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ANIMAL MODELS OF *CHLAMYDIA PNEUMONIAE* AND ATHEROSCLEROSIS: DISSEMINATION TO AND PERSISTENCE IN ATHEROMATOUS LESIONS

by

Teresa C. Moazed

A dissertation submitted in partial fulfillment of
the requirements for the degree of

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Chairperson of Supervisory Committee

Program Authorized
to Offer Degree Pathobiology

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Abstract

ANIMAL MODELS OF *CHLAMYDIA PNEUMONIAE* AND ATHEROSCLEROSIS: DISSEMINATION TO AND PERSISTENCE IN ATHEROMATOUS LESIONS

by Teresa C. Moazed

Chairperson of the Supervisory Committee
Professor Lee Ann Campbell
Department of Pathobiology

Chlamydia pneumoniae has been associated with atherosclerosis in humans. The goal of this dissertation is to evaluate animal models of both *C. pneumoniae* and atherosclerosis to investigate the pathogenesis of *C. pneumoniae* in atherosclerosis. New Zealand White (NZW) and Watanabe heritable hyperlipidemic (WHHL) rabbits and apolipoprotein, (apo)E-deficient transgenic and C57BL/6J mice were evaluated for susceptibility to *C. pneumoniae* infection.

Following inoculation, NZW rabbits developed moderate self-resolving interstitial pneumonia. Chlamydial DNA was detected intermittently in the nasopharynx and lung through 42 days post-inoculation. In WHHL rabbits,

C. pneumoniae was detected by PCR and/or immunocytochemistry in the nasopharynx and lung, but not within aortic atheromas, suggesting that rabbits, although susceptible to infection, were not appropriate for future studies.

Following inoculation of apoE-deficient mice, *C. pneumoniae* was detected in lung, aorta and spleen for 20 weeks post-inoculation by PCR and within atherosclerotic lesions at 12 and 16 weeks post-inoculation by immunocytochemistry, indicating the suitability of this mouse model to study the role of *C. pneumoniae* in the progression of atherosclerosis. In C57BL/6J mice in the absence of a high fat diet, *C. pneumoniae* was detected in the aorta for 5 weeks after inoculation, suggesting this mouse model should be useful for studying the role of *C. pneumoniae* in the induction of atherosclerosis.

To determine whether macrophages are a vehicle of dissemination, macrophages from C57BL/6J mice infected with *C. pneumoniae* were analyzed for their ability to support growth and transfer infection. *C. pneumoniae* was detected in alveolar and peritoneal macrophages and peripheral blood mononuclear cells of intranasally and/or intraperitoneally inoculated mice. When macrophages were transferred from infected to uninfected mice, *C. pneumoniae* DNA was detected in lung, spleen, abdominal lymph nodes and/or thymus.

These results demonstrate the ability of *C. pneumoniae* to disseminate via macrophages *in vivo*. Persistence of *C. pneumoniae* within atheromas of apoE-deficient mice suggests tropism of the organism to the lesion indicating this animal model should be of benefit in examining whether infection alters the progression of atherosclerosis. The presence of *C. pneumoniae* in the aorta of C567BL/6J mice indicates this mouse model will be useful to investigate whether infection can induce atherosclerosis.

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DEDICATION

I wish to dedicate this dissertation to my husband, David, who fed me when I'd forgotten to eat, soothed me when I required calming and hugged me when I needed it most.

INTRODUCTION

Chlamydiae are a diverse group of organisms causing a wide range of disease in both human and animal hosts. Members of the genus Chlamydia are obligate intracellular bacteria with a unique, biphasic developmental life cycle consisting of a noninfectious intracellular reticulate body (RB) stage and an infectious extracellular elementary body (EB) form (56). Infection of host cells with chlamydiae is initiated when the EB attaches to the host cell membrane and enters the cell by receptor mediated endocytosis in a phagosome. Once within the cell, the metabolically inactive EB reorganizes into the metabolically active RB losing its infectivity with walls becoming porous and fragile and increasing ribosomal activity. The RB divides by binary fission within the phagosome or inclusion vesicle which is composed of the host plasma membrane and chlamydial proteins. An as yet uncharacterized factor produced by the elementary body appears to modify the membrane of the phagosome preventing phagosome-lysosome fusion and allowing the intracellular survival of chlamydial particles (55). Division and growth continues as the inclusion vesicle expands in size to accommodate the increasing numbers of RBs, EBs and intermediate bodies produced. After 48 to 72 hours, chlamydial particles are released, either by cell lysis or fusion of the inclusion membrane with that of the host (29). Other unique characteristics of the genus Chlamydia include the lack of a peptidoglycan layer between the outer and inner membranes, the presence of hexagonally arrayed cylindrical projections on the outer membranes of EBs, utilization of host ATP for synthesis of chlamydial proteins and a small genome, second in size only to Mycoplasma (56).

The genus Chlamydia is currently separated into four species, *Chlamydia trachomatis*, *Chlamydia psittaci*, *Chlamydia pneumoniae* and *Chlamydia pecorum,* of which *C. trachomatis*, and *C. pneumoniae* are primarily human pathogens. Isolates of *C. trachomatis* are divided into 3 distinct biovars, trachoma, lymphogranuloma venereum (LGV) and mouse, based on host specificity, the types of cells infected and disease syndrome produced (54). The trachoma and lymphogranuloma biovars are further divided into serovars based on surface protein antigens. In *C. trachomatis* disease, serovars A, B, Ba and C infect conjunctival epithelia and cause trachoma, the leading cause of preventable blindness in developing countries (75). Serovars D through K primarily infect urogenital epithelia which, if undetected and untreated, can result in pelvic inflammatory disease, a major cause of infertility in women (86). Serovars L1, L2 and L3 initally infect urogenital epithelia, but spread to deeper lymphatic tissues developing into lymphogranuloma venereum, an invasive systemic disease (75). *C. psittaci* is primarily a disease of psittacine birds and nonhuman mammals, but infection can spread to man resulting in pneumoniae and, rarely, fatal meningoencephalitis (75).

Chlamydia pneumoniae was first isolated in 1965 from the conjunctiva of a child in Taiwan (35). This isolate was designated TW-183 and was observed to form dense oval shaped inclusions containing no glycogen similar in morphology to *C. psittaci*. Based upon the similarity of its phenotypic and morphologic characteristics, the isolate was initially considered a strain of *C. psittaci*. The serological association of this strain with respiratory disease led to the isolation in 1983 of the first isolate, designated AR-39, from the respiratory tract of a student with pharyngitis (17). The strain designation, TWAR, was derived from the first conjunctival (TW-183) and respiratory isolates (AR-39).

Based upon DNA restriction endonuclease analysis and DNA-DNA hybridization, the TWAR isolate was established as the third separate species of Chlamydia, i.e., *C. pneumoniae* (7). Quantitative DNA liquid hybridization studies have shown that *C. pneumoniae* isolates have ≥94% homology with each other, but <10% with *C. psittaci* and *C. trachomatis* isolates. *C. pneumoniae* also have a uniquely characteristic pear-shaped EB morphology with a large periplasmic space which is distinct from the round EB of other Chlamydia species.

C. pneumoniae is a significant cause of human respiratory disease accounting for 10% of community acquired pneumonia (20). Infection with *C. pneumoniae* can result in both acute upper (pharyngitis, sinusitis) and lower (bronchitis, pneumonia) respiratory disease (20, 79). Pneumonia and bronchitis are the most commonly observed signs of infection, but asymptomatic or mildly symptomatic disease are the most common result of infection. Other clinical signs include prolonged, nonproductive cough and fever. Routine laboratory diagnosis of respiratory disease is based primarily on serology and isolation. *C. pneumoniae* grows poorly and is difficult to isolate from clinical samples, therefore diagnosis of infection is often based on serology. The microimmunofluorescence test is a specific and sensitive serological assay used to distinguish recent from past infection and reinfection from primary infection. The criteria for serological diagnosis of an acute infection is a four fold rise in titer between two sera collected over a 3-4 week period, an IgG titer ≥ 512 or an IgM titer ≥16. Pre-existing antibody suggesting current or persistent infection is indicated by an IgG titer between 8 and 256 (39). The difficulty in culturing *C. pneumoniae* has led to the development of alternative molecular methods of detection of the organism. Fluorescent antibody or enzyme linked immunosorbent assay detection has been used for swab and smear samples, but is not sensitive. Immunocytochemistry (ICC) using genus-specific and species-specific monoclonal antibodies can localize chlamydial antigen to tissue and cell types (38). *C. pneumoniae*-specific polymerase chain reaction (PCR) has been successfully used for the detection of organisms in routine upper respiratory specimens (39). In these studies, PCR appeared to be more sensitive than isolation for detection of the organism (8, 13).

Seroepidemiological studies using the *C. pneumoniae* specific MIF test have revealed a high prevalence of antibody to *C. pneumoniae* indicating the universal nature of infection worldwide (85). In Europe and the US, serological evidence of infection is uncommon in children less than 5 years of age. Prevalence increases from ages 5 through 14 years of age, reaching approximately 50% by 20 years of age (24). Seroprevalence continues to increase slowly through old age, reaching approximately 75% in individuals aged 60 years and above. Infection rates are high (5-10% per year) in children 5 to 15 years of age with new infections occurring in adults of all ages at rates of 1-2% a year (1). *C. pneumoniae* antibodies are usually lost in 3-5 years following the initial infection (62). These prevalence and infection rates, together with the time-limited antibody response, suggests that during their lifetime, all persons are infected and usually reinfected with *C. pneumoniae*. Also, a persistent carrier state may follow asymptomatic infection. The organism is transmitted human to human via respiratory secretions and can be spread by asymptomatic carriers (39). Humans are the only known reservoir of infection as no evidence of an animal intermediate host has been proven (24). The incubation period is long, 21 days, and the apparent low infectiousness of *C. pneumoniae* has led to the slow spread of infection even in favorable epidemic conditions (53).

All chlamydial species have a tendency to cause chronic infections. Mild, acute infection with *C. trachomatis* can progress to severe, chronic inflammation leading to blindness and infertility up to 50 years after primary infection (42). Chronic cases of LGV and *C. psittaci* have persisted in humans for up to 10-20 years (42). *C. pneumoniae* has also been associated, at least serologically, with several chronic diseases, including bronchial asthma, chronic obstructive pulmonary disease, Guillian-Barré syndrome, cystic fibrosis, erythema nodosum and sarcoidosis (24, 26, 73). The chronic disease state which has accumulated the most extensive body of evidence for an association with *C. pneumoniae* is atherosclerosis and coronary heart disease (24).

The first association between *C. pneumoniae* and atherosclerosis was made in 1988 in Finland by Saikku *et al.* (72), who compared antibody titers of paired sera from 40 patients with chronic coronary heart disease (CCHD) and 30 patients with acute myocardial infarction (AMI) to controls matched for age, sex, social status and geographical area. Significantly elevated antibody levels of IgG and/or IgA to chlamydial antigens were found in patients with CCHD and AMI as compared to control patients. This study first established a possible relationship between chronic chlamydial infection and CCHD and AMI. This investigative group also found circulating immune complexes containing chlamydial antigens in the sera of patients immediately following AMI (42) and in patients with CCHD (45).

This study was soon followed by many seroepidemiological studies from different groups of investigators, all of which have shown a significant association between TWAR antibody and coronary artery disease and coronary heart disease (45, 50, 51, 73, 80, 81). Five of these studies were compared (24) and the associations expressed as an adjusted odds ratio, which approximates the relative risk of atherosclerosis in those with antibody to *C. pneumoniae* to those without antibody. The odds ratios were adjusted for such risk factors for atherosclerosis as hypertension, smoking and serum lipid levels. An adjusted odds ratio of 1.5 to 2.6 was consistently found. Untreated hypertension has a similar risk factor for atherosclerosis (24). These findings suggest a moderate, independent cross-sectional association between antibody to *C. pneumoniae* and atherosclerotic disease.

A direct correlation between *C. pneumoniae* and atherosclerotic disease was found when *C. pneumoniae* organisms were observed by electron microscopy (EM) in coronary atheromas of 7 autopsy patients by Allan Shor, a pathologist (77). He described structures similar to the pear-shaped elementary bodies which are characteristic of *C. pneumoniae* in fibrolipid plaques from atheromatous coronary arteries of gold miners in South Africa. Five of these cases were later confirmed by ICC using both Chlamydia genus-specific and *C. pneumoniae*-specific monoclonal antibodies by Kuo *et al.*, at the University of Washington (77).

This finding was followed by several more studies at the University of Washington in which *C. pneumoniae* was found in arterial tissues from many arterial sources, geographical regions and populations and in both early and late atherosclerotic lesions by a variety of different techniques including EM, ICC and PCR. Further evidence of a physical association of *C. pneumoniae* was found in a study by Kuo *et al.*, (37), in which 36 South African autopsy cases were examined by PCR as well as ICC and EM. Samples included coronary atheromas from cases ranging in age from 20 to 83 years in which death in the majority of cases was due to trauma. Lesions ranged from early fatty streaks to later fibrolipid plaques. Control coronary tissues was included from 11 individuals in which no atheroma was found. *C. pneumoniae* was detected by ICC in 15 of the 36 cases and by PCR in 13 of 30 cases examined. In all, the organism was found by ICC or PCR or both methods in 20 of the 36 cases. TWAR was detected in 7 of 16 fatty streak lesions and in 13 of 20 fibrolipid plaques. By ICC, the organism was localized to the site of tissue damage, in foam cells and in the central necrotic core of the plaque and not in adjacent nondiseased arterial tissues. All eleven control coronary artery tissues were negative by ICC and PCR. Amplified sequences from two cases positive by PCR representing the rRNA gene were cloned and sequenced and confirmed the DNA sequence as identical to *C. pneumoniae*.

In a retrospective aortic atherosclerotic study (38), *C. pneumoniae* antigens were detected by ICC in 1 of 4 fatty streaks and 6 of 17 fibrous plaques. Double-label immunocytochemical staining using *Chlamydia*- and tissue type-specific monoclonal antibodies showed *C. pneumoniae* antigen in the cytoplasm of macrophages and smooth muscle cells of the atheromatous lesion.

In a study of directional coronary atherectomy tissue (9), 38 specimens, 23 from primary lesions and 15 from restenotic lesions, were examined for *C. pneumoniae* by ICC, PCR and EM. The organism was detected by either ICC or PCR or both in 20 of 38 specimens with localization of chlamydial antigen to macrophages within the lesions. Interestingly, of positive lesions, *C. pneumoniae* was found in 10 of 15 (67%) of patients with restenotic lesions as compared to 10 of 23 (43%) with primary lesions.

In a study conducted to detect *C. pneumoniae* in arterial sites other than coronary or aortic (23), 5 fresh or formalin fixed carotid endarectomy specimens were examined by ICC and PCR and 56 archived formalin-fixed paraffin-embedded carotid endarectomy tissues were examined by ICC. Five of 5 fresh/formalin-fixed samples were positive by ICC and 3 of 5 were positive by PCR. Thirty -two of the 56 archived samples were positive by ICC. Thirteen normal carotid artery tissue samples were negative by ICC, however these specimens were not age-matched to the case specimens. These studies are significant because they extended the finding of *C. pneumoniae* to another major artery in which atherosclerotic disease is important.

In a multicenter study (40), "Pathobiological determinants of atherosclerosis in Youth", age- and sex-matched left anterior descending coronary artery specimens from autopsy cases of young persons, aged 15-34 years of age, with and without evidence of atherosclerosis, were examined by PCR and ICC. Of 49 patients, eight specimens were positive for *C. pneumoniae* by PCR and/or ICC. Six of 7 atheromas and 2 of 11 intimal thickenings were positive. None of the 31 coronary samples without lesions were positive. This study showed that *C. pneumoniae* can be found in a high proportion of coronary atheromatous lesions of young people. It also showed that in tissue from age- and sex-matched controls with no evidence of atherosclerosis, *C. pneumoniae* is not detected, adding further circumstantial evidence that *C. pneumoniae* influences the atherosclerotic process. In a study to demonstrate *C. pneumoniae* in a variety of atherosclerotic arteries (59), the organism was detected by PCR in 11 of 25 aortas, 5 of 9 iliac arteries, 2 of 5 femoral arteries and 1 of 2 iliac veins. Notably, of 23 samples in which moderate to severe inflammatory infiltrates were present, 56% were positive for *C. pneumoniae* by PCR as compared to 31% positive in which there was no or minimal infiltrates.

In summary, cumulative results of these studies showed that *C. pneumoniae* was detected in aorta, surgical and atherectomy tissues in persons of different race and from different geographical regions. The organism was detected in early and late lesions of young and older patients of both genders. It is important to note that in studies where tissues were available, the organism was not detected in normal internal mammary, carotid and aorta tissues.

The studies at the University of Washington have been confirmed by other investigative groups worldwide (57, 59, 60). In a study by Muhlestein *et al.*, (57), 90 atheromatous coronary atherectomy specimens were analyzed by direct immunofluorescence for *C. pneumoniae* and compared to 12 nondiseased coronary specimens and 12 diffusely diseased coronary samples associated with ischemia-induced transplant rejection coronary heart disease. Seventy-nine per cent of the atheromatous specimens were positive as compared to only 1% of the nonatherosclerotic specimens. This was the first study to compare the presence of *C. pneumoniae* between not only atherosclerotic tissue and nondiseased arterial tissue, but also to arteries diseased by processes other than atherosclerosis. The presence of *C. pneumoniae* was expanded to iliac vessels when the organism was found in 11 of 25 (44%) of aortas, 5 of 9 (55%) of iliac arteries, 2 of 5 (40%) femoral arteries and 1 of 2 iliac veins from 32 patients undergoing vascular surgery by PCR in a study by Ong *et al.*, (59). Of the 23 positive specimens, there was a tendency for the organism to be present in tissues exhibiting moderate to severe inflammation (56%) as compared to specimens that had no or minimal inflammation (31%). In Japan, *C. pneumoniae* has also been found by ICC and/or PCR in 16 of 29 coronary arteries and 4 of 10 large arteries (60).

In all the molecular studies described above, *C. pneumoniae* was detected in atheromatous lesions by techniques other isolation. The difficulty in obtaining fresh human arterial samples in a timely fashion, the presence of necrotic tissue debris in atherectomy specimens, combined with the technical expertise required to successfully culture chlamydia even under the most optimal circumstances has resulted, until recently, in the inability to culture viable organisms from atherosclerotic specimens. *C. pneumoniae* has been isolated from the coronary artery of a recipient heart removed prior to heart transplantation (68) and from carotid atheroma removed during carotid endarterectomy (32). In spite of these recent successes, PCR remains the method of choice in the detection of *C. pneumoniae* in chronic infections due to its greater sensitivity over isolation (39). Also, detection of chlamydial DNA by PCR or ICC may indicate persistence of a quiescent infection when the organism is no longer in a culturable state. Two studies using mouse models of pneumonitis were done to answer the question of whether PCR detects persistent, nonculturable, but viable, organisms (41, 46). In both studies, immunosuppression with cortisone treatment reactivated lung infection of experimentally inoculated mice in which lung tissue was culture negative, but remained PCR positive. These studies indicate that *C. pneumoniae* persists *in vivo* in a viable, but culture negative, state which can be detected by PCR.

Three animal models of *C. pneumoniae* infection have been described: mouse (87, 88, 89), monkey (4, 30) and rabbit (Chapter 1, 34). *C. pneumoniae* is of low virulence in baboons, cynomolgus macaques and rhesus macaques. Nasopharyngeal, oropharyngeal and intranasal inoculation resulted in no clinical signs of disease attributable to experimental infection although *C. pneumoniae* could be cultured from the nasopharynx. Inoculation of *C. pneumoniae* into the conjunctiva resulted in only mild inflammation. In contrast, mice are exquisitely susceptible to infection with *C. pneumoniae* with intranasal inoculation resulting in moderate to severe respiratory disease and systemic dissemination. Lung pathology in mice is characterized by interstitial pneumonia with a predominately granulocytic infiltrate initially, followed by mononclear cell infiltration. A striking pathologic feature is the perivascular and peribronchiolar accumulation of lymphoid cells in the intersitium which persists for up to 60 days after inoculation. Ultrastructure examination showed *C. pneumoniae* inclusions in ciliated bronchial epithelial cells and interstitial macrophages. Organisms could be isolated from the lungs of Swiss Webster mice for 42 days after inoculation. *C. pneumoniae* was also shown to spread systemically to the spleen and peritoneal macrophages following intranasal inoculation of mice. The rabbit model of *C. pneumoniae* infection is fully described in Chapter 1 and is characterized by a mild self-resolving pneumonitis. As in the mouse model, systemic dissemination to peripheral blood mononuclear cells and spleen was also seen. A systematic nature of *C. pneumoniae* is supported by the finding of the organism in lungs, liver and lymph node of patients with severe pneumonia and the finding of the organism in atheromatous lesions.

