Age Alterations in Extent and Severity of Experimental Intranasal Infection with *Chlamydomphila pneumoniae* in BALB/c Mice

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The intracellular bacterium *Chlamydomphila* (“*Chlamydia*”) *pneumoniae* is a pathogen for several respiratory diseases and may be a factor in the pathogenesis of chronic diseases of aging including atherosclerosis and Alzheimer’s disease. We assessed whether aging is coupled with increased burden of infection in BALB/c mice after intranasal infection by *C. pneumoniae*. Six- and twenty-month-old BALB/c mice were infected intranasally with 5×10^4 inclusion forming units (IFU) or 5×10^5 IFU of *C. pneumoniae*. Lung, brain, and heart tissue were analyzed for infectious *C. pneumoniae* and for *Chlamydomphila* antigen by immunohistochemistry. At both doses, aging was associated with a decreased proportion of animals that cleared infection from the lung and greater burden of infectious organism within the lung. We observed dose-dependent spread to the heart/ascending aorta in animals infected with *C. pneumoniae*. In mice given 5×10^4 IFU, spread to the heart by day 14 was only observed in old mice. By day 28, all animals inoculated with 5×10^4 IFU showed evidence of spread to the heart, although higher *C. pneumoniae* titers were observed in the hearts from old mice. In mice inoculated with 5×10^5 IFU, spread of *C. pneumoniae* to the heart was evident by day 14, with no discernible age effect. *C. pneumoniae* was also recovered from the central nervous system (brain and olfactory bulb) of all mice by day 28 postinfection, with higher *C. pneumoniae* titers in old animals than in young animals. Our results suggest that infection with *C. pneumoniae* may be more severe in old animals.

With the life expectancy increasing over this century and predicted to continue to rise over the course of the next half century, it is expected that, by the middle of the 21st century, more than 20% of the population will be over the age of 65 (19). Thus, a major challenge to medicine will be addressing the health problems of this growing proportion of the population, particularly the prevention and treatment of infectious disease. Complications from bacterial infection as well as pneumonia are included in the ten most common causes of death in the elderly (48).

Infection with *Chlamydomphila* (“*Chlamydia*”) *pneumoniae* may represent an important pathogen in the elderly. Human infection by this obligate intracellular bacterium appears to be ubiquitous. Epidemiologic studies show that infection rates are high and increase with age. In both eastern and western industrialized countries, it is estimated that 50% of middle-aged individuals are seropositive (32, 37, 38, 49). In males 60 years of age and older, seropositivity is estimated at 60 to 70% (25, 40).

The route of entry for *C. pneumoniae* appears to be the oral and nasal mucosa (20, 21). Respiratory infection with *C. pneumoniae* causes pneumonia and bronchitis (15, 16, 26). Infection with *C. pneumoniae* has also been linked to acute exacerbations of asthma (27) and may be involved in the etiology of chronic obstructive pulmonary disease (14, 51) and sarcoidosis (18, 30, 36). *C. pneumoniae* has also been implicated in several

diseases that are not respiratory, including reactive arthritis (23) and atherosclerosis (6, 13).

C. pneumoniae infection has also been associated with several diseases of the central nervous system (CNS). These include meningoencephalitis (4, 22, 28), Alzheimer’s disease (5, 33), and multiple sclerosis (9, 50), although a direct causal relationship of the organism to these diseases remains to be conclusively established. The infection of nasal mucosa, accepted as the typical route of infection, may be the first step in the infection of multiple organs systems, including the CNS.

Many of these diseases linked to *C. pneumoniae*, including respiratory infections whose etiology is firmly linked to *C. pneumoniae* and nonrespiratory diseases in which the role of *C. pneumoniae* remains to be fully elucidated, are more common or more serious in aged individuals. Therefore, the role that aging plays in altering the outcome of an acute infection by the organism or the ongoing balance between the organism and the host in a chronic *C. pneumoniae* infection becomes quite relevant.

An important aspect of aging that may influence the outcome of infection is immunosenescence. Several parameters of immune function change with age and appear to correlate with both morbidity and mortality, especially that caused by infectious diseases (19, 43, 53). In a mouse model studying age-related alterations in the immune response to influenza virus, Po et al. demonstrated an age-associated delay in clearance of influenza virus that was linked to a decrease in influenza-specific cytotoxic-T-lymphocyte (CTL) activity and the frequency of influenza-specific CD8⁺ lymphocytes, as well as delayed expansion of influenza-specific clones (42). There was also a significant decrease in intracellular gamma interferon

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(IFN- γ) production in response to in vitro stimulation with nucleoprotein peptide in CD8⁺ cells from old relative to young mice. In humans, a study performed by Ferguson et al. (17) associated "nonsurvival" with the clustered parameters of poor T-cell proliferative response, a high CD8⁺ cytotoxic/suppressor cell fraction, and low CD4⁺ helper/delayed-type hypersensitivity cells and CD19⁺ B cells. These studies, as well as several others (19, 43, 53), support the rationale that immunosenescence is a major factor in increased morbidity and mortality from infectious diseases in the elderly.

It is therefore important to establish whether aging (and the associated senescence of the immune response) is linked to increased severity and extent of *C. pneumoniae* infection, since pneumonia is an important cause of morbidity and mortality in the elderly. In addition, because of the putative links between infection with this organism and several chronic diseases of aging, including atherosclerosis and Alzheimer's disease, it is also important to establish whether the organism can spread to the relevant organs systems after infection through the respiratory tract, and additionally, whether the kinetics of dissemination or burden of infection at these extrapulmonary sites is altered by aging.

