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Analysis of *Chlamydia pneumoniae* and AD-like Pathology in the Brains of BALB/c Mice Following Direct Intra-cranial Infection

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Abstract

Alzheimer's disease (AD) is an age-related progressive neurodegenerative disorder and the most common form of dementia. The pathology in the central nervous system (CNS) impairs memory and cognition, hindering the capabilities and the quality of life of the individual. This project continues studying the role of infection and Alzheimer's disease, as previous studies in this laboratory have done, and contributes to the overall understanding of the possible causes of this disease. In this study, BALB/c mice were infected, via direct intracranial injection, with a respiratory isolate (AR-39) of Chlamydia pneumoniae. Their brains were analyzed at 7 and 14 days post-infection, via immunohistochemistry, for the presence of C. pneumoniae, amyloid deposits and activated glial cells. The goal of this project was to measure the location and degree of C. pneumoniae burden, amyloid deposition and glial cell activation in the CNS following direct intracranial injection and to compare this data with results obtained from previous studies in this laboratory. We hypothesized that C. pneumoniae antigen and activated inflammatory cells will be observed in the infected mouse brains following direct intracranial injection and Aβ deposition will be observed in areas where inflammation occurs. C. pneumoniae, amyloid deposits and activated glial cells were detected in the brains following direct intracranial infection with C. pneumoniae. In infected mice there was an approximate 3.5-fold increase of C. pneumoniae antigen burden compared to uninfected mice at day 7 and there was an approximate 5.5-fold increase of C. pneumoniae antigen burden compared to uninfected mice at day 14. The burden of C. pneumoniae antigen, in the infected mice, increased 1.009-fold (no change) from day 7 to day 14 post-infection. The amyloid burden in infected mice increased approximately 3-fold compared to uninfected mice at day 7 and increased greater than10-fold compared to uninfected mice at day 14. The burden of amyloid, in the infected mice, increased 7-fold from day 7 to 14. From 7 to 14 days post-infection the C. pneumoniae and amyloid deposits located near the injection site spread distally from this location to other regions of the brain. Global activation of glia was observed in the CNS of infected mice at both 7 and 14 days post-infection. This data confirms that C. pneumoniae is capable of establishing an infection in the CNS. Although deposits were observed, the lack of a substantial amount of amyloid deposits suggested that the generation of deposits may require longer than 14 days following C. pneumoniae infection. As early as 7 days post-infection, inflammation is observed in response to the presence of C. pneumoniae and/or soluble amyloid in the CNS and the contribution of both infection with C. pneumoniae and the presence of soluble amyloid elicit the inflammatory response that presumably precedes and contributes to amyloid deposition.

Introduction

Alzheimer's disease (AD), the most common form of dementia in elderly individuals, is an age-related, progressive neurodegenerative disease that produces memory loss and severe cognitive impairment [1-3]. The onset of sporadic, late-onset AD (LOAD), which accounts for approximately 95% of all AD, is not primarily due to a genetic disorder, in contrast to familial AD (FAD), and instead it increases with age, generally occurring after age 65 [3,4]. The neuropathology shared by the two forms of AD presents as two defining hallmarks of the disease: neurofibrillary tangles (NFTs) and neuritic (senile) plaques (NSPs). Tangles are comprised of the abnormally hyperphosphorylated form of the tau protein whereas plaques are extracellular accumulations of amyloid β (Aβ) peptide [3, 5-7].

Late-onset AD seems to have multiple factors contributing to its development and ultimately leading to an inflammatory response and the generation of Aβ resulting in neurodegeneration. LOAD is recognized as a multifactorial disease, including the role of infectious organisms. Chronic inflammation is a hallmark of AD, however, whether the inflammatory response results from the over expression of Aβ or other stimuli is yet to be determined [11]. Research in this laboratory suggests that an infection with Chlamydia pneumoniae may be the initial stimulus for inflammation and thus the development of AD pathology [6, 9]. C. pneumoniae is an obligate intracellular bacterial pathogen that infects mucosal surfaces of the respiratory tract [12-14]. C. pneumoniae is generally transmitted person-to-person through aerosolized droplets to the respiratory tract and can disseminate systemically, typically infecting and "hitching a ride" inside monocytic cells, and has been shown to be capable of infecting an array of human cell types [9, 10, 15, 16]. Chlamydia pneumoniae infection has been associated with the onset and progression of several chronic diseases, including AD [9, 11, 17, 18].

This lab has developed a mouse model of AD-like pathology in which amyloid deposits have been experimentally induced following infection with a human AD-isolate of the organism C. pneumoniae [4, 6]. This model is useful for exploring the early events that take place in LOAD as well as the role of infection, particularly with the organism C. pneumoniae, in the induction of neuroinflammation and AD pathogenesis. Additionally, experiments utilizing a respiratory isolate of C. pneumoniae (AR-39) show that the greatest amount of C. pneumoniae was detected at 1 month post infection (earliest time analyzed), and then decreased at subsequent time points. The amount of Aβ deposition peaked at 2 months post-infection, which suggests that C. pneumoniae is capable of establishing a CNS infection and promoting amyloid deposition. This may serve as a stimulus for inflammation in the brain.

