MONTANA STATE UNIVERSITY

Adaptive immunity to Chlamydia pneumoniae respiratory infection
by Thomas Gordon Day, III

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Microbiology
Montana State University
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Abstract:
Chlamydia pneumoniae is a significant cause of respiratory infection in humans and has been associated with cardiovascular disease. Many studies have investigated the relationship between C. pneumoniae infection and cardiovascular disease, but few have examined host immunity to respiratory infection. Using a murine model of respiratory infection, we sought to characterize the course of primary and secondary C. pneumoniae infection, and to determine the role of humoral and cell mediated immune responses in adaptive immunity to infection. Intranasal inoculation with C. pneumoniae resulted a self-limiting respiratory infection that resolved within 21 days. Histological evaluation of lung sections revealed an interstitial pneumonia composed of polymorphonuclear neutrophils and mononuclear cells, and the association of C. pneumoniae inclusions with bronchial epithelial cells. Inflammation peaked at 10-14 days post infection and had completely resolved by five weeks following primary infection. The inflammatory response consisted primarily of CD4+ T cells and B cells when analyzed by immunohistochemistry. Anti-C pneumoniae serum and bronchial antibody titers were determined by enzyme-linked immunosorbent assay (ELISA), and were characterized by a predominance of IgG2a and IgG2b and the lack of IgA. Cytokine production by immune splenocytes was analyzed as a measure of cell mediated immunity, and was characterized by the production of IFN-γ and IL-10. Mice were susceptible to reinfection, but showed a level of protective immunity as evidenced by a shortened course of infection. Reinfection was also characterized by anti-C. pneumoniae IgA in bronchial washes, and a marked CD4+ T cell and B cell response in lung tissues. To assess the role of B cells in protective immunity to C. pneumoniae respiratory infection, primary and secondary infections were evaluated in B cell gene knockout mice. Infection of B cell deficient mice was indistinguishable from that of wild-type C57BL/6 mice.

Thus, a level of adaptive immunity developed following resolution of primary infection, and B cells appeared unnecessary for the resolution of primary or secondary infection. If C. pneumoniae is proven to cause cardiovascular disease, then vaccination against respiratory infection is a logical goal, and an understanding of host immunity to respiratory infection will prove paramount in achieving this goal.

ADAPTIVE IMMUNITY TO *CHLAMYDIA PNEUMONIAE* RESPIRATORY

INFECTION

by

Thomas Gordon Day III

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APPROVAL

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Thomas Gordon Day III

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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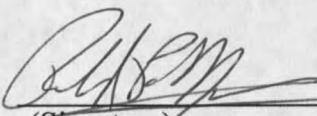
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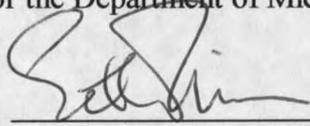
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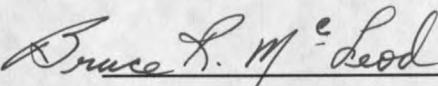
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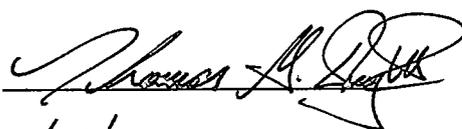
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I dedicate the completion of this thesis and this degree to the memory of my father, Dr. Thomas Gordon Day Jr. M.D., whose strength and courage serve as a constant source of inspiration and motivation. He taught me so much without ever knowing it.

In recognition of their tireless support of my efforts as a graduate student, I would like to acknowledge the following people. Dr. Richard Morrison for serving as my research advisor, teaching me a great deal in the lab, and always being there to answer my questions. Dr. Seth Pincus and Dr. Jim Cutler for serving as committee members, and for being good teachers in every capacity. Finally, I would like to thank my wife Jessica for always providing warmth and support, and without whom I would have struggled mightily.

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ABSTRACT

Chlamydia pneumoniae is a significant cause of respiratory infection in humans and has been associated with cardiovascular disease. Many studies have investigated the relationship between *C. pneumoniae* infection and cardiovascular disease, but few have examined host immunity to respiratory infection. Using a murine model of respiratory infection, we sought to characterize the course of primary and secondary *C. pneumoniae* infection, and to determine the role of humoral and cell mediated immune responses in adaptive immunity to infection. Intranasal inoculation with *C. pneumoniae* resulted a self-limiting respiratory infection that resolved within 21 days. Histological evaluation of lung sections revealed an interstitial pneumonia composed of polymorphonuclear neutrophils and mononuclear cells, and the association of *C. pneumoniae* inclusions with bronchial epithelial cells. Inflammation peaked at 10-14 days post infection and had completely resolved by five weeks following primary infection. The inflammatory response consisted primarily of $CD4^+$ T cells and B cells when analyzed by immunohistochemistry. Anti-*C. pneumoniae* serum and bronchial antibody titers were determined by enzyme-linked immunosorbent assay (ELISA), and were characterized by a predominance of IgG2a and IgG2b and the lack of IgA. Cytokine production by immune splenocytes was analyzed as a measure of cell mediated immunity, and was characterized by the production of IFN-γ and IL-10. Mice were susceptible to reinfection, but showed a level of protective immunity as evidenced by a shortened course of infection. Reinfection was also characterized by anti-*C. pneumoniae* IgA in bronchial washes, and a marked $CD4^+$ T cell and B cell response in lung tissues. To assess the role of B cells in protective immunity to *C. pneumoniae* respiratory infection, primary and secondary infections were evaluated in B cell gene knockout mice. Infection of B cell deficient mice was indistinguishable from that of wild-type C57BL/6 mice. Thus, a level of adaptive immunity developed following resolution of primary infection, and B cells appeared unnecessary for the resolution of primary or secondary infection. If *C. pneumoniae* is proven to cause cardiovascular disease, then vaccination against respiratory infection is a logical goal, and an understanding of host immunity to respiratory infection will prove paramount in achieving this goal.

INTRODUCTION

The genus *Chlamydia* consists of three species of obligate intracellular bacterial pathogens known to be infectious to humans: *C. pneumoniae, C. trachomatis, and C. psittaci.* Members of the genus *Chlamydia* are considered Gram-negative and have a unique biphasic developmental cycle that consists of two distinct forms: the elementary body (EB), the infectious, non-replicative form; and the reticulate body (RB), the non-infectious, replicative form. Following attachment to host cells EBs are internalized into a vacuole that avoids fusion with the phagolysosomal pathway of the host cell. Within hours EBs differentiate into RBs. RBs undergo binary fission, and by 48-72 hours the RBs reorganize back to EBs, which are released from the cell to begin a new infectious cycle. The manner in which the intracellular inclusion interacts with the host cell is still somewhat of a mystery, but the ability of the vacuole to avoid fusion with the lysosomal pathway may contribute to *Chlamydia* being such elusive pathogens.

