens, respectively (Zhu et al., 2000). A second group recently corroborated this finding in a patient population of 572 (Espinola-Klein et al., 2002). Animal models may help to identify potential mechanisms accounting for this phenomenon. While we have not yet pursued these studies in our rabbit model explicitly, we have shown that repeated *C. pneumoniae* inoculations (Fong et al., 1999a) produce more severe atherosclerosis than a single inoculation (Fong et al., 1997). We started using repeat inoculations for all our subsequent animal studies and this practice has also been adopted for the mouse model of *C. pneumoniae*-enhanced atherosclerosis (Moazed et al., 1999). However, more experiments and

improved diagnostics are required to determine to what extent the bacterial load increases in the vascular tree following repeated inoculations compared with a single inoculation.

Immunopathology of chlamydial disease

The fact that atherosclerosis is an immune-mediated disease in the vessel wall (Ross, 1999) is congruous with the evidence suggesting that chlamydial-induced disease is largely immunopathological (Ward, 1995, 1999). That is, the host's immune response to the bacteria induces a prolonged chronic disease that may or may not heal with complications of scarring and tissue damage. This is exemplified by the fact that *C. trachomatis*-induced female infertility and ectopic pregnancy are caused by multiple infectious episodes followed by post-inflammatory fibrosis and scarring of the upper genital tract and fallopian tubes (Van Voorhis et al., 1997).

To date, no observational studies have furnished conclusive evidence of immunopathology due to *C. pneumoniae* infection, but some circumstantial evidence is available. For example, several groups have identified immunoreative T lymphocytes from human atherosclerotic plaques that respond specifically to *C. pneumoniae* antigen (Curry et al., 2000; de Boer et al., 2000; Mosorin et al., 2000). Also, *C. pneumoniae* infection induces (i) mRNA for proinflammatory cytokines and chemokines (Molestina et al., 1998; Coombes and Mahony, 2001), (ii) enhanced transendothelial cell migration of neutrophils and monocytes (Molestina et al., 1999) and (iii) the secretion of proinflammatory cytokines such as TNFα, IL-1 and IFN-γ from monocytes (Kol et al., 1998). Together, these data are consistent with a possible immunopathogenic link between *C. pneumoniae* infection and atherosclerosis.

A recent animal study added insight into the debate on the possible role of *C. pneumoniae* in the immunopathogenesis of CAD. Bachmaier et al. (1999) reported that a peptide motif in the

60-kDa outer membrane protein of *C. trachomatis*, and to a lesser extent *C. pneumoniae*, mimicked a 30 amino acid sequence of the cardiac tissue-specific α-myosin heavy-chain molecule known to induce myocarditis in BALB/c mice. While this finding was reported with strong resolve by the authors, upon closer inspection of the data it appeared unconvincing (to this author) that this type of antigenic mimicry was the 'at long last' discovered molecular mechanism of chlamydia's involvement in heart disease. First, there is no clinical or molecular evidence for such a mechanism in humans. Secondly, the authors did not attempt to determine whether this response was *atherogenic*, rather than merely *immunogenic*. Third, it is possible that other bacterial species harbor peptide sequences analogous to the region of alpha- myosin (only four amino acids were implicated) and yet are not associated with CAD. Finally, the *C. trachomatis* peptide was more effective than the *C. pneumoniae* counterpart at inducing myocarditis in mice when paradoxically, *C. pneumoniae* and not *C. trachomatis* is associated with human heart disease. In a second study, Kol et al. (1999) suggested that antigenic mimicry between chlamydial and human heat shock protein (Hsp)-60 was a potential mechanism of *C. pneumoniae*-induced atherogenesis. This is intriguing in light of the recent finding of anti-chlamydial antibodies to Hsp-60 in 99% of patients with CAD and 0% in controls (Ciervo et al., 2002). However, the latter finding should be tempered with the fact that the ability of recombinant chlamydial Hsp-60 to capture anti-human Hsp-60 IgG (also associated with CAD) was not assessed in cross-reactivity experiments by the authors.

A clear and unified mechanism remains to be fully elucidated. Human studies to date have not adequately discriminated between *C. pneumoniae* as a cause of CAD, a contributing factor in CAD, or a consequence of CAD itself. Sorting out such complicated issues are extremely difficult since the temporal order of events in humans is unknown. Atherogenesis is a

dynamic process involving a consortium of molecular players and representing a continuum of disease progression and possibly regression. This is further complicated by the difficulty in determining when primary *C. pneumoniae* infection took place and by the fact that persistent infections smoldering in tissue can escape our current methods of detection. Animal models have overcome some of these difficulties in that the timing of infection and order of events are controlled, being introduced by design rather than by chance. Based on the current body of literature, it can be suggested that the host cell response to *C. pneumoniae* infection could result in a local inflammatory response that may have implications for the progression of atherosclerosis. Recently, we reviewed the immunopathophysiology of atherosclerosis in the context of *C. pneumoniae* infection with reference to current data from the literature (Mahony and Coombes, 2001). In that essay we presented an integrated model that included the involvement of *C. pneumoniae* in all stages of atherogenesis – initiation, inflammation, fibrous plaque formation, plaque rupture and thrombosis. We speculated that acute and persistent infection of professional immune cells (T-cells, monocytes and macrophages) and non-immune cells (endothelial cells and smooth muscle cells) contributes to a sustained inflammatory response mediated by extensive cellular cross-communication via cytokines, chemokines and growth factors. This cascade of inflammatory mediators may contribute to cellular dysfunction and tissue remodeling of the arterial intima.

LOOKING FORWARD: SUGGESTIONS FOR FUTURE INVESTIGATION

C. pneumoniae invasion

Work presented herein demonstrated that activation of MEK-ERK and PI 3-kinase signaling, together with actin polymerization was necessary for *C. pneumoniae* entry but that

these signaling pathways were independent of attachment to host cell membranes. Potential links between attachment to the host cell surface and activation of host signaling pathways have been discussed above. Identifying adherence mechanisms is difficult to accomplish in obligate intracellular bacteria, but creative use of molecular biology techniques such as expression of dominant-negative mutants, recombinant protein synthesis in the host cell and immunofluorescent confocal microscopy techniques should foster investigation into the bacterial components and host cell receptors implicated in cellular invasion. Of particular interest will be to determine whether activation of host cell signaling during invasion is a feature of bacterial attachment per se, or whether type III effector molecules secreted into host cells after attachment are implicated (or whether both are involved). Identification of chlamydial effector molecules and their intracellular targets will reveal much about chlamydial-host cell interactions. Importantly, because *C. pneumoniae* can infect many different cell types in culture and *in vivo*, comparative studies examining invasion mechanisms in different cell types seems warranted.

C. pneumoniae and atherosclerosis

As described above, many seroepidemiological studies have reported an association between past *C. pneumoniae* infection and the risk of CAD. Numerous pathological studies have corroborated this finding and animal models have substantiated the biological plausibility of this claim. Consistently, laboratories with extensive experience in culturing and detecting *C. pneumoniae* report that this organism can be found in well over 50% of all atherosclerotic arteries but almost never from normal arteries. These findings do not leave much room for reasonable doubt (in this author's opinion) that an association between *C. pneumoniae* and CAD indeed exists. However, the difficult experiments remain: sorting out what role, if any, *C. pneumoniae*

has in this disease process. Since observations of the bacteria in diseased arteries are plentiful, a more constructive research direction might involve understanding the host response to *C. pneumoniae* at the basic molecular level and then applying lead mechanisms to an improved animal model of *C. pneumoniae*-induced atherosclerosis. As mentioned above, the responses of host cells infected with persistent *C. pneumoniae* may reveal differences from acute infections that are pertinent to the discussion of chronic disease.

Towards an improved animal model

Animal models can be considered the proving ground for a biological hypothesis. Reassuringly, outbred rabbits have proven useful for studying potential mechanisms by which *C. pneumoniae* could contribute to atherogenesis. Improved versions of this and other animal models should continue to be applied to this effort. Extensive work by our laboratory using this model (Fong et al., 1997, 1999a, 1999b, Coombes et al., 2002) has revealed that atherosclerosis can ensue following intranasal administration of *C. pneumoniae*. While the percentage of normocholesterolemic rabbits that develop disease in response to infection has, at most, been ~40%, this number is likely due to the contribution of host genetics in conferring a genetic predisposition to disease or increased susceptibility to infection. That only a percentage of animals develop disease following infection may necessitate larger numbers of animals for future studies. In addition, current animal studies have been relatively short in duration, measured on the order of a few months. Longer observation periods seem warranted in order to address the impact of long-term bacterial persistence and chronic infection on the development of more severe arterial pathology. Longer studies aimed at defining the natural history of infection on the

order of years may also be more clinically relevant and directly applicable to the human counterpart.

Animal studies should focus on improving the diagnostic rate of *C. pneumoniae* detection in vascular tissue. Our current methods of detection are hampered by sampling error of the affected tissue and the inability to culture persistent *C. pneumoniae* out of tissue known (by the presence of bacterial antigens, DNA and mRNA transcripts) to harbor viable bacteria. Molecular-based tests to identify a persistent bacterial infection may be useful in this regard, and pursuing ways to reactivate persistent chlamydia particles in culture should be encouraged. Furthermore, additional animal studies are needed to investigate the additive or synergistic effect of *C. pneumoniae* infection superimposed with additional risk cofactors such as smoking, hypercholesterolemia and hypertension. These studies could be initiated in the rabbit, as well as other models using larger mammals such as the miniature pig (de Smet et al., 1998) or primates (Clarkson, 1998). Larger mammals have the added value of applying non-invasive imaging techniques to the coronary arteries, such as B-mode Doppler ultrasound and magnetic resonance imaging to track atherosclerotic changes *in situ*. Application of these techniques may also reduce the number of animals required for a given set of experiments, which may offset the increased cost of working with larger animals.

Future therapeutic applications

Studies at the cellular and molecular level are expected to provide insight into the essential host-pathogen interactions dictating productive chlamydial infection and fundamental disease processes. Elucidating essential host-pathogen interactions that allow a persistent infection to take hold should also be a primary objective of future investigations because it is

these very infections that are so difficult to treat, let alone eradicate. The hope is that elucidation of these essential interactions may lead to the development of new adjunctive therapeutic modalities to supplement our current arsenal of anti-chlamydial antibiotics that arguably have little value in treating chronic, non-replicating chlamydial infections. An obvious target for neutralization would be a *C. pneumoniae*-specific antigen that conferred the ability to invade host cells, or intracellular chlamydia antigens responsible for persistent infection. While we did not find such bacterial proteins in the current studies, we did establish several host cell signal transduction components that were required for bacterial invasiveness. Others have suggested that mammalian signaling molecules might be viable targets for therapeutic intervention of hyperproliferative diseases (Dalgarno et al., 2000; Stein and Waterfield, 2000; Shakespeare, 2001). However, this field is still in its early infancy and key issues of toxicity and overcoming inherent selectivity problems of interfering with a non-binary signaling system are beginning to be debated. The direct application of our data toward a clinical utility awaits further investigation but strategic interference of specific signaling pathways may offer a second tiered approach to supplement the current arsenal of antichlamydial antibiotics.

CLOSING PERSONAL REMARKS

It is clear that chlamydiae are not forthcoming when it comes to revealing their secrets. At times, it appears as if these bacteria know more about mammalian cell biology than cell biologists. Chlamydia's ability to usurp many host cell pathways in order to invade, disseminate and cause disease has been forged out of an intimate relationship with their hosts. Indeed, the wide phylogenic distribution of host species infected with chlamydiae in the wild (including

humans, koalas, horses, boa constrictors, puff adders, African barred frogs, sea turtles and chameleons) argues for an extensive coevolutionary history with host animals.

Studying the cellular microbiology of such a fascinating and complex bacteria is a rewarding, if not arduous business. With no cell-free growth system and no genetic transformation systems, the chlamydia field is not for the faint of heart or for the short of patience. The chlamydia genome sequences have helped to overcome at least some of the limitations plaguing this field, providing scientists with the blueprints for chlamydia virulence – their clandestine ploy revealed, at least in code. Forgive me if I have indulged the reader with too much anthropomorphic reference in the discussion of chlamydia-host interactions. This comes from my enthusiasm for understanding pathogenic traits of microorganisms and from my continual wonderment of the strategies used by pathogens in their interactions with the host.