MATERIALS AND METHODS

Mice. Female BALB/c mice were obtained from the National Institute on Aging (Bethesda, Md.). Mice were approximately 6 months of age (young) or 20 months of age (old) at the time of inoculation. Mice were housed in groups of three in HEPA filter caged racks, with infected mice housed separately from uninfected mice, within the containment facility at Philadelphia College of Osteopathic Medicine. All animal husbandry was performed using biosafety level 2 precautions and in a class II biosafety cabinet. Mice were fed food and water ad libitum. Organs from a total of 60 mice, in two independent experiments, were used to quantify bacterial load in lung, heart, and CNS tissue. Organs from an additional 14 mice were collected after perfusion and immersion fixation with 4% paraformaldehyde.

HEp-2 cell line. The human epithelial cell line HEp-2 (American Type Culture Collection [ATCC], Rockville, Md.) was maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 5 mM L-glutamine (Fisher Mediatech) at 37°C and 5% CO₂. A total of 1×10^5 to 2×10^5 cells were plated in a T25 tissue culture flask (Fisher Scientific, Pittsburgh, Pa.) and passaged every 3 days into a new T25 flask. Cells were washed with Hanks balanced salt solution (HBSS) and removed from the flask by using $1 \times$ trypsin-EDTA (Fisher Mediatech) for 3 to 5 min at 37°C, followed by neutralization of trypsin with fresh complete growth medium. Cells were centrifuged (10 min at 400 $\times$ g), and the pelleted cells were washed once in HBSS. Cells were then resuspended in complete MEM, counted, and plated at 1×10^5 to 2×10^5 cells per flask.

Experimental *C. pneumoniae* infection of mice. The *C. pneumoniae* AR-39 isolate (ATCC) was propagated in the HEp-2 cell line and concentrated by using a technique previously described by Campbell et al. (12). *C. pneumoniae* was then divided into aliquots and frozen until dilution for experimental infection of mice under biosafety level 2 precautions. Animals were inoculated intranasally with 5×10^4 (low dose) or 5×10^5 (high dose) inclusion forming units (IFU) of mycoplasma-free *C. pneumoniae* AR-39 (ATCC) in HBSS. Control mice in each age group received HBSS alone. For the recovery of viable *C. pneumoniae*, mice were euthanized at 14 and 28 days after infection by CO₂ asphyxiation. Organs were removed and snap-frozen in liquid nitrogen followed by storage at -80°C until thawed for quantitation of the organism. For immunohistochemical analysis, mice were anesthetized and perfused with 4% paraformaldehyde, followed by immersion fixation for more than 24 h.

Recovery and quantitation of *C. pneumoniae*. Frozen tissue removed from euthanized mice was thawed and homogenized. A 10% (wt/vol) homogenate was prepared in serum-free MEM (Gibco RPL, Grand Island, N.Y.) supplemented with 2 mM glutamine. Serial 10-fold dilutions (in 200 μ l) were added to four-well Lab Tech chamber slides (Naperville, Ill.) that had been coated with poly-L-lysine (Sigma) and plated to confluence with HEp-2 cells. Negative control wells contained cells mock infected with medium alone. The chamber slides were incubated at 37°C in 5% CO₂ for 2.5 h, washed with HBSS, and refilled with fresh complete medium supplemented with 2 μ g of cycloheximide/ml. The slides were

then incubated for another 48 h as described above. After incubation, slides were washed with HBSS and fixed in 50% methanol at room temperature for 20 min. After fixation, slides were washed twice in HBSS. After the last wash, the cells in the wells were stained with a 1:10 dilution of fluorescein isothiocyanate-conjugated *Chlamydia*-specific antibody (Imagen; Dako, Carpinteria, Calif.) in 100 μ l of phosphate-buffered saline (PBS) for 90 min in the dark at 37°C. Slides were washed in PBS and counterstained with a 1:1,000 dilution of bisBenzamide (Sigma, St. Louis, Mo.) in PBS for 1 min. Slides were washed with PBS and covered with aqueous mounting medium (Imagen) and stored in the dark until viewed and counted on a Nikon Eclipse E800 microscope. All titers are calculated as IFU/milliliter of 10% (wt/vol) tissue homogenate.

Organ sample preparation for immunohistochemical analysis. After euthanasia, brains, lungs, and hearts were removed, immersion fixed in 4% paraformaldehyde, and embedded in paraffin. Brains were cut coronally before further processing. Serial sections of 7- to 10- μ m thickness were cut from the paraffin-embedded block by using a Spencer 820 microtome (American Optical, Southbridge, Mass.). Coronal sections were cut from the most medial aspects of brains. Likewise, 7- to 10- μ m serial sections were taken from the lung and heart of each mouse. One section from each tissue was placed on individual Fisher Brand Superfrost Plus slides (Fisher Scientific).

Immunohistochemistry. Slides were deparaffinized by using xylene three times, rehydrated in graded alcohols (100, 90, 80, and 70% ethanol), and finally placed in distilled water for 3 min each at room temperature. Slides were rinsed three times for 10 min each time in PBS. Endogenous peroxidases were quenched by incubation in 3.0% hydrogen peroxide (H₂O₂) in PBS for 5 min at room temperature and then rinsed in PBS for 5 min. Antigen retrieval was performed by flooding samples with $1 \times$ Citra antigen retrieval buffer (Biogenex, San Ramon, Calif.), followed by heating in a microwave for 30 s on high power. Sections were then rinsed with PBS for 5 min and blocked three times for 15 min each time in 2% fetal bovine serum in PBS (blocking buffer) at room temperature. The slides were blotted and incubated with a *C. pneumoniae*-specific antibody, Dako 6600, at a working dilution of 1:20 that was applied to the samples with incubation overnight at 4°C. Negative control slides, one per group of antibody-labeled tissue, received secondary antibody only and were incubated overnight with blocking buffer alone.

