Comparison of Chlamydia antigen and AD-like pathology in the brains of BALB/c mice following intranasal infection with *Chlamydia muridarum* or *Chlamydia pneumoniae*

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Abstract

Previous research indicates BALB/c mice inoculated with *Chlamydia pneumoniae* (*Cpn*) demonstrated AD-like pathology which suggests that this mouse model is valid for studying the pathogenesis implicated in Alzheimer's disease (AD). Studies have demonstrated that *Chlamydia trachomatis* (*Ctr*) can disseminate from its primary site of infection and plays a major role in the induction of reactive arthritis. The objectives of this lab are: (1)to identify and localize Chlamydia antigens in the brains of BALB/c mice infected with *C. muridarum* and (2) to determine if infection with *C. muridarum* induces AD-like pathology comparable to *Cpn*. Using mouse adapted respiratory isolates of *C. muridarum*, we investigated whether *C. muridarum* disseminated from the respiratory tract to the brain. Mice were intranasally infected with plaqued C small Weiss (CSW) or plaqued mouse pneumonitis Weiss (MoPn weiss). Brain tissue was isolated at 2 months post-infection. Serial sections from brains infected mice were analyzed for amyloid or Chlamydia antigens. Preliminary analysis of brain tissue demonstrated no detectable difference in *C. muridarum antigen* between mice receiving 1 x10^5 IFU and mice receiving 1 x10^3 IFU, whereas a small but detectable difference was identified in amyloid-specific labeling between these two experimental groups. In contrast, prominent *Chlamydia*-specific labeling was identified in the brains of *Cpn*-infected mice as well as substantial amyloid deposition at 2 months p.i.. These data suggest that, relative to *Cpn* AR-39 infection, *C. muridarum infection* is a weaker stimulus for inflammation, resulting in decreased amyloid deposition in the brains of BALB/c mice.

Introduction

Alzheimer's disease (AD) is progressive neurodegenerative disease that affects one in eight older Americans. It is the most common form of dementia accounting for sixty to eighty percent of all cases and is the fifth leading cause of death for individuals age 65 and older. [1]. Due to its high prevalence, continued research on AD will be important in not only determining causes and risk factors but also developing treatment and improving patient's quality of life.

Alzheimer's disease is characterized by a progressive loss in cognitive function with the accumulation of the defining neuropathology amyloid (senile) plaques and neurofibrillary tangles in brain tissue. Decline in cognitive function is caused by neuronal cell death in areas of the brain associated with memory such as the medial temporal lobe and cortical structures. Neuronal cell death is induced by extracellular amyloid accumulation which disrupts normal synaptic communication within the brain. The formation of tangles within the neuron induces apoptosis by interfering with normal cellular function and transport of nutrients. Early AD pathology involves the medial temporal lobe which includes the hippocampus and the entorhinal cortex. [1,2]. Both of these regions are important for memory [3]. As the disease progresses, motor and sensory function become compromised and the disease is ultimately fatal [1].

There are two different forms of AD, sporadic and familial. Familial AD (FAD) accounts for less than 5% of AD cases and is caused by genetic mutations. Patients with FAD develop AD pathology before the age of 65. Genetic mutations associated with FAD involve mutations of the amyloid precursor protein and presenilin 1 and 2 [4]. A mutation in any of these proteins will lead to an early accumulation of amyloid plaques within brain tissue. [2,5]. While there is a known cause for FAD, the cause of sporadic AD which accounts for more than 95% of AD cases has not been identified. Several risk factors have been recognized including mild cognitive impairment, cardiovascular disease, head trauma, and inflammation. [2,6]. In 1998 Balin and coworkers identified and infectious agent, *Chlamydia pneumoniae* (Cpn) in 17 of 19 AD brains, suggesting the potential role of infection in the induction or progression of AD [7].

Two members of the genus Chlamydiae, *C. trachomatis* and *C. pneumoniae,* play a role in acute and chronic human illness [8] and have been shown to induce a chronic inflammatory response. Following the establishment of a urogenital infection by *Chlamydia trachomatis*, this organism has also been implicated in reactive arthritis, a chronic inflammatory joint condition [9, 10]. *C. trachomatis* can also ascend from its primary site of infection in the lower genital tract to the fallopian tubes and cause complications such as pelvic inflammatory disease which may result in infertility [11,12]. *Chlamydia pneumoniae*, a respiratory pathogen, has been shown to establish chronic infection and has been implicated in coronary artery disease [13], multiple sclerosis [14], and AD [7]. Mouse models have shown that this strain of chlamydia can disseminate after intranasal inoculation. The bacterium travels via peripheral blood monocytes and macrophages and has been found in areas not typically associated with *C. pneumoniae*- respiratory infection such as the spleen, aorta, and abdominal lymph nodes [7,15]. Thus, the ability of *C. pneumoniae* to disseminate and establish chronic infection may promote pathology in tissue other than respiratory epithelium.