C. trachomatis has been studied as a human pathogen for years. Although it is most commonly associated with sexually transmitted diseases, *C. trachomatis* is also associated with trachoma, the leading cause of preventable blindness in the world. *C. psittaci* infection is limited primarily to lower animals, and is not a major cause of human disease. *C. pneumoniae* causes a mild form of pneumonia that can be asymptomatic or present as acute respiratory disease in the form of bronchitis and/or pharyngitis. *C. pneumoniae* has also been associated with a number of other diseases including, but not

limited to, reactive arthritis and atherosclerosis. Although there is far less known about

C. pneumoniae, it has emerged as a very relevant human pathogen.

<u>Classification of *C. pneumoniae*</u>

C. pneumoniae was recognized formally by Grayston and colleagues in 1989 as a

distinct species of *Chlamydia* (15). The first step in differentiating *C. pneumoniae* as a

new species was taken in 1986 when, in an effort to determine if *C. psittaci* was an

important respiratory pathogen, a unique isolate of *Chlamydia* was isolated from the

oropharynx of several university students that presented with acute respiratory infection

(16). Symptoms of the students included pneumonia, bronchitis, and pharyngitis. The

unique chlamydial isolate was termed TWAR, and through the use of genus and species

specific monoclonal antibodies the unique antigenic identity of the TWAR isolates was

demonstrated (16). *Chlamydia* specific antibodies react with *C. trachomatis*, *C. psittaci*,

and TWAR, whereas TWAR specific antibodies react only with TWAR isolates (16).

Previous studies had postulated a role for certain strains of *C. psittaci* in epidemics of

pneumonia (16), but this was the first study in which the organism was isolated from

multiple patients that presented with pneumonia or other respiratory illnesses. Due to its

morphological similarity to *C. psittaci* and *C. psittaci*'s potential to cause human disease,

Grayston et al hypothesized that the respiratory infections had arisen from bird to human

contact, but they were unable to verify that mode of transmission. Despite the inability to

establish a mode of transmission for TWAR outside of human-to-human contact, this

study laid the groundwork for a causative relationship between this unique isolate and human respiratory infection.

Inclusion morphology has been used to distinguish TWAR from *C. trachomatis* (30). The inclusion bodies formed by TWAR, when cultured in HeLa cells, are more characteristic of *C. psittaci* than *C. trachomatis*. *C. pneumoniae* inclusions are oval in shape and dense, and do not contain glycogen, which is similar to the inclusion morphology of *C. psittaci* (30). *C. trachomatis* inclusions are more vacuolar and contain glycogen (30). *C. pneumoniae* has also been differentiated from the other two species by its unique pear-shaped EB. Electron microscopy demonstrates pear-shaped EBs that are typically .38 micrometers with a large periplasmic space. *C. trachomatis* and *C. psittaci* generally exhibit round EBs with narrow periplasmic spaces (15).

C. trachomatis and *C. psittaci* share <10% DNA homology, and as such are easily differentiated by restriction endonuclease analysis and DNA-DNA hybridization (4). TWAR isolates are readily distinguishable from *C. trachomatis* and *C. psittaci* by restriction endonuclease analysis (4), and different TWAR isolates have identical restriction endonuclease banding patterns. Thus, TWAR isolates appear to be of a single strain (4). Finally, when TWAR DNA is used to probe *C. trachomatis*, *C. psittaci*, and TWAR DNA, only DNA from TWAR isolates hybridizes. These data suggest that there is a single strain of TWAR, and that it is distinct from both *C. trachomatis* and *C. psittaci*. In addition, TWAR does not contain a plasmid, a trait shared by the other two *Chlamydia* species (4). In 1988 Cox et al confirmed that TWAR strains possess between 94-100% DNA homology with each other and less than 10% homology with either *C.*

trachomatis or *C. psittaci* (various strains) (7). Collectively the molecular data suggest

that TWAR is a unique species within the genus *Chlamydia*, and in 1989 *Chlamydia*

pneumoniae was proposed and accepted as a new species of *Chlamydia* (15).

In addition to the lack of DNA relatedness between *C. pneumoniae* and the other

chlamydial species, *C. pneumoniae* is also differentiated serologically based on the

observation that TWAR specific monoclonal antibodies react with TWAR antigen, but

not with *C. trachomatis* or *C. psittaci,* and vice versa (15, 16, 30). The recent sequencing

of the *C. trachomatis* and *C. pneumoniae* genomes (22, 60) provides a great deal of

insight into the genotypic commonalities and differences of the two human pathogens.

Genomic sequences confirm the lack of DNA relatedness between the two species.

Based on additional nucleic acid analysis, it has recently been proposed that the

organisms presently contained in the genus *Chlamydia* be reclassified. The new

classification would place *C. pneumoniae* into a new genus *Chlamydophila* (i.e.

Chlamydophila pneumoniae)(10). Since the official reclassification has yet to occur,

Chlamydia pneumoniae will continue to be used throughout the current thesis.

C. pneumoniae and Human Disease

First isolated as a human respiratory pathogen in 1983 (16), *C. pneumoniae* has

since been associated with a number of other human diseases including reactive arthritis,

atherosclerosis, erythema nodosum, Reiter syndrome, aortic aneurysm, stroke (5),

malignant lymphoma (2), and cerebellar dysfunction (28). Of these associations, the

connection between *C. pneumoniae* and cardiovascular disease has received the most

attention. Initial evidence linking *C. pneumoniae* and cardiovascular disease was based upon seroepidemiological studies (57). Saikku et al were the first to make the association between elevated *C. pneumoniae* antibody levels and acute myocardial infarction, and between elevated anti-*C. pneumoniae* antibody titers and the increased likelihood of cardiovascular disease (56, 57). Subsequently, numerous reports have described the presence of *C. pneumoniae* specific antibodies in patients with atherosclerosis (1, 11, 33, 42, 56, 65). However, these associations are confounded by the finding that in some studies matched controls demonstrated titers similar to those seen in patients suffering from cardiovascular disease (57). Approximately 54% of the adult population exhibits a positive antibody titer for *C. pneumoniae* serology. Since so many adults have a positive *C. pneumoniae* antibody titer, it is difficult to determine serologically if the heart disease is an unrelated event.