Atherosclerosis is a complex and multifactorial disease process associated with many risk factors, including hypercholesterolemia, hypertension, cigarette smoke, and family history (33). The current understanding of the pathogenesis of atherosclerosis involves the following sequence of events: (a) vascular injury or activation; (b) monocyte/macrophage adherence to the endothelium, migration across the endothelium to the subendothelial space; (c) alteration in lipid metabolism of the macrophage due to an undefined cause leading to increased uptake and accumulation of oxidized low density lipoproteins within the macrophage transforming them into foam cells; (d) the presence of chemically modified lipoproteins activate the release of cytokines by macrophage/foam cells inducing the upregulation of endothelial cell adhesion molecules resulting in further monocyte and lymphocyte adhesion and subendothelial migration; (e) platelet aggregation at the site of endothelial activation resulting in the release of platelet derived growth factor, a mitogen, which induces directed migration and stimulates vascular smooth muscle cell migration and proliferation; (f) smooth muscle cells dedifferentiate from a mature contractile phenotype to a less mature synthetic phenotype secreting increased amounts of collagen, elastin and proteoglycans leading to the formation of fibrous tissue and progression of the atheroma; (g) organization of platelet thrombus and continued extracellular matrix synthesis result in the further development of the atherosclerotic lesion. (33, 34, 49)

The presence of inflammatory cells, notably mononuclear phagocytes and T lymphocytes, within the atherosclerotic plaque has led to the investigation of infection and the immune system as a component of disease (43). Inflammation and immune mechanisms may be actively engaged in the development of atherosclerosis and may also serve as the initiating event in atherogenesis. Activation of cells by infectious agents followed by the expression of surface antigens can cause cytokine secretion, upregulation of leukocyte adhesion molecules and alterations in lipid metabolism resulting in the cascade of events leading to atherogenesis. Candidate antigens for activation include viruses and bacteria. An avian herpesvirus, Marek's disease virus (MDV), stimulates cholesterol and phospholipid accumulation in the aortic wall of normocholesterolemic chickens and atherosclerosis in diet-induced hypercholesterolemic chickens (26, 27). Human herpesviruses, Cytomegalovirus and Herpes simplex virus, have been associated with early atheromatous changes and accelerated atherosclerosis in cardiac allograft recipients (32). Although these studies have indicated herpesviruses are associated with atheroclerosis, the establishment of a causal role for these viruses is hampered by the ubiquitousness of the organism in the human population and the presence of the virus in normal nondiseased areas of arterial tissue in the absence of atherosclerotic changes. As has been discussed earlier in this dissertation, *C. pneumoniae* is consistently present in atherosclerotic lesions and not in normal control arterial tissues or in inflamed arterial tissues due to diseases other than atherosclerosis. The absence of *C. pneumoniae* within arteries diseased by processes other than atherosclerosis suggests that *C. pneumoniae* plays an active role in the pathogenesis of atherosclerosis.

Three possible roles exist for *C. pneumoniae* in the pathogenesis of atherosclerosis. First, the organism may play no role, persisting in cells within the atherosclerotic lesion, but causing no pathology. Second, the organism may act as an inducer by initiating cellular injury at the lesion site. Third, the organism may act as a cofactor and exacerbate the disease process and contribute to the immunopathology of the developing lesion.

In a study to test the null hypothesis that *C. pneumoniae* was found as frequently in other tissues as in atherosclerotic lesions, the frequency of the organism in cardiovascular tissue was compared to noncardiovascular tissue in multiple tissues from 38 autopsy patients (31). The proportion of patients with coronary artery specimens (34%) positive by PCR and/or ICC was significantly higher than the proportion with lung (13%) (p=.03), liver (10%) (p=.01), or spleen

(5%) specimens positive. In the same study, 33 granulomatous tissue specimens from different disease states were screened for the presence of *C. pneumoniae* PCR and ICC. The organism was not found in granulomatous tissues except in cases of sarcoidosis, which is known to be associated with *C. pneumoniae*. These studies indicate that C. pneumoniae has a predilection for cardiovascular tissue.

Although, the seroepidemiological and direct detection studies described previously do not differentiate a causal or effectual relationship of *C. pneumoniae* in atherosclerosis, these data exemplify the large body of evidence that links the immunopathology of *C. pneumoniae* infection with the process of atherosclerosis. Because *C. pneumoniae* has been found only within atheromatous lesions of vascular tissues and not in normal tissue, its presence implies that it may play a pathogenic role in the atherosclerotic process. The accumulation of infammatory cells including macrophages and lymphocytes within atherosclerotic lesions has led to several investigative groups to consider atherosclerosis to be the result of an immune mediated chronic inflammatory response. Many characteristics of *C. pneumoniae* immunopathology are similar to those seen in atherosclerosis. These characteristics, as discussed earlier in the dissertaton, include the following:

- Chlamydiae are known to establish persistent chronic infections and stimulate an immune response consisting initially of mononuclear cells, predominately macrophages and lymphocytes, which leads ultimately to fibrosis and scarring (75);
- *C. pneumoniae* disseminates systemically in mouse models to sites other than respiratory following intranasal inoculation and can infect macrophages *in vivo* (89);

- *in vitro* studies have shown *C. pneumoniae* can infect and replicate in endothelial cells, human macrophages and smooth muscle cells - all cellular components of atherosclerosis (15, 16);
- human studies have shown a tropism of C. pneumoniae for cardiovascular tissue (31).

Our hypothesis for an etiological association of atherosclerosis with *C. pneumoniae* is that following infection and respiratory disease, *C. pneumoniae* infected macrophages disseminate from the lung to the cardiovasculature or other major arteries such as the aorta or carotid artery. The pathway utilized would either be hematogenous or lymphatic and the organism transported within a carrier cell such as a monocyte/macrophages. Once at an appropriate arterial site, infected monocytes/macrophages would be attracted to a site of lesion formation and enter the arterial wall from the vessel lumen by either adherence to the endothelium and migrating to the subendothelium or from the adventitia via the vaso vasorum. There, persistent infection by *C. pneumoniae* would result in either the initial injury and/or induction of chronic immunopathology characteristic of chlamydial infection. Once present, the immune response to *C. pneumoniae* would be augmented by other atherogenic factors such as hypertension and hypercholesterolemia known to contribute to the progression of atherosclerosis.

Many animal models of atherosclerosis exist, each with its predominant features of lesion development which make it suitable for studying different aspects of atherosclerosis (2). Nonhuman primates, particularly the rhesus and cynomolgus macaques, are considered to be one of the best animal models of atherosclerosis due to the rapid induction of lesions when fed a high fat diet and histopathological similarity to human lesions. However, the disadvantage of this model include expense, limited availability and considerable variability of lesion formation. Porcine models develop atheromas of the aorta and coronary arteries when fed a high fat diet and lesions closely resemble human lesions. However, the disadvantages of this model are similar to those of nonhuman primates in that the species is expensive to maintain and variability of lesion formation due to the lack of inbred strains. Also, lesions rarely occur spontaneously and require induction by an atherogenic diet in both primate and porcine models. Several avian species develop spontaneous atherosclerosis of the thoracic aorta and iliac bifurcation. Although these lesions are milder than those seen in humans, they can be accentuated by high cholesterol diets. White Carneau pigeons are used extensively to study coronary atherosclerosis and coronary infarction which closely resembles human disease. Selective breeding of Japanese quails has resulted in resistant and susceptible strains when fed a cholesterol diet. Although foam cells accumulate in the arteries of this avian model, no inflammation or necrosis is seen, limiting the use of this model to studies of cholesterol metabolism. Rats, hamsters and guinea pigs have also been used to study atherosclerosis, but due to the restricted development of lesions in morphology, size and location, their use has also been limited.

Rabbit models offer the advantage of providing both spontaneously occurring genetic strains (WHHL) and diet-induced strains (cholesterol-fed NZW). Developed in 1980, the WHHL rabbit was the first animal model of natural endogenous hypercholesterolemia. The trait is due to a single gene defect consisting of an in frame deletion of 12 nucleotides in the LDL receptor gene. This deletion results in a poorly glycosylated mature protein which impairs transport from the endoplasmic reticulum thus preventing its localization to the cell membrane. The clinical and pathological expression of this defect closely resembles the disease in humans with familial hypercholesterolemia (3, 4). Atherosclerotic lesions of WHHL rabbits resemble those seen in humans, but a disadvantage of this strain is its low reproductive rate. Atherosclerosis can also be induced in rabbits by feeding an atherogenic diet consisting of cholesterol, fat and casein. However, the kinds, amounts and ratios of fat in reported diets vary widely and may have an effect on the time of lesion onset and size. The effects of age, sex and breed on the susceptibility of rabbits to diet-induced atherosclerosis is not clear. Also, a criticism of diet-induced rabbit models is that lesions do not progress to advanced or complicated plaques.

Mice demonstrate a wide genetic variation in susceptibility to atherosclerosis, but will develop lesions when fed an atherogenic diet supplemented with cholic acid. The most susceptible strains include the C57BL/J, DBA/2J, AKR/J and the 129/J (65). Development of lesions requires 4-5 months of feeding to induce lesion formation. Although lesions do eventually form along the arterial tree, the most advanced lesions are confined to the coronary sinus, are not progressive and morphologically resemble large fatty streaks composed predominately of foam cells. Also, the cholic acid diet necessary to induce these lesions is largely nonphysiologic, containing 10-20 times the amount of cholesterol in human diet, and is toxic on long term feeding. Recent advances in mouse genetics has resulted in the generation of transgenic mouse strains which either overexpress or are completely deleted of a specific gene of interest. Several transgenic mouse lines have been established that overexpress and underexpress the human lipoprotein transport genes (6). Most develop limited atherosclerotic lesions and are used mainly in studies of lipoprotein metabolism. Two genetically engineered mouse models of that develop spontaneous atherosclerosis on a low fat diet have been reported (67).

In the apoA-II transgenic mouse which overexpresses the apoA-II, one of the major proteins of HDL, lesions were immature and located solely at the coronary sinus. In the apoE-deficient mouse, the apoE gene has been deleted by insertional inactivation and homologous recombination. In mice, apoE is the primary ligand for LDL receptor-mediated removal of lipoproteins from the circulation (65). The deletion of this ligand results in the accumulation of cholesterol in the peripheral circulation due to the lack of uptake by cells. ApoE-deficient mice show severe hypercholesterolemia from birth even when fed a normal chow diet. These mice exhibit all the known phases of the fibroproliferative responses of atherogenesis. Monocyte adherence is seen at 8 weeks of age, fatty streaks by 10 weeks of age and intermediate lesions and fibrous plaques by 15-20 weeks of age. They are unique among mouse models of atherosclerosis in that not only do lesions contain all the cellular components of human lesions, but also progressively form over time throughout the thoracic and abdominal aorta. In all cases, lesions form principally at artery branch points or bifurcations. In all, the pathogenesis of atherosclerosis observed in the apoE-deficient mouse model bears bears many similarities to the human disease process (58). Due to its relatively low cost and the advantage of an homologous genetic background, it is extensively used as a model of atherosclerosis (65, 66, 67).

The collective purpose of this dissertation is to establish and characterize effective animal models of both *C. pneumoniae* infection and atherosclerosis to investigate the etiological role of *C. pneumoniae* in the pathogenesis of atherosclerosis according to the proposed hypothesis. Animal models to be evaluated include WHHL and NZW rabbits and apoE-deficient transgenic and C57BL/6J mice. These models were chosen because they offer the advantage of both spontaneously occurring and diet induced models within the same species. Also, they are well-characterized animal models of atherosclerosis. The murine model offers the added advantage of a homologous genetic background.

CHAPTER 1: EXPERIMENTAL RABBIT MODELS

CHAPTER SECTION: PURPOSE

An animal model of both *C. pneumoniae* infection and atherosclerosis is essential to study the role of *C. pneumoniae* in the pathogenesis of atherosclerosis. Of the many animal models of atherosclerosis, the New Zealand White (NZW) rabbit, a high cholesterol diet-induced model and the Watanabe heritable hyperlipidemic (WHHL) rabbit, a spontaneous genetic model, are considered to be the best characterized. The WHHL rabbit has also proven to be a valuable model of human familial hypercholesterolemia. The WHHL rabbit exhibits a natural spontaneously occurring single gene mutation in the LDL receptor which results in the deletion of 4 amino acids in the third repeat unit of the receptor binding domain (34). Although the receptor is synthesized, it is not glycosylated and therefore, is nonfunctional. WHHL rabbits are hypercholesterolemic from birth. Homozygous WHHL rabbits have serum cholesterol concentrations that are 8-14 times normal. Atherosclerotic lesions develop in the aorta by 3 months of age and diffuse aortic atherosclerosis is observed after 1 year of age. Lesions also occur in coronary arteries. Lesion morphology is very similar to humans and ranges from fatty streaks to plaques with fibrous capsules, necrotic cores and extracellular cholesterol crystals. Atherosclerotic lesions can also be diet induced in NZW rabbits by feeding an atherogenic diet. Depending upon the cholesterol and fat content of the diet and the amount of time the animals are fed the diet, lesion morphology can vary from predominately foam cell fatty streaks to mature atheromas similar to human lesions consisting of foam cells and smooth muscle cells with necrotic cores, calcification and fibrous plaques can be produced. Rosenfeld *et al.* (70, 71), compared the development of atherosclerosis between WHHL rabbits and cholesterol-fed NZW rabbits and found no differences in the initial development and subsequent expansion of fatty streaks. NZW and WHHL rabbits were chosen for evaluation based on the similarity of lesion morphology to that of human lesions and the availability of both spontaneously occurring genetic and diet induced models of atherosclerosis in the same species.

Therefore, we evaluated NZW and WHHL rabbits to determine whether they can be used to study the role of *C. pneumoniae* in atherosclerosis. In order to be acceptable models of *C. pneumoniae* infection, animals must be susceptible to infection with demonstrable signs of respiratory disease, seroconversion, pulmonary inflammation and recovery of viable organisms from the upper and lower respiratory tract. Most importantly, *C. pneumoniae* must disseminate from the respiratory tract to the site of atherosclerotic lesion development and be detected by either culture, PCR or ICC. Also, we sought to develop a rabbit model of *C. pneumoniae* pneumonitis similar to previously developed murine models (87, 88, 89) to investigate the immunopathology of chronic *C. pneumoniae* infection.

CHAPTER SECTION: MATERIALS AND METHODS

CHAPTER SUBSECTION: *C. PNEUMONIAE* STRAIN AND INOCULUM PREPARATION

C. pneumoniae, strain AR-39 (18), was used for inoculation of animals. TWAR organisms were grown in HL cells(22) and purified by diatrizoate meglumine centrifugation (Hypaque-76; Winthrop-Breon Laboratories, New York, NY) (8) The inoculum preparations were resuspended in sucrose phosphate glutamic acid (SPG) medium and frozen at -70°C until use.

CHAPTER SUBSECTION: EXPERIMENTAL ANIMALS

Animals used were *Pasteurella multocida*-free (four to five months old) male and female NZW rabbits, purchased from Western Oregon Rabbit Company (Philomath, OR), and eight months old male and female homozygous WHHL rabbits, obtained from the breeding colony at the University of Washington (70, 71).

CHAPTER SUBSECTION: INOCULATION OF NZW AND WHHL RABBITS

Rabbits were anesthetized with a cocktail of ketamine (Fort Dodge Laboratories, Fort Dodge, IA), xylazine (Lloyd Laboratories, Shenandoah, IA) and acepromazine (Fermenta Animal Health Company, Kansas City, MO). Rabbits were inoculated with a total volume of 1.0 ml of organisms suspended in SPG medium. Sham-inoculated negative controls were inoculated with either 1.0 ml of sterile SPG medium or nonviable organisms which were heat-inactivated at 56°C for 60 min. Intratracheal inoculations were delivered by a tuberculin syringe through a 20-gauge 11/4 inch catheter (Angiocath; Becton Dickinson, Sandy, UT) inserted into the trachea just distal to the thyroid cartilage. Intranasal inoculation was done by administering 1.0 ml of inoculum to the nostrils using a tuberculin syringe.

To determine the optimal infectious dose and route of inoculation, NZW rabbits were divided into 3 groups: intratracheal inoculation at 4×10^7 inclusion forming units (IFUs) per rabbit (IT/low dose) or 5×10^8 IFUs per rabbit (IT/high dose) and intranasal inoculation at 4×10^8 IFUs per rabbit (IN/high dose). Samples were taken at various time points post-inoculation.

Repeated intratracheal inoculations of high dose *C. pneumoniae* organisms were used to attempt to establish long-term infection in NZW rabbits. Rabbits were inoculated two or three times at weekly intervals with 4.6×10^8 IFUs each time. Tissues were collected at various time points after inoculation.

To evaluate the susceptibility of WHHL rabbits to *C. pneumoniae* infection, animals were inoculated intratracheally three times at weekly intervals with 4.6 X 10^8 IFUs each time. Samples were collected at various time points after the last inoculation.

CHAPTER SUBSECTION: SAMPLE COLLECTION

Rabbits were sedated with acepromazine and heparinized blood was collected from the auricular vein. After blood collection, each rabbit was euthanized by intravenous injection of T-61 (American Hoechst Corp., Somerville, NJ). Nasopharyngeal and tracheal swabs were collected using sterile cotton swabs on aluminum shafts (Spectrum Laboratories, Inc., Houston, TX) and placed in sterile glass vials containing 1.0 ml of either SPG medium for culture or lysis buffer (100mM Tris Cl, pH 8.0, 25 mM EDTA, 2% Triton X-100) for PCR. After vortexing, swabs were removed and the remaining sample was frozen at -70°C. Portions of the lung, liver, spleen, heart and ascending aorta were removed using a separate set of sterile instruments for each tissue, placed in sterile glass vials and immediately placed on ice. Tissues were later weighed and frozen at -70°C. Peripheral blood mononuclear cells (PBMC) were purified using a sodium diatrizoate gradient (Histopaque-1077; Sigma, St. Louis, MO), washed twice in 1X phosphate buffered saline (PBS), resuspended in 1.0 ml lysis buffer and frozen at -70°C.

CHAPTER SUBSECTION: SAMPLE PREPARATION FOR PCR

Tissue samples were thawed and homogenized with a sterile mortar and pestle in 3.0 ml of lysis buffer. Nasopharyngeal, tracheal and PBMC samples were thawed and 500 µg/ml Proteinase K (Amresco, Solon, OH) was added to each sample. After vortexing, samples were incubated overnight at 55°C. Lysates were extracted with phenol, phenol/chloroform: isoamyl alcohol (24:24:1) and chloroform: isoamyl alcohol (24:24:1). DNA was precipitated with

0.7 volumes of 100% isopropanol followed by washing with ice cold 70% ethanol. DNA pellets were air dried, resuspended in 50-100 ml of sterile water (Baxter Healthcare Corp., Deerfield, IL), and purified by drop dialysis (31) to remove possible inhibitors in tissue and swab specimens. An extraction control of lysis buffer alone was included with each set of tissue extractions to detect cross-contamination of samples during the extraction process. All tissues were collected at necropsy with a separate set of sterile instruments. Tissue homogenization, DNA extractions and PCR amplification were performed in separate rooms equipped with germicidal lighting. All bench surfaces were prepared prior to and after use with bleach and alkaline solutions. Procedures were performed using gloves, face masks and lab coats. Gel electrophoresis and confirmation of amplification products were performed in areas separate from tissue processing and extraction of DNA

CHAPTER SUBSECTION: POLYMERASE CHAIN REACTION

DNA samples were amplified using *C. pneumoniae*-specific primers HL-1 and HR-1 according to previously described parameters (8). The nucleotide sequences of the primers used, from the 5' to 3' end, are as follows:

HL-1, GTTGTTCATGAAGGCCTACT;
HR-1, TGCATAACCTACGGTGTGTT.

