Mouse Models of \textit{C. pneumoniae} Infection and Atherosclerosis

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Mouse models were used to determine whether \textit{Chlamydia pneumoniae} establishes chronic infection of the aorta and contributes to atherogenesis. Persistent infection of the aorta occurred in 11 of 31 hyperlipidemic apolipoprotein E-deficient (apoE\textsuperscript{−/−}) mice but not in C57BL/6J mice fed a normal diet after a single inoculation and in both models following repeated inoculation with \textit{C. pneumoniae}. Repeated inoculation of C57BL/6J mice resulted in inflammatory changes in the heart and aorta in 8 of 40 of mice; however, no atherosclerotic lesion development was observed. Repeated inoculation of apoE\textsuperscript{−/−} mice resulted in a statistically significant increase in lesion area (\(n = 43; P = .05\)). Although \textit{Chlamydia trachomatis} disseminated to the aorta, persistent infection was not established and no statistically significant increase in lesion area occurred. These studies suggest that persistent infection of the aorta can lead to inflammatory changes in the absence of hyperlipidemia and accelerate lesion progress in concert with hyperlipidemia.

Previous studies that used these experimental models showed that following intranasal inoculation, lung macrophages are infected and monocytes/macrophages can disseminate infection to the vasculature [6]. The studies summarized below investigated whether \textit{C. pneumoniae} establishes persistent infection of the normal or diseased aorta and, if established, whether persistent infection induces atherosclerotic-like changes or affects disease progression.

Materials and Methods

\textit{Chlamydial strains and inoculum preparation}. \textit{C. pneumoniae} strain AR-39 [7] or \textit{Chlamydia trachomatis} E/UW-5/Cx were used for inoculation of mice. The organisms were grown in HL and HeLa cells, respectively [8], and were purified by diatrizoate meglumine (Hypaque-76; Winthrop-Breon Laboratories, New York, NY) density gradient using centrifugation. The inoculum preparations were resuspended in sucrose phosphate glutamic acid (SPG) medium and frozen at \(−70°C\) until use.

\textbf{Mouse strains.} Male homozygous apoE\textsuperscript{−/−} or C57BL/6J mice, aged 6–8 weeks, were obtained from Jackson Laboratories (Bar Harbor, ME). All mice were fed a normal mouse chow diet ad libitum. Animals were housed under biosafety level 2 conditions and cared for by standard and specific practices as outlined by the National Institutes of Health. All mouse experiments were performed under barrier conditions with sentinel mice monitored by a comprehensive quality assurance program.

\textbf{Inoculation of mice.} Mice were mildly sedated by subcutaneous injection of a mixture of ketamine (Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (Lloyd Laboratories, Shenandoah, IA). Mice were inoculated intranasally with 3 \(×\) 10\(^{7}\) inclusion-forming units (ifu) per mouse per inoculation in a volume of 0.03 mL as previously described [9, 10]. Titers of purified organism were determined in cell culture and diluted appropriately to yield a standard inoculum of 3 \(×\) 10\(^{5}\) ifu. For a single inoculation, depending on the experiment, mice were inoculated at 8 or 16 weeks of age. For multiple inoculations, apoE\textsuperscript{−/−} mice were inoculated at 8, 10, and 12 weeks of age and C57BL/6J mice at 8, 9, and 10 weeks of age. Control mice were sham inoculated with 0.03 mL of sterile SPG medium.
Tissue collection. Tissue collection was done as previously described [9, 10] with minor modifications. In brief, mice were heavily sedated with Avertin (2,2,2-tribromoethanol), and whole blood was collected by exsanguination from the femoral arteries. Perfusion fixation of the hearts and aortas was done using 10% buffered formalin administered through the left ventricle. Lung, hearts, and the aorta with its main branches were removed with a separate set of sterile instruments for each tissue, placed in sterile glass vials, and immediately placed on ice. For histopathology studies in C57BL/6J mice, the base of the heart was collected and sectioned at the aortic sinus. Thoracic aortas were embedded in paraffin and sectioned longitudinally. The heart and aorta with its main branches were dissected out intact, and the aorta was cleaned of surrounding adventitial tissue. The aortic arch was separated from the heart at the aortic sinus and from the rest of the aorta distal to the first intercostal artery branch.