The work presented here aims to expand on the knowledge and data gained from our previous studies, where mice were infected intranasally. Following direct intracranial infection, we analyzed brain tissue for the presence of C. pneumoniae antigen, activated glial cells and Aβ deposition in fixed and embedded mouse brain tissue. This project focused on determining the burden of pathogen, the mobility of the pathogen and the presence and location of activated glial cells in the CNS.

Material and Methods

Chlamydia pneumoniae: Female BALB/c mice were infected with 1 x 105 infectious units, of a human respiratory isolate of C. pneumoniae, AR-39, obtained from the American type Culture Collection (ATCC), and propagated in HEp-2 cells, isolated for these experiments. Infectious units were administered via direct intracranial injection. Hank's balanced salt solution (HBSS) vehicle alone was given intracranially for age and sex matched uninfected control mice.

Infection of Mice and Brain Removal: The injection site is located at –Bregma -2.12mm on the anatomical right side of the mouse brain. At 7 or 14 days post-infection the mice were sacrificed and perfused with 4% paraformaldehyde, immersion fixed in 4% paraformaldehyde , paraffin embedded and then sectioned at 7-10 microns.

Mouse Brain: 50 coronal sections were labeled per mouse: 4 sets (1 per primary antibody) and 1 set (secondary antibody only) for rostral and caudal regions – 5 sections were labeled for each set. Samples represent regions spanning from rostral (bregma + 2.22mm) to caudal (bregma – 5.88mm).

Primary Antibodies Specific for C. pneumoniae: A mouse monoclonal RDI-PROAC1p used at a working concentration of 1:10, mouse monoclonal M6600 also used at a working concentration of 1:10, and mouse monoclonal 10C-27 used at a working concentration of 1:100.

Primary Antibodies Specific for Amyloid Beta 1-42 antigens: A mouse monoclonal A 1-42 (6E10) used at a working concentration of 1:500.

Primary Antibody Specific for Glial Fibrillary Acidic Protein (GFAP): A mouse, anti-human monoclonal GFAP used at a working concentration of 1:25.

Secondary Antibodies: Alkaline phosphatase conjugated secondary antibodies were utilized to visualize the respective antigens of interest. All antibodies were diluted to working concentration in phosphate buffer saline – blocking buffer.

Immunohistochemistry: The basic protocol consisted of re-hydrating sections in xylene , and then a series of graded alcohol solutions followed by DI H_2O. Slides were placed in Citra antigen retrieval buffer, rinsed with phosphate buffer saline (PBS) pH 7.4. Endogenous peroxidase activity was quenched utilizing a 3% solution of H_2O_2/PBS,then rinsed in PBS and blocked in 2% FBS/PBS. Slides were incubated in primary antibodies in a humidified chamber at 37oC. Sections were rinsed in 2% FBS/PBS, then incubated with 2° antibodies in a 37°C humidified chamber. Following incubation, sections were rinsed with distilled water and developed using alkaline phosphatase new magenta, rinsed in DI H_2O and PBS followed by acidified Harris's Hematoxylin. Sections were rinsed thoroughly in DI H_2O and then were contrasted in PBS and rinsed with DI HO, air dried, crystal mounted and coverslipped.

Microscopic Analysis: Images were captured using NIS-Elements F 2.20 Imaging System software on a Nikon Eclipse 50i microscope equiped with a Nikon Digital Sight DS-SM Camera.

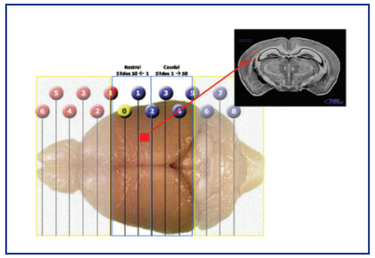


Figure 1. Anatomic Locations of Representative Coronal Sections

The numbers and corresponding lines above are 1 mm apart from one another. The regions boxed in red indicate the location of the injection site. The brains were sliced coronally starting, at line 2 above, approximately, and then separated into two halves – rostral (BrA) and caudal (BrB). From this initial slice (at line 2) the two halves were serially sectioned, every 35-50 microns and at a thickness of 7-10 microns, in both the rostral and caudal directions – see small arows at the top of the figure [6].

Results

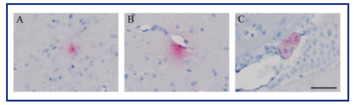


Figure 2. *C. pneumoniae*-**specific labeling in the brains of intracranially infected mice**

These images represent *C. pneumoniae* labeling, which includes extracellular and intracellular, observed in the brains of experimental mice at both 7 and 14 days post-infection. A) Representative image of extracellular labeling of *C. pneumoniae*. B) Representative image of labeling of *C. pneumoniae* associated with a blood vessel. C) Representative image of labeling of intracellular *C. pneumoniae*. (Size bar = 100 µm)

Table 1. Total amount of *C. pneumoniae*-specific labeling

	7 Days Post-Infection	14 Days Post-Infection
Control	47	16
Control	9	19
Control	22	15
Infected	220	153
Infected	10	4
Infected	143	47
Infected	30	118
Infected	52	137

The total amount of C. pneumoniae-specific immunoreactive sites (Cpn), intracellular, observed at both 7 and 14 days post-infection is displayed above.