After incubation with primary antibody or blocking buffer alone, specimens were rinsed three times for 10 min each time in PBS, blocked three times, and incubated with 25 μ l of horseradish peroxidase-conjugated goat anti-mouse IgH+L (1:300 in blocking buffer; Amersham Biosciences, Piscataway, N.J.) for 2 h at room temperature. Samples were rinsed three times in PBS, followed by incubation with 25 μ l of fresh Sigma Fast 3,3'-diaminobenzidine according to the manufacturer's directions (Sigma) for 12 min at room temperature. Samples were then rinsed once with water, washed three times in PBS, counterstained with Harris' Alum Hematoxylin (EM Sciences, Fort Washington, Pa.) for 30 s, and rinsed with distilled water. Samples were dehydrated by using graded alcohols (70, 80, 90, and 100% ethanol), followed by xylene two times (3 min each time). Samples were allowed to air dry, and a coverslip was applied by using Permount mounting medium (Fisher Scientific).

Hematoxylin-and-eosin-stained sections were prepared as described by the Rosen Lab protocol (website). Briefly, slides were deparaffinized and rehydrated, followed by staining with hematoxylin for 1 min. Slides were then stained in eosin for 30 s, followed by clearance of alcohol, air drying, and mounting in nonaqueous mounting medium.

Microscopic analysis. Microscopic examination of tissue was completed by using $\times 10$, $\times 20$, and $\times 100$ objective lenses. Digital still pictures were captured by using Image-Pro Plus Phase 3 Imaging System software with a Nikon Eclipse E800 Camera. The degree of inflammation was assessed by a pathologist who was blinded to the experimental protocol. The degree of leukocyte infiltration was examined for lung, heart, and brain from each of the 14 mice examined by histology. The degree of infiltration was scored (0 to +3) as follows: 0, indistinguishable from normal healthy tissue; +1, a limited focal infiltrate; +2, multiple foci of leukocytes; or +3, diffuse accumulation(s) of leukocytes. For each organ no fewer than four slides, evenly distributed across the entire series of serial sections, were examined.

Statistical analysis. A multifactorial analysis of variance was performed to compare the bacterial load recovered from each tissue relative to both the age at the time of infection and the dose of *C. pneumoniae* used to infect mice.

RESULTS

The ability of aged mice to resolve an acute *C. pneumoniae* respiratory infection is impaired. We compared the ability of

FIG. 1. Recovery of *C. pneumoniae* from the lung at days 14 and 28. The number of IFU of *C. pneumoniae*/milliliter, on a log scale, recovered from lung tissue homogenate of young and aged mice is shown on the y axis. The x axis displays the age of the animal at inoculation, the infectious dose, and the time of sacrifice. Each dot represents the results from a single animal. The bars show the arithmetic mean of all animals in each group ($\log_{10}$). (A) Recovery of infectious *C. pneumoniae* at days 14 and 28 after intranasal inoculation of 5×10^4 IFU. The "+" symbol indicates a statistically significant difference ($P = 0.026$) at day 28 p.i. between the arithmetic means of the groups of young and old mice inoculated with 5×10^5 IFU. The "++" symbols indicate a statistically significant difference ($P = 0.04$) at day 28 p.i. between the arithmetic means of the groups of young and old mice inoculated with 5×10^4 IFU. (B) Recovery of infectious *C. pneumoniae* at days 14 and 28 after intranasal inoculation of 5×10^5 IFU.

young and aged BALB/c mice to resolve an acute respiratory infection initiated by intranasal inoculation of 5×10^4 or 5×10^5 IFU of *C. pneumoniae* (AR-39) at 14 and 28 days postinfection. For these experiments, lung tissue was excised and homogenized as described in Materials and Methods.

Five of six young mice inoculated intranasally with 5×10^4 IFU of *C. pneumoniae* appeared to clear infection from the lungs within 14 days. At day 14, the *C. pneumoniae* titer for the remaining young animal was 5×10^2 IFU, representing a 100-fold drop in titer compared to the inoculating dose (Fig. 1A). When tested 28 days after infection, complete clearance of organism from the lung was observed in four of seven young animals. The remaining mice had low, but detectable *C. pneumoniae* titers ranging from 5×10^1 to 2×10^3 (Fig. 1A). The results from young mice given the lower dose of *C. pneumoniae*, taken together, suggest that infection initiated by 5×10^4 IFU clears in the majority of young animals, probably within the first 2 weeks of infection. In the remaining animals, low numbers of *C. pneumoniae* persist, possibly establishing a low grade, chronic infection in the lung.

Infection established by intranasal inoculation of 5×10^4 IFU *C. pneumoniae* was not cleared as efficiently from the lungs of old mice relative to young mice. By day 14, only two of six old animals were able to clear infection initiated by 5×10^4 IFU (compared to five of six young mice); the remaining four old animals had titers that ranged from 5×10^2 to 2×10^3 IFU (Fig. 1A). When the mean *C. pneumoniae* recovery from the lungs of young animals at day 14 was compared to that from comparably treated old animals, the 10-fold increase in mean titer in old animals was not statistically significant. Only two of seven aged mice, compared to four of seven young mice, completely cleared the lower dose of *C. pneumoniae* from the lung by day 28 (Fig. 1A). The old animals that failed to clear the organism had *C. pneumoniae* lung titers ranging from 5×10^3

to 3×10^6 IFU. The mean lung titer at day 28 in old animals (4.8×10^5) was approximately 1,000-fold higher than that in young animals (3.0×10^2) and was statistically significant ($P = 0.026$).

These results, taken together, suggest that old animals may clear lung infection after intranasal inoculation of 5×10^4 IFU *C. pneumoniae* less frequently than young animals. Moreover, in those animals still harboring infection in the lung at day 28, the amount of infectious *C. pneumoniae* recovered from old animals is greater than that recovered from young animals.