Microbiological and molecular techniques have been used to provide additional evidence linking *C. pneumoniae* with cardiovascular disease. *C. pneumoniae* DNA has been detected in atherosclerotic plaques by PCR (6, 18, 31, 32, 42, 64), and *C. pneumoniae* mRNA has been detected in atheromas by RT-PCR (9). However, some studies attempting to detect *C. pneumoniae* in association with atherosclerotic plaques have yielded disparate results, ranging from 1.7% to 100% of diseased tissues being culture positive or nucleic acid positive for *C. pneumoniae* (51). Specimens examined include coronary arteries, aorta, carotid arteries, iliac arteries, and smooth muscle (6, 32, 51). In most cases *C. pneumoniae* nucleic acid is not detected in explanted heart tissues without overt heart disease (49). However, in one study 9% of control tissues were

positive for *C. pneumoniae* (58). The accuracy and precision of molecular detection methods is still somewhat questionable, and as a result most studies still depend on serology as a correlative aspect of evaluation. In one such study (6) of 38 patients presenting with overt atherotic disease, 72% were positive for *C. pneumoniae* specific antibodies. This percentage is similar to that seen in population studies of older adults without heart disease, which reiterates the difficulty in interpreting seroepidemiological studies. However, despite this ambiguity 20/38 of the atherectomy specimens were positive for *C. pneumoniae* by immunocytochemical (ICC) staining and/or PCR. A large percentage of specimens (9/12), were positive by PCR and by ICC. There was no correlation between a positive serologic titer and detection of *C. pneumoniae* antigen (by ICC) or DNA (by PCR). In fact, the patient with the highest titer of *C. pneumoniae* specific IgG, was not positive for *C. pneumoniae* by either of the other methods. This study highlights some of the inconsistencies linking *C. pneumoniae* infection and cardiovascular disease.

Data from other studies (18, 33, 41) suggest that the relationship between *C. pneumoniae* infection and heart disease is arguable. In those studies the investigators were unable to establish a positive correlation between *C. pneumoniae* antibody titer and atherogenesis (18, 33, 41, 49). In addition, only 8% of coronary atherectomy specimens tested by RT-PCR were positive for *C. pneumoniae* (18), and *C. pneumoniae* DNA was not detected in any of the samples by in situ hybridization (18). In a separate study using PCR (8), *C. pneumoniae* DNA was not detected in the atherosclerotic plaques of the 29

patients tested. Collectively, these studies highlight the inconsistent nature of associating *C. pneumoniae* and coronary artery disease.

To further examine the inconsistencies associating *C. pneumoniae* with cardiovascular disease, specimens from 12 patients undergoing heart transplant were sent to different laboratories for analysis (49). Ten of the 12 patients presented overt atheromas. Specimens were sent to seven different labs where a series of tests were performed to isolate and identify *C. pneumoniae*. One patient provided a convincing argument that *C. pneumoniae* was associated with heart disease. This male patient was culture positive for *C. pneumoniae* by all three labs that performed the test, PCR positive for *C. pneumoniae* by two of three labs tested, positive by ICC in one of two labs tested, and positive by transmission electron microscopy (TEM) and in situ hybridization by 1 lab. Unfortunately, the remaining patients involved in the study demonstrated inconsistent detection of *C. pneumoniae* by different methods in different labs. In fact, none of the other labs involved in the study were able to culture *C. pneumoniae* from the specimens they studied. These inconsistencies may be attributed to the fact that experimental protocols varied between some of the labs.

Examining the nature of atherosclerosis may provide some insight into the role of *C. pneumoniae* in the development of atherosclerotic plaques. The current paradigm regarding the development of atherosclerosis is the "response to injury" hypothesis (53), in which the protective inflammatory response associated with injury to the endothelium persists, and eventually becomes a chronic inflammatory process. The initiating step in atherosclerosis is endothelial cell dysfunction, which is often due to cell injury.

Endothelial cell injury may include the infection of resident endothelial cells by *C. pneumoniae*. Infection of human aorta derived endothelial cells (HAEC) and human umbilical vein endothelial cells (HUVEC) with *C. pneumoniae* promotes endothelial cell activation characterized by enhanced rolling, adhesion, and transmigration of monocytes and PMNs (29). Endothelial cell activation is also evidenced by the up regulation of cell surface adhesion molecules on leukocytes associated with the activated endothelial cells. Endothelial cell injury leads to increased adherence and diapedesis of leukocytes, increased transcytosis of low-density lipoprotein (LDL), and subsequent oxidation of LDL by macrophages and smooth muscle cells. While native LDL is not considered atherogenic, oxidized LDL is highly atherogenic, and accumulation of ox-LDL, smooth muscle cells, and macrophages in the arterial intima leads to the progression of atherosclerotic plaques.

Macrophages are very important cells in the progression of atherogenesis and are present throughout plaque development in the atherosclerotic microenvironment. Studies of the interaction of *C. pneumoniae* and macrophages have provided further insight into possible mechanisms of *C. pneumoniae* induced atheroma formation. Macrophages extravasate to the intima where they phagocytose excessive lipids (LDL). Phagocytosed LDLs reside in cytoplasmic vacuoles, and due to their histological appearance, macrophages containing an abundance of LDLs are termed foam cells. Foam cells make up the initial fatty streak in atherogenesis (53). It has been shown that *C. pneumoniae* infected macrophages more readily phagocytose LDL, and take on the appearance of foam cells (19, 20). Data suggest that foam cell formation is increased an average of

30%-50% in *C. pneumoniae* infected macrophages, and that lipid accumulation occurs through dysregulation of the native LDL receptor (20). In vitro inhibition of chlamydial lipopolysaccharide with lipid X results in reduced foam cell formation, indicating that chlamydial LPS induces macrophage foam cell formation (19). LDLs activate macrophages, and mediators released by these activated cells attach additional macrophages, smooth muscle cells, and lymphocytes to the intima causing the fatty streak to progressively form a fibrous plaque (53).

Chlamydial and human heat shock protein 60s have both been shown to localize in human atheromas and up-regulate vascular cell functions including the expression of adhesion molecules E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) (24), and macrophage/monocyte production of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and IL-6 (25, 26). Furthermore, *C. pneumoniae* infected monocytes and purified chlamydial HSP-60 (in the presence of monocytes) have been shown to induce oxidation of LDL (21). Thus of the cell types involved in the development of atheromatous plaques *C. pneumoniae* has been shown to infect endothelial cells, smooth muscle cells, and macrophages. While *C. pneumoniae* growth in these cell types is not as robust or as consistent as that of *C. pneumoniae* growth in epithelial cells, infection and replication do occur (13). Together those findings suggest that *C. pneumoniae* infected macrophages may participate in foam cell formation and oxidation of LDL, both of which contribute to the pathogenesis of atherosclerosis.