Histopathologic examination. Immunocytochemical (ICC) staining of tissue sections was done using chlamydial genus (CF-2) or C. pneumoniae species-specific (TT-401) monoclonal antibodies on formalin-fixed tissue as described by Moazed et al. [10] or on frozen tissue. For frozen tissue preparation, lungs, hearts, and thoracic aortas were perfused with 10 mL of sterile PBS administered through the left ventricle. Tissues were kept in 30% sucrose overnight and embedded in OCT medium. Perfusion fixation of the hearts and aortas was done using 10% buffered formalin administered through the left ventricle. Lung, hearts, and the aorta with its main branches were removed with a separate set of sterile instruments for each tissue, placed in sterile glass vials, and immediately placed on ice. For histopathology studies in C57BL/6J mice, the base of the heart was collected and sectioned at the aortic sinus. Thoracic aortas were embedded in paraffin and sectioned longitudinally. The heart and aorta with its main branches were dissected out intact, and the aorta was cleaned of surrounding adventitial tissue. The aortic arch was separated from the heart at the aortic sinus and from the rest of the aorta distal to the first intercostal artery branch.

Results

C. pneumoniae establishes persistent infection of the atheroma more readily than the normal aorta [9, 12]. In both C57BL/6J and apoE−/− mice, C. pneumoniae disseminates from the lungs to the aorta; however, the organism appears to have an affinity for atheromatous tissues. Specifically, in C57BL/6J mice inoculated once at age 8 weeks, C. pneumoniae was detected by PCR in the normal aorta in 2 of 26 mice for up to 2 weeks after inoculation and was not detected subsequently. No aortas from sham-inoculated mice were positive (0/20). In apoE−/− mice inoculated in the same manner, the organism could still be detected 20 weeks after inoculation. The overall percentage of mice infected in the aorta in apoE−/− mice in this experiment was 35% (11/31). When apoE−/− mice were given a single inoculation at age 16 weeks when intermediate atherosclerotic lesions were well formed, 100% of the animals (8/8) were positive in the aorta up to 5 weeks after inoculation, the last time point examined (figure 1). Repeated inoculation of C57BL/6J mice on a normal chow diet resulted in longer persistence than in mice inoculated once: 20% of the mice (4/20) were still infected in the aorta 5 weeks after inoculation (figure 2). In apoE−/− mice, persistent infection of the aorta was established in a higher percentage of mice (13 [72%] of 18) by repeated inoculation than by single inoculation (figure 3). In apoE−/− mice, the organism was detected by ICC staining in foam cells within the aortic atheroma [10].

C. pneumoniae infection accelerates the progression of atherosclerosis in apoE−/− mice [10]. To study whether C. pneumoniae infection accelerates the development of atherosclerotic lesions, apoE−/− mice fed a chow diet were inoculated intranasally three times and analyzed 6 and 10 weeks after the third inoculation. The lesion area was 140% greater (P = .05) 6 weeks after inoculation and 64% greater (P = .05) 10 weeks after inoculation in infected mice than in control mice (n = 18–22 per time point). The heart weight was greater 8 weeks after inoculation (9% increase; P = .02) than at 12 weeks after inoculation.
C57BL/6J and apoE<sup>−/−</sup> mice fed normal chow diet were inoculated once intranasally at ages 8 and 16 weeks. Aortas were examined for presence of <i>C. pneumoniae</i> by polymerase chain reaction with HL-1, HR-1 primer set. C57BL/6J (<i>n</i> = 76) and apoE mice (<i>n</i> = 8) were followed <i>n</i> ≤ 4 weeks after inoculation. ApoE<sup>−/−</sup> mice inoculated at age 8 weeks were examined for <i>n</i> < 20 weeks after inoculation.

(2.9% increase; <i>P</i> = .32; <i>n</i> = 21–22 per time point). Cardiac wall thickness and chamber size were not increased nor was there any evidence of cardiac pathology. Because the changes were transient and reversible, these results suggest that the initial increase in heart weight was a non-specific response to acute lung infection. There were no differences in total cholesterol levels between infected and sham-inoculated mice. This study demonstrated that <i>C. pneumoniae</i> infection accelerates the progression of atherosclerosis in the aortic arch of apoE<sup>−/−</sup> mice.

<i>C. pneumoniae</i> infection induces inflammatory reactions in the aorta in C57BL/6J mice on a chow diet. As stated above, repeated intranasal inoculation of C57BL/6J mice resulted in longer persistence of <i>C. pneumoniae</i> in the aorta of C57BL/6J mice than in mice given a single inoculation. To determine whether persistent <i>C. pneumoniae</i> infection of the normal aorta induces histopathologic changes, C57BL/6J mice fed a chow diet were repeatedly inoculated intranasally with <i>C. pneumoniae</i> and analyzed at 1, 4, and 8 weeks after inoculation. Severe inflammatory infiltrates in the lungs were noted 3 days after the primary inoculation; however, no obvious inflammatory changes were noted in the aorta in the few mice examined (0/4). For this reason, we focused our studies on repeated inoculations.