> The goal of cellular microbiology is to know the enemy
> and its interactions with human cells.
>
> -Dr. Julian Davies, University of British Columbia

APPENDIX A

APPENDIX A

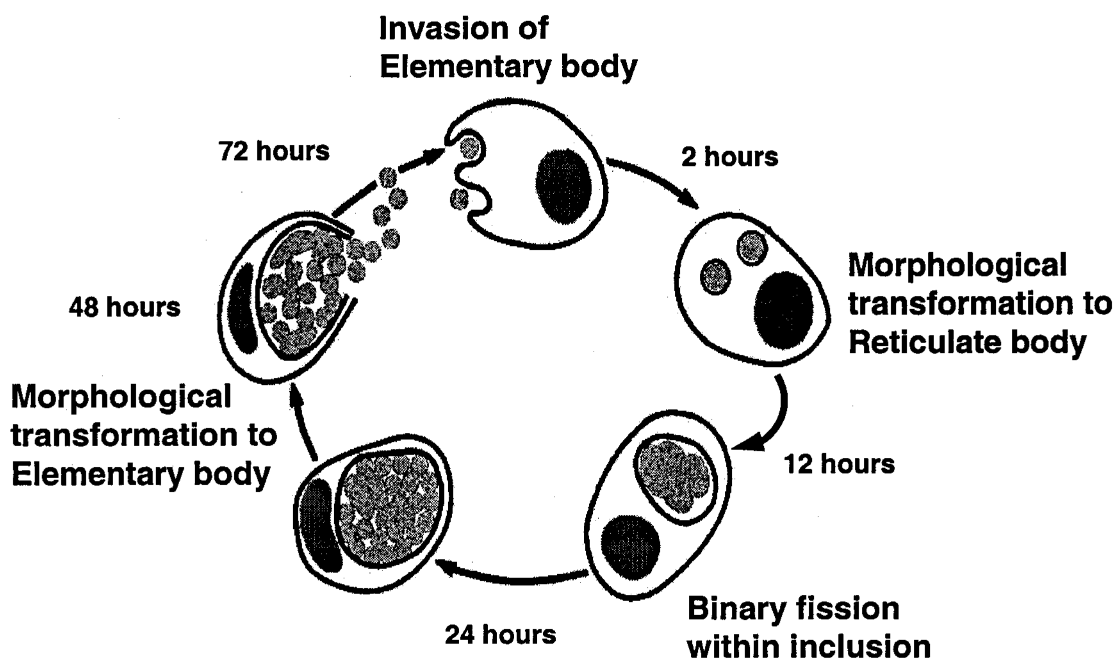
C. PNEUMONIAE REPLICATION CYCLE AND MORPHOLOGICAL FORMS

The following section contains figures describing the *C. pneumoniae* replication cycle and the different morphological forms of the bacteria. Reference to these figures is given in the introductory text in Chapter 1.

Figure A.1 – Developmental cycle of *Chlamydiae*

Figure A.2 – Morphological forms of *C. pneumoniae*

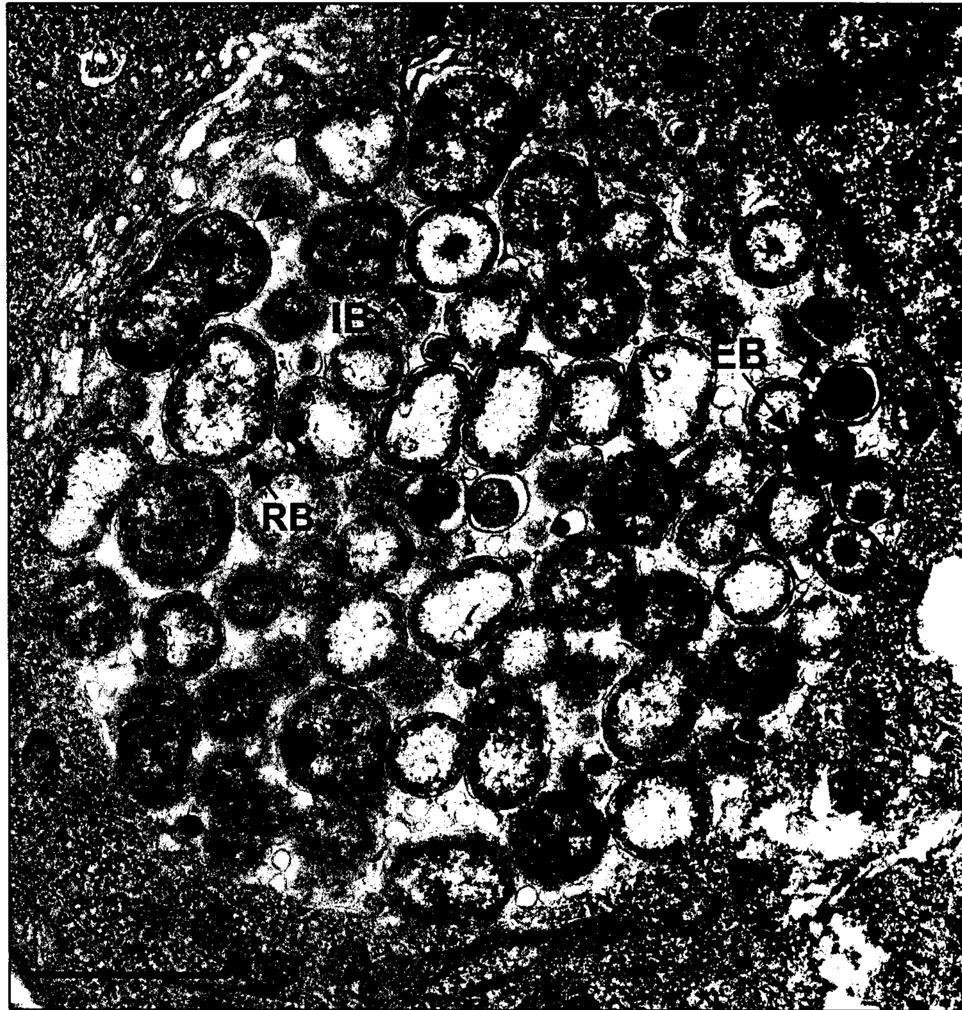
Figure A.1 Developmental Cycle of *Chlamydiae*



Chlamydia invasion is initiated when the extracellular EB attaches to the host cell surface. This event induces host cell changes that facilitate uptake of the EB into a membrane-bound endosome called the inclusion. EB differentiate into RB and begin to divide by binary fission for approximately 48-60 hours. RB then differentiate back into EB prior to lysis of the inclusion and plasma membrane of the host cell. Released EB initiate a new round of development in neighboring host cells.

Figure A.2

Morphological forms of *C. pneumoniae*



A transmission electron photomicrograph depicting the various morphological forms of *C. pneumoniae.* EB, small electron dense elementary body of approximatly 300 nm in diameter. RB, larger less condensed reticulate body that undergoes division by binary fission, approximately 700-1000 nm in diameter. IB, intermediate bodies undergoing morphological transformation either from an EB to RB, or RB to EB. RB undergoing binary fission are highlighted with an arrowhead. Bar, 500 nm. Photographed by Brian K. Coombes

APPENDIX B

APPENDIX B

SUPPLEMENTAL MATERIALS AND METHODS

The following section is a more descriptive Materials and Methods supplement to complement those found in Chapters 2 and 3 of this thesis. The Material and Methods contained in Chapters 4 and 5 are adequately explained for reproducibility purposes.

Supplement to Chapter 2.

***C. pneumoniae* propagation.** *C. pneumoniae* strain VR1310 was purchased from the American Type Culture Collection (ATCC; ATCC-1310VR) and propagated by serial passage in HEp2 cells. HEp2 cells were grown as monolayers in 75-cm^2 culture flasks and inoculated with *C. pneumoniae* in 8 ml of MEM, 10% FBS. Inoculated cells were centrifuged for 45 minutes at 1500 $\times g$ at room temperature, then incubated for 1 hour at 37°C. After 1 hour of incubation, the inoculum was removed and replaced with 15 ml of MEM, 10% FBS containing 1 μg/ml cycloheximide. Infected cells were cultured for 72 hours at 37°C, 5% CO_2. To harvest *C. pneumoniae*, infected cells were lysed with 3 mm sterile glass beads and the crude cell lysate was transferred to a 50 ml centrifuge tube on ice. Cells were further bead-lysed and disrupted in a water bath sonicator using three 8-second pulses, and placed on ice briefly in-between pulses. Cell debris was removed from the lysate by centrifugation at 1000 $\times g$ for 10 minutes at 4°C. The supernatant containing suspended *C. pneumoniae* EB was transferred to a new sterile centrifuge tube. EB were then collected by high-speed centrifugation at 38,000 $\times g$ for 45 minutes at 4°C. The high-speed supernatant was removed and discarded and the EB pellet was resuspended in either MEM; 10% FBS (for serial passage onto fresh HEp2 cells), or was resuspended in a

mixture of 50% MEM and 50% sucrose-phosphate-glutamic acid buffer (SPG; 100% SPG contains 75 g sucrose, 0.52 g KH_2PO_4, 1.22 g Na_2HPO_4, 0.72 g glutamic acid per 1L; pH 7.4, sterilized by filtration through 0.2 μM pore filters). For titration of *C. pneumoniae*, log-dilutions of the inoculum were prepared in MEM, and 0.25 ml of each dilution was inoculated onto HEp2 cells grown in 1-cm^2 glass shell vials as described above. At 48 hours post-infection, the medium was removed from the shell vials and the infected cells were washed once with PBS. Cells were then fixed and permeabilized in 100% ethanol for 10 minutes at room temperature. To stain intracellular inclusions, fixed cells were rinse briefly with distilled water, then incubated for 30 minutes in a humid chamber with a monoclonal antibody directed against the lipopolysaccharide (LPS) of chlamydia, which was directly conjugated with the fluorochrome fluorescein isothiocyanate (FITC). Cells were rinsed briefly with distilled water, wet-mounted onto glass slides and visualized with an epi-fluorescent microscope. Green intracellular inclusions were enumerated from each appropriate dilution of inoculum and expressed as inclusion forming units (IFU) per ml.

Testing of all cultures for Mycoplasma. All stocks of mammalian cell lines and *C. pneumoniae* isolates were routinely tested for the presence of *Mycoplasma* spp. using a genus-specific PCR method developed by van Kuppeveld et al. (van Kuppeveld et al., 1994). Supernatant samples and whole-cell fractions from actively growing cultures were centrifuged at 20,000 ×*g* for 20 minutes to pellet cellular material. The supernatant was removed and the pellet resuspended in 0.2 ml of PBS supplemented with 100 units of Proteinase K. Samples were incubated at 56°C for 30 minutes and then DNA was extracted using either phenol-chloroform extraction with alcohol precipitation of the aqueous phase, or using Qiagen DNA spin-columns according to the

manufacturer's instructions. DNA was eluted in a final volume of 50 µl with distilled / deionized water and 5 µl was used for Mycoplasma genus-specific PCR. PCR reactions contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate, 0.5 µM of each primer and 1 unit of Taq Gold DNA polymerase. Oligonucleotide primers were as follows: forward, 5' GGG AGC AAA CAG GAT TAG ATA CCC T 3' and reverse, 5' TGC ACC ATC TGT CAC TCT GTT AAC CTC 3'. All primers were synthesized by the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University, Hamilton, ON. After an initial incubation at 95°C for 10 minutes to activate the DNA polymerase, reactions were subjected to 40 cycles of the following thermal program: 95° C for 1 min, 55° C for 1 min and 72°C for 75 seconds. This was followed by a final incubation at 72° C for 8 minutes. 10 µl of the reaction was resolved on 2% agarose gels containing 0.25 mg/ml ethidium bromide and visualized under ultraviolet light. Specific reaction products migrate as a 280 bp species. Positive controls consisted of purified DNA from *M. hominis*, *M. pneumoniae*, or *M. genitalium*.

Preparation of cells for transmission electron microscopy- HEp2 cells were grown in 24-well plates and infected according to a modified procedure to that described above. For inoculation, HEp2 cells were overlaid with a suspension of EB in 0.25 ml volume to achieve a multiplicity of infection of ~50. Cells were not centrifuged, or exposed to cycloheximide during the infection procedure. After the desired time of infection (15 to 120 minutes), the inoculum was removed and the cells were washed briefly with sodium cacodylate buffer. Cells were then fixed with 2% glutaraldehyde in sodium cacodylate buffer, pH 7.4 for 1 h at 4°C, and then rinsed twice for 5

min with sodium cacodylate buffer. Fixed cells were post-fixed with 50% osmium tetroxide for 30 min at room temperature. The sample was then dehydrated in a graded ethanol series (2 × 5 min in 70% EtOH; 3 × 10 min in 95% EtOH; 4 × 10 min in 100% EtOH) at room temperature. After the last alcohol incubation, the cells were overlaid with 100% propylene oxide and the plates were gently shaken. Immediately using a Pasteur pipette, the sample was aspirated from the plastic dish and transferred to a 1.5 ml microcentrifuge tube. The sample was gently pelleted by centrifugation at 5000 rpm in a microcentrifuge and the propylene oxide layer was replaced with fresh propylene oxide for 5 min. The sample was then overlaid with a mixture of 50% propylene oxide/50% Spurr's resin and mixed in a sample spinner for 30 min. The solution was removed and replaced with a 3:1 mixture of resin: propylene oxide and mixed for 30 min. The final mixture contained 100% resin, which was mixed with the sample for 1 h. The sample was then transferred to a bean capsule and filled with resin to the top. Filled capsules were tightly sealed and centrifuged for 20 min at 2000 × *g* to sediment the sample. Plastic resin was polymerized overnight at 40°C.

Determination of protein concentration of cell lysates – The Bradford method for protein determination was used, which is a dye-binding assay in which an acidic solution of Coomassie Brilliant Blue G-250 is added to the protein in solution. The absorbance maximum for an acidic solution of G-250 dye shifts from 465 nm to 595 nm when bound to basic and aromatic amino acid residues. Protein standards of 1.8, 4.5, 9.0, 14.4, and 23.4 μg.ml were prepared in water with bovine γ-globulin. For each unknown sample, 2.5 or 5 μl was used for protein determination. To separate tubes, an aliquot of the unknown samples and the standards were brought up to 0.8 ml

final volume with distilled water. 0.2 ml of concentrated dye reagent (BioRad catalog number 500-0006) was added to each tube and solutions were mixed immediately with a vortex mixer. Reactions were incubated 5 min at room temperature and the absorbance of the solution was read immediately in a spectrophotometer, pre-warmed and set at 595 nm (VIS). The 'blank' consisted of 0.8 ml of distilled water containing 0.2 ml of dye reagent.