Samples were amplified for 40 cycles. Each cycle consisted of the following: denaturation at 94°C for 1 min, annealing at 48°C for 1 min and primer extension at 72°C for 1 min. The amplification product, a 437 bp *C. pneumoniae*-specific DNA sequence, was analyzed by gel electrophoresis through a 1.5% agarose gel according to standard protocols (74). The amplification product was transferred by Southern blot to a nylon membrane (Qiagen, Chatsworth, CA) and hybridized to a digoxigenin-dUTP-labeled probe consisting of a 474 bp *PstI* fragment which spanned the target sequence (8). DNA probes were labeled using the Genius DNA labeling and detection kit (Boehringer Mannheim Biochemical, Indianapolis, IN). Hybridized amplification products were detected by immunochemiluminescence using Lumiphos 530 (Boehringer Mannheim Biochemical, Indianapolis, IN) according to the manufacturer's instructions, followed by autoradiography. Controls for each amplification consisted of serial dilutions of purified *C. pneumoniae* DNA as positive controls and sterile water in place of sample DNA as a negative control. Total volume for each amplification reaction equaled 50 µl. Positive and negative controls were amplified, analyzed and confirmed concurrently with each set of samples. All DNA samples were amplified undiluted and diluted 1:10 at 1 and 5 µl each in order to ensure sufficient target DNA for amplification. All positive samples were confirmed by a second amplification and hybridization. Selected tissue DNA samples were screened for inhibition of PCR amplification by seeding samples with 1 µl of *C. pneumoniae* genomic DNA diluted 10^{-7} and comparing the amplification product with the positive control of the same strength.

CHAPTER SUBSECTION: SEROLOGY

Antibody to *C. pneumoniae* was assayed by the microimmunofluorescence test using formalin-fixed whole EBs of *C. pneumoniae*, AR-39, as antigen (80). Serum antibody was detected with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (whole molecule; Sigma Company, St. Louis, MO). Pre- and post-inoculation sera were titrated by serial 2 fold dilutions. IgM antibody was not tested because anti-rabbit IgM conjugate was not available commercially.

CHAPTER SUBSECTION: ISOLATION

Lung tissue was thawed and homogenized with a sterile mortar and pestle in SPG medium to make a 10% (wt/vol) suspension. Lung homogenates were centrifuged at 500 X g for 5 min at 4°C to remove coarse tissue debris and the supernatant was aliquotted for isolation. Pharyngeal and tracheal swab samples were thawed prior to use. Isolation was done in HL cell culture and inclusions were detected by the direct fluorescent antibody stain using the *Chlamydia* genus-specific monoclonal antibody (CF-2) (35) or a *C. pneumoniae* species-specific monoclonal antibody (TT401).

CHAPTER SUBSECTION: HISTOPATHOLOGICAL AND ULTRASTRUCTURAL STUDIES.

Histopathology

Tissues from 1 infected apoE-deficient mouse and 1 sham-inoculated control mouse were analyzed by histopathology and immunocytochemistry at the same time points as tested for isolation and PCR. Lung, spleen and ascending aorta were fixed in 10% phosphate buffered formalin (Fisher Scientific, Pittsburgh, PA) immediately after removal. Lungs were perfused through the trachea with 10-15 ml of 10% formalin using a blunt 18-gauge needle and syringe. Tissues were embedded in paraffin, sectioned and stained with hematoxylin and eosin.

Immunocytochemistry

Paraffin embedded tissues were sectioned for immunocytochemical (ICC) staining. For detection of chlamydial antigens, sectioned tissues were stained with the *Chlamydia* genus-specific murine monoclonal antibody CF-2 and detected by the avidin-biotin-peroxidase method (38). For identification of cell type, serially-sectioned tissues were immunostained with a murine monoclonal antibody specific for rabbit macrophages (RAM-11) (16, 84). Negative antigen controls consisted of consecutive tissue sections from infected animals and tissues from sham-inoculated animals reacted with normal mouse ascitic fluid as a negative control antibody. Positive antigen controls consisted of *C. pneumoniae* infected HL cell pellets which were formalin-fixed and processed as tissue sections.

Electron Microscopy

Lung tissue was dissected and fixed in Karnovsky's glutaraldehyde-paraformaldehyde and post-fixed in osmium tetroxide. Tissue sections were embedded in Epon (Ernest F. Fullman, Schenectady, NY). Thin sections were then stained with uranyl acetate, lead citrate counterstain and viewed in a transmission electron microscope (CM 10: Philips Electronic Instruments, Mahwas, NJ).

CHAPTER SECTION: RESULTS

CHAPTER SUBSECTION: PRIMARY INOCULATION OF NZW RABBITS

Clinical signs

No clinical signs of disease were observed in singly inoculated NZW rabbits. By auscultation, lung sounds were slightly increased immediately after inoculation, but returned to normal within 24 hours. Food intake, hydration and defecation were normal throughout the course of infection. Oculonasal discharge indicative of upper respiratory disease was not seen.

Histopathology

Gross observations revealed lung lesions consisting of multifocal to locally extensive areas of congestion located bilaterally and dorsoventrally. Microscopic findings were characterized by a mild to moderate interstitial pneumonia with bronchiolitis. In rabbits inoculated intratracheally, lesions were most severe at day 7 after inoculation gradually decreasing in severity and returning to normal by day 28 post-inoculation. Histological findings consisted of a moderate to severe cell infiltrate within the interstitium, alveolar space and bronchiolar lumen. The infiltrating cells consisted of heterophilic granulocytes (referred to as heterophils in rabbits) at 3 days after infection, which changed to a mixture of lymphocytes and heterophils by day 7 (Fig. 1B). At day 14 of infection, the cell infiltrate consisted of primarily mononuclear cells with few heterophils. Perivascular and peribronchiolar lymphoid cell accumulations were most severe at day 7 of infection (Fig. 1C). Inflammatory cells were also present in the media of some pulmonary vessels at days 3 and 7 of infection. Extravasation of macrophages and lymphocytes into bronchiolar lumens was seen with some bronchioles completely filled with inflammatory cells and cell debris. Focal areas of alveolar collapse with alveolar septal thickening were noted to gradually increase from day 7 through day 14 of infection. Inflammatory reactions were greatly reduced in severity by day 21 with complete resolution by day 28. Rabbits inoculated once intranasally demonstrated a similar, but milder, lung pathology of only 7 to 14 days in duration. Lymphoid hyperplasia of the splenic corpuscles was seen at 7 days following a single inoculation of *C. pneumoniae* by both intranasal and intratracheal inoculation. This lymphoid reaction was greatly diminished by 14 days post-inoculation, returning to normal by 21 and 28 days. No inflammation was noted in the aorta. No difference was seen in lung or spleen histopathology between high and low inoculum size. Rabbits sham-inoculated with SPG medium showed no inflammatory response in lung or spleen tissue 7 days after inoculation (Fig. 1A).

Serology

Rabbits seroconverted 28 days after inoculation with a serum IgG titer against *C. pneumoniae* of 1:64. Pre-inoculation sera had no *C. pneumoniae* antibody (data not shown).

CHAPTER SUBSECTION: DETECTION OF ORGANISMS

Isolation

C. pneumoniae was isolated from the nasopharynx of IN/high dose animals 2 and 3 days after inoculation (Table 1). Inclusions were detected in cell culture and confirmed by staining with CF-2 and TT401, respectively. Attempts to isolate organisms from the nasopharynx, trachea and lung tissue from other animals at different time points were unsuccessful.

Polymerase Chain Reaction

C. pneumoniae DNA was detected intermittently by PCR in the nasopharynx, trachea and lung tissue through day 21 (Table 1). Spleen tissue at day 14 and PBMC at days 3 and 21 from IN/high dose animals were positive by PCR. All ascending aorta were negative by PCR. Tissues from animals inoculated with SPG medium and heat-inactivated organisms were negative by PCR.

The presence of inflammation did not consistently correlate with PCR results. Only mild diffuse pulmonary lesions were present at 14 days following infection, but lung tissue remained positive by PCR through days 21 and 28 (Table 1). Similarly, spleen tissue was positive by PCR at 14 days when histopathology was resolving. This localization of inflammatory infiltrates may result in a lack of correlation between PCR and histopathology. Only a limited area of tissue can be extracted for PCR, while multiple sections of tissue can be examined histologically.

Immunocytochemistry

Immunoperoxidase staining of lung tissue for *C. pneumoniae* antigen was positive at days 2, 7, 14 and 28 post-inoculation in NZW rabbits inoculated intratracheally with a single high dose of organism. The pattern of staining within the lung tissue was not uniform, ranging from focal to locally extensive in distribution. Chlamydial antigen was present in alveolar macrophages, interstitial cells and peribronchiolar associated lymphoid tissue. Macrophages, as indicated by RAM-11 monoclonal antibody staining, were present within the alveolar space, bronchiolar lumen, lung interstitium and bronchiolar associated lymphoid tissue. Some macrophages within these areas were also positive for chlamydial antigen when examined in serial lung tissue sections (Fig. 2). Negative controls consisting of consecutive tissue sections stained with normal mouse ascitic fluid and tissue sections from sham-inoculated animals were negative. Ascending aorta from NZW rabbits with a single inoculation was negative by immunocytochemical staining.

Electron microscopy

Chlamydial inclusions were seen in interstitial cells of lung tissue at 2 and 3 days after inoculation (Fig. 3). Inclusions were of normal size and shape, but contained few particles. All particles observed were RBs. Infected interstitial cells had the ultrastructural morphology characteristic for macrophages. These cells contained oblong nuclei with diffuse nuclear chromatin and prominent nucleoli. Chlamydial inclusions were not found by EM later than 3 days after inoculation.

CHAPTER SUBSECTION: REPEATED INFECTION OF NZW RABBITS

Clinical signs

No clinical signs of disease were observed in multiply inoculated NZW rabbits. Lung sounds were slightly increased immediately after inoculation, but returned to normal within 24 hours. Food intake, hydration and defecation were normal throughout the course of infection.

Histopathology

At days 14, 21 and 28 post-inoculation, diffuse multifocal mononuclear cell infiltrates containing a mixture of lymphocytes and macrophages were present in the lung interstitium (Fig. 1D). Mononuclear foci gradually decreased in size and number with time, but persisted up to day 42 post-inoculation (the last time point monitored).

Serology

Serum IgG antibody titers against *C. pneumoniae* were 1:256 at day 28 and >1:1024 at day 42 after inoculation (data not shown).

CHAPTER SUBSECTION: DIRECT DETECTION

Isolation

C. pneumoniae was isolated from the nasopharynx and trachea at days 7 and 14 after the last inoculation in rabbits inoculated twice and the trachea at day 14 after the last inoculation in rabbits inoculated three times (Table 2). All other swabs and tissues were negative.

Polymerase Chain Reaction

C. pneumoniae DNA was detected intermittently in the nasopharynx, trachea and lung tissues up to day 42 after inoculation in animals inoculated three times (Table 2). Spleen, ascending aorta and PBMC were negative at all time points (data not shown).

Immunocytochemistry

C. pneumoniae antigen was detected in lung tissue at day 21 in animals inoculated twice and at days 14 and 28 in animals inoculated three times. Differential immunoperoxidase staining with CF-2 or RAM-11 revealed chlamydial antigen in interstitial cells of the alveolar septum and alveolar macrophages. Ascending aorta was negative by immunocytochemical staining in all repeatedly inoculated NZW rabbits.

CHAPTER SUBSECTION: WHHL RABBITS

Four animals, inoculated intratracheally 3 times at weekly intervals with 1.2 X 10^8 inclusion forming units per dose, were analyzed at 7, 14, 21 and 28 days after inoculation. *C. pneumoniae* DNA was detected by PCR in lung tissue at days 7 and 14 post-inoculation. *C. pneumoniae* antigen was found in lung tissue at days 7, 14 and 21 post-inoculation by ICC. Lung pathology was similar to that seen in NZW rabbits. No increase in inflammation was seen in atheromas of infected rabbits as compared to uninfected controls. Rabbits seroconverted with an IgG antibody titer against *C. pneumoniae* of 1:256 at day 28 after inoculation. Antibody in pre-inoculation sera was negative. All attempts to isolate *C. pneumoniae* from the nasopharynx, trachea and lung tissue were unsuccessful. PCR and ICC testing of ascending, thoracic and abdominal aorta were negative in all rabbits at all time points. All tissues from 2 WHHL rabbits sham-inoculated with SPG medium and analyzed 3 and 7 days after inoculation were negative by PCR.

CHAPTER SECTION: DISCUSSION

In this study, we have demonstrated that the rabbit is susceptible to infection with *C. pneumoniae*. Evidence for the establishment of infection includes recovery of infectious organisms by isolation in the upper respiratory tract of repeatedly inoculated animals; detection of chlamydial DNA by PCR and chlamydial antigen by ICC in the upper and lower respiratory tract; and visualization of chlamydial inclusions by EM within the lung interstitium.

The respiratory disease induced in the rabbit model by *C. pneumoniae* is characterized by mild multifocal interstitial pneumonia with bronchiolitis. Microscopically, the location of inflammatory cells within the media of small pulmonary vascular tissues suggests a mild vasculitis. Pulmonary inflammation persisted for up to 42 days after infection in the form of small, multifocal lymphoid aggregates. These findings are similar to that described for the murine model of *C. pneumoniae* pneumonitis (87). Clinically, *C. pneumoniae* infection in the rabbit produces a mild self-resolving respiratory disease. Clinical signs of respiratory disease observed in these rabbit models were less severe than that observed in murine models of *C. pneumonia* pneumonitis (87). Respiratory disease due to *C. pneumoniae* in humans varies from inapparent to mild to severe bronchitis and pneumonia (10, 19). A *C. pneumoniae* epidemic in military trainees showed seven of eight patients were asymptomatic (11). The rabbit model also exhibits a subclinical form of pneumonia upon infection with *C. pneumoniae*.

Unlike previously described mouse models (87), recovery of viable *C. pneumoniae* organisms from the lung was difficult, suggesting that rabbits are less susceptible than mice to *C. pneumoniae* infection. Chlamydial DNA and/or antigen are detected as early as 2 days and as late as 42 days after inoculation indicating the presence of *C. pneumoniae*. Electron microscopic examination of rabbit lung 2 days after inoculation revealed inclusions in macrophages containing small numbers of RB particles and no EBs. In contrast, inclusions that were detected in the mouse lung contained all developmental stages (88). The paucity of infectious EB particles within inclusions may explain the lack of success in isolating organisms from rabbit lung tissue. Inclusions were not seen by EM in other phagocytic cells in the rabbit model. Positive PCR results of the nasopharynx and trachea indicated that nasal and tracheal cells are infected. In the mouse model (88), inclusions are readily detected by EM in ciliated bronchial epithelial cells and also interstitial macrophages. This difference can be explained by the greater susceptibility of mice to *C. pneumoniae* and a higher inoculum dose relative to total body weight in the mouse model. Also, rabbit tissues were not examined exhaustively for inclusions because large regions of rabbit lung tissue relative to total area could not be analyzed by EM.

In both NZW and WHHL rabbits, *C. pneumoniae* was detected by PCR and ICC in tissues other than the lung following respiratory infection. Detection of *C. pneumoniae* in peribronchiolar lymphoid tissue and PBMC by PCR and/or ICC suggests that *C. pneumoniae* may be disseminated by lymphatic or hematogenous routes. This conclusion is supported by the isolation of viable organisms from spleen tissue and peritoneal macrophages in the murine model of TWAR pneumonitis (88).

C. pneumoniae was isolated from the upper respiratory tract of NZW rabbits early in infection, however viable organisms were never recovered from WHHL rabbits. Also, chlamydial DNA was repeatedly detected in the upper respiratory tract of NZW rabbits, but was not detected in the upper respiratory tract of the WHHL rabbits. These results indicate that the WHHL rabbit may not be as susceptible to *C. pneumoniae* infection as the NZW strain despite repeated inoculations. Several reasons may be considered. Age at the time of infection may be a factor. NZW rabbits were inoculated at 3 to 4 months of age as compared to WHHL rabbits at 8 months of age. Differing susceptibilities to infectious disease between rabbit strains are known to be influenced by genetic factors, but the mechanisms involved are unclear (44). The WHHL rabbit strain may be less genetically susceptible than the NZW strain to chlamydial disease. Unlike NZW rabbits which were *Pasteurella multocida*-free, the WHHL colony at the University of Washington had a past history of *Pasteurella* infection. Prior exposure to this well-characterized bacterial respiratory pathogen of rabbits (48) may have altered susceptibility of the rabbits to *C. pneumoniae* by inducing a heightened immune response. The apparent resistance of the WHHL rabbit to *C. pneumoniae* infection may account for the inability to detect the organism within atherosclerotic lesions. At 8 months of age, mature atheromatous lesions were fully formed. It is possible that infection of the WHHL rabbit at a younger age, when atherosclerotic lesions are in earlier stages of development and more actively growing, may result in detection of the organism within the atheroma.

In conclusion, we have shown that *C. pneumoniae* infection in rabbits results in a mild, self-resolving interstitial pneumonia with bronchiolitis. Infection persisted, as demonstrated by PCR, for up to 6 weeks after inoculation. The rabbit model of *C. pneumoniae* mimics human respiratory infection and will prove beneficial in studies of the cellular and immunological pathogenesis of chlamydial disease. However, because *C. pneumoniae* could not be detected in the atheromatous lesions in the WHHL rabbit model by any of the methods available, it does not appear to be a useful model to investigate the role of *C. pneumoniae* in atherosclerosis. Due to the lack of demonstrable organism in the lesion, alternative animal models of atherosclerosis, the apoE-deficient and C57BL/6J mice, were evaluated.

TABLE 1. Direct detection of *C. pneumoniae* from the respiratory tract of NZW rabbits following a single inoculation.

Inoculation (dose)	Days after inoculation	Detection of organisms[a]			
		Nasopharynx	Trachea	Lung	
		PCR[b]	PCR	PCR	ICC[c]
IT[d]/low dose (4 X 10^7 IFUs[f])	3	+	+	+	-
	7	+	+	+	-
	14	+	+	-	-
IN[e]/high dose (4 X 10^8 IFUs)	3	+[g]	+	-	-
	7	+	-	+	-
	14	+	-	-	-
	21	-	-	-	-
IT/high dose (5.5 x 10^8 IFUs)	2	-	+	+	+
	7	-	-	-	+
	14	+	-	-	+
	21	+	-	+	-
	28	-	-	+	+

[a] One animal was analyzed at each time point.
[b] PCR, polymerase chain reaction.
[c] ICC, immunocytochemistry.
[d] IT, intratracheal.
[e] IN, intranasal.
[f] IFUs, inclusion forming units.
[g] Site was also isolation positive at this time point.

TABLE 2. Direct detection of *C. pneumoniae* from the respiratory tract of NZW rabbits following multiple inoculations.

		Detection of organisms[a]			
		Trachea	Nasopharynx	Lung	
No. of Inoculations[b]	Days after last inoculation	PCR[c]	PCR	PCR	ICC[d]
2	7	+[e]	+[e]	-	-
	14	+	+[e]	-	-
	21	+	-	-	-
3	14	+[e]	-	+	+
	28	+	-	-	+
	42	-	-	+	-

[a] One animal was analyzed at each time point.

[b] Animals were inoculated intratracheally at 1 week intervals with 1.2 X 10^8 inclusion forming units per rabbit each time.

[c] PCR, polymerase chain reaction.

[d] ICC, immunocytochemistry.

[e] Sites were also isolation positive at this time point.