The inflammatory infiltrates in the lungs were much milder after repeated infections than after the primary infection. However, histopathologic changes were observed in the heart, aorta, or both in 8 (20%) of 40 mice after repeated inoculations and intimal thickening in 2 (5%) of 40 mice. Inflammatory changes were noted in the heart (myocardium, aortic root, sinus, and valves) in 6 (15%) of 40 mice: mononuclear infiltrates on the valvular leaflet suggestive of endocarditis (1), mononuclear infiltrates in the myocardium suggestive of myocarditis (1), and coronary periarteritis with mononuclear infiltrates (4). Inflammatory changes were noted in the aorta in 6 of 40 mice: intimal thickening (2) and mononuclear cell infiltrates in the adventitia (4). Foam cell lesions, the early lesion of atherosclerosis, were not observed. All of the sham-inoculated animals (<i>n</i> = 24) exhibited normal morphology. No differences in plasma cholesterol levels were noted between infected and non-infected animals. Thus, although <i>C. pneumoniae</i> infection alone induced arteritis in a small number of animals, it did not induce definitive atherosclerosis during the observational period.

The trachoma biovar of <i>C. trachomatis</i> also disseminates to the aorta following intranasal inoculation but does not establish persistent infection in the aorta. To study whether dissemination to and establishment of persistent infection in the cardiovasculature are virulence properties of <i>C. pneumoniae</i> but not of other chlamydiae, C57BL/6J and apoE<sup>−/−</sup> mice fed a chow diet were inoculated intranasally with <i>C. trachomatis</i> strain E/UW-5/Cx. C57BL/6J mice were inoculated once or three times starting at age 8 weeks. ApoE<sup>−/−</sup> mice were inoculated once at age 16 weeks. As with <i>C. pneumoniae</i>, <i>C. trachomatis</i> also disseminated from the lungs to the aorta. In C57BL/6J mice inoculated three times, the organism could be isolated from the lungs and aorta (3/3) 3 days after a single inoculation and in lungs (3/3) and aorta (1/3) 7 days after the
third inoculation. The organism was not cultured from the lungs or aorta of sham-inoculated mice (3/3). In apoE<sup>−/−</sup> mice, C. trachomatis was also isolated from the lungs and aorta 3 days after infection (3/3). The organism was cultured from the lungs (5/5) and aorta (4/5) 7 days after inoculation but from the lungs only (1/5) 14 days after inoculation. Isolation was negative from tissues of 5 sham-inoculated mice. Longer follow-up by PCR showed that C. trachomatis infection persisted in the lungs 8 weeks after inoculation but did not persist in the aorta more than 1 week. All sham-inoculated mice were PCR negative in both the lungs and the aorta. This study showed that both C. pneumoniae and C. trachomatis disseminate from the lungs to the normal aorta and atheromatous lesions in the aorta. However, unlike C. pneumoniae, C. trachomatis does not establish persistent infection in atheromatous lesions.

Preliminary studies suggest that C. trachomatis infection does not significantly accelerate the progression of atherosclerosis in apoE<sup>−/−</sup> mice. To study whether C. trachomatis infection accelerates the atherosclerotic process, apoE<sup>−/−</sup> mice were inoculated intranasally three times with E/UW-5/OT. Although there was an increase in the lesion area in C. trachomatis–infected mice at both 16 (2.5 ± 2.4 μm<sup>2</sup> × 10<sup>3</sup> [n = 10] vs. 1.2 ± 1.2 μm<sup>2</sup> × 10<sup>3</sup> [n = 9]) and 20 (4.0 ± 2.7 μm<sup>2</sup> × 10<sup>3</sup> vs. 2.9 ± 1.8 μm<sup>2</sup> × 10<sup>3</sup> [n = 12]) weeks of age, unlike C. pneumoniae, the increase was not statistically significant (P = .16 and .23, respectively). We are currently repeating this experiment to see if the finding is reproducible and to have sufficient numbers of mice to make a valid conclusion.

Discussion

Mouse models of C. pneumoniae infection and atherosclerosis were used to determine whether in normolipidemic or hyperlipidemic states C. pneumoniae establishes persistent infection of the aorta, induces atherosclerotic changes, or exacerbates disease progression. The results suggest that while transient infection of the normal aorta can be produced following primary infection at age 8 weeks in C57BL/6J mice, chronic infection is readily established in the aorta when the atherosclerotic process in hyperlipidemic apoE<sup>−/−</sup> mice is progressing in the aorta. This apparent tropism of C. pneumoniae for atheromatous tissues was further demonstrated by inoculation of apoE<sup>−/−</sup> mice at age 16 weeks, a time by which all mice had developed early to intermediate atheromas. Inoculation of mice at age 16 weeks resulted in 100% infection in the aorta compared with 35% aortic infection in mice inoculated at age 8 weeks [9]. In the apoE<sup>−/−</sup> mouse, monocyte adherence occurs at age 8 weeks and foam cell (lipid-laden macrophages) lesions begin to develop at age 9 weeks; these progress to intermediate lesions at age 15 weeks and to developed plaques at about 20 weeks of age [5]. Thus, lesions are more frequent and advanced in mice inoculated at age 16 weeks than at age 8 weeks. These
findings suggest that C. pneumoniae has a tropism to developing lesions or that C. pneumoniae–laden monocytes are directed to the lesion as a result of the inflammatory response elicited by injured endothelium.