Activation of sodium orthovanadate – Na_3VO_4 (inhibitor of ATPase, alkaline phosphatase and tyrosine phosphatase) was included in the Triton X-100 lysis buffer used for immunoblotting and immunoprecipitation. Stock solutions of 1mM Na_3VO_4 were prepared in distilled water and adjusted to pH 10.0 with NaOH. The yellow solution was heated to boiling until it appeared translucent, at which point the pH was re-adjusted to pH 10 after the solution had returned to room temperature. If the solution returned to yellow in colour, it was re-heated and the pH was adjusted to 10 as described above until the solution remained colourless at pH 10. The activated solution was stored in aliquots at -20°C and used within 4 months.

SDS-PAGE gels and Western transfer – The running buffer for SDS-PAGE gels was diluted fresh from a 10× stock. 10× SDS-PAGE running buffer stock consisted of 0.25 M Tris base, 1.92 M glycine and 1% SDS. Conditions for protein separation was typically 20-30 mA for 3 h. The buffer used for electro-transfer of proteins from SDS-PAGE gels to nitrocellulose membranes contained 25 mM Tris base, 192 mM glycine and 20% (v/v) methanol. This buffer was prepared fresh from stock reagents for each use. Western transfers were typically performed at 70 V for 3-5 h, or at 20 V overnight.

Supplement to Chapter 3.

DNase 1 digestion of RNA samples – RNA samples isolated for cDNA synthesis were first treated with RNase-free DNase 1 to remove traces of genomic DNA. To 0.5 ml microcentrifuge tubes on ice, the following was added: 1-2 μg of total RNA, 1 μl 10× reaction buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl, 20 mM $MgCl_2$], 1 μl amplification-grade DNase 1, RNase-free distilled water to a final volume of 10 μl. Reactions were incubated at room temperature for 15-20 minutes. To stop reactions, 1 μl of 25 mM EDTA was added and the reactions were heated at 65°C for 15 minutes. The reactions were placed on ice for 5 minutes, then extracted with phenol-chloroform-isoamyl alcohol and precipitated with 2.5 volumes of absolute ethanol, 0.1 volume of 2 M sodium acetate, pH 4.5. Total RNA was collected by centrifugation, washed with 75% ethanol and resuspended in distilled water. mRNA was purified from total RNA samples as described in Chapter 3.

Denaturing agarose-formaldehyde gels for RNA electrophoresis – The gel apparatus was pretreated with 3% H_2O_2 and rinsed with RNase-free water prior to use. Gels were prepared by combining 1.2 g agarose, 10 ml of 10× MOPS buffer [200 mM MOPS, 50 mM sodium acetate, 10 mM EDTA], and 72 ml of RNase-free water. The agarose was melted in a microwave and allowed to cool to 65°C. Formaldehyde was added to 2.2 M final concentration, mixed well and polymerized in gel molds for 45 min. Gels were equilibrated in 1× MOPS buffer for 15 min prior to loading RNA samples. For preparation of RNA samples, 5 μl of RNA was mixed with 1 μl of 10× MOPS buffer, 3.5 μl of formaldehyde and 10 μl formamide. Tubes were heated for 10 min

at 65°C, placed on ice and 4 µl of 6× bromophenol blue/xylene cyanol loading buffer was added to each sample [6× bromophenol blue/xylene cyanol buffer contains 0.25% bromophenol blue, 0.25% xylene cyanol FF, 40% (w/v) sucrose in distilled water]. Samples were loaded onto gels and electrophoresed at 50 V in 1× MOPS buffer until the dye front migrated near the bottom of the gel. Gels were stained with 0.25 µg/ml ethidium bromide, rinsed with distilled water and photographed under ultraviolet illumination.

APPENDIX C

APPENDIX C

INVASION ASSAY SUPPLEMENT

The following section contains data to supplement the invasion assay experiments described in Chapter 2.

HEp2 cells were infected with [^{35}S]-radiolabeled *C. pneumoniae* EB as described in the Experimental Procedures section in chapter 2. The counts per minute (CPM) of radioactivity contained in the HEp2 cell-associated fraction – representing trypsin-resistant, internalized EB – was determined by lysing the HEp2 cell pellet and counting the cell lysate in the liquid scintillation counter. The CPM in the supernatant fraction – representing trypsin-sensitive, surface attached EB – was also determined by liquid scintillation counting. The data presented in Table C.1 is representative of an invasion assay performed in duplicate at a multiplicity of infection of ~ 10. Under these conditions, approximately 35%-45% of total EB were typically cell-associated (either surface attached or internalized) after 2 hours of invasion. Table C.1 shows the distribution of cell-associated bacteria after 2 hours of infection.

Table C.1 – Invasion assay supplemental data

Figure C.1 – [^{35}S]-*C. pneumoniae* invasion assay: distribution of cell-associated bacteria

These data are graphically presented in Figure C.1. In this figure, the distribution of internalized bacteria (solid bars) or surface attached bacteria (hatched bars) is displayed as a fraction of the total cell-associated bacteria (100%) in the presence or absence of the indicated inhibitors.

Table C.1 Invasion assay supplemental data

Internalized bacteria

SAMPLE	CPM (internalized) [a]		Avg CPM (internal)
control (DMSO)	64,448.00	57,058.00	60,753.00
1 um U0126	36,798.00	28,087.00	32,442.50
10 uM U0126	30,666.00	17,030.00	23,848.00
10 uM LY29	27,488.00	24,519.00	26,003.50
50 uM LY29	18,935.00	14,814.00	16,874.50
LY29+U0126	10,933.00	7,165.00	9,049.00
cytoD	17,510.00	9,345.00	13,427.50

SAMPLE	% of total bacteria internalized		(Avg)
control (DMSO)	72.76	64.42	68.59
1 um U0126	40.82	31.16	35.99
10 uM U0126	38.66	21.47	30.07
10 uM LY29	35.96	32.08	34.02
50 uM LY29	29.17	22.82	25.99
LY29+U0126	15.93	10.44	13.18
cytoD	23.68	12.64	18.16

Attached bacteria

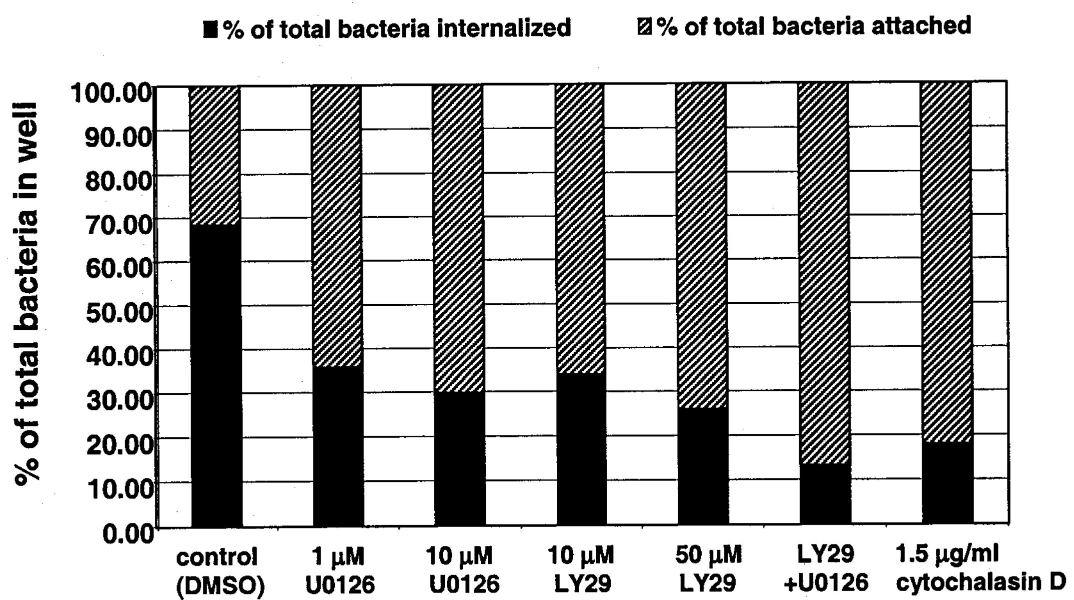
SAMPLE	CPM (attached)		Avg CPM (attached)
control	27,322.00	28,320.00	27,821.00
1 um U0126	67,930.00	47,460.00	57,695.00
10 uM U0126	59,881.00	51,061.00	55,471.00
10 uM LY29	49,947.00	50,914.00	50,430.50
50 uM LY29	49,945.00	46,146.00	48,045.50
LY29+U0126	56,401.00	62,782.00	59,591.50
cytoD	53,273.00	67,781.00	60,527.00

SAMPLE	% of total bacteria attached		(Avg)
control (DMSO)	30.85	31.97	31.41
1 um U0126	75.36	52.65	64.01
10 uM U0126	75.49	64.37	69.93
10 uM LY29	65.35	66.61	65.98
50 uM LY29	76.93	71.08	74.01
LY29+U0126	82.17	91.46	86.82
cytoD	72.03	91.65	81.84

SAMPLE	Total CPM
control (DMSO)	88,574.00
1 um U0126	90,137.50
10 uM U0126	79,319.00
10 uM LY29	76,434.00
50 uM LY29	64,920.00
LY29+U0126	68,640.50
cytoD	73,954.50

[a] samples measured in duplicate
CPM, counts per minute

Figure C.1 [^{35}S]-*C. pneumoniae* invasion assay: Distribution of cell-associated bacteria



APPENDIX D

APPENDIX D

EFFECT OF *C. PNEUMONIAE* DOSE ON THE INDUCTION OF HOST MEMBRANE MICROVILLI: AN ELECTRON MICROSCOPIC STUDY

The following section describes the results of a scanning electron microscopic study that examined the effect of *C. pneumoniae* dose on the formation of host membrane microvilli.

HEp2 cells were cultured in 8-well chamber slides (0.64 cm^2 growth area per well) for ~20 h at 37°C, 5% CO2. For infection with *C. pneumoniae*, HEp2 cells were overlaid with a suspension of *C. pneumoniae* EB prepared in SF-MEM to achieve the desired multiplicity of infection (MOI) per host cell. Cells were infected without centrifugation and in the absence of cycloheximide. After incubation at 37°C, 5% CO2 for the specified time interval, infected cells were processed for scanning electron microscopy (SEM). For SEM, HEp2 cells were fixed in 2% glutaraldehyde for 1 h at 4°C, post-fixed in osmium tetroxide for 1 h at room temperature, and then dehydrated with a graded series of ethanol *in situ* (the dehydration protocol was as follows: 2 × 5 min 50% ethanol, 2 × 5 min, 75% ethanol, 3 × 10 min, 95% ethanol, 3 × 10 min 100% ethanol). Dehydrated samples were critical point dried, sputter-coated with gold and viewed with a Jeol JSM-840 scanning electron microscope.

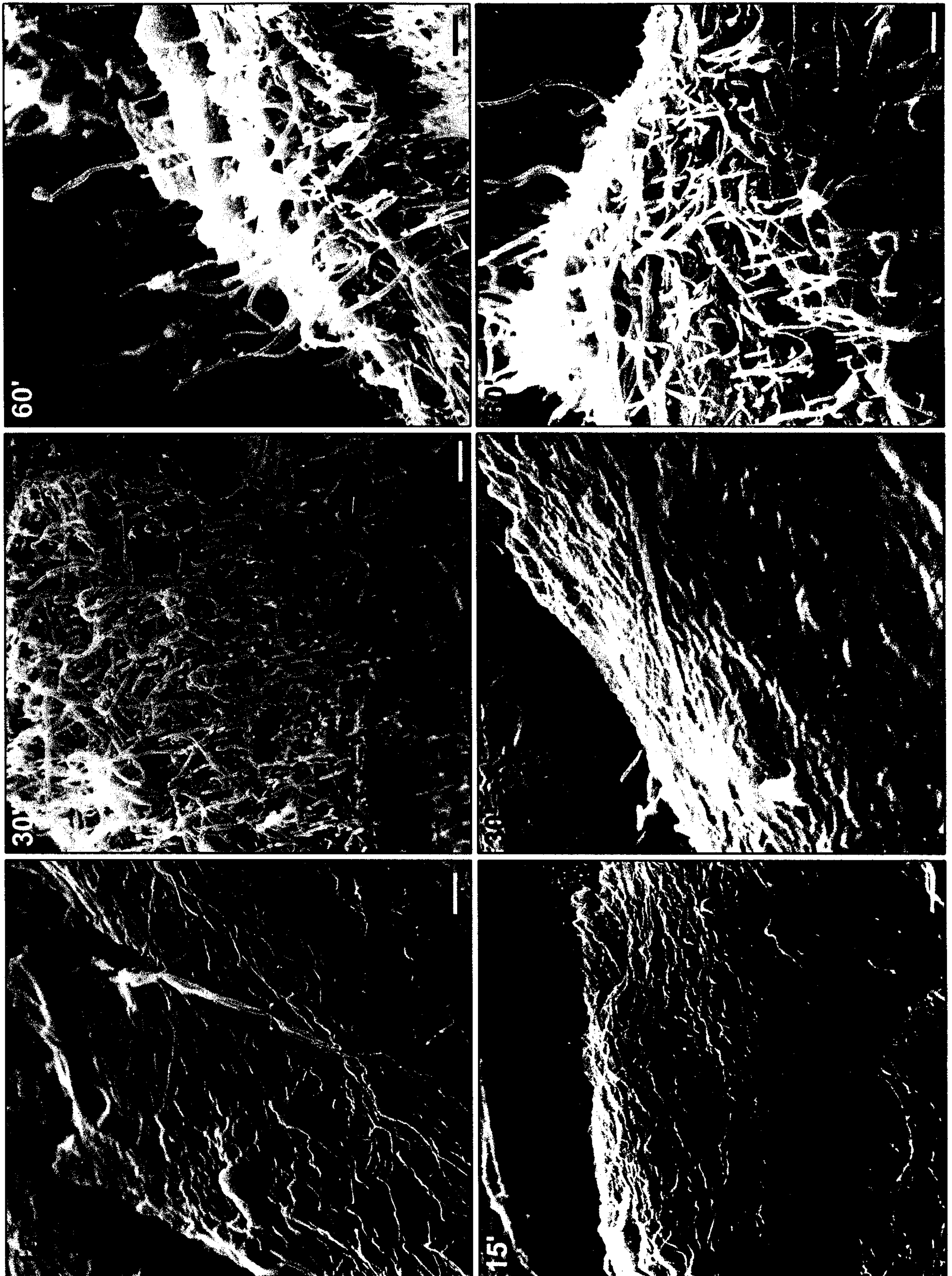
Figure D.1 – Effect of *C. pneumoniae* dose on the formation of membrane microvilli

HEp2 cells were infected with *C. pneumoniae* as described in the 'Materials and Methods' section of chapter 2, then processed for SEM as described.