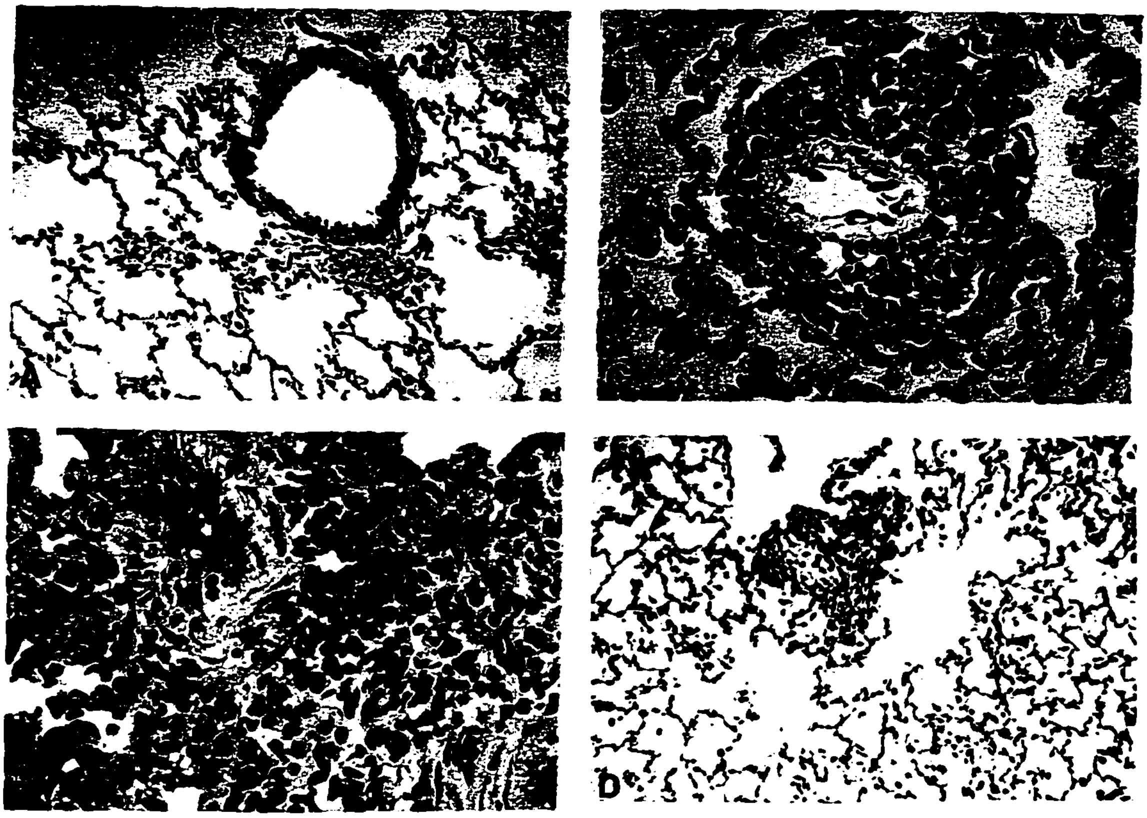

Figure 1. Time course of inflammatory lesions in the lung of NZW rabbits. **A**, Section collected 7 days after inoculation with sterile SPG medium demonstrating normal lung histology. Hematoxylin and eosin; magnification, X16. **B**, Section of lung collected 7 days after a single inoculation with *C. pneumoniae* showing moderate interstitial inflammatory infiltrates of heterophils and mononuclear cells. Note the presence of inflammatory cells in the vessel wall of 2 small arteries. Hematoxylin and eosin; magnification, X40. **C**, Section of lung collected 7 days after a single inoculation with *C. pneumoniae*. Note perivascular lymphoid cell accumulation and the presence of an inflammatory cell in the media of the vessel wall. Hematoxylin and eosin, magnification, X40. **D**, Section of lung taken 21 days after repeated inoculation with *C. pneumoniae*. Occasional mononuclear cell aggregates are present throughout the interstitium. Hematoxylin and eosin, magnification, X16.

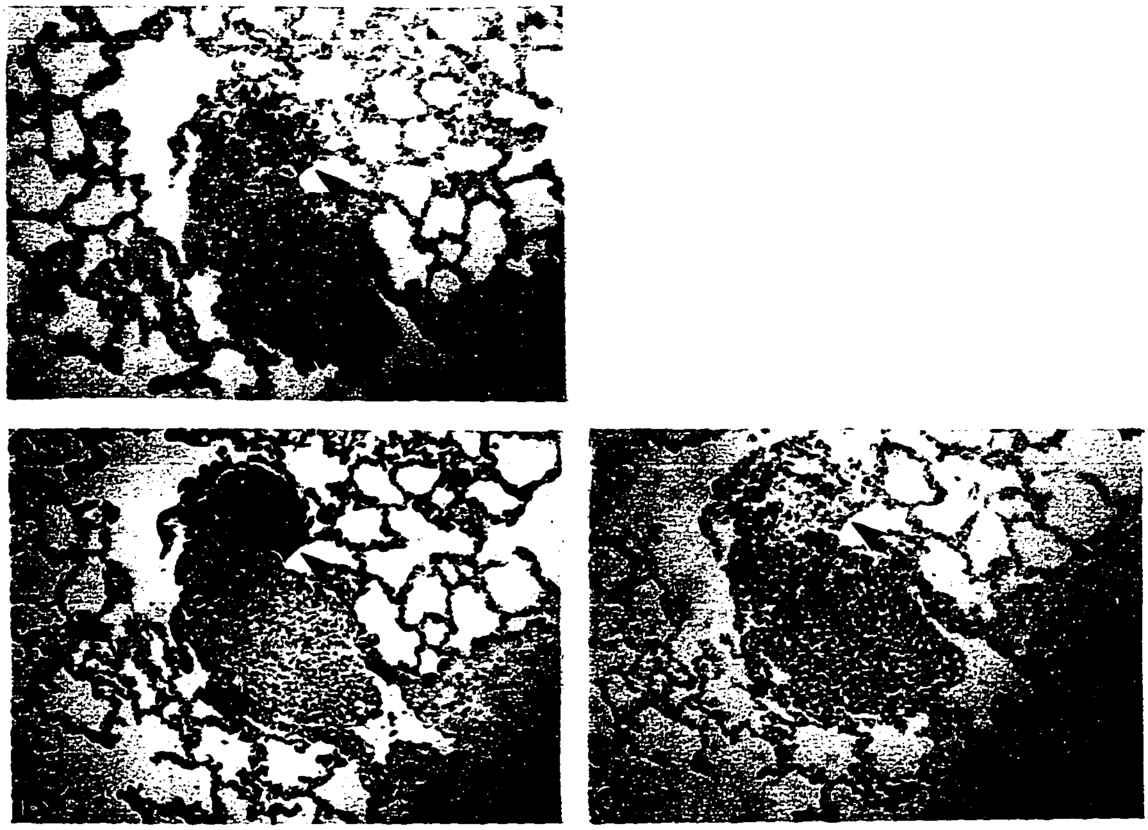

Figure 2. Cellular composition of inflammatory infiltrates of the lung of a NZW rabbit 7 days after a single inoculation with *C. pneumoniae*. **A**, Interstitial lung lesion consisting of mononuclear cells immunoreactive with anti-rabbit macrophage antibody, RAM-11. The arrow shows a focal accumulation of macrophages within the lesion. Avidin:biotinylated enzyme complex (ABC) immunocytochemistry, methyl green counterstain; magnification, X40. **B**, The same lesion in a serial section reacted with *Chlamydia* genus-specific antibody (CF-2). Note the same area of the interstitial lesion is immunoreactive demonstrating the presence of chlamydial antigen within lung macrophages (arrow). ABC immunocytochemistry, methyl green counterstain; magnification, X40. **C**, A serial lung section reactive with normal mouse ascitic fluid as a negative control antibody. Note the same area within the interstitial lesion is not stained (arrow). ABC immunocytochemistry, methyl green counterstain; magnification, X40.

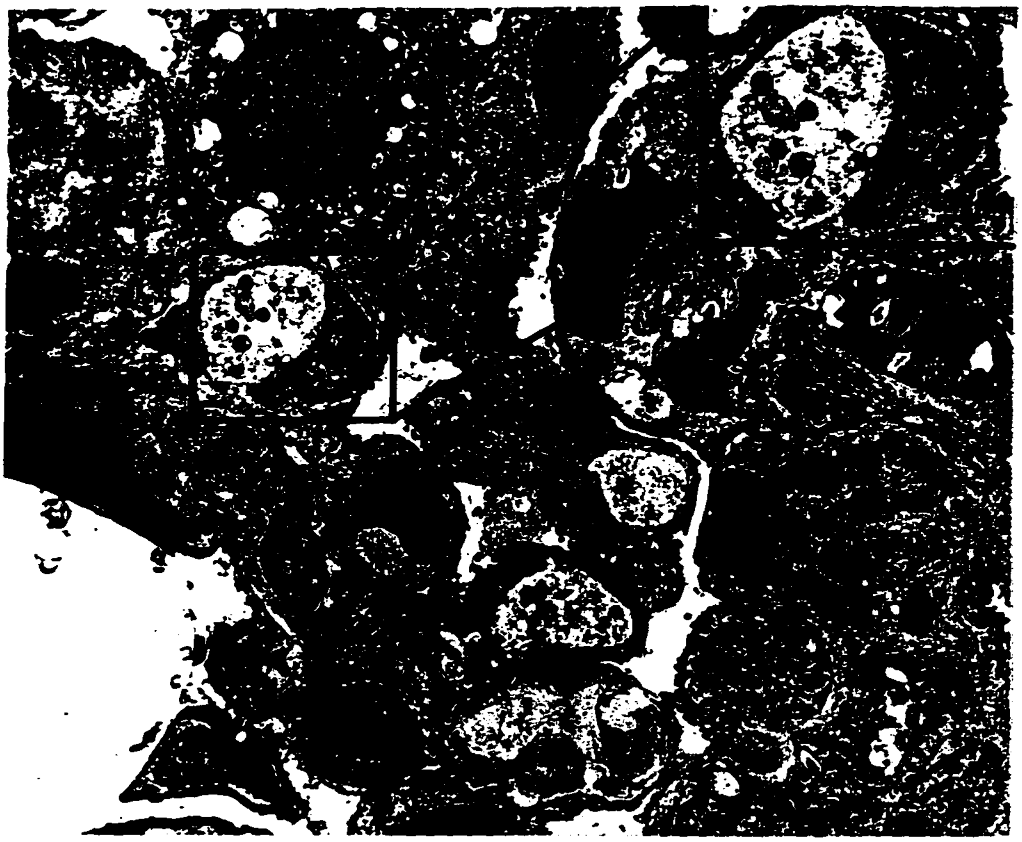

Figure 3. Ultrastructure of NZW rabbit lung interstitium collected 2 days after inoculation depicting a *C. pneumoniae* inclusion within a macrophage. The inclusion is of normal size and shape containing only a few developmentally immature, but no mature, particles. Transmission electron microscopy, Karnovsky's fixative, uranyl acetate; insert magnification, X8600; bar represents 1 μm.

CHAPTER 2: EXPERIMENTAL MURINE MODELS

CHAPTER SECTION: PURPOSE

Due to the inability to detect *C. pneumoniae* organisms in the atheromas of WHHL rabbits following repeated inoculation, an alternative model of atherosclerosis was needed for evaluation as an appropriate animal model. Previous studies had established that mice, both inbred and outbred strains, were highly susceptible to *C. pneumoniae* infection (87). In these models, respiratory disease resulted in severe pneumonitis with recovery of viable organisms from the respiratory tract for up to 42 days after inoculation. Also, infection disseminated to the spleen and peritoneal macrophages, thus fulfilling 2 of the criteria for an acceptable model of *C. pneumoniae* infection stated as in Chapter 1. Mice offered a further advantage over rabbits because inbred strains are available providing a more genetically defined and homozygous model.

The apoE-deficient mouse (63, 64), a transgenic strain constructed on the C57BL/6J background, develops atherosclerosis in the absence of a high fat/high cholesterol diet. These animals exhibit a severe and spontaneous hypercholesterolemia and develop, in a time dependent manner, a complete range of atherosclerotic lesions, from fatty streaks to fibrous plaques (58, 74). The apoE-deficient mouse constitutes an ideal model to study the biochemical and immunological processes in atherosclerosis (61, 65). Because lesions develop spontaneously in the apoE-deficient mice, it can be used to study the affects of infection on the progression of lesions in the absence of dietary factors.

Traditionally, mice are resistant to atherosclerosis, but several inbred strains, including the C57BL/6J mouse, can be induced to develop atherosclerosis when fed a diet consisting of 15% fat, 1.24% cholesterol and 0.5% cholic acid. When fed this diet for 3-4 months, C57BL/6J mice develop foam cell lesions at the base of the aorta in the region of the aortic valves. After extensive feeding, small fatty streaks may also develop in the abdominal aorta. Because the development of lesion formation can be controlled and manipulated by diet, the effect of *C. pneumoniae* infection can be studied on more slowly developing lesions than in the apoE-deficient mouse. Also, the C57BL/6J mouse can be used to analyze if and how dietary lipids may function as a cofactor with *C. pneumoniae* infection in atherosclerosis. Therefore, the C57BL/6J mouse can be used to study the effect of *C. pneumoniae* infection on the induction and acceleration of atherosclerosis in conjunction with dietary factors.

The purpose of this study was to evaluate the susceptibility of apoE-deficient transgenic and C57BL/6J mice to *C. pneumoniae* and appraise the feasibility of these mice as animal models of atherosclerosis in association with *C. pneumoniae* infection. For these mouse strains to be acceptable models for further study, it is of paramount importance to observe dissemination of *C. pneumoniae* from the respiratory tract to the site of lesion development in the aorta and to localize the organism within the atherosclerotic plaque.

CHAPTER SECTION: MATERIALS AND METHODS

CHAPTER SUBSECTION: *C. PNEUMONIAE* STRAIN AND INOCULUM PREPARATION.

C. pneumoniae, strain AR-39 (19), was used for inoculation of animals. The organism was grown in HL cells (23) and purified by density gradient using diatrizoate meglumine centrifugation (Hypaque-76; Winthrop-Breon Laboratories, New York, NY) (7). The inoculum preparations were resuspended in chlamydia transport media (sucrose phosphate glutamic acid) and frozen at –70°C until use.

CHAPTER SUBSECTION: EXPERIMENTAL ANIMALS.

Sixty-eight male homozygous apoE-deficient mice and 74 male C57BL/6J mice, aged 6 to 7 weeks of age, were obtained from the Jackson Laboratories (Bar Harbor, Maine). All mice were fed a regular mouse chow diet ad libitum. Animals were housed under Biosafety Level 2 conditions and cared for by standard and specific practices as outlined by the National Institutes of Health (HHS Publication No. 88-8395). Experiments on all mice were performed under barrier conditions with sentinel mice monitored by a comprehensive quality assurance program.

CHAPTER SUBSECTION: INOCULATION OF MICE

Mice were mildly sedated by subcutaneous injection of a mixture of ketamine (Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (Lloyd Laboratories, Shenandoah, IA). All mice were inoculated intranasally with a total volume of 0.03-0.05 mL of organisms suspended in SPG medium or SPG medium alone. Inoculum doses were 3×10^7 to 1×10^8 inclusion forming units (IFUs) per mouse for apoE-deficient mice inoculated once at 8 weeks and 16 weeks of age. Multiply inoculated apoE-deficient mice were inoculated with 3×10^7 IFUs per mouse at 8, 10 and 12 weeks of age. Singly inoculated C57BL/6J mice were inoculated at 8 weeks of age with 3×10^7 IFUs per mouse. Multiply inoculated C57BL/6J mice were inoculated at 8, 9 and 10 weeks of age with 3×10^7 IFUs per mouse. Control mice were sham-inoculated with 0.03 mL of sterile SPG medium.

CHAPTER SUBSECTION: TISSUE COLLECTION

Mice were heavily sedated with Avertin (2,2,2-tribromoethanol) and whole blood was collected by exsanguination from the femoral arteries. The chest and abdomen were opened and the heart and aorta were perfused with PBS. Lung, spleen, heart, ascending and abdominal aorta were removed with a separate set of sterile instruments for each tissue, placed in sterile glass vials and immediately placed on ice. Tissues were later weighed and frozen at -70°C. Sera was separated from clotted blood and also frozen at -70°C.

Lung, spleen, ascending and abdominal aorta were collected from apoE-deficient mice from 1-20 weeks after a single inoculation at 8 weeks of age and from 1-8 weeks after a single inoculation at 16 weeks of age. Additionally, heart was collected from multiply inoculated apoE-deficient mice from 2-20 weeks after infection. Tissues from 2-4 apoE-deficient mice were analyzed by culture and PCR at each time point. Lung, heart, ascending and abdominal aorta were collected from C57BL/6J mice at 3, 5, 7, 14, 21 and 28 days after a single inoculation and at 1, 2, 3, 4 and 5 weeks after the last inoculation in multiply inoculated mice. Tissues from 9-14 singly inoculated and 5 multiply inoculated C57BL/6J mice were analyzed by culture and/or PCR at each time point.

CHAPTER SUBSECTION: TISSUE PREPARATION FOR ISOLATION AND DNA EXTRACTION

Tissue samples were thawed and homogenized with a sterile mortar and pestle in 1.0-2.0 mL of SPG medium to make a 10% (wt/vol) suspension. Tissue homogenates were centrifuged at 500 × *g* for 5 min at 4°C to sediment tissue debris and the supernatant aspirated for inoculation of cultures. Tissue pellets were resuspended in 1.0 mL of lysis buffer (100mM Tris Cl, pH 8.0, 25 mM EDTA, 2% Triton X-100) and 200 µg/mL Proteinase K (Amresco, Solon, OH) was added to each sample. Samples were incubated overnight at 55°C and extracted with hexadecyltrimethyl ammonium bromide (CTAB) according to standard methods (3). Lysates were extracted twice with phenol/chloroform:isoamyl alcohol (24:24:1) and once with chloroform:isoamyl alcohol (24:24:1). DNA was precipitated with 0.7 volumes of 100% isopropanol at room temperature followed by washing with ice cold 70% ethanol. DNA pellets were air dried, resuspended in 50-100 μl of sterile water (Baxter Healthcare Corp., Deerfield, IL). An extraction control of lysis buffer alone was included with each set of tissue extractions to detect cross-contamination of samples during the extraction process. Tissue homogenization, DNA extractions and PCR amplification were performed in separate rooms equipped with germicidal lighting.

CHAPTER SUBSECTION: ISOLATION AND INFECTIVITY ASSAY

Human line (HL) cell monolayers were inoculated with 100 μl of 10% tissue homogenate according to previously described methods (36). Cultures were passed once, 4 days after inoculation. Inclusions were detected by direct fluorescent antibody stain using a *Chlamydia* genus-specific monoclonal antibody (CF-2) conjugated to fluorescein isothiocyanate (36). The infectious titer was assayed by titration of tissue homogenates in HL cells and expressed as $\log_{10}$ IFUs per gram of lung tissue.

CHAPTER SUBSECTION: SEROLOGY

C. pneumoniae-specific antibody titers were determined by the microimmunofluorescence test using formalin-fixed AR-39 elementary bodies as antigen (85). Both immunoglobulins G (IgG) and M (IgM) serum antibody were measured using fluorescein isothiocyanate-conjugated goat anti-mouse IgG and IgM (heavy-chain specific; Sigma Company, St Louis, Mo.). Sera was pooled from 2-4 animals per time point and titrated by serial two-fold dilutions.

CHAPTER SUBSECTION: POLYMERASE CHAIN REACTION

DNA samples were amplified using *C. pneumoniae*-specific primers HL-1 and HR-1 exactly as described in Chapter 1. The amplification product, an expected 437 bp *C. pneumoniae*-specific DNA sequence, was analyzed by gel electrophoresis through a 1.5% agarose gel according to standard protocols (75) and transferred by Southern blot to a nylon membrane (Qiagen, Chatsworth, CA). The product was confirmed by hybridization to a digoxigenin-dUTP-labeled probe consisting of a 474 bp *PstI* fragment which spanned the target sequence (8). DNA probes were labeled using the Genius DNA labeling and detection kit (Boehringer Mannheim Biochemical, Indianapolis, IN). Hybridized amplification products were detected by immunochemiluminescence using Lumiphos 530 (Boehringer Mannheim Biochemical, Indianapolis, IN) according to the manufacturer's instructions, followed by autoradiography. Controls for each amplification consisted of serial dilutions of purified *C. pneumoniae* DNA as positive controls and sterile water in place of sample DNA as a negative control. Positive and negative controls were amplified, analyzed and confirmed concurrently with each set of samples. Aortic tissues samples which were negative for *C. pneumoniae* DNA were tested for the presence of mouse genomic DNA using primers specific for the mouse β globin gene (12). The nucleotide sequences of the mouse β globin gene primers used from the 5' to the 3' end are as follows:

MβG-1: CTGGCACGCGCTGGACGCG;
MβG-2: GGGGTCTTTGTCTGTAGTACC.

All aortic tissues tested positive indicating the presence of mouse genomic DNA.