It has been postulated that macrophages have the ability to relocate from the respiratory tract to coronary arteries by way of arterial and venous transmission, and that *C. pneumoniae* could infect alveolar macrophages that in turn travel to coronary arteries and undergo foam cell formation (36). Subsequent oxidation of LDL by *C. pneumoniae* infected macrophages generates an atherogenic environment in which respiratory pathogen and host immune response combine to create a persistent state of inflammation. Thus dissemination of *C. pneumoniae* infected macrophages provides a link between respiratory infection and cardiovascular disease, and corroborates the role of *C. pneumoniae* infected macrophages in atherogenesis.

Thus far *C. pneumoniae* has been linked to atherosclerosis by a variety of means. In vivo, the two have been associated by serology and the detection of *C. pneumoniae* in atherosclerotic plaques by RT-PCR, in situ hybridization, and immunocytochemistry, and by culturing and propagating *C. pneumoniae* from atherosclerotic plaques. In vitro, *C. pneumoniae* has been associated with atherosclerosis by its ability to infect the cell types associated with atherogenesis, induce foam cell formation, oxidize LDL, and stimulate a local inflammatory response via chlamydial HSP60. Although there remains a large degree of uncertainty regarding the role of *C. pneumoniae* in atherogenesis, if a causal relationship is proven, a more complete understanding of the host immune response to respiratory infection will be required if vaccination is deemed a reasonable approach to controlling this infectious disease.

Animal Models of *C. pneumoniae* Cardiovascular Infection

The rabbit and mouse have been evaluated as animal models for studying the influence of *C. pneumoniae* on the development of experimental atherosclerosis. Rabbits fed a moderately enhanced cholesterol diet and challenged intranasally with *C. pneumoniae* develop intimal thickening of coronary arteries similar to that associated with atherosclerotic plaques of humans (40). Because cholesterol is a well-established risk factor in the development of atherosclerosis, it is not known whether chlamydiae home to sites of lipid accumulation and exacerbate atherosclerotic plaque formation, or if chlamydiae initiate the pathologic process. However, in a separate study *C. pneumoniae* infected rabbits on a noncholesterol diet developed atherosclerotic pathology (12). Furthermore, *C. pneumoniae* is detected in aortic sections of infected rabbits by PCR and immunocytochemistry (40). Lastly, azithromycin reduces intimal thickening in *C. pneumoniae* infected rabbits (40). That finding is particularly relevant because of the ongoing studies in humans to reduce the number of cardiac events associated with atherosclerosis through antibiotic therapy (14). Thus, studies using the rabbit model of *C. pneumoniae* infection suggest that *C. pneumoniae* may not only have a predilection for fatty streaks, but also induce atherosclerotic pathology.

Although studies from the rabbit model have provided some encouraging results concerning *C. pneumoniae* infection and atherosclerosis, the usefulness of the model for detailed microbiological, immunological and pathological studies is limited. Alternatively, mice provide an experimental system that is more amenable to

experimental manipulation. Apolipoprotein (apo)E-deficient mice develop atherosclerotic lesions spontaneously in the absence of an atherogenic diet, and thus provide a model to test the ability of *C. pneumoniae* to enhance atherosclerotic development. In apoE-deficient gene knockout mice challenged intranasally at eight weeks of age, *C. pneumoniae* DNA persists for 20 weeks in atheromatous lesions, compared to two weeks in wild type C57BL/6 mice. However, *C. pneumoniae* DNA was only detected in 35% of the apoE aorta samples by PCR. Interestingly though, atheromatous lesions from apoE-deficient mice challenged at an older age (16weeks) were consistently positive for *C. pneumoniae* DNA at eight weeks following infection, implying perhaps, that older mice develop more severe atherosclerotic plaques (37). ApoE-deficient mice challenged intranasally with *C. pneumoniae* also develop atherosclerotic lesions at an accelerated rate compared to sham inoculated apoE-deficient mice (35). Collectively, these data suggest that *C. pneumoniae* is associated with fatty streaks, and has the ability to enhance atherosclerotic pathology in a murine model.

Animal Models of *C. pneumoniae* Respiratory Infection

The majority of research involving *C. pneumoniae* to date has focused primarily on the possible role of *C. pneumoniae* in atherogenesis. For example, positive serology for *C. pneumoniae* correlates with an increased risk of heart disease; *C. pneumoniae* infects vascular endothelial cells, macrophages, and smooth muscle cells; *C. pneumoniae* antigen and/or DNA is present in atherosclerotic plaques; and *C. pneumoniae* has been isolated and propagated from atherosclerotic plaques. These observations have generated

a considerable body of information regarding the association of *C. pneumoniae* and atherosclerosis. What is less clear is how respiratory infection relates to atherosclerosis.

It is generally believed that *C. pneumoniae* attains residency in the heart following respiratory infection. In a study by Moazed et al. (36) *C. pneumoniae* infected alveolar and peritoneal macrophages were collected from mice that had been challenged intranasally or intraperitoneally, with *C. pneumoniae*, respectively. Naïve mice were then injected intraperitoneally with infected macrophages, and tissues were analyzed three days later for the presence of *C. pneumoniae*. Using this model the lung, spleen and thymus of mice inoculated with *C. pneumoniae* infected alveolar or peritoneal macrophages contain *C. pneumoniae* DNA. *C. pneumoniae* was detected in peripheral blood mononuclear cells (PBMC) by culture and by PCR indicating dissemination of *C. pneumoniae* in the peripheral circulation. In addition, viable organisms were re-isolated from macrophages of infected mice. This study demonstrates the ability of *C. pneumoniae* to remain infectious in macrophages and to disseminate systemically. The route of initial inoculation with the *C. pneumoniae* EBs appeared to have a dramatic effect on the subsequent recovery of *C. pneumoniae*. *C. pneumoniae* was found in alveolar macrophages of mice inoculated intranasally, but not intraperitoneally. Similarly, *C. pneumoniae* was recovered from peritoneal macrophages of mice inoculated intraperitoneally but not intranasally. This study begins to address the route of *C. pneumoniae* infection as it relates to atherosclerosis. Thus, examining the basic immunology surrounding the respiratory infection may prove to be critical in developing intervention strategies for preventing the subsequent pathology in the heart.