To address the potential role of Chlamydia in the induction of sporadic AD, this laboratory has developed a mouse model in which AD-like pathology is induced following the introduction of *C. pneumoniae*. This model involves intranasally infecting non-transgenic BALB/c mice with *C. pneumoniae* [16]. In contrast with transgenic mice, the use of BALB/c mice more accurately models the predominant form of AD (sporadic/late onset) because these mice do not harbor any (human) genes. Pathology is induced following infection with *C. pneumoniae* [16]. This bacterium is commonly associated with respiratory infections worldwide in humans and was first associated with AD following identification and isolation from post-mortem brain tissue of individuals with AD in 1998 [17]. This laboratory has shown that following intranasal infection of mice, this organism establishes a chronic infection in the olfactory bulbs and induces substantial pathological deposition of Aβ in the brain [16].

The work presented here continues previous studies involving the BALB/c mouse model of AD-like pathology following *C. pneumoniae* infection. The focus of this research is to investigate the presence of the respiratory adapted biovar of *C. muridarum*, in fixed and embedded mouse brain tissue previously infected with this organism. Analysis was conducted using immunohistochemistry to label *Chlamydia*-specific antigens and beta amyloid deposits followed by a semi-quantitative microscopic analysis of brain tissue. The goal of this work was to investigate the relationship between the relative amount and location of *Chlamydia* antigens to that of AD-like pathology and to determine whether infection with *C. muridarum* induces AD-like pathology comparable to *C. pneumoniae*.

Methods

Chlamydia muridarum: Two different isolates of mouse adapted *Chlamydia trachomatis* Weiss strain were used to intranasally infect BALB/c mice at various concentrations. Group A mice were intranasally infected with 1 x 10^2 or 1 X 10^5 IFU's of C Small Weiss (MoPn *in vivo* passage). Group B mice were intranasally infected with 1 x 10^1 or 1 x 10^5 IFU of mouse pneumonitis Weiss (MoPn stock strain).

Infection of Mice and Tissue Preparation: Mice were intranasally infected with either C Small Weiss or mouse pneumonitis. Mice were sacrificed 60 days p.i, brains were removed and immersion fixed in 4% paraformaldehyde, embedded in paraffin, and coronally sectioned at 7-10 micron thickness.

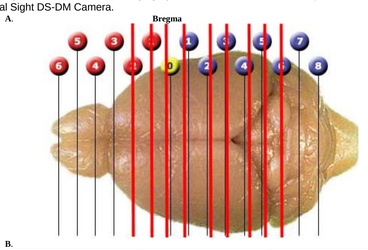
Mouse Brain Tissue: A total of 50 coronal brain sections were immunolabeled per mouse. Five sets of 10 sections: two sets were labeled with *C. trachomatis* specific antibody (10C-CR2104M1, 10C2104M3) , two sets were labeled with beta-amyloid specific antibody (AB1-16 6E10, AB (H-43) SC9129) and one set did not receive primary antibody and was used as a negative control. In order provide representative samples from areas throughout the whole brain sections were selected every 70-100 microns from rostral (bregma + 2.22mm) to caudal (bregma -5.88mm) (Figure 1).

Antibodies: The primary antibodies specific for *C. trachomatis* used were: mouse monoclonal 10C-CR2104M1 at a working concentration of 2 micrograms/mL(Fitzgerald, Acton, MA), mouse monoclonal 10C-CR2104M3 at a working concentration of 2 micrograms/mL((Fitzgerald, Acton, MA), and Anti-C muridarum (Sera mouse Anti-MoPn) at a working concentration of 1:500. The primary antibodies that were used to detect B-Amyloid are: rabbit polyclonal sc-9129 at a working concentration of 4 micrograms/mL (Santa Cruz Biotechnology) and mouse monoclonal 6E10 at a working concentration of 2 micrograms/mL (Covance). To visualize the respected antigen alkaline phosphatase conjugated goat anti-mouse and anti-rabbit secondary antibodies were utilized (Life Technologies, Carlsbad, CA) at a dilution of 1:400 (1.25 micrograms/mL). Antibodies were diluted to working concentration using phosphate buffer saline+2% fetal bovine serum.