CHAPTER SUBSECTION: HISTOPATHOLOGY AND IMMUNOCYTOCHEMISTRY

Abdominal and ascending aorta were fixed *in situ* prior to removal by perfusion with 10 mL of PBS followed by 10-15 mL of 10% phosphate buffered formalin (Fisher Scientific, Pittsburgh, PA) injected through the left ventricle of the heart. Lungs were perfused through the trachea with 10% formalin prior to removal. The base of the heart was collected and sectioned at the level of the aortic sinus. Ascending aorta was sampled from the junction of the aorta with the heart at the end of the aortic sinus through the aortic arch and thoracic aorta. Abdominal aorta was sampled from the mesenteric artery branch through the iliac bifurcation. Aorta from singly inoculated and sham-inoculated animals were cut into 2-3 mm pieces, embedded in paraffin, then cross-sectioned for staining. Aorta from multiply inoculated animals was cut into 6-8 mm pieces, embedded in paraffin and sectioned longitudinally. Tissues for histopathology were stained with hematoxylin and eosin.

For detection of chlamydial antigens, tissue sections were reacted with a *Chlamydia* genus-specific mouse monoclonal antibody, (CF-2), which is directed against chlamydial lipopolysaccharide. Lung sections were stained by the direct avidin-biotin-peroxidase method using CF-2 conjugated to biotin at 1:250 dilution as previously described (23). However, direct staining of mouse arterial tissues with biotinylated CF-2 resulted in nonspecific reaction with areas of the vessel wall besides the atherosclerotic lesion. Cross-reaction of the mouse-derived monoclonal antibody CF-2 with smooth muscle cells in the tunica media and nerve cell bundles in the adventitia was observed in aorta sections by the direct staining method. In an effort to overcome nonspecific staining, aorta sections were stained by the indirect method with CF-2 at 1:1000 dilution using the HistoMouse™ Kit (Zymed Laboratories, San Francisco, CA). Tissue sections were counterstained with either methyl green or hematoxylin. Duplicate tissue sections and tissues from sham-inoculated mice were reacted with CF-2 and normal mouse ascitic fluid as negative controls. *C. pneumoniae* infected HL cell pellets were formalin fixed, embedded in paraffin and sectioned for positive controls.

CHAPTER SECTION: RESULTS

CHAPTER SUBSECTION: CLINICAL RESPONSE TO INFECTION

Clinical signs in singly inoculated apoE-deficient animals included increased respiration, decreased body weight, dehydration and nasoocular-ocular discharge (data not shown). Signs were most severe 3-6 days after inoculation and resolved within 10-12 days. No mortality was seen with an inoculum dose of 3×10^7 IFUs per mouse. However, 20-40% mortality during the first 6 days after inoculation was observed in apoE-deficient mice as the inoculum dose was increased to 1×10^8 IFUs per mouse. C57BL/6J mice received a single inoculation of 3×10^7 IFUs per mouse and exhibited clinical signs of disease similar to those seen in apoE-deficient mice. Gross examination of the lungs revealed bilateral, multifocal irregular dark red to purple lesions which were most severe 10-14 days after infection and resolved gradually by 28 days. Beginning at 8 weeks after inoculation, gross bright red hemorrhagic lung and kidney lesions were observed intermittently in both infected and sham-inoculated animals. This finding may be attributed to the presence of mouse hepatitis virus (MHV) which was discovered by serology in singly inoculated apoE-deficient mice beginning at 12 weeks after inoculation with *C. pneumoniae*, the result of an outbreak in the animal facility As indicated below, hemorrhagic lesions were not seen in subsequent experiments with apoE-deficient mice that were MHV seronegative.

Clinical signs seen in multiply inoculated animals were similar, but more severe, than those seen in singly inoculated animals. Signs were most severe 2-6 days after the first and second inoculations at 8 and 10 weeks of age.

Only mild to moderate clinical signs were observed after the third inoculation at 12 weeks of age and resolved within 2 weeks after the third inoculation at 14 weeks of age. Total mortality for all inoculations was 10-15% and occurred within 6 days following the first and second inoculations. Gross lung lesions consisted of severe, diffuse pulmonary inflammation at 10 and 12 weeks of age, decreased to moderate degree by 14 weeks of age, and resolved gradually by 20 weeks of age. No evidence of MHV was observed in the multiply inoculated apoE-deficient mice.

CHAPTER SUBSECTION: SEROLOGICAL RESPONSE AND BACTERIAL LOAD

ApoE-deficient mice developed an *C. pneumoniae* IgG titer of 1:16 at day 10 after a single inoculation (Table 3), which increased to 1:256 at 4 weeks, then subsided gradually. Following multiple inoculations, IgG antibody titers ranged from 1:512 to 1:1024 (data not shown) at 2 to 12 weeks after inoculation. IgM antibody was not detected. No antibodies were detected in sham-inoculated animals. *C. pneumoniae* was isolated from lung tissue for 4 weeks after a single inoculation. Infectivity titers were highest on day 2 and then gradually decreased through week 4 (Table 3). Organisms were not isolated from multiply inoculated animals after 2 weeks post-inoculation, therefore infectivity titers were not done.

CHAPTER SUBSECTION: ISOLATION AND POLYMERASE CHAIN REACTION

In apoE-deficient mice inoculated once at 8 weeks of age, *C. pneumoniae* was isolated from several tissue sites including lung, spleen and aorta during the first week of infection (Table 4A). Organisms continued to be isolated from lung tissue at 2 and 4 weeks and aorta at 4 weeks after inoculation. No other tissues were positive by isolation at any other time points. Lung and the aorta were intermittently positive for *C. pneumoniae* DNA by PCR through 20 weeks after inoculation. When detected, the per cent of animals whose aorta (either ascending or abdominal) was positive for *C. pneumoniae* varied widely and ranged from 33-100%.

In apoE-deficient mice inoculated once at 16 weeks of age, *C. pneumoniae* was isolated from lung tissue at 1, 2 and 4 weeks and the aorta at 1 week after inoculation (Table 4B). *C. pneumoniae* DNA was consistently detected by PCR in lung and aorta (either ascending or abdominal) through 12 weeks after inoculation. The per cent of animals inoculated at 16 weeks of age whose aorta was positive for *C. pneumoniae* was 100% at all time points.

In apoE-deficient mice multiply inoculated at 8, 10 and 12 weeks of age, *C. pneumoniae* was isolated from lung and aorta (either ascending or abdominal) at 2 weeks after inoculation (Table 5). Similar to singly inoculated animals, lung and aorta were consistently positive for *C. pneumoniae* DNA for up to 20 weeks after inoculation. The per cent of multiply inoculated animals whose aorta was positive ranged from 50-100%. Tissues from negative control apoE-deficient control mice sham-inoculated once at 8 weeks or three times at 8, 10 and 12 weeks of age were negative for *C. pneumoniae* by PCR at all time points.

In C57BL/6J mice inoculated once at 8 weeks of age, *C. pneumoniae* was isolated from lung tissue up to 14 days and detected by PCR until 28 days after inoculation, the final time point tested (Table 6). Aorta (either ascending or abdominal) was positive by PCR for *C. pneumoniae* in 21% of animals at 3 and 5 days, decreasing to 8% at 14 days after inoculation. *C. pneumoniae* was isolated from the ascending aorta of 1 animal at 3 days after inoculation. Heart tissue was negative by culture and PCR at all time points.

In multiply inoculated C57BL/6J mice infected at 8, 9 and 10 weeks of age, *C. pneumoniae* was detected by PCR in lung and ascending aorta for up to 35 days after inoculation. Ascending aorta was positive in 80% of animals at

7 days, 60% at 21 days and 40% at 28 and 35 days after the last inoculation. Heart was negative at all time points.

CHAPTER SUBSECTION: HISTOPATHOLOGY AND IMMUNOCYTOCHEMISTRY

In singly inoculated apoE-deficient mice 1 week after inoculation, lung histopathology was characterized by moderate to severe pneumonitis with an initial polymorphonuclear cell infiltrate, followed by predominantly mononuclear cells at 2 weeks after inoculation. Lung lesions decreased to few multifocal interstitial mononuclear cell aggregates at 4 weeks and resolved by 6 weeks after inoculation. In multiply inoculated apoE-deficient mice, moderate to severe pneumonitis with occasional interstitial mononuclear cell aggregates developed at 2 and 4 weeks after final inoculation. Pneumonitis gradually decreased from 6 through 12 weeks and resolved by 16 weeks after inoculation.

The earliest atherosclerotic lesions were observed at 10 weeks of age (2 weeks after inoculation) and consisted of macrophage adherence to the endothelium (data not shown). Early foam cell lesions were seen at 12, 14 and 16 weeks of age and fatty streaks at 18 and 20 weeks of age. Lesions progressed to early atheromas at 24 weeks of age and mature atheromas with subendothelial foam cell migration at 28 weeks of age. Lesions in the abdominal aorta followed a similar, but slower development, progressing to only foam cell lesions by 24 and 28 weeks of age. Histopathology of C57BL/6J mouse tissues was not done.

Immunocytochemistry of lung sections in singly inoculated apoE-deficient mice was positive for chlamydial antigen at 1, 2 and 8 weeks after inoculation, but negative at all later time points (data not shown). At 1 and 2 weeks after inoculation, chlamydial antigen was distributed multifocally throughout the lung interstitium and peribronchiolar and alveolar mononuclear cells. At 8 weeks after inoculation, chlamydial antigen was sparsely present in focal interstitial cells. Aortic tissue from singly inoculated apoE-deficient mice was weakly positive for chlamydial antigen at 16 and 20 weeks after inoculation (data not shown). Immunocytochemistry of the aorta at other time points was difficult to interpret due to nonspecific reaction of the monoclonal antibody, CF-2, with smooth muscle cells in the tunica intima of the aorta and nerve cells of the surrounding adventitia. Subsequent retesting was not possible due to loss of tissue and/or lesion upon resectioning.

In multiply inoculated apoE-deficient mice, chlamydial antigen was distributed multifocally throughout the lung interstitium and peribronchially from 2-10 weeks post-inoculation. Lung tissue remained focally positive for chlamydial antigen for up to 20 weeks post-inoculation, the last time point analyzed (Figure 4A-C). Aortic tissue was positive by ICC at 12 and 16 weeks after inoculation. Results from aortic tissue at 20 weeks after inoculation could not be interpreted due cross-reaction of the genus-specific monoclonal antibody, CF-2, with regions in these later more advanced atherosclerotic lesions which were necrotic and highly inflammatory. Chlamydial antigen was present within atherosclerotic plaques and appeared confined to foam cells both in atheromas and the subendothelium (Figure 5A-C).

CHAPTER SECTION: DISCUSSION

The findings of this study have determined that the apoE-deficient mouse fulfills all the criteria necessary for a useful model to continue further studies in the investigation of a pathogenic role of *C. pneumoniae* in atherosclerosis. Evidence to support this conclusion includes induction of moderate to severe clinical signs of respiratory disease and production of gross and microscopic pulmonary inflammation following intranasal inoculation. Infection resulted in stimulation of a *C. pneumoniae*-specific serum IgG antibody response and seroconversion within 3 weeks post-inoculation. Viable organisms were isolated from lung, spleen and aortic tissue. Importantly, *C. pneumoniae* DNA was intermittently detected in the aorta of singly inoculated apoE-deficient mice and consistently detected in the aorta of multiply inoculated mice for up to 20 weeks after inoculation, indicating chronic persistence of the organism after infection. Most significant was the detection of *C. pneumoniae* antigen by ICC within the atheroma of a 24 week old apoE-deficient mouse, thus, not only confirming persistence, but localizing the organism to within the developing atherosclerotic lesion.

Clinical disease, lung pathology and infectivity titers seen in apoE-deficient mice are similar to that observed in the Swiss-Webster mouse model described previously (87). *C. pneumoniae* could be isolated from the lung of ApoE-deficient mice up to 28 days after infection as compared to 42 days in the Swiss Webster mouse. Pulmonary mononuclear cell aggregates resembling lymphoid follicles, a prominent feature in the Swiss-Webster model, are also observed in multiply inoculated apoE-deficient mice. The spectrum of human clinical disease due to *C. pneumoniae* is broad ranging from asymptomatic and subclinical to severe systemic disease; however, infection generally results in mild to moderate upper respiratory disease lasting from days to weeks in duration (39). The syndrome of signs observed in these mouse models represent a slightly more severe and rapid course of disease than that commonly seen in human disease.

Infection of apoE-deficient mice with *C. pneumoniae* results in pulmonary disease which disseminates to and persists in other tissue sites as previously observed in the Swiss-Webster mouse model (88). Single inoculation of the apoE-deficient mouse resulted in infection of mouse tissues which, by PCR, was intermittent with some, but generally not all, animals infected in a particular tissue at each time point. Many factors can account for the varying levels of PCR positivity such as uneven distribution of chlamydial organisms within tissues, the presence of inhibitors in tissue extracts and, most importantly, biological variability in immune response and other host factors between individual mice.

The most important finding of this study is the detection of *C. pneumoniae* antigen directly by ICC within developing atherosclerotic lesions of both singly and multiply inoculated apoE-deficient mice, thus localizing the organism at the lesion site. At the time points *C. pneumoniae* was detected by ICC, mice were 20 weeks of age or older with lesions ranging from well-developed fatty streaks to early atheromas. The persistence of the organism during the development and maturation of atherosclerotic lesions is significant because chronic infection with chlamydiae commonly results in an inflammatory response associated with tissue damage. Atherosclerosis is a complex and multifactorial disease process in which inflammation is thought to play an important role. These data suggest that *C. pneumoniae* may contribute to the atherosclerotic process. In human atherosclerotic plaques, *C. pneumoniae* has been found in both macrophages and smooth muscle cells by double immunocytochemical staining using cell type specific antibodies. Future studies to determine the exact cell type infected in the apoE-deficient model are needed to fully characterize this model.

Also consistent with the predilection of *C. pneumoniae* for infection of atheromas was the finding that the percentage of mice infected was higher when mice were infected at an older age or multiple times. Specifically, infection was intermittent and variable in apoE-deficient mice singly inoculated at 8 weeks of age. This contrasts with apoE-deficient mice singly inoculated at 16 weeks of age in which *C. pneumoniae* was consistently detected in the aorta of all animals at all time points. Inoculation of apoE-deficient mice at 16 weeks of age, when intermediate atherosclerotic lesions are actively growing due to a constant influx of macrophages and smooth muscle cells to the developing lesion, may explain the homing of *C. pneumoniae* to the aorta of these animals. Additionally, multiple inoculations were performed in apoE-deficient mice at 8, 10 and 12 weeks of age when monocyte adhesion and foam cell lesion formation were occurring. As in mice receiving a single inoculation at 16 weeks of age, multiple inoculations at 8, 10 and 12 weeks of age may have resulted in a more consistent and higher percentage of mice infected in the aorta due the presence of the organism during intermediate stages of lesion formation when the plaque is actively growing.

Another equally important finding was the transient presence of C. pneumoniae in the aorta of the C57BL/6J mouse following a single inoculation. This mouse strain is the background strain upon which the apoE-deficient mouse was constructed. As stated earlier, it does not develop atherosclerosis unless fed a high fat diet. Because the C57BL/6J mice used in this study were fed a normal chow diet and not exposed to other predisposing factors of atherosclerosis, the presence of *C. pneumoniae* in the aorta is highly significant. The transent presence of C. pneumoniae in the aorta of the C57BL/6J is in sharp contrast to the persistence of the organism within the developing atherosclerotic plaque of the apoE-deficient mouse. Similar to human studies (31), these findings also suggest that *C. pneumoniae* has a predilection for the atherosclerotic plaque.

The number of C57BL/6J mice infected in the aorta and the length of infection was increased following multiple inoculations showing that repeated inoculations result in chronic infection of the aorta of the C57BL/6J mouse. Dissemination to and persistence of *C. pneumoniae* in the aorta of C57BL/6J mice in the absence of an atherogenic diet suggests that *C. pneumoniae* may play a role in the initiation of atherosclerosis and indicates this animal model will be useful in studying whether chronic infection with *C. pneumoniae* can act as an initiator of atherosclerosis by causing vascular changes as a result of infection of the arterial wall. Also, because atherosclerosis can be induced in this mouse strain by feeding a high fat diet, the C57BL/6J mouse can also be used to study the whether *C. pneumoniae* infection is a cofactor with lipid for atherogenesis.

Both the apoE-deficient and C57BL/6 mice can be used as models of persistent infection and chronic disease. Chronic and/or repeated infection with *C. trachomatis* results in the severe pathology of trachoma and pelvic inflammatory disease (75). These animal models can be used to determine whether similar chronic immune responses are stimulated by *C. pneumoniae* within the developing atherosclerotic lesion.

We have evaluated and characterized the apoE-deficient transgenic mouse and the C57BL/6J mouse strains as animal models of *C. pneumoniae* infection of atheromatous lesions. *C. pneumoniae* has been observed to disseminate from the lungs to the aorta and infect and persist in the atherosclerotic lesions of the apoE-deficient mouse thus fulfilling all the criteria required for an animal model to determine whether *C. pneumoniae* affects the severity or progression of atherosclerosis. The long term persistence of *C. pneumoniae* in the developing atherosclerotic plaques of these mice indicates that this model is exquisitely suited for the evaluation of the pathogenic role of the organism in the advancing atherogenic process. The persistence of *C. pneumoniae* DNA in the aortic tissue of multiply inoculated C57BL/6J mice in the absence of predisposing factors for atherosclerosis indicate that C57BL/6J mice may be used to study whether *C. pneumoniae* induces atherosclerosis independent of other factors. Also, by feeding infected C57BL/6J mice an atherogenic diet, it can be determined whether *C. pneumoniae* is a cofactor to lipids for atherogenesis. These studies show that both of these animal models will serve distinctly different, but equally important, purposes in confirming that *C.*

pneumoniae infection plays a role in the initiation, development and progression of atherosclerosis.

Table 3. Serum antibody levels and bacterial lung load in apoE-deficient mice after a single intranasal inoculation with *C. pneumoniae*.

Time after inoculation (days)	Age (weeks)	Serum IgG Titer[a]	Mean titer ± SD ($\log_{10}$IFU/g lung)[b]
2	8	<1:8	6.42 ± .04
7	9	1:8	6.15 ± .18
10	9.5	1:16	5.48 ± .09
14	10	1:64	5.32 ± .65
28	12	1:256	4.57
70	18	1:32	0
84	20	1:16	0
112	24	1:16	0
140	28	1:8	0

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[a] Serum IgG titers were assayed from pooled serum samples collected from 2-4 animals at each time point.

[b] Mean titer of $\log_{10}$ IFUs/g lung was calculated for 2-3 animals at days 2, 7, 10 and 14 and 1 animal at day 28 after inoculation.

Table 4A. Detection of *C. pneumoniae* in tissues of apoE-deficient mice following a single intranasal inoculation at 8 weeks of age.

Time after inoculation (weeks)	Age (weeks)	Tissues					
		Lung		Spleen		Aorta	
		Isol.[b]	PCR[c]	Isol.	PCR	Isol.	PCR
1	9	3/3[a]	3/3	3/3	3/3	2/3	3/3
2	10	3/3	2/3	0/3	0/3	0/3	0/3
4	12	1/3	2/3	0/3	1/3	1/3	1/3
6	14	0/3	1/3	0/3	1/3	0/3	1/3
8	16	0/4	3/4	0/4	0/4	0/4	3/4
10	18	0/3	0/3	0/3	0/3	0/3	1/3
12	20	0/4	1/4	0/4	0/4	0/4	0/4
16	24	0/4	0/4	0/4	0/4	0/4	0/4
20	28	0/4	3/4	0/4	0/4	0/4	2/4
Total		7/31	15/31	3/31	5/31	3/31	11/31
(%)		(23)	(48)	(1)	(16)	(1)	(35)

[a] Fractions indicate the number positive over the number tested.
[b] Isol., isolation.
[c] PCR, polymerase chain reaction.

Table 4B. Detection of *C. pneumoniae* in tissues of apoE-deficient mice following a single intranasal inoculation at 16 weeks of age.