Initial studies involving *C. pneumoniae* respiratory infection established the basic parameters of the murine model (66, 67, 70). Intranasal inoculation results in a primary course of infection that is self limiting, generally resolving by three weeks (67, 70). However, *C. pneumoniae* organisms have been recovered from lung samples of some strains of mice until day 42 post challenge (70). Mice develop a *C. pneumoniae* specific humoral immune response characterized by IgM and IgG antibodies. Anti-*C. pneumoniae* IgG antibodies become apparent between days 9 and 11 post infection and the titer continues to rise until day 40 (67). IgM has been detected by some investigators until day 17 post challenge (67), but not detected by others (70). The role of *C. pneumoniae* specific antibodies in protective immunity to respiratory infection has not been clearly established, but in one report immune sera conferred a level of protection to naïve recipient mice as demonstrated by fewer recoverable infectious chlamydiae following intranasal challenge (66). Histologically, respiratory infection causes interstitial pneumonia, characterized by alveolar septae thickening and the absence of exudate in the alveolar spaces. Some studies report the presence of PMNs two days post challenge, followed by mononuclear cells during the resolution of infection (69, 70), while others detect only mononuclear cells (67). All histopathological studies however, note perivascular and peribronchial aggregation of inflammatory cells. Mice that resolve primary infection are partially resistant to reinfection as evidenced by fewer recoverable infectious chlamydiae following secondary challenge (66).

More recent studies have examined the local immune response to *C. pneumoniae* respiratory infection. The number of mononuclear cells in lungs of infected mice is

approximately three times that of uninfected mice (44). In addition, a novel cell

population (CD3$^-$, CD4$^+$, CD8$^+$) increases by as much as 70% during infection (46).

While those data begin to examine the role of cell mediated immunity in *C. pneumoniae*

infection, the role of the CD3$^-$, CD4$^+$, CD8$^+$ cell population remains unclear. The nature

of the histological and cytokine response during secondary challenge has also been

examined (44). Fewer cultivatable bacteria characterize reinfection, and there is a

significant increase in the magnitude of the cellular response characterized by a moderate

perivascular and peribronchial lymphocytic infiltration. T cells are the predominant

lymphocyte population during the resolution of secondary infection (44), yet the

importance of this increase in T cell number in imparting immunity is unclear. Consistent

with the in vivo data, in vitro proliferation assays reveal a marked increase in

mononuclear cell proliferation and cytokine (IFN-γ) production following rechallenge

compared to primary infection (44).

CD4$^+$ T cells are critical for acquired immunity to *C. trachomatis* infection,

whereas CD8$^+$ T cells play more subordinate roles (38). Cellular immunity to *C.*

pneumoniae infection is less well understood. In recent studies, investigators have

concluded that unlike *C. trachomatis* infection, CD8$^+$ T cells contribute to protective

immunity in *C. pneumoniae* infection (45, 54). A variety of gene knockout mice have

also been used to analyze the cellular response to *C. pneumoniae* respiratory infection.

Mice deficient for the following molecules, among others, have been studied: CD4$^{-/-}$,

CD8$^{-/-}$, IFN-γR$^{-/-}$, perforin$^{-/-}$, TAP-1$^{-/-}$, β2 microglobulin(μ)$^{-/-}$, MHC-II$^{-/-}$, CD4$^{-/-}$//CD8$^{-/-}$,

and TAP-1$^{-/-}$//β2 microglobulin(μ)$^{-/-}$. Mice genetically depleted for CD8$^+$ T cells

experience a 10-fold increase in IFU recovery compared to normal mice, 14 and 24 days following primary infection with *C. pneumoniae*, while mice depleted for CD4$^+$ T cells resolve in a manner similar to normal mice (54). Mice genetically depleted for MHC-II experienced a course of infection similar to that seen in CD4$^{-/-}$ and wild type mice, while mice genetically depleted for TAP-1/β2μ experienced an enhanced recovery of *C. pneumoniae*, corroborating the relative importance of CD4$^+$ T cells and CD8$^+$ T cells respectively. Early during the course of infection, CD4$^{-/-}$//CD8$^{-/-}$ and severe combined immunodeficient (SCID) mice challenged intranasally develop an infection similar to that seen in normal mice, but fail to resolve infection and remain culture positive to day 60 post primary challenge. In addition, SCID mice reconstituted with CD4$^+$ T cells and challenged intranasally, shed higher numbers of IFUs following primary challenge than non-reconstituted mice. Collectively, those data suggest that CD8$^+$ T cells are critical in the resolution of primary infection, and that CD4$^+$ T cells have a detrimental effect early in infection. However, reconstituted SCID mice were only tested at one time point (14 days) following primary challenge, and thus it is difficult to assess the overall effect on the course of infection. If CD8$^+$ T cells play a role in the resolution of infection, it appears they do so without using perforin, as perforin deficient mice resolve infection without incident (54). The role of T cells is further implicated by the fact that mice deficient in IFN-γ experience a 100-fold increase in bacterial burden throughout the course of primary infection. Helper T cells are generally considered requisite for the development of an adaptive immune response, and as such these data are counterintuitive. In addition, the data from several of the experiments was collected at a single time point,

and thus the significance of those data is arguable. Others have examined the role of CD8^{+} T cells and CD4^{+} T cells in *C. pneumoniae* respiratory infection, and shown that nude mice experience a prolonged period of primary infection that does not resolve, while normal mice resolve infection within 3 weeks (45). These data once again suggest a role for T cells in the resolution of primary *C. pneumoniae* respiratory infection. In vivo depletion of CD4^{+} T cells and CD8^{+} T cells has also been used to study the contribution of these cell populations to immunity. Mice depleted of CD4^{+} T cells resolve infection normally, and show enhanced clearance of bacteria at one time point, while mice lacking CD8^{+} T cells have an increased bacterial load. Both groups of mice however, resolve primary infection in a time span similar to that seen in normal mice. The course of secondary infection is exacerbated in CD8 depleted mice (compared to wild type mice), yet once again infection resolves within 3 weeks. Past literature supports the need for MHC-II restricted T cell responses in the development of adaptive immunity. These data from the *C. pneumoniae* infection model seem to contradict that paradigm, and clearly additional work is required to clarify the existing data and to elucidate the mechanisms involved in the host immune response to *C. pneumoniae* respiratory infection.

A growing body of evidence supports the role of *C. pneumoniae* infection in the development of atherosclerosis, although a causal relationship has not been precisely defined. If *C. pneumoniae* is shown to cause coronary artery disease, then vaccination against respiratory infection is a logical goal, and an understanding of host immunity to respiratory infection will prove paramount in achieving this goal. Our current

understanding of the elements of the immune response that contribute to the resolution of

C. pneumoniae respiratory infection is quite meager, and the relative importance of the

various cell populations is arguable. The current study seeks to characterize the course of

primary and secondary *C. pneumoniae* infection of C57BL/6 mice, and to determine the

role of adaptive immunity in the resolution of infection. We have confirmed and

expanded upon previous studies by analyzing the evolution of the humoral and cell

mediated immune responses during the course of primary and secondary *C. pneumoniae*

respiratory infection, and demonstrate that specific antibody is not necessary for the

resolution of respiratory infection.