The formation of membrane microvilli on the surface of HEp2 cells could be delayed with decreasing doses of *C. pneumoniae*. Figure D.1 shows that in cells treated with *C. pneumoniae* at an MOI of 10, (top panels) the formation of membrane microvilli was first noted at 30 minutes after addition of bacteria and remained present until 60 minutes after infection when the experiment was stopped. In cells infected with a 10-fold decreased dose of *C. pneumoniae* (bottom panels, MOI ~1), the formation of membrane microvilli was delayed further until 60 minutes after infection. Recall that in cells infected at a high MOI (chapter 2, Figure 2.1, MOI ~50), the formation of membrane microvilli was observed very early after addition of bacteria (within 15 minutes) and was largely attenuated by 60-120 minutes after initial infection.

In Figure D.1, the numbers in individual panels represent the time in minutes of *C. pneumoniae* infection. Bars represent 1 μm.

Figure D.1



REFERENCES

REFERENCES

Ades, E.W., Candal, F.J., Swerlick, R.A., George, V.G., Summers, S., Bosse, D.C., Lawley, T.J. (1992) HMEC-1: establishment of an immortalized human microvascular endothelial cell line. J. Invest. Dermatol. 99:683-690.

Apfalter, P., Loidl, M., Nadrchal, R., Makristathis, A., Rotter, M., Bergmann, M., Polterauer, P., Hirschl, A.M. (2000) Isolation and continuous growth of *Chlamydia pneumoniae* from arterectomy specimens. Eur. J. Clin. Microbiol. Infect. Dis. 19:305-308.

Apfalter, P., Blasi, F., Boman, J., Gaydos, C.A., Kundi, M., Maass, M., Makristathis, A., Meijer, A., Nadrchal, R., Persson, K., Rotter, M.L., Tong, C.Y., Stanek, G., Hirschl, A.M. (2001) Multicenter comparison trial of DNA extraction methods and PCR assays for detection of *Chlamydia pneumoniae* in endarterectomy specimens. J. Clin. Microbiol. 39:519-524.

Aplin, A.E., Stewart, S.A., Assoian, R.K., Juliano, R.L. (2001) Integrin-mediated adhesion regulates ERK nuclear translocation and phosphorylation of Elk-1. J. Cell Biol. 153:273-282.

Auge, N., Escargueil-Blanc, I., Lajoie-Mazenc, I., Suc, I., Andrieu-Abadie, N., Pieraggi, M.T., Chatelut, M., Thiers, J.C., Jaffrezou, J.P., Laurent, G., Levade, T., Negre-Salvayre, A., Salvayre, R. (1998) Potential role for ceramide in mitogen-activated protein kinase activation and proliferation of vascular smooth muscle cells induced by oxidized low density lipoprotein. J. Biol. Chem. 273: 12893-12900.

Bachmaier, K., Neu, N., de la Maza, L.M., Pal, S., Hessel, A., Penninger, J.M. (1999) Chlamydia infections and heart disease linked through antigenic mimicry. Science 283:1335-1339.

Barnes, P.J., Karin, M. (1997) Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. N. Engl. J. Med. 336:1066-1071.

Beatty, W.L., Morrison, R.P., Byrne, G.I. (1994) Persistent chlamydia: from cell culture to a paradigm for chlamydial pathogenesis. Microbiol. Rev. 58: 686-699.

Bell, T.A., Kuo, C.C., Wang, S.P., Grayston, J.T. (1989) Experimental infection of baboons (papio-cynocephalus arubis) with *Chlamydia pneumoniae* strain TWAR. J. Infect. 19:47-49.

Berger, M., Schroder, B., Daeschlein, G., Schneider, W., Busjahn, A., Buchwalow, I., Luft, F.C., Haller, H. (2000) *Chlamydia pneumoniae* DNA in non-coronary atherosclerotic plaques and circulating leukocytes. J. Lab. Clin. Med.136:194-200.

Berliner, J. A., Navab, M., Fogelman, A.M., Frank, J.S., Demer, L.L., Edwards, P.A., Watson, A.D., Lusis, A.J. (1995) Atherosclerosis: basic mechanisms; oxidation, inflammation and genetics. Circulation. 91: 2488-2496.

Birkelund, S., Johnsen, H., Christiansen, G. (1994) *Chlamydia trachomatis* serovar L2 induces protein tyrosine phosphorylation during uptake by HeLa cells. Infect Immun **62**: 4900-4908.

Blasi, F., Boman, J., Esposito, G., Melissano, G., Chiesa, R., Cosentini, R., Tarsia, P., Tshomba, Y., Betti, M., Alessi, M., Morelli, N., Allegra, L. (1999) *Chlamydia pneumoniae* DNA detection in peripheral blood mononuclear cells is predictive of vascular infection. J. Infect. Dis. 180: 2074-2076.

Boleti, H., Benmerah, A., Ojcius, D.M., Cerf-Bensussan, N., Dautry-Varsat, A. (1999) Chlamydia infection of epithelial cells expressing dynamin and Eps15 mutants: clathrin-independent entry into cells and dynamin-dependent productive growth. J. Cell Sci. 112 (Pt 10):1487-1496.

Bondeva, T., Pirola, L., Bulgarelli-Leva, G., Rubio, I., Wetzker, R., Wymann, M.P. (1998) Bifurcation of lipid and protein kinase signals of PI3-K gamma to the protein kinase PKB and MAPK. Science. 282:293-296.

Burnett, M.S., Gaydos, C.A., Madico, G.E., Glad, S.M., Paigen, B., Quinn, T.C., Epstein, S.E. (2001) Atherosclerosis in apoE knockout mice infected with multiple pathogens. J. Infect. Dis. 183: 226-231.

Byrne, G.I., Moulder, J.W. (1978) Parasite-specified phagocytosis of *Chlamydia psittaci* and *Chlamydia trachomatis* by L and HeLa cells. Infect. Immun. 19: 598-606.

Byrne, G.I., Ouellette, S.P., Wang, Z., Rao, J.P., Lu, L., Beatty, W.L., Hudson, A.P. (2001) *Chlamydia pneumoniae* expresses genes required for DNA replication but not cytokinesis during persistent infection of HEp-2 cells. Infect. Immun. 69: 5423-5429.

Caldwell, H.D., Kromhout, J., Schachter. J. (1981) Purification and partial characterization of the major outer membrane protein of *Chlamydia trachomatis*. Infect. Immun. 31: 1161-1176.

Caligiuri, G., Rottenberg, M., Nicoletti, A., Wigzell, H., Hansson, G.K. (2001) *Chlamydia pneumoniae* infection does not induce or modify atherosclerosis in mice. Circulation 103: 2834-2838.

Campbell, J.H, Campbell, G.R. (1989) Biology of the vessel wall and atherosclerosis. Clin. Exp. Hypertens. 11: 901-913.

Campbell, J.H., Black, M.J., Campbell, G.R. (1989) Replication of smooth muscle cells in atherosclerosis and hypertension. *In P. Meyer and P. Marche (eds).* New York Raven Press. Atherosclerosis Rev. vol 19. pp15-33.

Campbell, L. A., O'Brien, E.R., Cappuccio, A.L., Kuo, C.C., Wang, S.P., Stewart, D., Patton, D.L., Cummings, P.K., Grayston, J.T. (1995) Detection of *Chlamydia pneumoniae* TWAR in human coronary atherectomy tissues. J. Infect. Dis. 172: 585-588.

Carabeo, R.A., Hackstadt, T. (2001) Isolation and characterization of a mutant Chinese hamster ovary cell line that is resistant to *Chlamydia trachomatis* infection at a novel step in the attachment process. Infect. Immun. 69: 5899-5904.

Cartun, R.W., Pedersen, C.A. (1989) An immunocytochemical technique offering increased sensitivity and lowered cost with a strepavidin-horseradish peroxidase conjugate. J. Histotechnol.12: 273-277.

Chang, L., Karin, M. (2001) Mammalian MAP kinase signalling cascades. Nature 401: 37-40.

Chen, H.C., Guan, J.L. (1994) Association of focal adhesion kinase with its potential substrate phosphatidylinositol 3-kinase. Proc. Natl. Acad. Sci. U S A. 91: 10148-10152.

Christerson, L.B., Vanderbilt, C.A., Cobb, M.H. (1999) MEKK1 interacts with alpha-actinin and localizes to stress fibers and focal adhesions. Cell Motil. Cytoskeleton.43:186-198.

Chuenkova, M., Furnari, F.B., Cavenee, W.K.,. Pereira, M.A. (2001) *Trypanosoma cruzi* tans-sialidase: a potent and specific survival factor for human Schwann cells by means of phosphatidylinositol 3-kinase/Akt signaling. Proc. Natl. Acad. Sci. USA. 98: 9936-9941.

Ciervo, A., Visca, P., Petrucca, A., Biasucci, L.M., Maseri, A., Cassone, A. (2002) Antibodies to 60-kilodalton heat shock protein and outer membrane protein 2 of *Chlamydia pneumoniae* in patients with coronary heart disease. Clin. Diagn. Lab. Immunol. 9:66-74.

Clarkson, T.B. (1998) Nonhuman primate models of atherosclerosis. Lab. Anim. Sci. 48:569-572.

Clausen, J.D., Christiansen, G., Holst, H.U., Birkelund, S. (1997) *Chlamydia trachomatis* utilizes the host cell microtubule network during early events of infection. Mol. Microbiol. 25: 441-449.

Cobb, M.H., Goldsmith, E.J. (1995) How MAP kinases are regulated. J. Biol. Chem. 270:14843-14846.

Coombes, B.K., Mahony, J.B. (2001) cDNA array analysis of altered gene expression in human endothelial cells in response to *Chlamydia pneumoniae* infection. Infect. Immun. 69: 1420-1427.

Coombes, B.K., Mahony, J.B. (1999) *Chlamydia pneumoniae* infection of human endothelial cells induces proliferation of smooth muscle cell via an endothelial cell-derived soluble factor(s). Infect. Immun. 67: 2909-2915.

Coombes, B.K., Chiu, B., Fong, I.W., Mahony, J.B. (2002) *Chlamydia pneumoniae* infection of endothelial cells induces transcriptional activation of PDGF-B: a potential link to intimal thickening in a rabbit model of atherosclerosis. J. Infect. Dis. *In press*.

Cossart, P., Boquet, P., Normark, S., Rappuoli, R. (1996) Cellular microbiology emerging. Science 271:315-316.

Cummings, C.A., Relman, D.A. (2000) Using DNA microarrays to study host-microbe interactions. Emerg. Infect. Dis. 6: 513-525.

Curry, A.J., Portig, I., Goodall, J.C., Kirkpatrick, P.J., Gaston, J.S. (2000) T lymphocyte lines isolated from atheromatous plaque contain cells capable of responding to Chlamydia antigens. Clin. Exp. Immunol. 121:261-269.

Czerucka, D., Dahan, S., Mograbi, B., Rossi, B., Rampal, P. (2001) Implication of mitogen-activated protein kinases in T84 cell responses to enteropathogenic *Escherichia coli* infection. Infect. Immun. 69: 1298-1305.

Dalgarno, D.C., Metcalf, C.A., Shakespeare, W.C., Sawyer, T.K. (2000) Signal transduction drug discovery: targets, mechanisms and structure-based design. Curr. Opin. Drug Discov. Dev. 3:549-564.

Danesh, J., Collins, R., Peto, R. (1997) Chronic infections and coronary heart disease: is there a link? Lancet 350: 430-436.

Datta, S.R., Brunet, A., Greenberg, M.E. (1999) Cellular survival: a play in three Akts. Genes Dev. 13: 2905-2927.