Time after inoculation (weeks)	Age (weeks)	Lung Isol.[b]	Lung PCR[c]	Spleen Isol.	Spleen PCR	Aorta Isol.	Aorta PCR
1	17	2/2[a]	2/2	0/2	1/2	2/2	2/2
2	18	2/2	2/2	0/2	2/2	0/2	2/2
4	20	1/2	2/2	0/2	0/2	0/2	2/2
8	24	0/2	2/2	0/2	2/2	0/2	2/2
Total (%)		5/8 (63)	8/8 (100)	0/8 (0)	5/8 (63)	2/8 (25)	8/8 (100)

[a] Fractions indicate the number positive over the number tested.
[b] Isol., isolation.
[c] PCR, polymerase chain reaction.

Table 5. Detection of *C. pneumoniae* in tissues of apoE-deficient mice after multiple intranasal inoculations at 8, 10 and 12 weeks of age.

		Tissues							
Time after		Lung		Spleen		Heart		Aorta	
inoculation (weeks)	Age (weeks)	Isol.[b]	PCR[c]	Isol.	PCR	Isol.	PCR	Isol.	PCR
2	10	2/2[a]	2/2	0/2	0/2	0/2	2/2	1/2	2/2
4	12	0/2	2/2	0/2	0/2	0/2	0/2	0/2	1/2
6	14	0/2	2/2	0/2	1/2	0/2	1/2	0/2	1/2
10	18	0/3	1/3	0/3	0/3	0/3	1/3	0/3	2/3
12	20	0/3	2/3	0/3	1/3	0/3	1/3	0/3	2/3
16	24	0/3	1/3	0/3	2/3	0/3	0/3	0/3	2/3
20	28	0/3	3/3	0/3	1/3	0/3	0/3	0/3	3/3
Total		2/18	13/18	0/18	5/18	0/18	5/18	1/18	13/18
(%)		(11)	(72)	(0)	(28)	(0)	(28)	(1)	(72)

[a] Fractions indicate the number positive over the number tested.
[b] Isol., isolation.
[c] PCR, polymerase chain reaction.

Table 6. Detection of *C. pneumoniae* in tissues of C57BL/6J mice after a single intranasal inoculation at 8 weeks of age.

Time after inoculation (days)	Tissues					
	Lung		Heart		Aorta	
	Isol.[b]	PCR[c]	Isol.	PCR	Isol.	PCR
3	14/14[a]	14/14	0/12	0/12	1/14	3/14
5	14/14	14/14	0/12	0/12	0/14	3/14
7	12/14	14/14	0/12	0/12	0/14	1/14[e]
14	5/12	10/12	ND[d]	0/12	ND	1/12
21	0/11	9/11	ND	0/11	ND	0/11
28	0/9	8/9	ND	0/9	ND	0/9
Total	45/74	69/74	0/36	0/74	1/42	7/74
(%)	(61)	(93)	(0)	(0)	(2)	(9)

[a] Fractions indicate the number of animals positive over the number of animals tested.

[b] Isol., isolation.

[c] PCR, polymerase chain reaction.

[d] ND, not done.

[e] Ascending and abdominal aorta from the same animal were positive.

Table 7. Detection of *C. pneumoniae* DNA by PCR in tissues of C57BL/6J mice after multiple intranasal inoculations at 8, 9 and 10 weeks of age.

Time after inoculation (days)	Tissues			
	Lung	Heart	Asc.[b] Aorta	Abd.[c] Aorta
7	5/5[a]	0/5	3/5	0/5
21	4/5	0/5	2/5	0/5
28	0/5	0/5	2/5	0/5
35	2/5	0/5	1/5	0/5
Total (%)	11/20 (55)	0/20 (0)	8/20 (40)	0/20 (0)

[a] Fractions indicate the number of animals positive over the number of animals tested.

[b] Asc., ascending.

[c] Abd., abdominal.

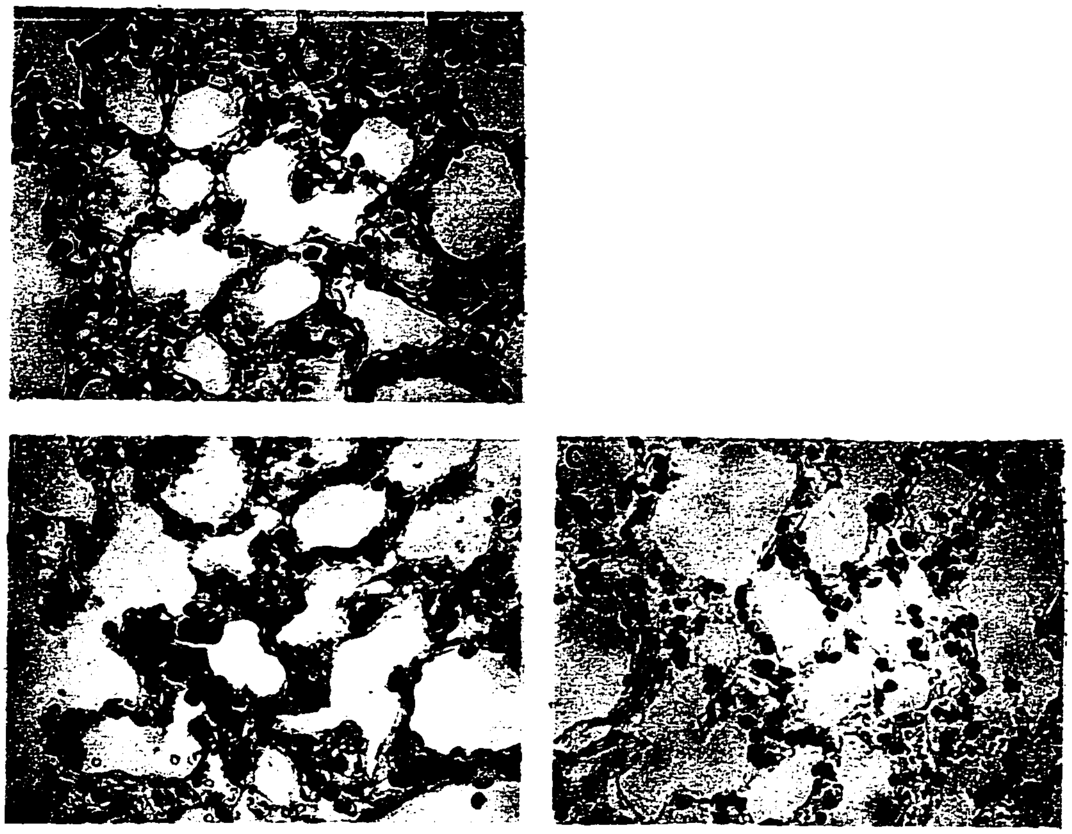

Figure 4. Consecutive sections of lung tissue from a 28 week old apoE-deficient mouse 20 weeks after a single intranasal inoculation at 8 weeks of age with *C. pneumoniae*. **A**, Histopathology of the pulmonary interstitium shows a mild inflammatory infiltrate consisting of mononuclear cells. Sections were stained with hematoxylin and eosin: original magnification = ×40. **B**, Immunocytochemistry of a consecutive section of lung tissue showing a focal area of black precipitate demonstrating the presence of chlamydial antigen. The section was stained with the *Chlamydia* genus-specific monoclonal antibody, CF-2, and counterstained with methyl green; original magnification = ×40. **C**, Consecutive lung tissue section stained with normal mouse ascites as a negative control. No chlamydial antigen is seen. The section was counterstained with methyl green; original magnification = ×40.

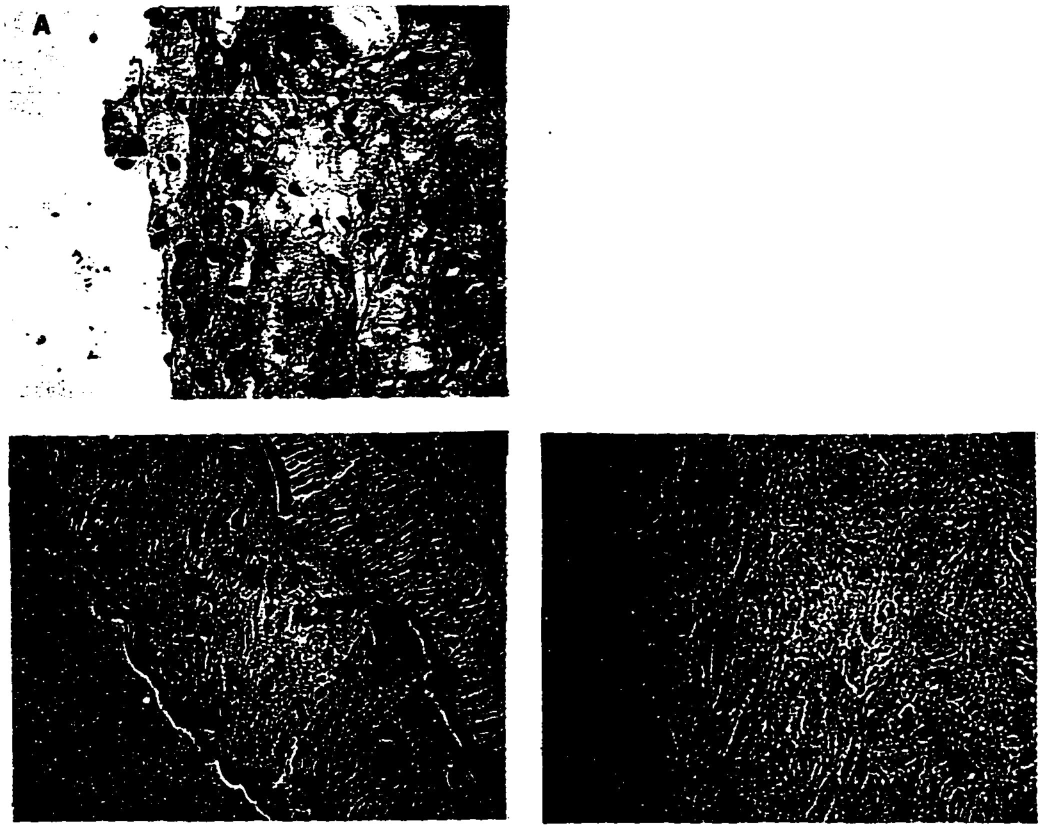

Figure 5. Consecutive sections of aorta tissue from a 24 week old apoE-deficient mouse 16 weeks after multiple intranasal inoculations at 8, 10 and 12 weeks of age with *C. pneumoniae*. **A**, Histopathology of the arterial wall shows an early atheroma consisting of a mixture of foam cells, smooth muscle cells and mononuclear cells. Sections were stained with hematoxylin and eosin: original magnification = ×40. **B**, Immunocytochemistry of a consecutive section of aorta showing several areas of brown precipitate throughout the atherosclerotic lesion demonstrating the presence of chlamydial antigen. The section was stained with the *Chlamydia* genus-specific monoclonal antibody, CF-2, and counterstained with hematoxylin; original magnification = ×40. **C**, Consecutive aorta tissue section stained with normal mouse ascites as a negative control. No chlamydial antigen is seen. The section was counterstained with hematoxylin; original magnification = ×40.

CHAPTER 3: SYSTEMIC DISSEMINATION

CHAPTER SECTION: PURPOSE

We hypothesize that systemic disease can occur following respiratory infection when *C. pneumoniae* infected macrophages disseminate from the lung to other tissues. Evidence for systemic dissemination of *C. pneumoniae* includes *in vivo* and *in vitro* studies of both natural human disease and experimental animal models of *C. pneumoniae* infection.

In humans, systemic disease can occur as a result of respiratory disease. Infection with *C. pneumoniae* in humans has been serologically associated with neurological and cardiovascular outcomes other than respiratory disease including lumbosacral meningoradiculitis, meningoencephalitis and myocarditis (39, 78, 83). In humans, *C. pneumoniae* has been detected by PCR in the liver, spleen and lymph nodes of a 10 year old boy with systemic infection and in spleen and lymph nodes of an adult with sarcoidosis indicating that *C. pneumoniae* can cause systemic disease (39). In human studies where vascular tissue was analyzed, *C. pneumoniae* was detected in approximately 50% of tissues tested suggesting systemic dissemination and a tropism for cardiovascular tissue (31, Introduction). *C. pneumoniae* has been detected by PCR and EM in atheromatous lesions and double ICC labeling with cell type-specific monoclonal antibodies has localized *C. pneumoniae* to within macrophages and smooth muscle cells within aortic atheromas (38).

In previous experimental mouse models, *C. pneumoniae* has been shown to spread to tissue sites distal to the site of inoculation, including spleen and peritoneal macrophages following intranasal, intraperitoneal and subcutaneous inoculations (89). In experimental rabbit models (52), *C. pneumoniae* can be detected in spleen and peripheral mononuclear cells following intranasal or intratracheal inoculation (52). In the apoE-deficient and C57BL/6J mouse models described in Chapter 2, the organism has been shown to spread systemically from the respiratory tract to spleen and aorta following intranasal inoculation.

In vitro studies have shown that *C. pneumoniae* can infect and replicate in both cell lines and primary cell cultures, including macrophage cell lines (RAW 264.7 and U937), endothelial cell lines (Hep-2), peripheral blood monocyte derived macrophages, human bronchoalveolar lavage macrophages and human aortic smooth muscle cells, demonstrating the ability of the organism to infect a wide range of cells.

The purpose of this study was to use the C57BL/6J mouse model to examine the pathogenesis of *C. pneumoniae* infection in macrophages *in vivo*. We specifically examined the ability of *C. pneumoniae* to infect and survive in macrophages and disseminate to tissues distal from the site of inoculation within the mouse model, the route of dissemination used by the organism *in vivo* and whether infection can be passed between animals by *C. pneumoniae*-infected macrophages.

CHAPTER SECTION: MATERIALS AND METHODS

CHAPTER SUBSECTION: INTRANASAL INOCULATION OF ANIMALS

Forty-eight male and female C57BL/6J mice (Jackson Laboratories, Bar Harbor, MA), aged 6-8 weeks of age, were inoculated, either intranasally or intraperitoneally, with *C. pneumoniae*, strain AR-39, suspended in chlamydia transport medium (SPG). Following light sedation with a mixture of ketamine and xylazine, mice were inoculated with 3 × 10^7 IFUs per mouse (0.05 mL total volume) by a 23 gauge needle and tuberculin syringe. Mice were inoculated with 5 × 10^7 IFUs per mouse (0.1 mL total volume) by intraperitoneal injection using a 25 gauge needle and tuberculin syringe. All infected mice were primed 2-3 days prior to infection with thioglycollate broth (1.5 mL total volume) injected intraperitoneally to recruit and activate peritoneal macrophages.

CHAPTER SUBSECTION: TISSUE SAMPLING

Alveolar and peritoneal macrophages were collected at 3, 5 and 7 days post-inoculation and peripheral blood was pooled from 2 mice in each group of intranasally and intraperitoneally inoculated animals. Mice were heavily sedated, exsanguinated from the femoral artery and heparinized blood was collected. Peritoneal macrophages were collected by washing the peritoneal cavity twice with Hanks balanced salt solution (HBSS), pH 7.2, containing 0.2% bovine serum albumin. Alveolar macrophages were collected by removing the lungs to a sterile petri dish and lavaging through the trachea with ice cold HBSS. Five mL of HBSS was injected into the lungs, allowed to diffuse out of the lung tissue and collected from the sterile petri dish. The procedure was repeated 2-3 times until a total of 10-15 ml of lavaged fluid was collected.

CHAPTER SUBSECTION: DIRECT PLATING

Alveolar and peritoneal macrophages were washed twice in 10 mL of Dulbecco's minimum essential medium, (DMEM), containing 1% nonessential amino acids (Gibco BRL, Grand Island, NY), 2 mM glutamine and 100 µg each of vancomycin and streptomycin. Macrophages were counted using a hemocytometer following staining with 0.4% Trypan blue dye (Sigma, St. Louis, MO). Based on morphology, approximately 80-90% of the cells collected were macrophages. Approximately one half of the collected cells were directly plated and the remainder were frozen at -70°C for culture and PCR. Macrophages were plated in 24 well microtiter plates containing 12 mm glass cover slips at a concentration of 2 - 5 × 10^5 cells per well in a total volume of 1 mL DMEM for up to 3 cover slips per sample. Cells were incubated at 37°C in 5% CO_2 overnight. Cover slips were fixed with acetone and stained with fluorescein isothiocyanate (FITC)-conjugated Chlamydia genus-specific (CF-2) monoclonal antibody for detection of chlamydial inclusions.

CHAPTER SUBSECTION: ISOLATION

Heparinized whole blood was diluted with an equal volume of phosphate buffered saline (PBS) and separated by Ficoll-Paque Plus (Pharmacia, Uppsala, Sweden) by centrifugation at 400 × *g* for 30 min at 20°C. The buffy coat, which contains mononuclear cells, was washed twice with PBS and resuspended in 2 mL of DMEM. Peripheral blood mononuclear cells (PBMC) and alveolar and peritoneal macrophages were lysed by sonication prior to culture. Plasma was aspirated and filtered through a 0.8 µm filter before culture. Isolation of *C. pneumoniae* organisms was done in HL cells by inoculating them with 200 µL of either PBMC, macrophages or plasma per vial (36). Monolayers were incubated at 35°C for 4 days, harvested by scraping, passed onto new HL cell monolayers and incubated another 4 days. The presence of chlamydial inclusions was detected as described above.

CHAPTER SUBSECTION: POLYMERASE CHAIN REACTION

DNA was extracted from macrophages, PBMC and plasma by centrifuging samples at 1250 × *g* for 30 min and resuspending the pellet in 100 µL lysis buffer (1% Tween, 1% Nonidet P-40 and 100 µg proteinase K). Lysis buffer alone was included in each extraction as a control to detect cross-contamination during the extraction process. Samples were incubated at 50°C for 1 h, then boiled for 10 min to inactivate the proteinase K. Ten to 15 µL of each sample were amplified using the *C. pneumoniae* specific primer set HL-1 and HR-1 (8, Chapter 2). Amplification products were viewed by agarose gel electrophoresis and confirmed by Southern hybridization to a cloned digoxigenin-labelled 474-bp *PstI* restriction fragment of *C. pneumoniae* according to previously described methods (47). Serial dilutions of purified *C. pneumoniae* organisms were used as positive controls for each amplification. Sterile water was used in place of sample DNA as a negative control.

CHAPTER SUBSECTION: ADOPTIVE TRANSFER

Peritoneal macrophages were collected from 2 donor mice 3 and 5 days after intranasal inoculation with 3×10^7 IFUs per mouse. Cells were prepared and counted as described above. Approximately 6×10^5 cells were transferred by intraperitoneal injection to 2 recipient mice at each time point. In a separate experiment, alveolar macrophages were collected from 10 donor mice 3 days after intranasal inoculation with 3×10^7 IFUs per mouse. Peritoneal macrophages were collected from 10 donor mice 7 days after intraperitoneal inoculation with 5×10^7 IFUs per mouse. Aliquots of 5×10^5 cells were analyzed for the presence of *C. pneumoniae* organisms by direct plating, culture and PCR. Approximately 2×10^6 cells, either alveolar or peritoneal macrophages, were transferred by intraperitoneal injection to 4 recipient mice. Three days after transfer, lung, spleen, thymus and abdominal lymph nodes were removed from all recipient mice and tested for the presence of *C. pneumoniae* by isolation and PCR.

CHAPTER SECTION: RESULTS

The frequency of detection of *C. pneumoniae* in alveolar and peritoneal macrophages and the peripheral blood is shown in Table 8. Staining of macrophages with fluorescein-labeled Chlamydia genus-specific antibody 24 hours after plating to glass cover slips detected the presence of chlamydial antigen, either as part of a developing viable inclusion or partially degraded nonviable organisms. The presence of viable organisms was indicated by growth and detection of chlamydial inclusions in HL cell culture. Productive infection was shown by passage of infectious particles from the initial cell monolayer to freshly grown cells. At 3 days post-inoculation, *C. pneumoniae* was consistently detected by direct plating, isolation and PCR in alveolar macrophages of all intranasally inoculated mice and peritoneal macrophages of all intraperitoneally inoculated mice. *C. pneumoniae* was detected in PBMC, but not in plasma, by isolation and/or PCR of both intranasally and intraperitoneally inoculated animals. Alveolar macrophages from intraperitoneally inoculated animals and peritoneal macrophages from intranasally inoculated animals were negative by isolation and PCR at all time points.