MATERIALS AND METHODS

Experimental Animals

Female C57BL/6 mice were purchased from the National Cancer Institute (Bethesda, Md.), and used at 8-10 weeks of age. B cell deficient C57BL/6-Igh-6 mice (23) were purchased from Jackson Laboratories (Barharbor, ME.), and were bred and maintained at the Montana State University Animal Resource Center. B cell maturation is arrested at the pre-B cell stage in B cell deficient mice, and they fail to produce detectable levels of immunoglobulin. B cell deficient mice were used at 8-14 weeks of age.

Growth and Purification of *Chlamydia pneumoniae*

HL cell (Washington Research Corporation, Seattle, Wa.) monolayers were grown in Dulbecco's Modification of Eagle's Medium supplemented with 10% heat inactivated fetal bovine serum (Hyclone, Logan, Ut.), L-glutamine (2mM) (Gibco Life Technologies, Grand Island, NY), HEPES (1mM)(Gibco Life Technologies, Grand Island, NY), sodium pyruvate (10μM) (Gibco Life Technologies, Grand Island, NY), beta mercaptoethanol (.6μM) (Gibco Life Technologies, Grand Island, NY), and gentamycin (10μg/ml) (Gibco Life Technologies, Grand Island, NY), (DMEM-10) at 35°C, in a humidified atmosphere containing 5% CO_2. *C. pneumoniae*, strain AR-39, was obtained from ATCC and stored at −80°C until used. *C. pneumoniae* was

propagated and EBs were purified following previously described methods for *C. trachomatis* (3). Briefly, 2.5ml of *C. pneumoniae* diluted in 250mM sucrose, 10mM sodium phosphate, 5mM L-glutamic acid (SPG) was inoculated onto HL cell monolayers in 150cm^2 flasks at a multiplicity of infection (MOI) of 2.5. Flasks were rocked for two hours at 35°C, and then 50ml of DMEM-10 containing 0.8μg/ml cyclohexamide was added. Infected monolayers were incubated for an additional 72 hours at 35°C. Media was removed and replaced with 10ml of Hank's Balanced Salt Solution (HBSS). Monolayers were disrupted with glass beads, and briefly sonicated (2 X 20 sec., 90 watts). EBs were pelleted by centrifugation at 30,000 X *g* for 60 minutes at 4°C. The pellets were resuspended in SPG, and sonicated as before. Sonicates were centrifuged over 30% renografin (Squibb Diagnostics, Princeton, NJ) at 80,000 X *g* for 30 minutes at 4°C. The pellets were resuspended in SPG, and briefly sonicated (4 X 5 sec., 56 watts). Sonicates were placed on a discontinuous renografin (Squibb Diagnostics, Princeton, NJ) gradient (40%, 44%, 54%) and centrifuged at 72,128 X *g* for 60 minutes at 4°C. EBs, which band at the interface of the 44% and 54% renografin layers, were collected, washed in SPG, and pelleted at 43,667 X *g* for 20 minutes at 4°C. Pellets were resuspended in SPG, aliquotted and frozen at –80°C until used. EBs purified as described above are referred to as density gradient purified EBs, and were used for antigen in ELISA and for in vitro stimulation of lymphocytes. For infection of mice, *C. pneumoniae* was propagated as described above, but EB preparations were purified by pelleting EBs through 30% renografin, resuspending in SPG, and freezing at –80°C until used.

Enumeration of Infectious Chlamydiae

C. pneumoniae were enumerated as described previously (38), with the following exceptions. Three hundred microliters of diluted, purified stocks or lung homogenates were added to triplicate wells of HL cell monolayers grown in 48 well plates. Plates were centrifuged at 540 X g for 60 minutes at 35°C, and then incubated at 35°C, 5% CO_2 for 30 minutes. The inoculum was removed and replaced with DMEM-10 containing 0.8μg/ml cycloheximide. Following 72 hours of incubation at 35°C, 5% CO_2, media was aspirated and monolayers were washed once with phosphate buffered saline (PBS) (10mM phosphate, 0.13M NaCl, pH 7.4), and fixed with 100% methanol at room temperature for ten minutes. Methanol was aspirated and monolayers were washed once with PBS. *C. pneumoniae* inclusions were visualized using indirect immunofluorescent staining. Anti-chlamydial LPS monoclonal antibody (EVI-H1) (a kind gift from Dr. Harlan Caldwell, NIH) diluted in PBS containing 2% BSA was added to fixed monolayers and incubated for 45 minutes at room temperature on a rocking platform. Monolayers were washed twice with 1.0ml of PBS. Fluoresceinated goat anti-mouse IgG (Sigma, St. Louis, Mo.), diluted 1:400 in PBS containing 2% BSA was added to each monolayer, and plates were rocked at room temperature for 45 minutes. Wells were washed twice with PBS and inclusion forming units (IFUs) were visualized using an inverted fluorescent microscope. IFUs were enumerated and expressed as $\log_{10}$ IFU.

Primary and Secondary Infectious Challenge

Initial challenge experiments were based on previous experiments by Yang et al. (70) in which Swiss Webster mice were challenged intranasally with 50μl of *C. pneumoniae* containing 3×10^7 IFUs. We found that intranasal inoculation of C57BL/6 with $>1 \times 10^7$ IFUs resulted in death of some mice. Therefore, an initial experiment was performed to determine a dose of *C. pneumoniae* that would result in a self-limiting respiratory infection. Mice were anesthetized with either Metofane or Isoflurane and inoculated intranasally with 40μl of diluted, partially purified (30%), *C. pneumoniae* EBs ($4 \times 10^7 - 1 \times 10^4$ IFUs). Mice were sacrificed seven days following infection, their lungs were removed, placed in 10ml of SPG and ground using glass homogenization tubes. Homogenates were centrifuged briefly (~1minute) at $200 \times g$ to remove tissue debris. Supernatants were transferred to sterile tubes and stored at $-80°C$ until IFUs were enumerated as described above. From our initial experiments, it was determined that intranasal infection with 5×10^6 IFUs, produced an infection that did not result in mortality. Thus, for primary infections, anesthetized mice were inoculated intranasally with 5×10^6 IFUs. Groups of five mice were sacrificed at various times following infection, lungs were removed, and IFUs were enumerated as described above. For secondary challenge experiments mice were allowed to resolve primary infection and then re-challenged with 5×10^6 IFUs on day 37 following primary infection. Lungs were removed at various times following secondary challenge and IFUs enumerated.