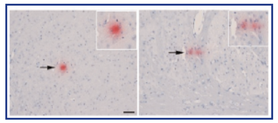


Figure 3. Amyloid deposits in the brains of intracranially infected mice

These images represent amyloid labeling observed for experimental mice at both 7 and 14 days post-infection. The top corners of each image are inset with a higher magnification image of amyloid deposits as designated by the low magnification arrow. Representative images of amyloid deposits observed using the Aβ6E10 antibody are presented. (Size bar = 100 µm)

Table 2. Total number of amyloid deposits

	Total Amount of Aβ (7 Days Post-Infection)	Total Amount of Aβ (14 Days Post-Infection)
Control	0	0
Control	5	1
Control	0	0
Infected	4	4
Infected	0	2
Infected	10	9
Infected	10	9
Infected	2	12

The total number of amyloid deposits observed at both 7 and 14 days post-infection is presented here.

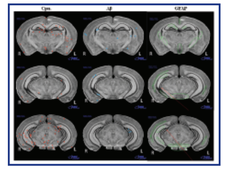


Figure 4. Distribution of *C. pneumoniae*-**specific immunoreactive sites, amyloid deposits and activated glial cells at 7 days post-infection**

C. pneumoniae immunoreactive sites (red dots), Aβ deposits (blue dots) and regions of the brain with glial cell labeling (green circles or red arrows) from individual slides in the day 7 group are presented here in areas of the hippocampus and dentate gyrus, as well as regions not affected early on in Alzheimer's disease.

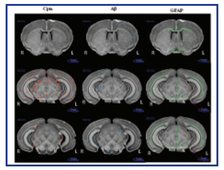


Figure 5: Distribution of *C. pneumoniae*-**specific immunoreactive sites, amyloid deposits and activated glial cells at 14 days post-infection**

C. pneumoniae immunoreactive sites (red dots), Aβ deposits (blue dots) and regions of the brain with glial cell labeling (green circles or red arrows) from individual slides in the day 14 group are presented here in areas of the frontal cortex, hippocampus and dentate gyrus, as well as regions not affected early on in Alzheimer's disease.

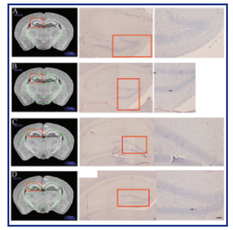
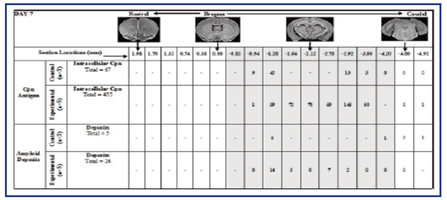
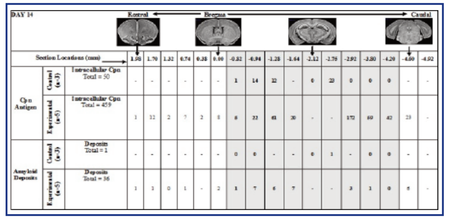


Figure 6. GFAP-specific immunoreactivity in brain tissue of infected and control mice

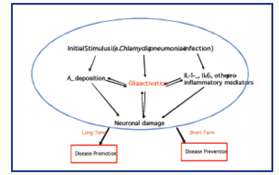
Regions of the brain – hippocampus and dentate gyrus, which are relevant structures in the AD brain – were selected to illustrate the difference in amount of glial cell labeling observed between a control and an experimental mouse at each time point. Substantial glial cell labeling was observed in the infected mice and comparable sections in controls were selected for comparison. The red boxes in the left column of images represent the region of the section observed at higher power, which are those located in the center column. The red boxes in the center column of images represent the region of the section observed at an additionally increased power, which are those located in the right column. A) This row represents day 7 data from an uninfected, vehicle injected control, mouse. B) This row represents day 7 data from an infected mouse. C) This row represents day 14 data from an uninfected, no-injection control, mouse. D) This row represents day 14 data from an infected mouse. (Size bar = 100 µm)





Conclusions

1) A C. pneumoniae antigen burden was detected in the mice at both days 7 and 14 following a dose of 10_5 infectious units of C. pneumoniae.
   - In infected mice there was an approximate 3.5-fold increase compared to uninfected mice at day 7.
   - In infected mice there was an approximate 5.5-fold increase compared to uninfected mice at day 14.
   - The burden of C. pneumoniae in infected mice increased 1.009-fold from day 7 to 14 (no change).
2) Following direct intracranial injection with 10_5 infectious units of C. pneumoniae amyloid was detected at all times analyzed post-infection.
   - In infected mice there was an approximate 3-fold increase compared to uninfected mice at day 7.
   - In infected mice there was a greater than 10-fold increase compared to uninfected mice at day 14.
   - The burden of amyloid in infected mice increased 7-fold from day 7 to 14.
3) Global activation of glia was observed in the CNS of infected mice at both 7 and 14 days post-in fection.



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