Results of the adoptive transfer of infected macrophages to uninfected mice are shown in Table 9. The presence of *C. pneumoniae* organisms was confirmed by direct plating, culture and PCR in alveolar macrophages transferred from intranasally inoculated mice and in peritoneal macrophages transferred from intraperitoneally inoculated mice, but not in peritoneal macrophages transferred from intranasally inoculated mice. In mice receiving peritoneal macrophages from intranasally inoculated donor mice, *C. pneumoniae* DNA was detected by PCR in spleen tissue of 1 recipient animal that received cells from donor mice 5 days post-inoculation. In mice receiving alveolar macrophages from intranasally inoculated donor mice, *C. pneumoniae* DNA was detected in lung, spleen and abdominal lymph nodes, but not thymus. In mice receiving peritoneal macrophages from intraperitoneally inoculated donor mice, *C. pneumoniae* DNA was detected in all tissues tested. All tissues were negative by isolation.

CHAPTER SECTION: DISCUSSION

This study demonstrates the ability of *C. pneumoniae* to infect, survive and multiply *in vivo* within murine alveolar and peritoneal macrophages. Also, the presence of chlamydial organisms in PBMC, and not in the plasma, shows that acute infection with *C. pneumoniae* results in a cell-associated bacteremia in the murine model. Finally, these results suggest that infection with *C. pneumoniae* can be transferred by infected alveolar or peritoneal macrophages and disseminate from the peritoneal cavity, the site of inoculation, to other tissue sites.

Infection of macrophages by *C. pneumoniae* was indicated by fluorescent staining of chlamydial antigen by direct plating, detection of chlamydial DNA by PCR and the presence of viable organisms by tissue culture. *C. pneumoniae* infection of murine macrophages was productive as evidenced by the passage of macrophage lysates from infected mice in tissue culture. Previous studies have demonstrated the *in vitro* growth of *C. pneumoniae* in human peripheral blood monocytes, endothelial and smooth muscle cells and alveolar macrophages as well as human and murine macrophage cell lines (14, 15). This is the first demonstration of the growth of *C. pneumoniae in vivo* in the macrophages of an experimental animal model.

We have shown that *C. pneumoniae* exists in monocytes within the blood stream following acute infection by both intranasal and intraperitoneal routes of inoculation. Demonstration of the presence of organisms in PBMCs and the adoptive transfer of infected macrophages shows that *C. pneumoniae* can disseminate systemically by both hematogenous and lymphatic routes. Organisms delivered directly to the lungs by intranasal inoculation may be carried by alveolar and/or interstitial macrophages to bronchiolar-associated lymphoid tissue. Infected lung macrophages then drain to the mediastinal lymph nodes and empty into the left subclavian vein via the thoracic duct (82). Infected peritoneal macrophages traverse the diaphragm by way of the transdiaphragmatic lymphatics, drain via the intrathoracic lymphatics to the mediastinal and parathymic lymph nodes and then empty into the bloodstream (82). In addition to the migration of peritoneal macrophages via the transdiaphragmatic lymphatic channels to the blood, peritoneal macrophages also migrate directly across the diaphragm through the pleural space to the lung interstitium (64). Both these pathways are likely in this model system.

The presence of *C. pneumoniae* in the peripheral circulation is the first evidence of a *C. pneumoniae*-induced bacteremia. Systemic disease has been reported in human cases in which *C. pneumoniae* was detected by PCR in lymph nodes and/or liver and spleen (39), but no evidence of blood-borne *C. pneumoniae* has been found. Rare human cases of bacteremia due to *C. psittaci* have been reported and were associated with endocarditis or sarcoidosis (28, 76).

These data support the hypothesis that macrophages infected with *C. pneumoniae* can disseminate to other tissue sites and result in systemic disease. The proposed modes of dissemination as the pathways by which viable chlamydial organisms are carried from the primary site of infection in the lungs to the peripheral circulation include both hematogenous and lymphatic routes. These studies indicate the macrophage as the vehicle of dissemination *in vivo*. In humans, *C. pneumoniae* was detected by double ICC in macrophages within aortic atheromas and in atheromas of experimentally inoculated apoE-deficient mice. These experiments support the hypothesis that *C. pneumoniae* preferentially localizes to cardiovascular tissue and disseminates to the atherosclerotic lesion following respiratory infection.

In conclusion, the ability of *C. pneumoniae* to infect and survive *in vivo* within macrophages and disseminate systemically via infected macrophages has important physiopathologic implications for chronic infection and the role of *C. pneumoniae* in atherosclerosis. These findings are significant because they substantiate the hypothesis that *C. pneumoniae*-infected macrophages can disseminate from the lung to other tissue sites resulting in systemic disease.

Table 8. Frequency of *C. pneumoniae* in alveolar and peritoneal macrophages and peripheral blood mononuclear cells in mice inoculated by intranasal and intraperitoneal routes.

	Days after inoculation					
	3		5		7	
	IN[a]	IP[b]	IN	IP	IN	IP
Alveolar macrophages						
Direct	4/4[c]	0/4	4/4	0/4	3/4	0/4
Isolation	4/4	0/4	3/4	0/4	2/4	0/4
PCR	4/4	0/4	4/4	0/4	3/4	0/4
Peritoneal macrophages						
Direct	0/4	4/4	1/4	4/4	0/4	4/4
Isolation	0/4	4/4	0/4	3/4	0/4	3/4
PCR	0/4	4/4	0/4	3/4	0/4	3/4
PBMC[d]						
Isolation	1/2	2/2	0/2	0/2	1/2	0/2
PCR	1/2	2/2	1/2	1/2	1/2	0/2
Plasma						
Isolation	0/2	0/2	0/2	0/2	0/2	0/2
PCR	0/2	0/2	0/2	0/2	0/2	0/2

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[a] IN, intranasal inoculation.
[b] IP, intraperitoneal inoculation.
[c] Fractions represent the number positive over the number tested.
[d] PBMC, peripheral blood mononuclear cells.

Table 9. Detection of *C. pneumoniae* DNA by PCR in tissues of C57BL/6J mice 3 days after intraperitoneal transfer of macrophages from infected mice.

	Routes of inoculation of donor mice		
	Intranasal		Intraperitoneal
	Cells transferred[a]		
Tissues of recipient mice	Peritoneal macrophages	Alveolar macrophages	Peritoneal macrophages
Lung	0/4[b]	1/4	3/4
Thymus	ND[c]	0/4	3/4
Spleen	1/4	3/4	4/4
Abdominal lymph nodes[d]	0/4	4/4	3/4

[a] Peritoneal macrophages from intranasally infected donor mice were collected 3 and 5 days after inoculation. Alveolar macrophages from intranasally infected donor mice were collected 3 days after inoculation. Peritoneal macrophages from intraperitoneally infected donor mice were collected 7 days after inoculation.

[b] Fractions represent the number positive over the number tested.

[c] ND, not done.

[d] Abdominal lymph nodes include the superior mesenteric, renal and para-aortic nodes.

CHAPTER 4: PERSPECTIVES AND FUTURE STUDIES

The hypothesis for an etiological association between *C. pneumoniae* and atherosclerosis is that following infection and respiratory disease, *C. pneumoniae* infected macrophages from the lung disseminate, either hematogenously or lymphatically, to other tissues causing systemic disease. As a result of dissemination, arterial sites of atherosclerosis, such as the cardiovasculature or other major arteries, are infected. Once at the arterial site, *C. pneumoniae* infection could induce the initial injury or contribute to the immunopathology of the atherosclerotic process, thus either inducing atherogenesis or exacerbating the ongoing atherosclerotic disease.

These studies have proven the following steps of the hypothesis stated above concerning how *C. pneumoniae* spreads from the respiratory tract to the atherosclerotic plaque. First, in the NZW rabbit model, we have shown by differential ICC staining using both Chlamydia genus-specific and a rabbit macrophage-specific monoclonal antibodies, that lung macrophages are infected with *C. pneumoniae* during respiratory disease. Using the C57BL/6J mouse model, we have shown that *C. pneumoniae* infects, survives and replicates within both alveolar and peritoneal macrophages *in vivo*. Second, in both rabbit and murine models, we have shown that lung macrophages disseminate to other tissue sites following respiratory disease. In the NZW rabbit, *C. pneumoniae* was detected in the spleen and PBMC following intranasal inoculation. In the apoE-deficient mouse, *C. pneumoniae* disseminated to the spleen and heart following intranasal inoculation. In the C57BL/6J mouse, we demonstrated that *C. pneumoniae* infected macrophages could disseminate from the peritoneal cavity to other tissue sites including the lung and that infection could be transferred between animals by *C. pneumoniae* infected macrophages. Third, that *C. pneumoniae* disseminates to and persists in the aorta during lesion development as indicated by the persistent detection of *C. pneumoniae* in the aorta of apoE-deficient mice for 20 weeks after inoculation. Multiple inoculations of the C57BL/6 mouse resulted in chronic *C. pneumoniae* infection of the aorta. Fourth, that *C. pneumoniae* is localized within the developing mature atheroma as shown by the presence of *C. pneumoniae* antigen by ICC in the atherosclerotic plaque of 24 week old mice.

This dissertation has achieved its goal of evaluating and characterizing both rabbit and murine models of *C. pneumoniae* infection and atherosclerosis. The study described in Chapter 1 showed that NZW and WHHL rabbits were susceptible to *C. pneumoniae* respiratory disease with infection resulting in a mild interstitial pneumonitis. However, these animal models were not appropriate for continued studies regarding the relationship between *C. pneumoniae* and atherosclerosis due to the lack of recovery of viable organisms from the lower respiratory tract and, most importantly, the inability to detect the organism in atherosclerotic lesions of WHHL rabbits. In spite of this finding, these rabbit models may prove useful in immunological studies of chlamydial pathogenesis and as models of mild or asymptomatic *C. pneumoniae* infection.

In Chapter 2, the presence of respiratory disease and recovery of viable organisms from the lower respiratory tract, together with persistence of *C. pneumoniae* at the site of aortic lesion development and localization of the organism within the atherosclerotic plaque of the apoE-deficient mouse indicates this model will be ideal for further investigation of the role of *C. pneumoniae* as a cofactor in the development and progression of atherosclerosis. The persistence of *C. pneumoniae* in the aorta of C57BL/6J mice following multiple inoculations in the absence of predisposing factors for atherosclerosis suggests *C. pneumoniae* is capable of inducing alterations in the vasculature which may lead to atherosclerosis. The C57BL/6J mouse will also serve as equally an important purpose as the apoE-deficient mouse by ascertaining whether *C. pneumoniae* can act as an inducer of atherosclerosis. Because the development of atherosclerosis can be controlled by diet in C57BL/6 mice, this model can be used to determine if diet acts as a cofactor with *C. pneumoniae* infection in the initiation of atherosclerosis. Both the apoE-deficient and C57BL/6J can be used as models of chronic *C. pneumoniae* infection to study the immunopathogenesis of chlamydial disease. These experiments have also established the optimal conditions necessary for future experiments to reproduce infection and persistence in murine models of disease.

In Chapter 3, studies using the C57BL/6J model provided valuable evidence to substantiate the hypothesis that *C. pneumoniae* can disseminate from the lung to other tissue sites by infected macrophages. *C. pneumoniae* was shown to infect, survive and replicate within macrophages *in vivo*. In addition, the systemic dissemination of *C. pneumoniae* by infected macrophages was demonstrated to occur by both hemoatogenous and lymphatic routes. Most importantly, infection could be transferred by *C. pneumoniae*-infected macrophages using the macrophage as the vehicle of dissemination.

Several possible roles exist for the involvement of *C. pneumoniae* in atherosclerosis. Either *C. pneumoniae* initiates atherogenesis and/or contributes to the progression of the disease or the organism has no effect at all on lesion pathogenesis and acts primarily as an "innocent bystander". A possible hypothesis for a role of *C. pneumoniae* in atherosclerosis involves dissemination of *C. pneumoniae* infected macrophages to the sites of lesion development where, due to direct activation and alteration of macrophage metabolism as a result of infection, the macrophage will adhere to and migrate into the vessel wall. Future goals should focus on proving an effectual role of *C. pneumoniae* infection in the initiation, development and/or progression of atherosclerosis.

To determine whether *C. pneumoniae* infection alters the progression or severity of atherosclerosis, morphometric analysis can be used to quantitatively compare lesion size between infected and non-infected control apoE-deficient mice. Comparative histopathology using special stains for collagen and fibrin and cell type-specific antibodies for inflammatory cells will enable study of the nature of the inflammatory response induced by *C. pneumoniae* within the atherosclerotic plaque and whether infection with *C. pneumoniae* stimulates a heightened immune response within the lesion. The apoE-deficient model can also be used to examine the pathophysiological mechanisms which may be induced by *C. pneumoniae* infection at the site of lesion development. *In situ* hybridization and ICC can be used to localize production of proatherogenic inflammatory enzymes such as collagenase, protease and cell adhesion molecules to sites of infection within the atherosclerotic plaque. These enzymes and factors have been shown to be involved in the atherogenic process. Infected cells located at the lesion site can also be examined for expression of cytokines such IL-1, IL-8 and chemoattractants to show whether infection results in cell activation. Previous studies have shown that *C. pneumoniae* can induce production of cytokines by macrophages and endothelial cells *in vitro*.

The C57BL/6J murine model is of particular importance in that the onset of atherosclerosis can be induced in this mouse by an atherogenic diet. The C57Bl/6 mouse also provides a unique opportunity to determine whether repeated infection with *C. pneumoniae* induces any early pre-atherosclerotic vascular changes which could lead to atherosclerosis. The effect of *C. pneumoniae* infection will be examined histologically prior to lesion formation in the absence of predisposing factors to determine if infection alone induces atherosclerosis, similar to MDV in chickens. Also, lesion formation and progression can be manipulated by diet, allowing the effect of *C. pneumoniae* infection to be analyzed in a controlled manner in conjunction with lipid as a cofactor. Future experiments could focus on determining where in the aorta of C57BL/6J mice *C. pneumoniae* resides and localizing the presence of the organism within the aortic wall using immunocytochemistry. Once this is known, specific areas can be analyzed to determine if any pre-atherosclerotic changes in the arterial wall such as vasculitis or subintimal thickening, are induced by *C. pneumoniae*.

If infection with *C. pneumoniae* is a factor in the initiation or progression of atherosclerosis, the result could be significant because of the high prevalence of *C. pneumoniae* infection and the importance of atherosclerosis and coronary heart disease as a source of morbidity and mortality. Eradication of *C. pneumoniae* infection could have a significantly beneficial effect on public health by reducing the incidence of atherosclerosis and coronary artery disease or delaying the progression and severity of the disease process. Intervention will take two forms - treatment of existing disease with therapeutic drugs and prevention of infection by vaccination. Intervention in the form of antibiotic therapy would break the cycle of chronic *C. pneumoniae* infection and disease and curtail its additive effect on the atherosclerotic process. If *C. pneumoniae* is found to induce atherosclerosis, prevention of infection in the form of vaccination would be necessary to effectively control the disease. Vaccination would have to be administered prior to exposure, preferably by 5 years of age, to be efficacious in preventing disease.

In conclusion, the murine models described in this dissertation, the apoE-deficient transgenic and the C57BL/6J mice, will serve as unique and essential tools to prove that infection by *C. pneumoniae* contributes to the initiation and/or progression of atherosclerosis.

BIBLIOGRAPHY

1. **Aldous, M.B., Grayston, J.T., Wang, S.P., and Foy, H.M.** 1992. seroepidemiology of *Chlamydia pneumoniae* TWAR infection in Seattle, 1966-1976. J. Infec. Dis. **166**:646-649.
2. **Armstrong, M.L. and Heistad, D.D.** 1990. Animal models of atherosclerosis. Arterioscler. **85**:15-23.
3. **Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (eds.).** 1991. Preparation of genomic DNA from bacteria *In*: Current Protocols, Molecular Biology. Vol 1. New York: Wiley Interscience, Supp. 9, Unit 2.4.1.
4. **Bell, T.A., Kuo, C.-C., Wang, S.-P., and Grayston, J.T.** 1989. Experimental infection of baboons (*Papio cynocephalus anubis*) with *Chlamydia pneumoniae* strain 'TWAR'. J. Infect. Dis. **19**:47-49.
5. **Black, C.M., Bullard, J.C., Staton Jr., G.W., Hutwagner, L.C., and Perez, R.L.** 1992. Seroprevalence of *Chlamydia pneumoniae* antibodies in patients with pulmonary sarcoidosis in north central Georgia. *In*: Mårdh, P.-A., La Placa, M., Ward, M.E. (eds.), Proceedings of the European Society for Chlamydial Research. Stockholm:Almquist & Wiksell International. 175.

6. **Breslow, J.** 1993. Transgenic mouse models of lipoprotein metabolism and atherosclerosis. Proc. Natl. Acad. Sci, USA. **90**:8314-8318.

7. **Campbell, L.A., Kuo, C.-C., and Grayston, J.T.** 1987. Characterization of the new *Chlamydia* agent, TWAR, as a unique organism by restriction endonuclease analysis and DNA-DNA hybridization. J. Clin. Microbiol. **25**:1911-1916.

8. **Campbell, L.A., Melgosa, M.P., Hamilton, D.J., Kuo, C.-C., and Grayston, J.T.** 1992. Detection of *Chlamydia pneumoniae* by polymerase chain reaction. J. Clin. Microbiol. **30**:434-439.

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

10. **Chirgwin, K., Roblin, P.M., Gelling, M., Hammerschlag, M.R., and Schachter, J.** 1991. Infection with *Chlamydia pneumoniae* in Brooklyn. J. Infect. Dis. **163**:757-761.

11. **Ekman, M.-R., Grayston, J.T., Visakorpi, R., Kleemola, M., Kuo, C.-C., and Saikku, P.** 1993. An epidemic of infections due to *Chlamydia pneumoniae* in military conscripts. Clin. Inf. Dis. **17**:420-425.

12. **Ellison, J., Dean, M., and Goldman, D.** 1993. Efficacy of fluorescence-based PCR-SSCP for detection of point mutations. BioTechniques. **4**:684-691.

13. **Gaydos, C.A., Roblin, P.M., Hammerschlag, M.R., Hman, C.L., *et al.*,** 1994. Diagnostic utility of PCR-enzyme immunoassay, culture, and serology for detection of *Chlamydia pneumoniae* in symptomatic and asymptomatic patients. J. Clin. Microbiol. **32**:903-905.

14. **Gaydos, C.A., Summersgill, J.T., Sahney, N.N., Ramirez, J.A., and 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.

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

16. **Gown, A.M., Vogel, A.M., Hoak, D., Gough, F., and McNutt, M.A.** 1986. Monoclonal antibodies specific for melanocytic tumors distinguish subpopulations of melanocytes. Am. J. Pathol. **123**:195-203.

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

18. **Grayston, J.T., Kuo, C.-C., Campbell, L.A., and Wang, S.-P.** 1989. *Chlamydia pneumoniae* sp. nov. for *Chlamydia* sp. strain TWAR. Int. J. Syst. Bacteriol. **39**:88-90.

19. **Grayston, J.T., Campbell, L.A., Kuo, C.-C, Mordhorst, C.H., Saikku, P., Thom, D.H., and Wang, S.-P.** 1990. A new respiratory tract pathogen: *Chlamydia pneumoniae* strain TWAR. J. Infect. Dis. **161**:618-625.

20. **Grayston, J.T.** 1992. Infections caused by *Chlamydia pneumoniae* strain TWAR. Clin. Infect. Dis. **15**:757-763.

21. **Grayston, J.T.** 1994. *Chlamydia pneumoniae* (TWAR) infections in children. Pediatr. Infect. Dis. J. **13**:675-685.

22. **Grayston, J.T., Thom, D.T., Kuo, C.-C., Campbell, L.A., and Wang, S.-P.** 1994. *Chlamydia pneumoniae* (TWAR) and atherosclerosis. *In*: Orfila, J. (ed.), Chlamydial Infections. Bologna, Italy:Societa Editrice Esculapio. 199-208.

23. **Grayston, J.T., Kuo C.-C., Coulson A.S., *et al.*** 1995. *Chlamydia pneumoniae* (TWAR) in atherosclerosis of the carotid artery. Circulation. **92**:3397-3400.

24. **Grayston, J.T.,** 1996. Epidemiology of *Chlamydia pneumoniae* (TWAR). *In*: Stary, A. (ed.), Chlamydial Research. Bologna, Italy:Societa Editrice Esculapio. 211-213.