Dean, D., Powers, V.C. (2001) Persistent *Chlamydia trachomatis* infections resist apoptotic stimuli. Infect. Immun. 69: 2442-2447.

de Boer, O.J., van der Wal, A.C., Houtkamp, M.A., Ossewaarde, J.M., Teeling, P., Becker, A.E. (2000) Unstable atherosclerotic plaques contain T-cells that respond to *Chlamydia pneumoniae*. Cardiovasc. Res. 48: 402-408.

Dechend, R., Maass, M., Gieffers, J., Dietz, R., Scheidereit, C., Leutz, A., Gulba, D.C. (1999) *Chlamydia pneumoniae* infection of vascular smooth muscle and endothelial cells activates NF-κB and induces tissue factor and PAI-1 expression. Circulation 100: 1369-1373.

Der, S.D., Zhou, A., Williams, B. R., Silverman, R.H. (1998) Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. Proc. Natl. Acad. Sci. USA. 95: 15623-15628.

de Smet, B.J., van der Zande, J., van der Helm, Y.J., Kuntz, R.E., Borst, C., Post, M.J. (1998) The atherosclerotic Yucatan animal model to study the arterial response after balloon angioplasty: the natural history of remodeling. Cardiovasc. Res.39:224-232.

DeVinney, R., Steele-Mortimer, O., Finlay, B.B. (2000) Phosphatases and kinases delivered to the host cell by bacterial pathogens. Trends Microbiol. 8:29-33.

De Waard, V., van den Berg, B.M.M., Veken, J., Schultz-Heienbrok, R., Pannekoek, H., van Zonneveld, A.J. (1999) Serial analysis of gene expression to access the endothelial cell response to an atherogenic stimuli. Gene 266: 1-8.

Dramsi, S., Cossart, P. (1998) Intracellular pathogens and the actin cytoskeleton. Annu. Rev. Cell Dev. Biol.14: 137-166.

Duggan, D.J., Bittner, M., Chen, Y., Meltzer, P., Trent, J.M. (1999) Expression profiling using cDNA microarrays. Nat. Genet. 21 (Suppl. 1): 10-14.

Eckmann, L., Smith, J.R., Housley, M.P., Dwinell, M.B., Kagnoff, M.F. (2000) Analysis by high density cDNA arrays of altered gene expression in human intestinal epithelial cells in response to infection with the invasive enteric bacteria *Salmonella*. J. Biol. Chem. 275: 14084-14094.

Ehrlich, L.C., Hu, S., Sheng, W.S., Sutton, R.L., Rockswold, G.L., Peterson, P.K., Chao, C.C. (1998) Cytokine regulation of human microglial cell IL-8 production. J. Immunol. 160: 1944-1948.

Engelse, M.A., Neele, J.M., van Achterberg, T.A., van Aken, B.E., van Schaik, R.H., Pannekoek, H., de Vries, C.J. (1999) Human activin-A is expressed in the atherosclerotic lesions and promotes the contractile phenotype of smooth muscle cells. Circ. Res. 85: 931-939.

Eramaa, M., Hurme, M., Stenman, U.H., Ritvos, O. (1992) Activin A/erythroid differentiation factor is induced during human monocyte activation. J. Exp. Med. 176: 1449-1452.

Ericson, K., Saldeen, T.G., Lindquist, O., Pahlson, C., Mehta, J.L. (2000) Relationship of *Chlamydia pneumoniae* infection to severity of human coronary atherosclerosis. Circulation 101:2568-2571.

Espinola-Klein, C., Rupprecht, H.J., Blankenberg, S., Bickel, C., Kopp, H., Rippin, G., Victor, A., Hafner, G., Schlumberger, W. (2002) Impact of infectious burden on extent and long-term prognosis of atherosclerosis. Circulation 105:15-21.

Everett, K.D., Bush, R.M., Andersen, A.A. (1999) Emended description of the order Chlamydiales, proposal of Parachlamydiaceae fam. nov. and Simkaniaceae fam. nov., each containing one monotypic genus, revised taxonomy of the family Chlamydiaceae, including a new genus and five new species, and standards for the identification of organisms. Int. J. Syst. Bacteriol. 49 (Pt 2):415-440.

Fan, B.T., Lu, H., Hu, H., Shi, L., McClarty, G.A., Nance, D.M., Greenberg, A.H., Zhong, G. (1998) Inhibition of apoptosis in Chlamydia-infected cells: blockade of mitochondrial cytochrome c release and caspase activation. J. Exp. Med. 187:487-496.

Favata, M.F., Horiuchi, K.Y., Manos, E.J., Daulerio, A.J., Stradley, D.A., Feeser, W.S., Van Dyk, D.E., Pitts, W.J., Earl, R.A., Hobbs, F., Copeland, R.A., Magolda, R.L., Scherle, P.A., Trzaskos, J.M. (1998) Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J. Biol. Chem. 273:18623-18632.

Fawaz, F. S., van Ooij, C., Homola, E., Mutka, S.C., Engel, J.N. (1997) Infection with *Chlamydia trachomatis* alters the tyrosine phosphorylation and/or localization of several host cell proteins including cortactin. Infect. Immun. 65: 5301-5308.

Fawaz, F., Mutka, S., Engel, J. (1993). Chlamydia-host cell interaction, abstr. D-150, p.121. *In* Abstracts of the 93rd General Meeting of the American Society for Microbiology. American Society for Microbiology, Atlanta, Georgia.

Fields, K.A., Hackstadt, T. (2001) Evidence for the secretion of *Chlamydia trachomatis* CopN by a type III secretion mechanism. Mol. Microbiol. 38:1048-1060.

Fincham, V.J., James, M., Frame, M.C., Winder, S.J. (2000) Active ERK/MAP kinase is targeted to newly forming cell-matrix adhesions by integrin engagement and v-Src. EMBO J. 19:2911-2923.

Finlay, B.B., Cossart, P. (1997) Exploitation of mammalian host cell functions by bacterial pathogens. Science 276:718-725.

Finlay, B.B., Falkow, S. (1997) Common themes in microbial pathogenicity revisited. Microbiol. Mol. Biol. Rev. 61136-1169.

Fong, I. W., Chiu, B., Viira, E., Fong, M. W., Jang, D., Mahony, J. (1997) Rabbit model for *Chlamydia pneumoniae* infection. J. Clin. Microbiol. 35: 48-52.

Fong, I.W., Chiu, B., Viira, E., Jang, D., Mahony, J.B. (1999a) De novo induction of atherosclerosis by *Chlamydia pneumoniae* in a rabbit model. Infect. Immun. 67:6048-6055.

Fong, I.W., Chiu, B., Viira, E., Jang, D., Fong, M.W., Peeling, R., Mahony, J.B. (1999b) Can an antibiotic (macrolide) prevent *Chlamydia pneumoniae*-induced atherosclerosis in a rabbit model? Clin. Diagnos. Lab. Immunol. 6:891-894.

Fryer, R.H., Schwobe, E.P., Woods, M.L., Rodgers, G.M. (1997) Chlamydia species infect human vascular endothelial cells and induce procoagulant activity. J. Investig. Med. 45:168-174.

Galan, J.E., Pace, J., Hayman, M.J. (1992) Involvement of the epidermal growth factor receptor in the invasion of cultured mammalian cells by *Salmonella typhyimurium*. Nature 357:536-537.

Galdiero, M., Cipolloaro, G., de L'ero, Marcatili, A. (1997) Cytokine and Adhesion Molecule expression in human monocytes and endothelial cells stimulated with bacterial heat shock proteins. Infect. Immun. 65: 699-707.

Gallo, R., Padurean, A., Jayaraman, T., Marx, S., Roque, M., Adelman, S., Chesebro, J., Fallon, J., Fuster, V., Marks, A., Badimon, J.J. (1999) Inhibition of intimal thickening after balloon angioplasty in porcine coronary arteries by targeting regulators of the cell cycle. Circulation 99:2164-2170.

Gaydos, C.A., Summersgill, J.T., Sahney, N.N., Ramirez, J.A., Quinn, T.C. (1996) Replication of *Chlamydia pneumoniae* in vitro in human macrophages, endothelial cells, and aortic artery smooth muscle cells. Infect. Immun. 64: 1614-1620.

Geng, Y., Shane, R.B., Berencsi, K., Gonczol, E., Zaki, M.H., Margolis, D.J., Trinchieri, G., Rook, A.H. (2000) *Chlamydia pneumoniae* inhibits apoptosis in human peripheral blood mononuclear cells through induction of IL-10. J. Immunol. 164:5522:5529.

Gerard, H.C., Krausse-Opatz, B., Wang, Z., Rudy, D., Rao, J.P., Zeidler, H., Schumacher, H.R., Whittum-Hudson, J.A., Kohler, L., Hudson, A.P. (2001) Expression of *Chlamydia trachomatis* genes encoding products required for DNA synthesis and cell division during active versus persistent infection. Mol. Microbiol. 41:731-741.

Gineitis, D., Treisman, R. (2001) Differential usage of signal transduction pathways defines two types of serum response factor target genes. J. Biol. Chem 276:24531-24539.

Gibbs, R. G. J., Carey, N., Davies, H. (1998). *Chlamydia pneumoniae* and vascular disease. Brit. J. Surg. 85: 1191-1197.

Gieffers, J., Fullgraf, H., Jahn, J., Klinger, M., Dalhoff, K., Katus, H.A., Solbach, W., Maass, M. (2001) *Chlamydia pneumoniae* infection in circulating human monocytes is refractory to antibiotic treatment. Circulation 103:351-356.

Godzik, K.L., O'Brien, E.R., Wang, S.P., Kuo, C.C. (1995) In vitro susceptibility of human vascular wall cells to infection with *Chlamydia pneumoniae*. J. Clin. Microbiol. 33: 2411-2414.

Grayston, J.T., Kuo, C.c., Wang, S.P., Altman, J. (1986). A new *Chlamydia psittaci* strain, TWAR, isolated in acute respiratory tract infections. N. Engl. J. Med. 315:161-168.

Grayston, J.T., Wang, S.P., Kuo, C.C., Campbell, L.A. (1989) Current knowledge on *Chlamydia pneumoniae*, strain TWAR, an important cause of pneumonia and other acute respiratory diseases. Eur. J. Clin. Microbiol. Infect. Dis. 8: 191-202.

Grayston, J.T. (2000) Background and current knowledge of *Chlamydia pneumoniae* and atherosclerosis. J. Infect. Dis. 181 (Suppl. 3): S402-S410.

Griffiss, J.M., Lammel, C.J., Wang, J., Dekker, N.P., Brooks, G.F. (1999) *Neisseria gonorrhoeae* coordinately uses Pili and Opa to activate HEC-1-B cell microvilli, which causes engulfment of the gonococci. Infect. Immun. 67:3469-3480.

Gupta, S. (1999) Chronic infection in the aetiology of atherosclerosis- focus on *Chlamydia pneumoniae*. Atherosclerosis 143: 1-6.

Gupta, S., Leatham, E.W., Carrington, D., Mendall, M.A., Kaski, J.C., Camm, J. (1997) Elevated *Chlamydia pneumoniae* antibodies, cardiovascular events, and azithromycin in male survivors of myocardial infarction. Circulation 96: 404-407.

Gurfinkel, E., Bozovich, G., Daroca, A., Beck, E., Mautner, B., ROXIS Study Group. (1997) Randomised trial of roxithromycin in non-Q-wave coronary syndromes: ROXIS pilot study. Lancet 350: 404-407.

Guthrie, R. (2001) Community-acquired lower respiratory tract infections. Chest 120:2021-2034.

Hall, A. (1998) Rho GTPases and the actin cytoskeleton. Science. 279:509-514.

Hammerschlag, M.R., Chirgwin, K., Roblin, P.M., Gelling, M., Dumornay, W., Mandel, L., Smith, P., Schachter, J. (1992) Persistent infection with *Chlamydia pneumoniae* following acute respiratory illness. Clin. Infect. Dis. 14:178-182.

Haranaga, S., Yamaguchi, H., Friedman, H., Izumi, S., Yamamoto, Y. (2001) *Chlamydia pneumoniae* infects and multiplies in lymphocytes in vitro. Infect. Immun. 69:7753-7759.

Hatch, T.P. (1996) Disulfide cross-linked envelope proteins: the functional equivalent of peptidoglycan in Chlamydiae? J. Bacteriol. 178: 1-5.

Hatch, G.M., McClarty, G. (1998). Phospholipid composition of purified *Chlamydia trachomatis* mimics that of the eukaryotic host cell. Infect. Immun. 66: 3727-3735.

Heinemann, M., Susa, M., Simnacher, U., Marre, R., Essig, A. (1996) Growth of *Chlamydia pneumoniae* induces cytokine production and expression of CD14 in a human monocytic cell line. Infect. Immun. 64: 4872-4875.

Higashiyama, S., Abraham, J.A., Miller, J., Fiddes, J.C., Klagsbrun, M. (1991) A heparin-binding growth factor secreted by macrophage-like cells that is related by EGF. Science. 251: 936-939.

Hirata, A., Igarashi, M., Yamaguchi, H., Suwabe, A., Daimon, M., Kato, T., Tominaga, M. (2000) Nifedipine suppresses neointimal thickening by its inhibitory effect on vascular smooth

muscle cell growth via a MEK-ERK pathway coupling with Pyk2. Br. J. Pharmacol.131: 1521-1530.