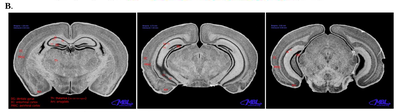
Immunohistochemistry: Mouse brain coronal sections were first rehydrated for 3 minutes in xylene 3x, followed by 100% ethanol x2, 90% ethanol x1, 70% ethanol x1 (Electron Microscopy Sciences, Fort Washington, PA). Next, slides were washed in DI H2O for 2 x 3 minutes, then placed in Citra antigen retrieval buffer (BioGenex, San Roman, CA) and steamed at high pressure for 15 minutes at 120 degrees Celsius in a 2010 Retreaver (Pick Cell Laboratories, Amsterdam, Netherlands). Slides remained in antigen retrieval buffer overnight at room temperature. Slides were washed with phosphate buffered saline pH 7.4 (PBS) 3 x 5 minutes. Next, Alkaline Phosphatase Horseradish Peroxidase was applied to the slides for 5 minutes to quench endogenous peroxidase activity, washed 3 x 5 minutes in PBS. Slides were incubated in mouse on mouse (MOM) blocking reagent (Vector Labs) was used 1 x 15 minutes. Following MOM, slides were blocked 1 x 15 minutes in PBS+2%FBS (Mediatech, Herndon, VA). Primary antibodies 10C-CR2104M1, 10C2104M3, Anti- C muridarum, AB1-16 6E10, AB (H-43) SC9129 were applied and slides placed in a humidified chamber at 37 degrees Celsius for 90 minutes. Next, slides were washed 3 x 5 minutes in PBS and blocked 2 x 15 minutes in 2% PBS+2%FBS. Secondary antibodies were applied to each slide and slides were incubated for 60 minutes in a humidified chamber at 37 degrees Celsius. Next, slides were washed 3 x 5 minutes in DI water (DI H2O). Slides were developed for 15 minutes using alkaline phosphatase new magenta (BioFX, Owings Mills, MD). Next, slides were washed in DI H2O 3 x 5 minutes and PBS 1 x 5 minutes. To stain the tissue Acidified Harris's Hematoxylin (Mercury free) was applied for 1 minute. The slides were rinsed briefly with DI H2O then washed with PBS for 5 minutes to produce a color change. Finally slides were washed 3 x 5 minutes w/ DI H2O then wiped down and air dried. Once dry slides were glycerol mounted and coverslipped.

Microscopic Analysis: Tissue was examined using 10x, 20x and 40x power objectives. Images were captured using NIS-Elements F 2.20 Imaging Systems software on a Nikon Eclipse 50i microscope using a Nikon Digital Sight DS-DM Camera.

A.



B.

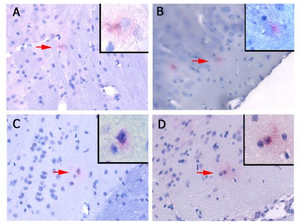


Anatomic Location of Representative Coronal Sections
In panel A, red lines indicate the location of selected Coronal sections from the Mouse Brain Library, providing an analysis of the whole brain. Sections rostral (red circles) to bregma (yellow circle) and caudal to bregma (blue circles) are displayed. Particular regions of interest, related to Alzheimer's disease, are identified in panel B.

Results

Chlamydia Labeling

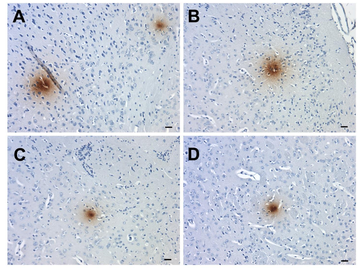
Chlamydia pneumoniae AR-39



Chlamydia-Specific Labeling in the Brains of Intranasally Infected Mice. Representative images of Chlamydia-specific antigen labeling in the brains of infected mice at 1 month (panels A and B) and 3 months (panels C and D) post infection. The upper right corner of each image is inset with a higher magnification image of Chlamydia-specific antigen labeling as designated by the low magnification arrow.

Beta Amyloid Labeling

Chlamydia pneumoniae AR-39



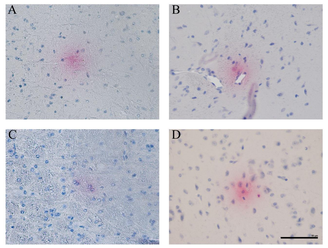
Amyloid beta 1-42 deposits in the brain of AR-39 infected mice. Brains were examined by light microscopy for the presence of A 1-42. Representative images of amyloid beta 1-42-specific labeling are shown. Bars = 20 microns.