In animals given a 10-fold-higher inoculum (5×10^5 IFU), an effect of age on clearance of *C. pneumoniae* from the lungs could not be demonstrated at day 14 (Fig. 1B). Only two of five young mice resolved the infection initiated by 5×10^5 IFU by day 14; the remaining three young animals had lung *C. pneumoniae* recoveries of 1×10^3 , 1×10^5 , and 2×10^6 IFU, respectively (Fig. 1B). *C. pneumoniae* could be recovered from the lung tissue of all (five of five) aged mice inoculated with 5×10^5 IFU of *C. pneumoniae*. Titers in lungs from old animals ranged from 5×10^2 to 1×10^6 IFU.

Although an age effect on burden of infection in the lung could not be demonstrated at day 14, there appeared to be a discernible age effect at day 28. By this time, six of nine young mice resolved infection from the lungs initiated by 5×10^5 IFU. The remaining three young animals had lung *C. pneumoniae* recoveries of 1×10^2 , 1×10^3 , and 2×10^4 IFU, respectively (Fig. 1B). In contrast, infection in old animals initiated by 5×10^5 IFU of *C. pneumoniae* cleared in only two of seven individuals (Fig. 1B). The remaining five old animals had lung *C. pneumoniae* recoveries of 5×10^3 , 1.5×10^4 , 1.5×10^4 , 2.5×10^4 , and 1×10^5 IFU, respectively. At day 28, the mean *C. pneumoniae* titer isolated from the lungs of aged mice given 5×10^5 IFU was 10-fold higher than comparably treated young mice (mean titer = 2.29×10^4 IFU versus 2.34×10^3

FIG. 2. Aged mice display a greater degree of inflammatory infiltration after infection with *Chlamydia pneumoniae*. After infection with *C. pneumoniae*, pulmonary inflammatory infiltrates were observed in all mice. The loss of typical lung architecture, including consolidation of alveoli and inflammatory infiltrates, was observed in all infected mice, but was more prominent in aged mice. A typical lung architecture was observed in all uninfected mice at 6 (A) and 20 (B) months. Inflammatory infiltrates in young mice at 6 months (5×10^4 IFU [C] and 5×10^5 IFU [E]) were less severe than those in aged mice at 20 months (5×10^4 IFU [D] and 5×10^5 IFU [F]). Bar, 100 μ m.

IFU, respectively). This difference is statistically significant ($P = 0.04$). These results suggest that infection induced by higher numbers of organism may eventually clear in a subset of both age groups but perhaps at a higher rate in young compared to old animals.

A total of eight uninfected mice (two old and two young animals at both day 14 and day 28) were tested for lung *C. pneumoniae* titers. None of the uninfected mice (i.e., zero of eight), regardless of age, had detectable *C. pneumoniae* titers (data not shown). This confirms that BALB/c mice are not natural reservoirs for *C. pneumoniae* infection and that the control animals used in the present study were not infected with *C. pneumoniae* before or during the experiment.

Aged BALB/c mice display more severe lung pathology during an acute *C. pneumoniae* infection. Histological analysis of lung tissue was performed in both young and old animals inoculated with 5×10^4 or 5×10^5 IFU or those that remained uninfected in order to determine whether aging was associated with profound changes in the inflammatory response or local

pathology induced by *C. pneumoniae*. Low-magnification analyses of lung tissue of the uninfected mice displayed normal lung architecture and no evidence of an inflammatory response (Fig. 2A and B). In contrast, low-magnification analyses of lung sections from young mice receiving either 5×10^4 IFU (Fig. 2C) or 5×10^5 IFU (Fig. 2E), and aged mice receiving 5×10^4 IFU (Fig. 2D) all show consolidation, alveolar thickening, various degrees of destruction of alveolar walls, and immune/inflammatory cell infiltration, although differences in the severity of pathology among these groups was difficult to ascertain. The lung pathology in aged mice receiving 5×10^5 IFU (Fig. 2F) appeared to be more severe. Sections examined from these latter mice showed significant destruction of alveoli and more prominent and extensive leukocyte infiltration. A semiquantitative analysis of the degree of leukocyte infiltration to the lungs was performed as described in Materials and Methods. Leukocytic infiltration in the lung was noted in most infected animals but may be more prominent in old animals (Table 1).

TABLE 1. Degree of leukocyte infiltration at day 28 postinfection^a

Age (mo)	Inoculum (IFU)	Degree of infiltration ^b		
		Lung	Heart	Brain
6	0	—	—	—
6	0	—	—	—
20	0	—	—	—
6	5×10^4	+	+	—
6	5×10^4	+	++	—
20	5×10^4	++	++	—
20	5×10^4	+	++	—
20	5×10^4	++	+++	+/-
6	5×10^5	+	+	—
6	5×10^5	+	++	—
6	5×10^5	—	—	—
20	5×10^5	—	+	—
20	5×10^5	++	+++	—
20	5×10^5	+++	++	—

^a The perfusion fixed tissues of all mice examined by histology evaluation were examined by a pathologist in order to determine the extent of leukocytic infiltration. Tissues were stained with hematoxylin and eosin and scored based on the degree of leukocyte infiltration.

^b Scoring: —, normal appearance; +, limited, focal distribution of leukocytes present in several fields of view; ++, multiple foci of leukocytes; +++, diffuse accumulations of leukocytes. Four sections were evaluated for each tissue.

The possibility existed that the local lung cellular infiltrates observed in animals infected with *C. pneumoniae* was, at least partially, induced by contaminating elements in the preparation derived from the HEP-2 cells used to cultivate the organism. To rule out this possibility, we examined lungs taken from two young (3-month-old) BALB/c mice that were inoculated with preparations derived from uninfected HEP-2 cells lysates 28 days previously. These animals displayed no histological evidence of leukocyte infiltration and were virtually indistinguishable from those taken from experimental animals inoculated with vehicle (HBSS) alone (data not shown).