Hobbie, S., Chen, L.M., Davis, R.J., Galán, J.E. (1997) Involvement of mitogen-activated protein kinase pathways in the nuclear responses and cytokine production induced by *Salmonella typhimurium* in cultured intestinal epithelial cells. J. Immunol. 159:5550-5559.

Hoffmann, I., Eugene, E., Nassif, X., Couraud, P.O., Bourdoulous, S. (2001) Activation of Erb2b receptor tyrosine kinase supports invasion of endothelial cells by *Neisseria meningitidis*. J. Cell. Biol. 155: 133-143.

Howe, A.K., Aplin, A.E., Juliano, R.L. (2002) Anchorage-dependent ERK signaling - mechanisms and consequences. Curr. Opin. Genet. Dev. 12:30-35.

Hu, H., Pierce, G.N., Zhong, G. (1999) The atherogenic effects of chlamydia are dependent on serum cholesterol and specific to *Chlamydia pneumoniae*. J. Clin. Invest. 103:747-753.

Hueck, C.J. (1998) Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol. Mol. Biol. Rev. 62:379-433.

Igarashi, M., Takeda, Y., Mori, S., Ishibashi, N., Komatsu, E., Takahashi, K., Fuse, T., Yamamura, M., Kubo, K., Sugiyama, Y., Saito, Y. (1997) Suppression of neointimal thickening by a newly developed HMG-CoA reductase inhibitor, BAYw6228, and its inhibitory effect on vascular smooth muscle cell growth. Br. J. Pharmacol. 120:1172-1178.

Inoue, S., Orimo, A., Hosoi, T., Ikegami, A., Kozaki, K., Ouchi, Y., Nomura, S., Muramatsu, M., Orimo, H. (1994) Demonstration of activin-A in arteriosclerotic lesions. Biochem. Biophys. Res. Commun. 205: 441-448.

Ireton, K., Payrastre, B., Chap, H., Ogawa, W., Sakaue, H., Kasuga, M., Cossart, P. (1996) A role for phosphoinositide 3-kinase in bacterial invasion. Science 274:780-782.

Ireton, K., Cossart, P. (1998) Interaction of invasive bacteria with host signaling pathways. Curr. Opin. Cell Biol. 10:276-283.

Ireton, K., Payrastre, B., and Cossart, P. (1999) The *Listeria monocytogenes* protein InlB is an agonist of mammalian phosphoinositide 3-kinase. J. Biol. Chem. 274:17025-17032.

Ishida, A., Sasaguri, T., Kosaka, C., Nojima, H., Ogata, J. (1997) Induction of the cyclin-dependent kinase inhibitor p21 (Sdi1/Cip1/Waf1) by nitric oxide-generating vasodilator in vascular smooth muscle cells. J. Biol. Chem. 272: 10050-10057.

Jackson, L.A., Campbell, L.A., Kuo, C.C., Rodriguez, D.I., Lee, A., Grayston, J.T. (1997) Isolation of *Chlamydia pneumoniae* from a carotid endarterectomy specimen. J. Infect. Dis. 176:292-295.

Jackson, L.A., Campbell, L.A., Schmidt, R.A., Kuo, C., Cappuccio, A.L., Lee, M.J., Grayston, J.T. (2000) Specificity of detection of *Chlamydia pneumoniae* in cardiovascular atheroma. J. Infect. Dis. 181 Suppl 3:S447-448.

Jahn, H.U., Krüll, M., Wuppermann, F.N., Klucken, A.C., Rosseau, S., Seybold, J., Hegemann, J.H., Jantos, C.A., Suttorp, N. (2000) Infection and activation of airway epithelial cells by *Chlamydia pneumoniae*. J. Infect. Dis.182: 1678-1687.

Jawien, A., Bowen-Pope, D.F., Lindner, V., Schwartz, S.M., Clowes, A.W. (1992) Platelet-derived growth factor promotes smooth muscle migration and intimal thickening in a rat model of balloon angioplasty. J. Clin. Invest. 89:507-511.

Jobin, C., Haskill, S., Mayer, L., Panja, A., Sartor, B. (1997) Evidence for altered regulation of IκBα degradation in human colonic epithelial cells. J. Immunol. 158: 226-234.

Johnson, D.L., Song, X., Coombes, B.K., Mahony, J.B. Characterization of a newly identified *C. pneumoniae* protein kinase (Cpn 0148). Submitted for publication.

Kalayoglu, M.V., Byrne, G.I. (1998) A *Chlamydia pneumoniae* component that induces macrophage foam cell formation is chlamydial lipopolysaccharide. Infect. Immun. 66: 5067-5072.

Kalayoglu, M.V., Hoernemen, B., LaVerda, D., Morrison, S.G., Morrison, R.P., Byrne, G.I. (1999) Cellular oxidation of low-density lipoprotein by *Chlamydia pneumoniae*. J. Infect. Dis. 180: 780-790.

Kalman, S., Mitchell, W., Marathe, R., Lammel, C., Fan, J., Hyman, R.W., Olinger, L., Grimwood, J., Davis, R.W., Stephens, R.S. (1999) Comparative genomes of *Chlamydia pneumoniae* and *C. trachomatis*. Nat. Genet. 21:385-389.

Karin, M. (1996) The regulation of AP-1 activity by mitogen-activated protein kinases. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 351:127-134.

Karn, T., Holtrich, U., Brauninger, A., Bohme, B., Wolf, G., Rubsamen-Waigmann, H., Strebhardt, K. (1993) Structure, expression and chromosomal mapping of TKT from man and mouse: a new subclass of receptor tyrosine kinases with a factor VIII-like domain. Oncogene 8: 3433-3440.

Kato-Maeda, M., Gao, Q., Small, P.M. (2001) Microarray analysis of pathogens and their interaction with hosts. Cell. Microbiol. 3: 713-719.

Kaukoranta-Tolvanen, S.S., Ronni, T., Leinonen, M., Saikku, P., Laitinen, K. (1996) Expression of adhesion molecules on endothelial cells stimulated by *Chlamydia pneumoniae*. Microb. Pathog. 21: 407-411.

Keely, P.J., Westwick, J.K., Whitehead, I.P., Der, C.J., Parise, L.V. (1997) Cdc42 and Rac1 induce integrin-mediated cell motility and invasiveness through PI (3)K. Nature. 390:632-636.

Khachigian, L.M., Resnick, N., Gimbrone, M.A. Jr, Collins, T. (1995) Nuclear factor-kappa B interacts functionally with the platelet-derived growth factor B-chain shear-stress response element in vascular endothelial cells exposed to fluid shear stress. J. Clin. Invest. 96:1169-1175.

Kol, A., Bourcier, T., Lichtman, A.H., Libby, P. (1999) Chlamydial and human heat shock protein 60s activate human vascular endothelium, smooth muscle cells, and macrophages. J. Clin. Invest. 103:571-577.

Kol, A., Libby, P. (1999) Molecular mediators of arterial inflammation: a role for microbial products? Am. Heart. J. 138:S450-S452.

Kol, A., Sukhova, G.K., Lichtman, A.H., Libby, P. (1998) Chlamydial heat shock protein 60 localizes in human atheroma and regulates macrophage tumour necrosis factor-alpha and matrix metalloproteinase expression. Circulation 98: 300-307.

Komukai, M., Wajima, Y.S., Tashiro, J., Shinomiya, M., Saito, Y., Morisaki, N. (1999) Carvastatin suppresses intimal thickening of rabbit carotid artery after balloon catheter injury probably through the inhibition of vascular smooth muscle cell proliferation and migration. Scand J. Clin. Lab. Invest. 59: 159-166.

Krüll, M., Klucken, A.C., Wuppermann, F.N., Fuhrmann, O., Magerl, C., Seybold, J., Hippenstiel, S., Hegemann, J.H., Jantos, C.A., Suttorp, N. (1999) Signal transduction pathways

activated in endothelial cells following infection with *Chlamydia pneumoniae*. J. Immunol. 162: 4834-4841.

Kubo, A., Stephens, R.S. (2000) Characterization and functional analysis of PorB, a Chlamydia porin and neutralizing target. Mol. Microbiol. 38:772-780.

Kubo, A., Stephens, R.S. (2001) Substrate-specific diffusion of select dicarboxylates through *Chlamydia trachomatis* PorB. Microbiology 147(Pt 11): 3135-3140.
Kuo, C.C., Campbell, L.A. (2000) Detection of *Chlamydia pneumoniae* in arterial tissues. J. Infect. Dis. 181 Suppl 3:S432-436.

Kuo, C.C., Shor, A., Campbell, L.A., Fukushi, H., Patton, D.L., Grayston, J.T. (1993a) Demonstration of *Chlamydia pneumoniae* in atherosclerotic lesions of coronary arteries. J. Infect. Dis. 167:841-849.

Kuo, C.C., Gown, A.M., Benditt, E.P., Grayston, J.T. (1993b) Detection of *Chlamydia pneumoniae* in aortic lesions of atherosclerosis by immunocytochemical stain. Aterioscler. Thromb. 13:1501-1504.

Kuo, C.C., Jackson, L.A., Campbell, L.A., Grayston, J.T. (1995) *Chlamydia pneumoniae* (TWAR). Clin. Microbiol. Rev. 8: 451-461.

Kuo, C.C., Grayston, J.T. (1998) Factors affecting viability and growth in HeLa 229 cells of *Chlamydia* sp. strain TWAR. J. Clin. Microbiol. 26:812-815.

Laitinen, K., Laurila, A., Pyhala, L., Leinonen, M., Saikku, P. (1997) *Chlamydia pneumoniae* infection induces inflammatory changes in the aortas of rabbits. Infect. Immun. 65:4832-4835.

Lee, Y.I., Kang-Park, S., Do, S.I., and Lee, Y.I. (2001) The hepatitis B virus-X protein activates a phosphatidylinositol 3-kinase-dependent survival signaling cascade. J. Biol. Chem. 276:16969-16977.

Leinonen, M. (1993) Pathogenetic mechanisms and epidemiology of *Chlamydia pneumoniae*. Eur. Heart J. 14 (supl.K): 57-61.

Lemstrom, K.B., Aho, P.T., Bruggeman, C.A., Hayry, P.J. (1994) Cytomegalovirus infection enhances mRNA expression of platelet-derived growth factor-BB and transforming growth factor-beta 1 in rat aortic allografts. Possible mechanism for cytomegalovirus-enhanced graft arteriosclerosis. Arterioscler. Thromb. 14:2043-2052.

Lemstrom, K.B., Bruning, J.H., Bruggeman, C.A., Lautenschlager, I.T., Hayry, P.J. (1993) Cytomegalovirus infection enhances smooth muscle cell proliferation and intimal thickening of rat aortic allografts. J. Clin. Invest. 92:549-558.

Levy, N.J., Moulder, J.W. (1982) Attachment of cell walls of *Chlamydia psittaci* to mouse fibroblasts (L cells). Infect. Immun. 37:1059-1065.

Lewis, T.S., Hunt, J.B., Aveline, L.D., Jonscher, K.R., Louie, D.F., Yeh, J.M., Nahreini, T.S., Resing, K.A., Ahn, N.G. (2000) Identification of novel MAP kinase pathway signaling targets by functional proteomics and mass spectrometry. Mol. Cell 6:1343-1354.

Libby, P., Egan, D., Sharlatos, S. (1997). Roles of infectious agents in atherosclerosis and restenosis: an assessment of the evidence and need for future research. Circulation 96: 4095-4103.

Lin, T.M., Campbell, L.A., Rosenfeld, M.E., Kuo, C.C. (2000) Monocyte-endothelial cell coculture enhances infection of endothelial cells with *Chlamydia pneumoniae*. J. Infect. Dis. 181:1096-1100.

Lin, Y., Gong, J., Zhang, M., Zue, W., Barnes, P.F. (1998) Production of monocyte chemoattractant protein 1 in tuberculosis patients. Infect. Immun. 66: 2319-2322.

Linnanmaki, E., Leinonen, M., Matilla, K., Nieminen, M.S., Valtonen, V., Saikku, P. (1993) Chlamydia *pneumoniae*-specific circulating immune complexes in patients with chronic coronary heat disease. Circulation 87:1130-1134.

Mabey, D.C., Robertson, J.N., Ward, M.E. (1987) Detection of *Chlamydia trachomatis* by enzyme immunoassay in patients with trachoma. Lancet 2:1491-1492.

Mahony JB, Coombes BK. (2001) *Chlamydia pneumoniae* and atherosclerosis: does the evidence support a causal or contributory role? FEMS Microbiol. Lett. 197:1-9.

Mansell, A., Khelef, N., Cossart, P., O'Neill, L.A. (2001) Internalin B activates nuclear factor-kappa B via Ras, phosphoinositide 3-kinase, and Akt. J. Biol. Chem. 276:43597-43603.

Mathews, S., George, C., Flegg, C., Stenzel, D., Timms, P. (2001) Differential expression of ompA, ompB, pyk, nlpD and Cpn0585 genes between normal and interferon-gamma treated cultures of *Chlamydia pneumoniae*. Microb. Pathog. 30:337-345.

Matter, M.L., Ruoslahti, E. (2001) A signaling pathway from the alpha5beta1 and alpha(v)beta3 integrins that elevates bcl-2 transcription. J. Biol. Chem. 276:27757-27763.

May, M.J., Ghosh, S. (1998) Signal transduction through NF-κB. Immunol. Today 19: 80-88.