Section Location (mm)	Rostral 2.22	1.70	0.38	Bregma -1.28	-2.75	-3.80	-4.92	-5.46	Caudal -5.88	Total
1 Mon Cpn	22	10	22	18	23	42	9	6	2	154
Amyloid	0	1	0	3	3	0	1	0	0	8
2 Mon Cpn	18	15	19	5	26	21	20	10	0	134
Amyloid	8	30	0	0	26	19	26	0	0	109
3 Mon Cpn	11	11	13	22	5	9	9	4	7	91
Amyloid	0	2	12	1	0	0	1	1	0	17
4 Mon Cpn	0	11	13	15	13	10	10	1	3	76
Amyloid	0	0	0	1	3	0	5	0	0	9
Cpn Totals	51	47	67	60	67	82	48	21	12	455
Amyloid Totals	8	33	12	5	32	19	33	1	0	143

***Chlamydia* (Cpn) Antigen and Amyloid Deposition (Dep)**
Location of *C. pneumoniae* immunoreactivity and amyloid deposits over 1, 2, 3, and 4 months post infection within brains of *C. pneumoniae*-infected mice. The location of immunoreactive label to amyloid deposits or *Chlamydial* antigen is designated in millimeters (Section location in mm) rostral or caudal to the mouse bregma. Gray, hatched, and aqua shaded areas highlight majority of *C. pneumoniae* antigen and the relationship to the majority of amyloid deposition 1 month later.

Chlamydia Labeling

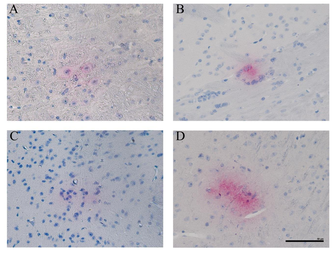
MoPn *in vivo* | MoPn stock strain



Chlamydia-specific labeling of CNS tissue of BALB/c mice inoculated.n. with 100,000 IFU of Mouse pneumonitis *in vivo* passaged (A and C) or Mouse pneumonitis stock strain (B and D) immunolabeled with10C-CR2104M1 *C. trachomatis*-specific antibody. Bar=50 microns.

Beta Amyloid Labeling

MoPn *in vivo* | MoPn stock strain



Amyloid-specific labeling CNS tissue of a BALB/c mouse inoculated i.n. with 100,000 IFU of Mouse pneumonitis *in vivo* passaged (A and C) or Mouse pneumonitis stock strain (B and D) immunolabeled with 6E10 amyloid-specific antibody. Bar = 50 microns.

Section Location (mm)	Bregma 0.00	-1.28	-2.75	-3.80	Caudal -4.82	Total	Total/Mouse
MoPn in vivo Day 60 p.i							
Titer Negative Infected Mice (n=2) *Cmu Ag*	0	4	11	7	-	22	11
Amyloid	0	3	2	1	-	6	3
Titer Positive Infected Mice (n=2) *Cmu Ag*	0	5	0	23	20	48	24
Amyloid	0	5	-	7	44	56	28
MoPn stock strain Day 60 p.i							
Titer Negative Infected Mice (n=1) *Cmu Ag*	-	0	0	7	-	7	7
Amyloid	-	0	0	2	-	2	2
Titer Positive Infected Mice (n=3) *Cmu Ag*	4	8	0	1	4	17	5.67
Amyloid	1	12	2	3	7	25	8.33

***Chlamydia* (Cmu) Antigen and Amyloid Deposition**
Location of *C. muridarum* immunoreactivity and amyloid deposition at day 60 post infection within the brains of mouse pneumonitis in vivo passage and mouse pneumonitis stock strain infected mice. The location of the immunoreactive label to amyloid or chlamydial antigen is designated in millimeters (section location in mm) rostral or caudal to the mouse bregma. The dash mark (-) indicates that no data was collected.

Conclusions

1.Substantial chlamydia and amyloid labeling at the 2 month time point following intranasal infection with *Chlamydia pneumoniae* suggests that the chlamydia infection induces amyloid pathology.

2.Amyloid deposition at the 2 month time point in mice intranasally infected with *Chlamydia muridarum* demonstrates that infection with this bacterium induces amyloid pathology.

3.The substantial increase in amyloid deposition and chlamydia labeling observed following infection with the C Small Weiss (MoPn *in vivo* passage) strain differs from the slight increase in amyloid labeling detected following infection with the mouse pneumonitis strain (MoPn stock strain) suggests that the degree of pathology induced following infection varies based upon the isolate of chlamydia introduced.

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