Immunohistochemical analyses of lung tissue taken from animals at day 28 after infection were performed with the *C. pneumoniae*-specific antibody Dako 6600 as described in Materials and Methods. Using this technique, we found no evidence of *Chlamydomphila* antigen in sections of lung from uninfected animals regardless of age (Fig. 3A and B). These results are consistent with our failure to demonstrate viable *C. pneumoniae* titers in these animals and provide further support that BALB/c mice are not natural reservoirs for *C. pneumoniae* infection and that the animals used in the present study were not infected during the experiment. Positive staining for *Chlamydomphila* antigen was found in sections taken from one of two young mice infected with 5×10^4 IFU, from all (three of three) young mice given 5×10^5 IFU, and from all (six of six) old infected mice, regardless of the inoculum (Fig. 3C to H). Cells staining positively for *Chlamydomphila* antigen appeared preferentially in areas of significant consolidation and to colocalize with mononuclear cell infiltration, which may be reflective of an acquired immune response against the organism.

The spread of *C. pneumoniae* to cardiac tissue of infected animals is age and dose dependent. Although *C. pneumoniae* infection of the respiratory system has been well documented, this organism has also been associated with several chronic infections involving other organ systems, particularly the car-

diovascular system where it has been linked etiologically to atherosclerosis (27, 31, 50). To determine whether there was an age effect on the incidence of spread to the heart and/or ascending aorta or an age effect on the burden of organism at these sites, hearts and/or ascending aortas were excised and homogenized as described in Materials and Methods. Extracts were then cultured on HEP-2 cell monolayers and viable *C. pneumoniae* was enumerated in each sample. Fixed heart tissue samples were also examined for the presence of *C. pneumoniae* antigens.

Viable *C. pneumoniae* could be recovered from heart tissue in both old and young animals (Fig. 4A and B), although the kinetics of spread appeared to vary with age and inoculum. After intranasal inoculation of 5×10^4 IFU, *C. pneumoniae* was not recovered from any of the three hearts from young animals (zero of three) tested at day 14. In contrast, viable *C. pneumoniae* was recovered from all (three of three) old animals given the same inoculum. Titters from old animals ranged from 2.0×10^4 to 5.5×10^4 IFU per ml of extract and differed significantly from the findings in young animals ($P = 0.0001$). By day 28, there was evidence of spread of *C. pneumoniae* to the heart in all animals (six of six total) receiving the lower inoculum, regardless of age (Fig. 4A). However, there was a significant ($P = 0.003$) age effect on mean *C. pneumoniae* titer (2.36×10^7 IFU/ml in old animals versus 1.48×10^7 IFU/ml in young animals). These results suggest that, at the lower inoculum of *C. pneumoniae*, the organism may disseminate to the heart of old animals within the first 2 weeks after infection. Dissemination to the heart in younger animals still occurs, although probably between 2 and 4 weeks postinfection.

A total of eight uninfected mice (two old and two young animals at each time point) were tested for *C. pneumoniae* titer in heart tissue. None of the uninfected mice (zero of eight), regardless of age, had detectable *C. pneumoniae* titers (data not shown).

At the higher inoculating dose of *C. pneumoniae* (5×10^5 IFU), spread to the heart in both young (three of three) and old (three of three) animals was evident at day 14 (Fig. 4B). At day 14, only a threefold age-related increase in titer, that was not statistically significant, was noted. Similarly, at this higher inoculum, there was still evidence of infection in the heart in both old (three of three) and young (three of three) animals at day 28. In fact, the mean *C. pneumoniae* titer from three young animals was 7.5×10^7 and 1.3×10^8 IFU for the old animals, a difference that was near statistical significance ($P = 0.07$). These results suggest that, after infection with a lower inoculum, *C. pneumoniae* may disseminate to the heart faster in older animals and that older animals may have an increased burden of organism in these tissues. However, when the intranasal inoculum is 10-fold higher, an age effect on spread to, or the burden of infection within, the cardiovascular system may not be as pronounced.

Immunohistochemical analysis of BALB/c heart tissue for *C. pneumoniae* antigen at 28 days postinfection indicated the presence of *Chlamydomphila* antigen perivascularly, possibly associated with the vascular endothelium (Fig. 5). By this methodology, *C. pneumoniae* antigen was not detected in heart tissue from uninfected (Fig. 5A, young, and Fig. 5B, aged) mice or from young mice receiving 5×10^4 IFU (Fig. 5C). Heart tissue from aged mice receiving 5×10^4 IFU of *C. pneumoniae*

FIG. 3. *Chlamydia pneumoniae* antigen is present in the lungs of both young and aged mice at day 28 after infection. *C. pneumoniae* antigens were detected in the lungs of young and aged mice by using *C. pneumoniae*-specific antibody (Dako M6600). Representative images of uninfected mice are shown in panels A (6 months) and B (20 months). Representative images of lung tissues from mice infected with *C. pneumoniae*, displaying immunoreactivity with *C. pneumoniae*-specific antibody, are shown in panels C (6 months, 5×10^4 IFU), D (20 months, 5×10^4 IFU), E (6 months, 5×10^5 IFU), and F (20 months, 5×10^5 IFU). Areas displaying *C. pneumoniae* immunoreactivity from panels E and F, respectively, are displayed in panels G (6 months, 5×10^5 IFU) and H (20 months, 5×10^5 IFU). Bar, 10 μ m.

showed some evidence of *C. pneumoniae* antigen (Fig. 5D). *Chlamydia pneumoniae* antigens were present in the heart tissue of both young and aged mice receiving a high dose inoculum (Fig. 5E and F). When examining the degree of leukocyte infiltration to cardiac tissue, at least some infiltration was noted in sections taken from almost all infected animals and was observed primarily within the pericardium. However, aged ani-

mals had more extensive leukocyte infiltration to cardiac tissue after *C. pneumoniae* infection than did young animals (Table 1).