McCormick, B.A., Nusrat, A., Parkos, C.A., D'Andrea, L., Hofman, P.M., Carnes, D., Liang, T.W., Madara, J.L. (1997) Unmasking of intestinal epithelial lateral membrane beta1 integrin consequent to transepithelial neutrophil migration in vitro facilitates inv-mediated invasion by *Yersinia pseudotuberculosis*. Infect. Immun. 65:1414-1421.

Mehta, S.J., Miller, R.D., Ramirez, J.A., Summersgill, J.T. (1998) Inhibition of *Chlamydia pneumoniae* replication in HEp-2 cells by interferon-gamma: role of tryptophan catabolism. J. Infect. Dis. 177: 1326-1331.

Melnick, S., Shahar, E., Folsom, A.R., Grayston, J.T., Sorlie, P.D., Wang, S.P. (1993) Past infection by *Chlamydia pneumoniae* strain TWAR and asymptomatic carotid atherosclerosis. Atherosclerosis risk in Communities (ARIC) Investigators. Am. J. Med. 95: 499-504.

Migliaccio, E., Mele, S., Salcini, A.E., Pelicci, G., Lai, K.M., Superti-Furga, G., Pawson, T., Di Fiore, P.P., Lanfrancone, L., Pelicci, P.G. (1997) Opposite effects of the p52shc/p46shc and p66shc splicing isoforms on the EGF receptor-MAP kinase-fos signalling pathway. EMBO J. 16:706-716.

Moazed, T.C., Kuo, C.C., Patton, D.L, Grayston, J.T. (1996) Experimental rabbit models of *Chlamydia pneumoniae* infection. Am. J. Pathol. 148:667-676.

Moazed, T.C., Kuo, C.C., Grayston, J.T., Campbell, L.A. (1997) Murine models of *Chlamydia pneumoniae* infection and atherosclerosis. J. Infect. Dis. 175: 883-890.

Moazed, T.C., Campbell, L.A., Rosenfeld, M.E., Grayston, J.T., Kuo, C.C. (1999) *Chlamydia pneumoniae* infection accelerates the progression of atherosclerosis in apolipoprotein E-deficient mice. J. Infect. Dis. 180:238-241.

Molestina, R.E., Dean, D., Miller, R.D., Ramirez, J.A., Summersgill, J.T. (1998) Characterization of a strain of *Chlamydia pneumoniae* isolated from a coronary atheroma by analysis of the *omp1* gene and biological activity in human endothelial cells. Infect. Immun. 66: 1370-1376.

Molestina, R.E., Miller, R.D., Ramirez, J.A., Summersgill, J.T. (1999) Infection of human endothelial cells with *Chlamydia pneumoniae* stimulates transendothelial migration of neutrophils and monocytes. Infect. Immun. 67:1323-1330.

Molestina, R.E., Miller, R.D., Lentsch, A.B., Ramirez, J.A., Summersgill, J.T. (2000) Requirement for NF-κB in transcriptional activation of monocyte chemotactic protein 1 by *Chlamydia pneumoniae* in human endothelial cells. Infect. Immun. 68: 4282-4288.

Montigiani, S., Falugi, F., Scarselli, M., Finco, O., Petracca, R., Galli, G., Mariani, M., Manetti, R., Agnusdei, M., Cevenini, R., Donati, M., Nogarotto, R., Norais, N., Garaguso, I., Nuti, S., Saletti, G., Rosa, D., Ratti, G., Grandi, G. (2002) Genomic approach for analysis of surface proteins in *Chlamydia pneumoniae*. Infect. Immun. 70:368-379.

Mosorin, M., Surcel, H.M., Laurila, A., Lehtinen, M., Karttunen, R., Juvonen, J., Paavonen, J., Morrison, R.P., Saikku, P., Juvonen, T. (2000) Detection of *Chlamydia pneumoniae*-reactive T lymphocytes in human atherosclerotic plaques of carotid artery. Arterioscler. Thromb. Vasc. Biol. 20:1061-1067.

Moulder, J.W. (1991) Interaction of chlamydiae and host cells in vitro. Microbiol. Rev. 55:143-190.

Muhlestein, J.B., Anderson, J.L., Hammond, E.H., Zhao, L., Trehan, S., Schwobe, E.P., Carlquist, J.F. (1997) Infection with *Chlamydia pneumoniae* accelerates the development of atherosclerosis and treatment with azithromycin prevents it in a rabbit model. Circulation 97:633-636.

Navab, M., Fogelman, A.M., Berliner, J.A., Territo, M.C., Demer, L.L., Frank, J.S., Watson, A.D., Edwards, P.A., Lusis, A.J. (1995) Pathogenesis of atherosclerosis. Am. J. Cardiol. 76:18C-23C.

Nobes, C.D, Hall, A. (1995) Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibres, lamellipodia and filopodia. Cell. 81:53-62.

Ogawa, K., M. Funaba, L.S. Mathews, T. Mizutani. (2000) Activin A stimulates type IV collagenase (matrix metalloproteinase -2) production in mouse peritoneal macrophages. J. Immunol. 165: 2997-3003.

Okada, S., Yamauchi, K., and Pessin, J.E. (1995) Shc isoform-specific tyrosine phosphorylation by the insulin and epidermal growth factor receptors. J. Biol. Chem. 270:20737-20741.

Ouchi, K., Fujii, B., Kudo, S., Shirai, M., Yamashita, K., Gondo, T., Ishihara, T., Ito, H., Nakazawa, T. (2000) *Chlamydia pneumoniae* in atherosclerotic and nonatherosclerotic tissue. J. Infect. Dis. 181 Suppl 3:S441-443.

Patnode, D., Wang, S.P., Grayston, J.T. (1990) Persistence of *Chlamydia pneumoniae* strain TWAR microimmunofluorescent antibody. In: Bowie, W.R., Caldwell, H.D., Jones, R.P., et al., eds. Chlamydial infections. Cambridge, UK: Cambridge University Press. p406-409.

Persad, S., Attwell, S., Gray, V., Mawji N., Deng, J.T., Leung, D., Yan, J., Sanghera, J., Walsh, M.P., Dedhar, S. (2001) Regulation of protein kinase B / AKT-serine-473 phosphorylation by integrin linked kinase (ILK): Critical roles for kinase activity and amino acids arginine-211 and serine-343. J. Biol. Chem. 276:27462-27469.

Porreca, E., DiFebbo, C., Reale, M., Castellani, M.L., Baccante, G., Barbacane, R., Conti, P., Cuccurullo, F., Poggi, A. (1997) Monocyte chemotactic protein 1 is a mitogen for cultured rat vascular smooth muscle cells. J. Vasc. Res. 34:58-65.

Quinn, T.C., Gaydos, C.A. (1999) In vitro infection and pathogenesis of *Chlamydia pneumoniae* in endovascular cells. Am. Heart J. 138(5 Pt 2):S507-511.

Raab, G., Klagsbrum, M. (1997) Heparin-binding EGF-like growth factor. Biochim. Biophys. Acta. 1333: F179-F199.

Ramirez, J., Ahkee, S., Summersgill, J., Ganzer, B., Ogden, L., Quinn, T., Gaydos, C., Bobo, L., Hammerschlag, M., Roblow, P., Lebar, W., Grayston, J., Campbell, L., Patton, D., Dean, D., Schachter, J. (1996) Isolation of *Chlamydia pneumoniae* from the coronary artery of a patient with coronary atherosclerosis. Ann. Intern. Med. 125:979-982.

Rao, K.M.K. (2001) MAP kinase activation in macrophages. J. Leukocyte. Biol. 69:3-10.

Raulston, J.E., Davis, C.H., Schmiel, D.H., Morgan, M.W., Wyrick, P.B. (1993) Molecular characterization and outer membrane association of a *Chlamydia trachomatis* protein related to the hsp70 family of proteins. J. Biol. Chem. 268:23139-21347.

Read, T.D., Brunham, R.C., Shen, C., Gill, S.R., Heidelberg, J.F., White, O., Hickey, E.K., Peterson, J., Utterback, T., Berry, K., Bass, S., Linher, K., Weidman, J., Khouri, H., Craven, B., Bowman, C., Dodson, R., Gwinn, M., Nelson, W., DeBoy, R., Kolonay, J., McClarty, G., Salzberg, S.L., Eisen, J., Fraser, C.M. (2000) Genome sequences of *Chlamydia trachomatis* MoPn and *Chlamydia pneumoniae* AR39. Nucleic Acids Res. 28:1397-1406.

Reddy, M.A., Prasadarao, N.V., Wass, C.A., Kim, K.S. (2000) Phosphatidylinositol 3-kinase activation and interaction with focal adhesion kinase in *Escherichia coli* K1 invasion of human brain microvascular endothelial cells. J. Biol. Chem. 275:36769-36774.

Roblin, P.M., Dunornay, W., Hammerschlag, M.R. (1992) Use of HEp-2 cells for improved isolation and passage of *Chlamydia pneumoniae*. J. Clin. Microbiol. 30:1968-1971.

Rödel, J., Woytas, M., Groh, A., Schmidt, K.H., Hartmann, M., Lehmann, M., Straube, E. (2000) Production of basic fibroblast growth factor and interleukin 6 by human smooth muscle cells following infection with *Chlamydia pneumoniae.* Infect. Immun. 68: 3635-3641.

Ross, R., Masuda, J., Raines, E.W., Gown, A.M., Katsuda, S., Sasahara, M., Malden, L.T., Masuko, H., Sato, H. (1990) Localization of PDGF-B protein in macrophages in all phases of atherogenesis. Science 248:1009-1012.

Ross, R. (1993) The pathogenesis of atherosclerosis: a perspective for the 1990's. Nature 362: 801-808.

Ross, R. (1999) Atherosclerosis is an inflammatory disease. Am. Heart. J. S419-S420.

Ross, R., Glomset, J.A. (1973) Atherosclerosis and the arterial smooth muscle cell. Science 180: 1332-1339.

Rothstein, N.M., Quinn, T.C., Madico, G., Gaydos, C.A., Lowenstein, C.J. (2001) Effect of azithromycin on murine arteriosclerosis exacerbated by *Chlamydia pneumoniae*. J. Infect. Dis. 183:232-238.

Saikku, P. (1993) Chlamydia *pneumoniae* infection as a risk factor in acute myocardial infarction. Eur. Heart J. 14 (suppl. K): 62-65.

Saikku, P., Mattila, K., Nieminen, M.S., Huttunen, J.K., Leinonen, M., Ekman, M.R., Makela, P.H., Valtonen, V. (1988) Serological evidence of an association of a novel chlamydia, TWAR, with chronic coronary heart disease and acute myocardial infarction. Lancet 2: 983-986.

Sambrook, J., Fritch, E.F., Maniatis, T. Molecular Cloning: A Laboratory Manual, second edition. Cold Spring Harbor Laboratory, Cold Spring Harbor, 1989.

Sasu, S., LaVerda, D., Qureshi, N., Golenbock, D.T., Beasley, D. (2001) *Chlamydia pneumoniae* and chlamydial heat shock protein 60 stimulate proliferation of human vascular smooth muscle cells via toll-like receptor 4 and p44/p42 mitogen-activated protein kinase activation. Circ. Res. 89:244-250.

Sato, K., Kimoto, M., Kakumoto, M., Horiuchi, D., Iwasaki, T., Tokmakov, A.A., Fukami, Y. (2000) Adaptor protein Shc undergoes translocation and mediates up-regulation of the tyrosine kinase c-Src in EGF-stimulated A431 cells. Genes Cells 5:749-764.

Schachter, J. (1978) Chlamydial infections. N. Engl. J. Med. 298:428-435.

Schachter, J., Stephens, R.S., Timms, P., Kuo, C., Bavoil, P.M., Birkelund, S., Boman, J., Caldwell, H., Campbell, L.A., Chernesky, M., Christiansen, G., Clarke, I.N., Gaydos, C., Grayston, J.T., Hackstadt, T., Hsia, R., Kaltenboeck, B., Leinonnen, M., Ocjius, D., McClarty, G., Orfila, J., Peeling, R., Puolakkainen, M., Quinn, T.C., Rank, R.G., Raulston, J., Ridgeway, G.L., Saikku, P., Stamm, W.E., Taylor-Robinson, D.T., Wang, S.P., Wyrick, P.B. (2001) Radical changes to chlamydial taxonomy are not necessary just yet. Int. J. Syst. Evol. Microbiol. 51(Pt 1): 251-3.

Schafer, D.A. (2002) Biochemical signals and biological responses elicited by the focal adhesion kinase. Biochem. Biophys. Acta. 1540:1-21.

Schaller, M.D. (2001) Biochemical signals and biological responses elicited by the focal adhesion kinase. Biochim. Biophys. Acta. 1540:1-21.

Schmidt, C., Hulthe, J., Wikstrand, J., Gnarpe, H., Gnarpe, J., Agewall, S., Fagerberg, B. (2000) *Chlamydia pneumoniae* seropositivity is associated with carotid artery intima-media thickness. Stroke 31:1526-1531.

Schramm, N., Bagnell, C.R., Wyrick, P.B. (1996) Vesicles containing *Chlamydia trachomatis* serovar L2 remain above pH 6 within HEC-1B cells. Infect. Immun. 64:1208-1214.

Schramm, N., Wyrick, P.B. (1995) Cytoskeletal requirements in *Chlamydia trachomatis* infection of host cells. Infect. Immun. 63:324-332.