This predilection for infection of developing lesions in mouse models is analogous to that seen in human infection. These findings are consistent with the high detection rate (50%) of C. pneumoniae in human atheromatous plaques by ICC and/or PCR but rarely (1%) in normal arteries (see Kuo CC and Campbell LA, elsewhere this supplement). The organism has not been detected in children less than 14 years old [13]. In humans, the organism is more frequently found in diseased cardiovascular tissue than in noncardiovascular tissue [14].

In contrast to single inoculations, repeated inoculations of C57BL/6J mice led to the establishment of longer-term infection of the aorta. In turn, following repeated inoculation, inflammatory changes were observed in the heart and the aorta. The percentage of C. pneumoniae–infected mice (20%) that exhibited inflammatory changes in the aorta, heart, or both was similar to that in the rabbit model of C. pneumoniae infection reported by Fong et al. [15], in which 22% of infected rabbits fed a normal diet developed inflammatory changes, but was less frequent than reported by Laitinen et al. [16]. However, unlike the studies in the rabbit model in which inflammatory changes suggestive of early or intermediate atherosclerotic lesions were noted as early as 7 and 14 days after inoculation, no foam cell lesions or more developed lesions were observed in the mouse model up to 8 weeks after inoculation. Due to the prolonged nature of the development of atherosclerosis, longer-term studies in both animal models are necessary to determine whether infection alone induces inflammatory changes that develop into definitive atherosclerosis.

In apoE−/− mice in which persistent infection of the aorta was established, infection accelerated the progression of lesion size. These findings are consistent with studies that used LDL receptor knockout mice [17]. These mice develop atherosclerosis readily if fed a high-fat, high-cholesterol diet. In this study, C. pneumoniae infection exacerbated lesion development induced by an atherogenic diet but did not cause any noticeable atherosclerotic-like changes in the mice fed a chow diet. Thus, C. pneumoniae infection accelerates lesion progression in hypercholesterolemic mice regardless of whether the hypercholesterolemic state is genetic or diet induced and provides evidence of a potential causal relationship between C. pneumoniae infection and atherosclerosis. C. pneumoniae also augments intimal thickening in New Zealand White rabbits fed a moderately hypercholesterolemic diet [18].

To determine whether the establishment of persistent infection within the atheroma and exacerbation of lesion progression is a characteristic of chlamydial infection or a virulence factor of C. pneumoniae, similar studies were performed in apoE−/− mice inoculated intranasally with the trachoma biovar of C. trachomatis. Although persistent infection of the lung with C. trachomatis was as prolonged as C. pneumoniae and C. tra-
chomatis disseminated to the aorta, C. trachomatis did not establish persistent infection of the atheroma. Preliminary studies found an increase in lesion size in C. trachomatis-infected apoE−/− mice, but the increase was not statistically significant. In LDL receptor knockout mice on an atherogenic diet, infection with the mouse biovar of C. trachomatis did not augment lesion size [17].

These preliminary results on C. trachomatis infection in apoE−/− mice suggest that lung infection with either organism may exert indirect effects by stimulating an inflammatory response in the lung that leads to the activation of the endothelium and to a limited increase in plaque size in the aorta. However, we hypothesize that persistent infection of the aorta by C. pneumoniae exerts an additional direct effect that results in a further augmentation of lesion development by stimulation of an inflammatory response in the artery. The hypothesis that the direct effects of persistent infection with C. pneumoniae are critical to the pathogenesis of atherosclerosis would be analogous to the local immunopathology that results from persistent C. trachomatis infection of the eye or salpinx leading to trachoma (blindness) and infertility (tubal obstruction), respectively [19, 20].

In conclusion, an etiologic role of C. pneumoniae infection has not yet been firmly established. However, animal studies on induction, progression, and intervention suggest that the organism is not an “innocent bystander” and that it contributes to atherogenesis. However, longer-term studies are required to determine whether the atherosclerotic-like inflammatory changes seen shortly after infection in mouse and rabbit models persist and further develop into classic atherosclerosis in the absence of hypercholesterolemia due to diet or predisposing genetic factors. In both mouse and rabbit models, infection appears to exacerbate atherosclerosis in conjunction with high serum cholesterol. Future studies should be directed to the pathogenetic and immunologic mechanisms underlying these observed histopathologic changes following respiratory tract infection.

References