Histopathology

Lungs were removed from infected and non-infected mice, and fixed in 10%

buffered formalin. Fixed tissues were forwarded to a veterinary pathologist for

embedding, sectioning, staining, and histological evaluation (William Quinn, DVM,

Bozeman, MT.)

In situ Detection of Chlamydial Inclusions

Formalin-fixed, paraffin embedded lung sections were deparaffinized, and stained

with an anti-chlamydial 60 kilodalton heat shock protein (hsp60) monoclonal antibody

(71) to visualize chlamydial inclusions as previously described (38). Briefly, paraffin

embedded lung sections were deparaffinized by heating at 60°C for 40 minutes, followed

by three successive xylene washes of 5 minutes each. Slides were placed in 100% ethanol

for 5 minutes, and endogenous peroxidase activity was blocked by a 30 minute

incubation in methanol containing 0.5% hydrogen peroxide. Slides were rehydrated by

sequential 3 minute washes in 100% ethanol, 70% ethanol, 50% ethanol; then washed 3

times in PBS for 3 minutes each, and washed a final time in PBS for 5 minutes. Tissues

were blocked with avidin, biotin and 5% normal mouse serum following the

manufacturer's recommendations (Vector Labs, Burlingame, CA.). Following blocking,

sections were rinsed with PBS, and incubated with biotinylated, anti-chlamydial hsp-60

antibody A57-B9 (71) diluted in PBS containing 5% normal mouse serum. Slides were

rocked in a humidified chamber for 2 hours at room temperature, and washed with PBS

for 5 minutes. Vectastain ABC complex (Vector Labs, Burlingame, CA.) was added and

tissue sections were incubated in a humidified chamber at room temperature for 30 minutes. Slides were rinsed in PBS for 2 minutes and developed with diaminobenzidine (DAB) substrate for 10 minutes. Slides were then rinsed in water for 2 minutes and counterstained with hematoxylin for 1-2 minutes. Slides were rinsed quickly with distilled water, followed by PBS for 1-2 minutes, and distilled water for 1-2 minutes. Finally, slides were de-hydrated with two successive washes of 100% ethanol, and xylene, and cover slides were added using Permount (Fisher Scientific, Pittsburgh, PA.).

Immunohistochemical Analysis of Cell Surface Phenotypes

Lungs were harvested, placed into OCT compound (Tissue-Tek ,Sakura, Torrance, CA.), and frozen in dry ice-cooled, 2-methyl butane. Thin sections (~5µm) were placed onto Superfrost slides (Fisherbrand, Pittsburgh, PA.), fixed with acetone for 5 minutes, and allowed to air dry. Tissue sections were re-hydrated in PBS for 15 minutes. Endogenous peroxidase activity was blocked by covering tissue sections with Peroxobloc (Zymed, San Francisco, CA.) for 40 seconds. Tissues were blocked with avidin, biotin and 5% normal mouse serum following the manufacturer's (Vector Labs, Burlingame, CA.) recommendations. Sections were rinsed with PBS for 2 minutes, and then incubated with primary antibody [rat anti-mouse CD4 (clone RM4-5), anti-CD8 (clone 53-6.7), anti-CD45R (clone RA3-6B2), anti-Ly6G clone (RB6-8C5), purchased from Pharmingen, San Diego, CA.] for 30 minutes in a humidified chamber at room temperature. Tissues were rinsed with PBS for 2 minutes, and incubated with biotin-labeled goat anti-rat Ig (Sigma, St. Louis, MO.) for 30 minutes at room temperature in a

humidified chamber. Sections were rinsed in PBS for 2 minutes, Vectastain ABC complex was added (following manufacturer's recommended procedure), and slides were incubated in a humidified chamber at room temperature for 30 minutes. Slides were developed, counterstained and de-hydrated as described above.

Analysis of Serum and Bronchiolar Alveolar Lavage (BAL) Fluid for Anti- *C. pneumoniae* Antibody

Sera and BAL were analyzed for anti-chlamydial class and sub-class specific antibody responses by enzyme linked immunosorbent assay (ELISA) as previously described (38). Briefly, 100μl of formalin-fixed, density gradient purified *C. pneumoniae* EBs (10μg/ml) diluted in buffer (50mM Tris, 150mM NaCl, pH 7.5) was aliquotted into wells of 96 well Immulon 2, U bottom plates (Dynatech Laboratories Inc., Chantilly, VA.) and incubated overnight at 4°C. Plates were washed three times with Tris HCl (12mM, pH 7.5) containing 0.5% Tween-20 (wash buffer), and blocked with Tris (12mM, pH 7.5) containing 2% bovine serum albumin (BSA) (blocking buffer) for 2 hours at 35°C. Wells were washed once with wash buffer, and fourfold serial dilutions of sera diluted in blocking buffer were added to the wells. Plates were covered and incubated at 35°C for 90 minutes. Wells were washed twice with wash buffer and alkaline-phosphatase labeled class and sub-class specific secondary antibodies for IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA (Southern Biotechnologies, Birmingham, AL.) were diluted 1:250 in blocking buffer and added to the appropriate samples. Plates were covered and incubated at 35°C for 75 minutes. Wells were washed twice with wash buffer, phosphatase substrate (Sigma, St. Louis MO.) was added, and the reaction was

terminated after 30 minutes with 5N NaOH. Absorbance was read at 405nm. Purified EVI-H1 anti-chlamydial LPS was used as a positive control, and wash buffer alone was used as a negative control in all experiments. Titer was defined as the inverse of the greatest dilution that generated an optical density (OD_{405}) >0.20. Sera from non-infected mice were consistently negative (OD_{405} <0.20) for anti-*C. pneumoniae* antibodies when diluted 1/16. Data were reported as mean serum titers log_2 of five mice at each time point ± standard error of the mean (SEM). For some experiments, BAL was collected by passing a nylon tube attached to a 21-gauge needle into resected lungs, and washing the lungs twice with 0.5ml of SPG. The BAL washes were centrifuged at 14,926 X g for 15 minutes to remove cellular debris, and the supernatant frozen at –20°C, until analyzed by ELISA as described.

Heat Inactivation of *C. pneumoniae* EBs

Density gradient purified *C. pneumoniae* EBs (3 x 10^9 IFUs/ml) were thawed in a 37°C water bath and diluted to a concentration of 1 x 10^9 IFUs/ml in DMEM-10 containing 10mM, 3-N-morpholino-propane sulfonic acid (MOPS). Diluted EBs were heat inactivated at 56 °C for 30 minutes. Heat inactivated EBs were nonviable as determined by in vitro culture.