Schulte, R., Grassl, G.A., Preger, S., Fessele, S., Jacobi, C.A., Schaller, M., Nelson, P.J., Autenrieth, I.B. (2000) *Yersinia enterocolitica* invasin protein triggers IL-8 production in epithelial cells via activation of Rel p65-p65 homodimers. FASEB J. 14:1471-1484.

Scidmore, M.A., Rockey, D.D., Fischer, E.R., Heinzen, R.A., Hackstadt, T. (1996) Vesicular interactions of the *Chlamydia trachomatis* inclusion are determined by chlamydial early protein synthesis rather than route of entry. Infect. Immun. 64:5366-5372.

Shakespeare, W.C. (2001) SH2 domain inhibition: a problem solved? Curr. Opin. Chem. Biol. 5:409-415.

Shen, Y., Naujokas, M., Park, M., Ireton, K. (2000) InlB-dependent internalization of Listeria is mediated by the Met receptor tyrosine kinase. Cell 103:501-510.
Shor, A. (2001) A pathologist's view of organisms and human atherosclerosis. J. Infect. Dis. 183:1428-1429.

Smieja, M., Mahony, J.B., Goldsmith, C.H., Chong, S., Petrich, A., Chernesky, M. (2001) Replicate PCR testing and probit analysis for detection and quantitation of *Chlamydia pneumoniae* in clinical specimens. J. Clin. Microbiol. 39:1796-1801.

Stary, H.C., Blankenhorn, D.H., Chandler, A.B., Glagov, S., Insull, W. Jr, Richardson, M., Rosenfeld, M.E., Schaffer, S.A., Schwartz, C.J., Wagner, W.D., et al. (1992) A definition of the intima of human arteries and of its atherosclerosis-prone regions. A report from the Committee on

Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Arterioscler. Thromb. 12:120-134.

Stary, H.C. (1990) The sequence of cell and matrix changes in atherosclerotic lesions of coronary arteries in the first forty years of life. Eur. Heart J. 11(Supl.E): 3-19.

Steele-Mortimer, O., Knodler, L.A., Marcus, S.L., Scheid, M.P., Goh, B., Pfeifer, C.G., Duronino, V., Finlay, B.B. (2000) Activation of Akt/protein kinase B in epithelial cells by the *Salmonella typhimurium* effector sigD. J. Biol. Chem. 275:37718-37724.

Stein, R.C., Waterfield, M.D. (2000) PI 3-kinase inhibition: a target for drug development? Mol. Med. Today 6:347-357.

Stephens, R.S., Fawaz, F.S., Kennedy, K.A., Koshiyama, K., Nichols, B., van Ooij, C., Engel, J.A. (2000) Eukaryotic cell uptake of heparin-coated microspheres: a model of host cell invasion by *Chlamydia trachomatis*. Infect. Immun. 68:1080-1085.

Stephens, R.S., Koshiyama, K., Lewis, E., Kubo, A. (2001) Heparin-binding outer membrane protein of chlamyidiae. Mol. Microbiol. 40:691-699.

Stephens, R.S., Kalman, S., Lammel, C., Fan, J., Marathe, R., Aravind, L., Mitchell, W., Olinger, L., Tatusov, R.L., Zhao, Q., Koonin, E.V., Davis, R.W. (1998) Genome sequence of an obligate intracellular pathogen of humans: *Chlamydia trachomatis*. Science 282:754-759.

Stenner-Liewen, F., Liewen, H., Zapata, J.M., Pawlowski, K., Godzick, A., Reed, J.C. (2002) CADD, a chlamydia protein that interacts with death receptors. J. Biol. Chem. JBC papers in ress, published online January 22, 2002 as MC100693200.

Su, H., Raymond, L., Rockey, D.D., Fischer, E., Hackstadt, T., Caldwell, H.D. (1996) A recombinant *Chlamydia trachomatis* major outer membrane protein binds to heparan sulfate receptors on epithelial cells. Proc. Natl. Acad. Sci. USA. 96:11143-11148.

Subtil, A., Parsot, C., Dautry-Varsat, A. (2001) Secretion of predicted Inc proteins of *Chlamydia pneumoniae* by a heterologous type III machinery. Mol. Microbiol. 39:792-800.

Taraktchoglou, M., Pacey, A.A., Turnbull, J.E., Eley, A. (2001) Infectivity of *Chlamydia trachomatis* serovar LGV but not E is dependent on host cell heparan sulfate. Infect. Immun. 69:968-976.

Thomas, K.W., Monick, M.M., Staber, J.M., Yarovinsky, T., Carter, A.B., Hunninghake, G.W. (2002) Respiratory syncytial virus inhibits apoptosis and induces NF-kappa B activity through a phosphatidylinositol 3-kinase-dependent pathway. J. Biol. Chem. 277:492-501.

Ting, L.M., Hsia, R.C., Haidaris, C.G., Bavoil, P.M. (1995) Interaction of outer envelope proteins of *Chlamydia psittaci* GPIC with the HeLa cell surface. Infect. Immun. 63:3600-3608.

Tjaden, J., Winkler, H.H., Schwoppe, C., Van Der Laan, M., Mohlmann, T., Neuhaus, H.E. (1999) Two nucleotide transport proteins in *Chlamydia trachomatis*, one for net nucleoside triphosphate uptake and the other for transport of energy.
J. Bacteriol. 181:1196-1202.

Ueda, M., Becker, A.E., Kasayuki, N., Kojima, A., Morita, Y., Tanaka, S. (1996) In situ detection of platelet-derived growth factor-A and -B chain mRNA in human coronary arteries after percutaneous transluminal coronary angioplasty. Am. J. Pathol. 149:831-843.

Vandahl, B.B., Birkelund, S., Demol, H., Hoorelbeke, B., Christiansen, G., Vandekerckhove, J., Gevaert, K. (2001) Proteome analysis of the Chlamydia pneumoniae elementary body. Electrophoresis 22:1204-1223.

van Kuppeveld, F.J., Johansson, K.E., Galama, J.M., Kissing, J., Bolske, G., van der Logt, J.T., Melchers, W.J. (1994) Detection of mycoplasma contamination in cell cultures by a mycoplasma group-specific PCR. Appl. Environ. Microbiol. 60:149-152.

Van Hal, N.L.W., Vorst, O., van Houwelingen, A., Kok, E.J., Peijnenburg, A., Aharoni, A., van Tunen, A.J., Keijer, J. (2000) The application of DNA microarrays in gene expression analysis. J. Biotechnol. 78: 271-280.

van Ooij, C., Homola, E., Kincaid, E., Engel. J. (1998) Fusion of *Chlamydia trachomatis*-containing inclusions is inhibited at low temperatures and requires bacterial protein synthesis. Infect. Immun. 66:5364-5371.

van Ooij, C., Kalman, L., van Ijzendoorn, Nishijima, M., Hanada, K., Mostov, K., Engel, J.N. (2000) Host cell-derived sphingolipids are required for the intracellular growth of *Chlamydia trachomatis*. Cell. Microbiol. 2: 627-637.

Vanhaesebroeck, B., Alessi, D.R. (2000) The PI3K-PDK1 connection: more than just a road to PKB. Biochem. J. 346:561-576.

Van Voorhis, W.C., Barrett, L.K., Sweeney, Y.T., Kuo, C.C., Patton, D.L. (1997) Repeated *Chlamydia trachomatis* infection of Macaca nemestrina fallopian tubes produces a Th1-like cytokine response associated with fibrosis and scarring. Infect. Immun. 65:2175-2182.

Vink A, Poppen M, Schoneveld AH, Roholl PJ, de Kleijn DP, Borst C, Pasterkamp G. (2001) Distribution of *Chlamydia pneumoniae* in the human arterial system and its relation to the local amount of atherosclerosis within the individual. Circulation 103:1613-1637.

Vlahos, C.J., Matter, W.F., Hui, K.Y., and Brown, R.F. (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). J. Biol. Chem. 269:5241-5248.

Wang, X., Yue, T.L., Ohlstein, E.H., Sung, C.P., Feuerstein, G.Z. (1996) Interferon inducible protein-10 involves vascular smooth muscle cell migration, proliferation and inflammatory response. J. Biol. Chem. 271:24286-24293.

Wang, Z.M., Liu, C., Dziarski, R. (2000) Chemokines are the main proinflammatory mediators in human monocytes activated by *Staphylococcus aureus*, peptidoglaycans and endotoxin. J. Biol. Chem. 275: 20260-20267.

Wanishsawad, C., Zhou,Y.F., Epstein, S.E. (2001) *Chlamydia pneumoniae*-induced transactivation of the major immediate early promoter of cytomegalovirus: potential synergy of infectious agents in the pathogenesis of atherosclerosis. J. Infect. Dis. 181:787-790.

Ward, M.E. (1995) The immunobiology and immunopathology of chlamydial infections. APMIS. 103:769-796.

Ward, M.E. (1999) "Mechanisms of Chlamydia-induced disease". In: Stephens, R.S. (ed)., *Chlamydia: Intracellular Biology, Pathogenesis and Immunity.* Washington, D.C.: ASM Press. 171-210.

Whitmarsh, A.J., Shore, P., Sharrocks, A.D., and Davis, R.J. (1995) Integration of MAP kinase signal transduction pathways at the serum response element. Science 269:403-407.

Wiernicki, T.R., Bean, J.S., Dell, C., Williams, A., Wood, D., Kauffman, R.F., Singh, J.P. (1996) Inhibition of vascular smooth muscle cell proliferation and arterial intimal thickening by a novel antiproliferative naphthopyran. J. Pharmacol. Exp. Ther. 278:1452-1459.

Wilkowsky, S.E., Barbieri, M.A., Stahl, P., Isola, E.L.D. (2001) *Trypanosoma cruzi* : phosphatidylinositol 3-kinase and protein kinase B activation is associated with parasite invasion. Exp. Cell. Res. 264: 211-218.

Wolf, K., Hackstadt, T. (2001) Sphingomyelin trafficking in *Chlamydia pneumoniae*-infected cells. Cell. Microbiol. 3:145-152.

Wolf, K., Fischer, E., Hackstadt, T. (2000) Ultrastructural analysis of developmental events in *Chlamydia pneumoniae*-infected cells. Infect. Immun. 68:2379-2385.

World Health Organization. (1996) Global prevalence and incidence of selected curable sexually transmitted diseases: overview and estimates. World Health Organization, Geneva, Switzerland.

Wright, S.D., Burton, C., Hernandez, M, Hassing, H., Montenegro, J., Mundt, S., Patel, S., Card, D.J., Hermanowski-Vosatka, A., Bergstrom, J.D., Sparrow, C.P., Detmers, P.A., Chao, Y.S. (2000) Infectious agents are not necessary for murine atherogenesis. J. Exp. Med. 191:1437-1442.

Wuppermann, F.N., Hegemann, J.H., Jantos, C.A. (2001) Heparan-sulfate-like glycosamioglycan is a cellular receptor for *Chlamydia pneumoniae*. J. Infect. Dis. 184:181-187.

Wyllie, S., Longbottom, D., Herring, A.J., Ashley, R.H. (1999) Single channel analysis of recombinant major outer membrane protein porins from *Chlamydia psittaci* and *Chlamydia pneumoniae*. FEBS Lett. 445:192-196.

Wymann, M.P., Bulgarelli-Leva, G., Zvelebil, M.J., Pirola, L., Vanhaesebroeck, B., Waterfield, M.D., Panayotou, G. (1996) Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction. Mol. Cell. Biol. 16:1722-1733.

Wyrick, P.B., Knight, S.T., Paul, T.R., Rank, R.G., Barbier, C. (1999) Persistent chlamydial envelope antigens in antibiotic-exposed infected cells trigger neutrophil chemotaxis. J. Infect. Dis. 179: 954-966.

Wyrick , P. (2000) Intracellular survival by Chlamydia. Cell. Microbiol. 2:275-282.

Yang, Z.P., Kuo, C.C., Grayston, J.T. (1993) A mouse model of *Chlamyida pneumoniae* strain TWAR pneumonitis. Infect. Immun. 61:2037-2040.

Yu, J., Dolter, K.E. (1997) Production of activin A and its roles in inflammation and hematopoiesis. Cytokines Cell. Mol. Ther. 3: 169-177.

Zhang, J.P., Stephens, R.S. (1992) Mechanism of *C. trachomatis* attachment to eukaryotic host cells. Cell. 69:861-869.

Zhang, Z.D., Cox, G. (1996) MTT assay overestimates human airway smooth muscle cell number in culture. Biochem. Mol. Biol. Internat. 38:431-436.

Zhou, Y.F., Yu, Z.X., Wanishsawad, C., Shou, M., Epstein, S.E. (1999) The immediate early gene products of human cytomegalovirus increase vascular smooth muscle cell migration, proliferation, and expression of PDGF beta-receptor. Biochem. Biophys. Res. Commun. 256:608-613

Zhu, J., Quyyumi, A.A., Norman, J.E., Csako, G., Waclawiw, M.A., Shearer, G.M., Epstein, S.E. (2000) Effects of total pathogen burden on coronary artery disease risk and C-reactive protein levels. Am. J. Cardiol. 85:140-146.

Zureik, M., Ducimetiere, P., Touboul, P., Courbon, D., Bonithon-Kopp, C., Berr, C., Magne, C. (2000) Common carotid intima-media thickness predicts occurrence of carotid atherosclerotic plaques. Aterioscler. Thromb. Vasc. Biol. 20:1622-1629.