Dissemination of *C. pneumoniae* to the brain and olfactory bulb is age and dose dependent. Viable *C. pneumoniae* could be recovered from the brains of both old and young animals (Fig. 6A and B). After intranasal inoculation of 5×10^4 IFU,

FIG. 4. Recovery of *C. pneumoniae* from the heart and/or ascending aorta at days 14 and 28. The numbers of IFU of *C. pneumoniae* per milliliter, on a log scale, recovered from heart tissue homogenate of young and aged mice are shown on the y axis. The x axis displays the age of the animal at inoculation, the infectious dose, and the time of sacrifice. Each dot represents the results from a single animal. The bars show the arithmetic mean of each group ($\log_{10}$). (A) Recovery of infectious *C. pneumoniae* at days 14 and 28 after intranasal inoculation of 5×10^4 IFU. The “++” symbols indicate a statistically significant difference ($P = 0.0001$) at day 14 p.i. between the arithmetic means of the groups of young and old mice inoculated with 5×10^4 IFU. The “+” symbol indicates a statistically significant difference ($P = 0.003$) at day 28 p.i. between the arithmetic means of the groups of young and old mice inoculated with 5×10^4 IFU. (B) Recovery of infectious *C. pneumoniae* at days 14 and 28 after intranasal inoculation of 5×10^5 IFU.

C. pneumoniae was not recovered from any (zero of six) brains, regardless of age, when tested at day 14. However, by day 28, there was evidence of spread of *C. pneumoniae* to the brains of all (six of six) animals receiving this inoculum, regardless of age, although it appeared that the mean *C. pneumoniae* titer was threefold higher in old compared to young animals (6.7×10^5 IFU/ml versus 2.3×10^6 IFU/ml, respectively; $P = 0.008$). These results suggest that, with lower inoculating numbers of *C. pneumoniae*, the organism spreads to the brain within 4 weeks postinfection, regardless of the age of the mice.

At the higher inoculating dose of *C. pneumoniae* (5×10^5 IFU), spread to the brain was evident in one of three young animals at day 14 (Fig. 6B). This animal had a titer in the brain of 4.5×10^5 IFU/ml. In comparison, at day 14, *C. pneumoniae* could be recovered from two of three brains taken from old animals given the same inoculum, with titers of 2.0×10^6 and 1.5×10^6 IFU/ml. The age difference in titer was not statistically significant. However, at day 28, it appears that older animals (3 of 3) given 5×10^5 IFU had an approximately 10-fold increase in *C. pneumoniae* in the brain than similarly treated young animals (three of three); (3.2×10^7 IFU/ml versus 3.3×10^6 IFU/ml, respectively; $P = 0.004$).

The brain and olfactory bulb tissues of eight uninfected mice (two old and two young animals at each time point) were tested for *C. pneumoniae* titers. None of the uninfected mice (zero of eight), regardless of age, had detectable *C. pneumoniae* titers (data not shown).

Immunohistochemistry of brains at day 28 postinfection (Fig. 7) revealed evidence of *C. pneumoniae*-specific staining in the brains of infected animals without prominent leukocyte infiltration. Antigen was detected in the brains of 10 of 11 infected mice but not in uninfected animals (zero of three brains positive). Staining appeared to preferentially localize to perivascular areas, particularly to the vascular endothelium. By

this methodology, differences in the degree of staining, as an indirect measure of organism load, could not be detected with regard to either the age of the animal or the inoculating dose of *C. pneumoniae*. In addition, evaluation by a pathologist indicated that although *C. pneumoniae* antigens were present little to no leukocyte infiltration was observed in consecutive serial sections of tissue stained with hematoxylin and eosin (Table 1).

DISCUSSION

C. pneumoniae is an obligate intracellular bacterial pathogen that causes pneumonia and bronchitis (16, 26). *C. pneumoniae* may be associated with acute exacerbations of asthma and in the etiology of chronic obstructive pulmonary disease (51). Interestingly, this organism may also be a factor in the pathogenesis of a number of nonrespiratory diseases, including atherosclerosis (11), Alzheimer's disease (5), multiple sclerosis (50), and possibly malignancy (lung cancer and cutaneous T-cell lymphomas) (1). The incidence and/or severity of several of these diseases (particularly pneumonia, Alzheimer's disease, and atherosclerosis) increase significantly with advancing age. However, the impact of age on the incidence, duration, and/or severity of respiratory disease caused by *C. pneumoniae* has not been addressed to date. Moreover, the role that age and/or immunosenescence play in the spread of infection and disease processes outside the respiratory system are also unknown.

Using a mouse model, we have demonstrated that clearance of *C. pneumoniae* from the lungs after intranasal infection is impaired in aged mice relative to young mice. By day 28 postinfection, 57 and 67% of young mice infected with 5×10^4 or 5×10^5 IFU, respectively, were able to clear *C. pneumoniae* from the lung. At this time, only 28% of old mice inoculated with either 5×10^4 or 5×10^5 IFU cleared the local infection.

FIG. 5. *Chlamydia* antigens are present in heart tissue at day 28 after infection. *Chlamydia* lipopolysaccharide antigens are present in heart tissue at day 28 postinfection. A majority of the immunoreactivity was localized to the endothelial cell layer. Images of heart tissue from young (6 months) mice are seen in panels A (uninfected), C (5×10^4 IFU), and E (5×10^5 IFU). Panels B (uninfected), D (5×10^4 IFU), and F (5×10^5 IFU) are representative images of aged (20 months) mice. Arrows indicate immunoreactivity for *C. pneumoniae* lipopolysaccharide antigens. Bar, 10 μ m.