Ultraviolet (UV) Inactivation of *C. pneumoniae* EBs

Partially purified *C. pneumoniae* EBs were thawed in a 37°C water bath and diluted to a concentration of 5 x 10^6 IFUs/ml in 0.5ml SPG. Diluted EBs were placed in

one well of a 24 well plate and exposed to an ultraviolet lamp in a biosafety cabinet for 30 minutes. UV inactivated EBs were nonviable as determined by in vitro culture.

Cytokine ELISA

Splenocytes (1×10^7/ml) from normal or infected mice were mixed with or without heat inactivated EBs at a splenocyte:EB ratio of 1:4. The splenocyte/EB suspensions were incubated at 35°C for 60 minutes with constant rotation, and then plated into wells of a 24 well plate at 1×10^7 splenocytes/well. Splenocyte cultures were incubated at 35°C, 5% CO_2 for 72 hours. Supernatants were collected, centrifuged at 350 X g for 15 minutes to remove cellular debris, and frozen at –80°C until analyzed. Frozen supernatants were thawed in a 37°C water bath and analyzed for IL-4, IL-10, IL-12, and IFN-γ using Opt-EIA cytokine kits (Pharmingen, San Diego, CA.), and following the manufacturer's instructions. Briefly, Maxisorp 96 well round bottom plates (NUNC) were coated with either anti-mouse IL-4, IL-10, IL-12, or IFN-γ overnight at 4°C. Plates were washed three times (five for IFN-γ) with wash buffer (PBS with 0.05% Tween-20) and blocked with assay diluent (Pharmingen, San Diego, CA.) for one hour at room temperature. Plates were washed three times with wash buffer (five for IFN-γ), and cytokine standards or sample was added and incubated for two hours at room temperature. All plates were washed five times with wash buffer. Biotin labeled anti-mouse cytokine antibody, and avidin labeled horseradish peroxidase conjugate were added to wells and incubated for one hour at room temperature. Plates were washed seven times (10 times for IFN-γ) and developed with substrate [H_2O_2, tetra-

methylbenzidene(TMB)] for 30 minutes in the dark. Reactions were terminated with 2N H_2SO_4 and read at 450nm.

Purification of T lymphocytes

Immunoaffinity chromatography columns were used to isolate normal and immune T lymphocytes from splenocyte preparations. Immune splenocytes were obtained from mice that had resolved primary *C. pneumoniae* infection and received a secondary challenge 20 days later. Ten days following secondary challenge splenocytes were harvested. T lymphocytes were purified according to the manufacturer's procedure (Cytovax Biotechnologies Inc., Edmonton, Alberta, Canada). Briefly, columns were washed with 15ml of HBSS containing 2%FBS (wash buffer), and then incubated with 1.5ml Column Reagent [polyclonal goat anti-mouse IgG (H+L)] for 1-4 hours at room temperature. Freshly isolated immune and normal splenocytes were adjusted to a concentration of 5×10^7 cells/ml in column wash buffer. Columns were washed with 20ml wash buffer, and the flow rate was adjusted to 6-8 drops per minute. Samples were added to the columns and non-adherent cells (T cells) were washed through with 10-15ml of wash buffer. T cell suspensions were centrifuged at 200 X *g* for 10 minutes. Supernatant was discarded and pellets were resuspended in HBSS. Cells were counted and adjusted to 2×10^7 cells/ml. Viability was >92% as determined by Trypan blue. Homogeneity of the T lymphocytes was >90% as determined by immunohistochemical staining for cell surface CD3e.

Adoptive Transfer of Immune T Lymphocytes

Purified immune or non-immue T lymphocytes were adjusted to 2×10^7 cell/ml in HBSS, and 0.5ml (1×10^7 cells) was injected intravenously into naïve mice. Recipient mice were subsequently challenged intranasally with 5×10^6 IFUs of *C. pneumoniae* 24 hours following the administration of T cells, and the course of infection was followed as described previously.

RESULTS

Determination of Infectious Dose

To establish a challenge inoculum that would result in a self-limiting respiratory infection, with manifestations similar to human infection, groups of mice were challenged intranasally with doses of *C. pneumoniae* ranging from 1×10^4 to 4×10^7 IFUs. Mice were sacrificed seven days following infection, lungs were removed and homogenized, and infectious chlamydiae were enumerated (Table 1). Mice challenged with 4×10^7 IFUs experienced greater mortality (7/16), but had a bacterial burden similar to that seen in mice challenged with 1×10^7. A challenge dose of 5×10^6 IFUs produced a non-lethal infection, and a bacterial burden that was ~10 fold less than that produced by higher challenge inoculums. Because the 5×10^6 IFU challenge inoculum did not result in death of experimental animals, and produced a respiratory infection having a bacterial burden only somewhat less than that found in animals challenged with a lethal dose, we chose the 5×10^6 inoculum to use in our studies of adaptive immunity.

Table 1. Determination of an Infectious Dose[a]

Challenge Inoculum[b]	Number of Deaths/ Number Tested[c]	Mean IFU Recovery ($\log_{10}$)	SEM[d] ($\log_{10}$)
1×10^4	0/5	2.69	0.11
1×10^5	0/5	3.54	0.12
1×10^6	0/5	4.45	0.24
5×10^6	0/5	5.50	0.48
1×10^7	1/5	5.80	0.69
4×10^7	7/16[e]	6.77	0.09

[a] Groups of mice were challenged intranasally with various doses of *C. pneumoniae*, and IFUs were enumerated 7 days following primary infection

[b] Mice were inoculated intranasally with the specified challenge inocula

[c] Number of mice that died from the challenge dose prior to day 7 post infection (# deaths/ # tested)

[d] Standard error of the mean of 5 mice per experimental group

[e] Data are pooled from two separate experiments

Time Course of *C. pneumoniae* Primary and Secondary Respiratory Infection

Mice were challenged intranasally with 5×10^6 IFUs, and on days 3, 7, 10, 14, 21, and 28 following primary challenge animals were sacrificed and infectious chlamydiae were enumerated. Mice developed a self-limiting respiratory infection that resolved within 21 days (Figure 1). Peak IFU recovery ($10^{6.7}$) occurred early during the course of infection, and IFUs were no longer detectable by three weeks. To determine if adaptive immunity developed following the resolution of primary infection, mice were infected, allowed to resolve primary infection, and then re-challenged with 5×10^6 IFUs on day 37 post primary infection. Lungs were harvested and infectious chlamydiae were enumerated on days 3, 7, 10, 14, and 21 following secondary challenge (Figure 2). Mice were not protected from reinfection. However, compared to primary infection, the course of secondary infection was characterized by a reduced bacterial burden at all sampling points ($P < 0.05$ at days 7 and 10), and a more rapid resolution of infection (Figure 2).