25. **Grönhagen-Risk, C., Saikku, P., Riska, H., Froseth, B., and Grayston, J.T.** 1988. Antibodies to TWAR-a novel type of Chlamydia in sarcoidosis. *In*: Grassi, C., Rizzato, G., Pozzi, E. (eds.), Sarcoidosis and other granulomatous disorders. Amsterdam:Excerpta Medica. 297-301.

26. **Hajjar, D., Fabricant, C.G., Minick, C.R., and Fabricant, J.** 1986. Virus-induced atherosclerosis: Herpesvirus infection alters aortic cholesterol metabolism and accumulation. Am. J. Pathol. **122**:62-70.

27. **Hajjar, D.** 1991. Viral pathogenesis of atherosclerosis: Impact of molecular mimicry and viral genes. Am. J. Pathol. **139**:1195-1211.

28. **Harris, A.A., Pottage, Jr., J.C., Kessler, H.A., Zeihen, M., and Levin, S.** 1984. Psittacosis bacteremia in a patient with sarcoidosis. Ann. Intern. Med. **101**:502-503.

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

30. **Holland, S.M., Taylor, H., Gaydos, C.A., Kappus, E.W., and Quinn, T.C.** 1990. Detection and differentiation of *Chlamydia pneumoniae* in nonhuman primates. Infect. Immun. **58**:593-597.

31. **Jackson, L.A., Campbell, L.A., Schmidt, R.A., Kuo, C.-C., Cappuccio, A.L., Lee, M.J., and Grayston, J.T.** 1996. Specificity of detection of *Chlamydia pneumoniae* in cardiovascular atheroma: Evaluation of the "innocent bystander" hypothesis. (submitted).

32. **Jackson, L.A., Rodriguez, D.I., Lee, A., Kuo, C.-C., Campbell, L.A., and Grayston, J.T.** 1996. Isolation of *Chlamydia pneumoniae* (TWAR) from a carotid atherosclerotic plaque specimen obtained by endarterectomy. 36th ICAAC. New Orleans, LA. Abstr. K121.

33. **Jang, I.K., Lassila, R., and Fuster, V.** 1993. Atherogenesis and inflammation. Eur. Heart J. **14** (Supp. K):2-6.

34. **Jayo, J.M., Schwenke, D.C., and Clarkson, T.B.** 1994. Atherosclerosis research. *In*: The biology of the laboratory rabbit. Manning, P., Ringler, D.H., and Newcomer, C.E. (eds.), 2nd edition. San Diego:Academic Press. 367-380.

35. **Kuo, C.-C., Chen, H.H., Wang, S.-P., and Grayston, J.T.** 1986. Identification of a new group of *Chlamydia psittaci* strains called TWAR. J. Clin. Microbiol. **24**:1034-1037.

36. **Kuo, C.-C., and Grayston, J.T.** 1990. A sensitive cell line, HL cells, for isolation and propagation of *Chlamydia pneumoniae* strain TWAR. J. Infect. Dis. **162**:755-758.

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

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

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

40. **Kuo, C.-C., Grayston, J.T., Campbell, L.A., Goo, Y.A., Wissler, R.W., and Benditt, E.P.** 1995. *Chlamydia pneumoniae* (TWAR) in coronary arteries of young (15 to 35 year) adults. Proc. Natl. Acad. Sci. USA. **92**:6911-6914.

41. **Laitinen, K., Laurila, L., Leinonen, M., and Saikku, P.** 1996. Reactivation of *Chlamydia pneumoniae* infection in mice by cortisone treatment. Infect. Immun. **64**:1488-1490.

42. **Leinonen, M.** 1994. Pathogenetic mechanism and epidemiology of *Chlamydia pneumoniae.* Eur. Heart J. **14**(supp):57-61.

43. **Libby, P., and Hansson, G.** 1992. Involvement of the immune system in human atherogenesis: current knowledge and unanswered questions. Lab. Invest. **64**:5-15.

44. **Lindsey, J.R., and Fox, R.R.** 1994. Inherited diseases and variations. *In*: The biology of the laboratory rabbit. Manning, P., Ringler, D.H., and Newcomer, C.E. (eds.), 2nd edition. San Diego:Academic Press. 293-319.

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

46. **Maliverni, R., Kuo, C.-C., Campbell, L.A., and Grayston, J.T.** 1995. Reactivation of *Chlamydia pneumoniae* lung infection in mice by cortisone. J. Infect. Dis. **172**:593-594.

47. **Maniatis, T., Fritsch, E.F., and Sambrook, J.** 1982. Molecular cloning: a laboratory manual, 1st ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

48. **Manning, P.J., DiGiacomo, R.F., and DeLongf, D.** 1989. Pasteurellosis in laboratory animals. *In*: Pasteurella and pasteurellosis. Academic Press:New York. 263-302.

49. **Melnick, J.L., and Schattner, A.** 1992. Viruses and atherosclerosis. Isr. J. Med. Sci. **28**:463-465.

50. **Melnick, S., Shahar, E., Folsom, A.E., Grayston, J.T., Wang, S.-P., and Szklo, M.** 1993. Past infection by *Chlamydia pneumoniae* strain TWAR and asymptomatic carotid atherosclerosis. Atherosclerosis Risk in Communities (ARIC) Study Investigations. Am. J. Pathol. **95**:499-504.

51. **Mendall, M.A., Carrington, D., Strachan, P., Molineaux, N., *et al.*,** 1995. *Chlamydia pneumoniae*: Risk factors for seropositivity and association with coronary heart disease. J. Infect. **30**:121-128.

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

53. **Mordhorst, C.H., Wang, S.-P., and Grayston, J.T.** 1994. Transmission of *C. pneumoniae* (TWAR). *In*: Orfila, J., (ed.), Chlamydial Infections. Bologna, Italy:Societa Editrice Esculapio. 488-491.

54. **Moulder, J.W.** 1984. Order II.Chlamydiales; Family Chlamydiae In: Bergeys Manual of Systematic Bacteriology. Baltimore and London:Williams and Wilkins. 729-739.

55. **Moulder, J.W.** 1985. Comparative biology of intracellular parasitism. Microbiol. Rev. **149**:298-337.

56. **Moulder, J.W.** 1988. Characteristics of Chlamydiae *In*: Microbiology of Chlamydia. Boca Raton, Florida:CRC Press. 3-19.

57. **Muhlestein, J.B., Hammond, E.H., Carlquist, J.F., Radicke, E., Thomson, M.J., *et al.*,** 1996. Increased incidence of Chlamydia species with in the coronary arteries of patients with symptomatic atherosclerotic versus other forms of cardiovascular disease. J. Am. Coll. Cardiol. **27**:1555-1561.

58. **Nakashima, Y., Plump, A.S., Raines, E.W., Breslow, J.L., and Ross, R.** 1994. ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. Arterioscler. Thromb. 133-140.

59. **Ong, G., Thomas, B.J., Mansfield, O.A., Davidson, B.R., and Taylor-Robinson, D.** 1996. Detection and widespread distribution of *Chlamydia pneumoniae* in the vascular system and its possible implications. J. Clin. Pathol. **49**:102-106.

60. **Ouchi, K., Jujii, B., Kanamoto, Y., Miyazaki, H., and Nakazawa, T.** 1996. Detection of *Chlamydia pneumoniae* in atherosclerotic lesions of coronary arteries and large arteries. 36th ICAAC. New Orleans, LA. Abstr. K37.

61. **Palinski, W., Ord, V.A., Plump, A.S., Breslow J.L., Steinberg, D., and Witztum, J.L.** 1994. ApoE-deficient mice are a model of lipoprotein oxidation in atherogenesis. Arterioscler. Thromb. 605-616.

62. **Patnode, D., Wang, S.-P., and Grayston, J.T.** 1990. Persistence of *Chlamydia pneumoniae*, strain TWAR, microimmunofluorescence antibody, p. 406-409. *In*: Bowie, W.R., *et al.* (eds.), Chlamydial Infections. Cambridge University Press:Cambridge, England.

63. **Piedrahita, J.A., Zhang, S.H., Hagaman, J.R., Oliver, P.M., and Maeda, N.** 1992. Generation of mice carrying a mutant apolipoprotein E gene inactivated by gene targeting in embryonic stem cells. Proc. Natl. Acad. Sci. USA **89**:4471-4475.

64. **Pitt, M.L.M., and Anderson, A.O.** 1988. Direct transdiaphragmatic traffic of peritoneal macrophages to the lung. Adv. Exp. Med. Biol. **237**:627-632.

65. **Plump, A.S., and Breslow, J.L.**, 1995. Apolipoprotein E and the apolipoprotein E-deficient mouse. Annu. Rev. Nutr. **15**:495-518.

66. **Plump, A.S., Smith, J.D., Hayek, T.** ***et al.*** 1992. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. Cell. **71**:343-353.

67. **Qiao, J.-H., Xie, P.Z., Fishbein, M.C., Jreuzer, J., Drake, T.A., Demer, L.L., and Lusis, A.J.** 1994. Pathology of atheromatous lesion in inbred and genetically engineered mice. Arterioscler. Thromb. **14**:1480-1497.

68. **Ramirez, J., Ahkee, S., Ganzel, B., Ogden, L., Gaydos, C., Quinn, T.,** ***et al.*****,** 1995. Isolation of *Chlamydia pneumoniae* from the coronary artery of a patient with coronary atherosclerosis. 35th ICAAC. San Francisco, CA. Abstr. B59.

69. **Reddick, R.L., Zhang, S.H., and Maeda, N.** 1994. Atherosclerosis in mice lacking apo E: Evaluation of lesion development and progression. Atheroscler. Thromb. **14**:141-147.

70. **Rosenfeld, M.E., Tsukada, T., Gown, A.M., and Ross, R.** 1987. Fatty streak initiation in Watanabe heritable hyperlipidemic and comparably hypercholesterolemic fat-fed rabbits. Arterioscler. **7**:7-23.

71. **Rosenfeld, M.E., Tsukada, T., Chait, A., Bierman, E.L., Gown, A.M., and Ross, R.** 1987. Fatty streak expansion and maturation in Watanabe heritable hyperlipidemic and comparably hypercholesterolemic fat-fed rabbits. Arterioscler. **7**:24-34.

72. **Saikku, P., Mattila, K., Niemenen, N.S., Huttunen, J.K., Leinonen, M., Ekman, M.R., Makela, P.H., and Valtonen, V.** 1988. Serological evidence of an association of a novel Chlamydia, TWAR, with chronic coronary heart disease and acute myocardial infarction. Lancet **2**:983-985.

73. **Saikku, P., Leinonen, M., Tenkanen, L., Linnanmaki, E., Ekman, M.R., Manninen, V., Mantarri, M., Frick, M.H., and Huttunen, J.K.** 1992. Chronic *Chlamydia pneumoniae* infection as a risk factor for coronary heart disease in the Helsinki Heart Study. Ann. Intern. Med. **116**:273-278.

74. **Sambrook, J., Fritsch, E.F., and Maniatis, T.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.

75. **Schachter, J.** 1988. Overview of human disease. In: Microbiology of Chlamydia. Boca Raton, Florida:CRC Press. 153-165.

76. **Shapiro, D.S., Kenney, S.C., Johnson, M., Davis, C.H., Knight, S.T., and Wyrick, P.B.** 1992. Brief report: *Chlamydia psittaci* endocarditis diagnosed by blood culture. N. Engl. J. Med. **236**:1192-1195.

77. **Shor, A., Kuo, C.-C., and Patton, D.L.** 1992. Detection of *Chlamydia pneumoniae* in coronary arterial fatty streaks and atheromatous plaques. S. Afr. Med. J. **82**:158-161.

78. **Socan, M., Beovic, B., and Kese, D.** 1994. *Chlamydia pneumoniae* and meningoencephalitis. N. Engl. J. Med. **331**:406.

79. **Thom, D.H., Wang, S.-P., Grayston, J.T., Siscovick, D.S., Stewart, D.K., Kronmal, R.A., and Weiss, N.S.** 1991. *Chlamydia pneumoniae* strain TWAR antibody and angiographically demonstrated coronary artery disease. Arterioscler. Thromb. **11**:547-55

80. **Thom, D.H., Grayston, J.T., Siscovick, D.S., Wang, S.-P., Weiss, N.S., and Daling, J.R.** 1992. Association of prior infection with *Chlamydia pneumoniae* and angiographically demonstrated coronary artery disease. JAMA **268**:68-72.1.

81. **Thom, D.H., Grayston, J.T., Campbell, L.A., Kuo, C.-C., Diwan, V.K., and Wang, S.-P.** 1994. Respiratory infection with *Chlamydia pneumoniae* in middle-aged and older adult outpatients. Eur. J. Clin. Microbiol. Infect. Dis. **113**:785-792.

82. **Tilney, N.L.** 1971. Patterns of lymphatic drainage in the adult laboratory rat. J. Anat. **109**:369-383.

83. **Tong, C.Y.W., Potter, F., Worthington, E., and Mullins, P.** 1995. *Chlamydia pneumoniae* myocarditis. Lancet **346**:710-711.

84. **Tsukada, T., Tippens, D., Gordon, D., Ross, R., and Gown, A.M.** 1987. HHF35, a muscle actin-specific monoclonal antibody. I. Immunocytochemical and biochemical characterization. Am. J. Pathol. **126**:51-60.

85. **Wang, S.-P., and Grayston, J.T.** 1986. Microimmunofluorescence serological studies with the TWAR organism. *In*: Oriel, J.D., Ridgway, G., Schachter, J., Taylor-Robinson, D., and Ward, M. (eds.), Chlamydial infections. Cambridge University Press :Cambridge, England.

86. **Westrom, L., and Wolner-Hanssen, P.** 1993. Pathogenesis of pelvic inflammatory disease. Genitourin. Med. **69**:9-17.

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

88. **Yang, Z.-P., Cummings, P.K., Patton, D.L., and Kuo, C.-C.** 1994. Ultrastructural lung pathology of experimental *Chlamydia pneumoniae* pneumonitis in mice. J. Infect. Dis. **170**:464-467.

89. **Yang, Z.-P., Kuo, C.-C., and Grayston, J.T.** 1995. Systemic dissemination of *Chlamydia pneumoniae* following intranasal inoculation in mice. J. Infect. Dis **171**:736-738.

VITA

Teresa Clark Moazed

University of Washington

1996

EDUCATION

University of Washington, Seattle, Washington
School of Public Health, Department of Pathobiology
Doctor of Philosophy in Pathobiology 1996
Masters of Science in Pathobiology, 1993
Laboratory Animal Medicine Residency, 1991
School of Medicine, Department of Comparative Medicine
North Carolina State University, Raleigh, North Carolina
College of Veterinary Medicine
Doctor of Veterinary Medicine with Honors, 1988
University of North Carolina at Chapel Hill, Chapel Hill, North Carolina
Certificate in Medical Technology, 1979
Bachelor of Science in Biology, 1978

BIBLIOGRAPHY

Published Articles

1. **Moazed, T.C.** and Wolff, A. The Raisin Board as an Environmental Enrichment Tool for Laboratory Primates. Laboratory Primate Newsletter 27(1):16, 1988.
2. **Moazed T.C.**, Deeb B. and DiGiacomo, R. Subcutaneous Abscess due to *Salmonella adelaide* in a Grey Collie with Cyclic Hematopoiesis. Laboratory Animal Science 40(66):639, 1990.
3. Hajjar A.M., DiGiacomo R., Weir E.C., Carpenter J.K., Bingel S.A., and **Moazed T.C.** Chronic Sialodacryoadenitis Virus (SDAV) Infection in Athymic Rats. Laboratory Animal Science 41(1):22, 1991.

4. **Moazed, T.C.** and Thouless, M.E. Viral Persistence of Simian Type D Retrovirus (SRV-2/W) in Naturally Infected Pigtailed Macaques (*Macaca nemestrina*). Journal of Medical Primatology 22:382, 1993.
5. Maggio-Price, L., Pollack, S.B., Shiota, F., Thouless, M., **Moazed, T.C.** and Grossman, A. Characterization of Natural Killer Cell Activity in *Macaca nemestrina.* American Journal of Primatology 39:251-261, 1996.
6. **Moazed, T.C.**, Kuo, C-C., Patton, D., Grayston, J.T. and Campbell, L.A. Experimental Rabbit Models of *Chlamydia pneumoniae* Infection. American Journal of Pathology, 148 (2):667, 1996.
7. **Moazed, T.C.**, Kuo, C.-C., Grayston, J.T. and Campbell, L.A. Murine Models of *Chlamydia pneumoniae* Infection and Atherosclerosis. Journal of Infectious Disease, (in press).
8. **Moazed, T.C.**, Maggio-Price, L., Shiota, F. and Thouless, M.E. In Vivo Reactivation of SRV-2/Wa in *Macaca nemestrina* by Chemically Induced Immunosuppression (manuscript in preparation).
9. **Moazed, T.C.**, Kuo, C.-C., and Campbell, L.A. Evidence of Systemic Dissemination of *Chlamydia pneumoniae* via Mononuclear Cells in a Mouse Model (submitted).

Abstracts

1. Spence C.D., **Moazed T.C.**, Ladiges W.C., Knitter G.L., Thouless M.T. and Morton W.P. Simian Retrovirus (SRV-2) Infection in Macaques: Altered Expression of Leukocyte Antigens. Laboratory Animal Science 39(5):496, 1989.
2. Thouless M.T., **Moazed T.C.**, Kuller, L. and Tsai, C.-C. Natural Transmission of SRV-2/W in *Macaca nemestrina* in an Infant Primate Nursery. Symposium on Nonhuman Primate Models for AIDS, 8th Annual Meeting, Portland, OR, November 28-30, 1990.
3. **Moazed, T.C** and Thouless, M.E. Tissue Distribution of Proviral SRV-2/W in Naturally Infected *Macaca nemestrina*. Symposium on Nonhuman Primate Models for AIDS, 9th Annual Meeting, Seattle, WA, November 6-9, 1991.
4. Maggio-Price, L., Grossman, A., Shiota, F. and **Moazed, T.C.** Evaluation of Immunosuppression with Cyclosporin A and Methylprednisolone in Seropositive (Type D Retrovirus SRV-2/W) and Seronegative Pig-tailed Macaques. Symposium on Nonhuman Primate Models for AIDS, 9th Annual Meeting, Seattle, WA, November 6-9, 1991.
5. **Moazed, T.C.**, Maggio-Price, L., Shiota, F., Kennedy B., and Thouless, M.E. In Vivo Reactivation of SRV-2/W in *Macaca nemestrina* by Chemically Induced Immunosuppression. Symposium on Nonhuman Primate Models for AIDS, 10th Annual Meeting, San Juan, Puerto Rico, November 17-20, 1992.

6. Welch, M. and **Moazed, T.C.** Infectious SRV in the Excreta of Viremic *Macaca nemestrina*. Symposium on Nonhuman Primate Models for AIDS, 10th Annual Meeting, San Juan, Puerto Rico, November 17-20, 1992.
7. **Moazed, T.C.**, Kuo, C.-C., Grayston, J.T. and Campbell, L.A. A Rabbit Model of *Chlamydia pneumoniae* Infection. American Society of Microbiology, 94th Annual Meeting, Las Vegas, Nevada May 19-22, 1994.
8. **Moazed, T.C.**, Kuo, C.-C., Grayston, J.T. and Campbell, L.A. Infection of ApoE-deficient Mice with *Chlamydia pneumoniae*. American Society of Microbiology, 95th Annual Meeting, Washington, DC, May 21-25, 1995.
9. **Moazed, T.C.**, Kuo, C.-C. and Campbell, L.A. A Pathogenic Role of Mononuclear Phagocytes in *Chlamydia pneumoniae* Infection in a Mouse Model. American Society of Microbiology, 96th Annual Meeting, New Orleans, LA, May 21-25, 1996.

Presentations

1. **Moazed, T.C.**, Ochs, H., Tsai, C.C., Kuller, L., Harrison, B., Kennedy, B., Morton, W.R., Bielitzki, J. and M.E. Thouless. What's Your Diagnosis: Weight Loss and Anemia in an Infant Pig-tailed Macaque. American Veterinary Medical Association, 128th Annual Meeting, Seattle, WA, July 27-31, 1991.
2. **Moazed, T.C.**, Kuo, C.-C., Grayston J.T. and Campbell, L. A. Murine Models of *Chlamydia pneumoniae* and Atherosclerosis. Japanese-American Chlamydia Research Symposium, 1st Annual Meeting, Seattle, WA, March 18-19, 1996.