Likewise, the burden of *C. pneumoniae* in the lungs from old animals was much higher than that in the lungs of younger animals. In the majority of older animals, local *C. pneumoniae* infection appeared to be progressive over the observed time period and was associated with more severe local pathology (Fig. 1 to 3). These results suggest that in elderly humans pneumonia and bronchitis induced by primary, acute infection by *C. pneumoniae* could be more severe and possibly associated with greater morbidity and mortality. Chronic local infection with *C. pneumoniae* in elderly humans may be associated with exacerbations of asthma or in initiation and/or progression of chronic obstructive pulmonary disease.

The underlying reason for the age-related increase in bacterial load in the lung may be immunosenescence. A link between immunosenescence and increased incidence and/or the severity of infectious disease has been established in experimental influenza virus infections in mice and from studies of influenza virus infection in humans. In mice, aging is associated with prolonged virus shedding from the lung and a significant decline in influenza virus-specific CTL activity (42). The de-

cline in influenza virus-specific CTL activity was due to decreased frequency and/or proliferative capacity of CTL precursors with age rather than decreased cytotoxic function within that population. Other potential age-associated defects that may contribute to more extensive disease may include a decrease in IFN- γ production (43). In fact, Po et al. (42) have shown decreased intracellular IFN- γ production in CD8 $^{+}$ cells from old compared to young mice in response to in vitro stimulation with influenza nucleoprotein peptide.

Similar age-sensitive, cell-mediated protective mechanisms may be operational in mice in response to *C. pneumoniae* infection. In BALB/c mice, intranasal inoculation of *C. pneumoniae* resulted in a mild, acute respiratory infection that was associated with a mild lymphoid reaction in the lungs and a weak in vitro lymphoproliferative response (41). Cytokines that promote cell mediated immunity, including IFN- γ , may be central to protective immunity against *C. pneumoniae*. In vitro studies of the role of IFN- γ in the control of infection within cells of the macrophage/monocyte series suggest an important role for this cytokine in restricting bacterial load (3, 44). Dur-

FIG. 6. Recovery of *C. pneumoniae* from the brain and olfactory bulb at days 14 and 28. The numbers of IFU of *C. pneumoniae* per milliliter, on a log scale, recovered from brain tissue homogenate of young and aged mice are shown on the y axis. The x axis displays the age of the animal at inoculation, the infectious dose, and the time of sacrifice. Each dot represents a single animal. The bars show the arithmetic mean of all animals in each group ($\log_{10}$). (A) Recovery of infectious *C. pneumoniae* at days 14 and 28 after intranasal inoculation of 5×10^4 IFU. The “++” symbols indicate a statistically significant difference ($P = 0.008$) at day 28 p.i. between the arithmetic means of the groups of young and old mice inoculated with 5×10^4 IFU. (B) Recovery of infectious *C. pneumoniae* at days 14 and 28 after intranasal inoculation of 5×10^5 IFU. The “+” symbol indicates a statistically significant difference ($P = 0.004$) at day 28 p.i. between the arithmetic means of the groups of young and old mice inoculated with 5×10^5 IFU.

ing primary infection, neutralization of IFN- γ increased the bacterial load and the severity of pneumonia in C57BL/6 mice but not in BALB/c mice (52). Anti-interleukin-12 treatment also resulted in less-severe pathological changes but higher bacterial titers and reduced IFN- γ production in the lung of infected animals (20). Mice genetically deficient in interleukin-12, IFN- γ , or IFN- γ R have higher bacterial loads in the lungs than the normal counterparts (3, 45, 47). Future studies will examine whether age-associated alterations in production of these cytokines are associated with the impaired bacterial clearance and more severe local pathology that we have observed in old infected animals.

Our data also suggest that spread of the organism to the cardiovascular and central nervous systems may be a natural outcome of respiratory infection, although dissemination to these sites may occur more rapidly in aged animals. The monocyte may be the principal peripheral blood cell in which *C. pneumoniae* is harbored (2). These cells are capable of harboring a chronic infection by *C. pneumoniae* (2) and may not only serve as a reservoir of the organism in a chronic infection but also be a major conduit by which the organism disseminates to the cardiovascular system (8).

With regard to spread of the organism into the CNS, recent evidence implicates both monocytes and human brain microvascular endothelial cells (HBMECs) in the entry of *C. pneumoniae* through an in vitro model of the blood-brain barrier (34). *C. pneumoniae* infection of HBMECs resulted in increased expression of surface adhesion molecules on the endothelial cells and increased integrin expression on human THP-1 monocytes. With this increased expression, a threefold increase in transmigration of the monocytes through the in vitro barrier was observed (35). In conjunction with these studies, zonula adherens junctional proteins of *C. pneumoniae*-infected HBMECs were shown to be upregulated with a con-

comitant transient downregulation of tight junctional proteins for up to 48 h postinfection (35). The transient changes increased the likelihood that transmigration of monocytes through the HBMEC barrier would occur (35). These alterations in blood-brain barrier could therefore lead to increased immune cell infiltration and pathogen entry into the brain.

Another important route of spread of organism to the CNS may be through the olfactory system. Since *C. pneumoniae* readily infects epithelial cells and has direct access to the olfactory neuroepithelium, this route of infection would be likely, given that *C. pneumoniae* is a respiratory pathogen. Evidence for this route of entry has been obtained in humans (24) and investigated in a mouse model in which *C. pneumoniae* was introduced into the animal through the nares (33). Analyses by PCR and reverse transcription-PCR of olfactory bulbs obtained at autopsy from patients with Alzheimer's disease show that *C. pneumoniae* genetic material was present in these structures (24). Ultrastructural analysis of the olfactory bulbs of mice infected intranasally with *C. pneumoniae* demonstrated the organism in the bulbs at 1, 2, and 3 months postinfection (33). These results suggest that infection of olfactory bulb occurs in humans and in mice infected intranasally with *C. pneumoniae*.

Interestingly, spread to the cardiovascular system and CNS was not associated with overt signs of illness in our study. The time required to establish an infection in the CNS seems to be dose dependent, but after establishment of an infection aged hosts seem to be more permissive to bacterial propagation. This pattern seems to be present in cardiac tissue as well, with one notable exception; at the lowest inoculum, *C. pneumoniae* was recovered from the heart of aged mice at day 14. Age, as well as the dose of inoculum, affects the rate at which *C. pneumoniae* disseminates to both the heart and CNS. In the present study, the bacterial burden recovered from the heart at

FIG. 7. *Chlamydia* antigens are present within endothelial cells of blood vessels in the brain at day 28 after infection. Immunoreactivity for *Chlamydia* antigens (Dako M6600) observed in brain tissue of *C. pneumoniae*-infected and mock-infected BALB/c mice at day 28 postinfection. Similar to the heart, the immunoreactivity was localized to the endothelial cells and the perivascular region. Representative images of brain tissue from young (6 months) mice are seen in panels A (uninfected), C (5×10^4 IFU), and E (5×10^5 IFU). Panels B (uninfected), D (5×10^4 IFU), and F (5×10^5 IFU) show representative images of aged (20 months) mice. Arrows indicate immunoreactivity for *Chlamydia* antigens. Bar, 10 μ m.

day 14 was similar to that reported by Rothfuchs et al. in double-knockout (RAG-1^{-/-} IFN- γ ^{-/-}) mice infected with *C. pneumoniae* (45). A high bacterial burden was reported in both lungs and heart at day 15 postinfection. The immune systems of these knockout mice were unable to limit the proliferation or the spread of *C. pneumoniae*, and it is likely that the CNS contained a high bacterial burden as well, similar to the aged mice in the present study.

In our study, the bacterial burden detected in the CNS and in cardiac tissue exceeded the burden in the lungs by day 28. The reasons for these findings are unclear, but it is most likely that either qualitative or quantitative differences in the immune response to the organism at various sites in the body may account for the increased burden observed in the cardiovascular system and CNS. Semiquantitative analysis of the degree of local cellular infiltration to the CNS of infected animals suggests minimal, if any, host cellular infiltration. This is consistent with the fact that the CNS is an immunoprivileged site.

Thus, following spread of the organism to the CNS, eradication of the organism may be more difficult. Conditions, instead, may favor the development of a persistent, chronic, or progressive infection in this system.

In contrast, strong cellular infiltration was observed in the cardiovascular tissue from infected animals, which may reflect an active inflammatory or immune response. It is likely, then, that qualitative differences in the host response in this area may allow the survival and/or replication of greater numbers of the bacteria. We are currently planning experiments to study whether there are differences in the nature of the cellular infiltrate or cytokines produced between the lung and the heart and/or vasculature that may account for these findings. We are also planning to study whether there are phenotypic differences in the organism found in infected lungs versus other sites in the body.

The ramifications of the age-associated increased dissemination of *C. pneumoniae* to the cardiovascular system and CNS

and/or the increased burden of infection at those sites remain to be conclusively established, although *C. pneumoniae* has been implicated in the pathogenesis of coronary artery disease in serologic studies demonstrating high-titer serum antibodies to *C. pneumoniae* in humans with atherosclerosis. In addition, the organism has been detected in atherosclerotic lesions in humans (29). The data obtained from mouse model studies suggest that infection by *C. pneumoniae*, together with hyperlipidemia, may be co-risk factors for atherosclerosis. Infection with *C. pneumoniae* appears to accelerate the progression of preexisting lesions in genetically hyperlipidemic animals and in animals fed high-fat, high-cholesterol diets (10, 39, 46). Even in otherwise healthy young (2-month-old) C57BL/6 mice, infection with *C. pneumoniae* induced inflammatory changes in the heart and aorta in a small percentage of chronically infected animals (7). It is important to note that, in the study conducted by Blessing et al. (7), no atherosclerotic changes were noted in the aortic sinus, the site of initial lesion formation, over a 20-week observation period. Our preliminary data suggest that intranasal infection of BALB/c mice also induces leukocyte infiltration in the heart and that the extent and degree of infiltration may be more severe in aged animals. Future studies will address whether the increased burden of infection within the cardiovascular system of aged BALB/c mice fed either normal or cholesterol-supplemented diets is associated with increased atherosclerotic changes in the aorta relative to younger animals fed the same diet.

The significance of increased spread to and/or burden of infection in the CNS of aged animals is intriguing. Previous work has demonstrated a very strong correlation between CNS infection by *C. pneumoniae* and Alzheimer's disease in humans (5) and demonstrated amyloid beta (A β) 1-42 immunoreactive deposits in the brains of *C. pneumoniae*-infected BALB/c mice at up to 3 months postinfection with the density, size, and number of deposits increasing with time (33). We are currently investigating whether there is increased A β 1-42 deposition in the CNS of aged animals, relative to young mice, after infection with *C. pneumoniae*.

In summary, intranasal inoculation of *C. pneumoniae* establishes an acute respiratory infection that appears to be more severe, as measured by bacterial load, in old compared to young mice. After respiratory infection, the organism is able to spread to and establish infections at distant sites, possibly via systemic circulation of infected monocytes. The spread of the organism to and burden of infection at extrapulmonary sites is modulated by the age of the host, but ultimately all mice in the present study became infected with *C. pneumoniae* in the two distant target organs investigated. Our results suggest that establishment of chronic *C. pneumoniae* infection in the CNS and heart may be a rather universal sequela of an acute, and often asymptomatic, respiratory infection. Persistent infection with this organism may accelerate or initiate the pathology observed in Alzheimer's disease or atherosclerosis.

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