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Chlamydia Pneumoniae as an Etiologic Agent for Late-Onset Alzheimer's Disease

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Abstract. Sporadic, late-onset Alzheimer's disease (LOAD) is a progressive neurodegenerative disease that is now the most common and severe form of dementia in the elderly. That dementia is thought to be a direct result of neuronal damage and loss associated with accumulations of abnormal protein deposits in the brain. Great strides have been made in the past 20 years with regard to understanding the pathological entities that arise in the AD brain, both for familial AD (∼5% of all cases) and LOAD (∼95% of all cases). The neuropathology observed includes: neuritic senile plaques (NSPs), neurofibrillary tangles (NFTs), neuropil threads (NPs), and often deposits of cerebrovascular amyloid. Genetic, biochemical, and immunological analyses have provided a relatively detailed knowledge of these entities, but our understanding of the "trigger" events leading to the biological processes resulting in this pathology and neurodegeneration remains limited. For this reason, the etiology of AD, in particular LOAD, has remained elusive. However, a number of recent and ongoing studies have implicated infection in the etiology and pathogenesis of LOAD. This review focuses specifically on infection with *Chlamydophila* (*Chlamydia*) *pneumoniae* in LOAD and how this infection may function as a "trigger or initiator" in the pathogenesis of this disease.

Keywords: Alzheimer's disease, amyloid, *APOE*, *Chlamydia pneumoniae*, etiology, infection, LOAD, neuroinflammation

INTRODUCTION

The idea that idiopathic chronic diseases might be caused by, or exacerbated by, microbial infection has long been a focus of attention in modern western medical thought. For one example, early in the 1900's rheumatoid arthritis was considered to be an infectious disease, an explanation which faded in the 1930's but re-emerged over the course of the century [1–4]. Similarly, the development of multiple sclerosis is generally agreed to involve an infectious component [5], and congruent arguments have been proposed in relation to several other idiopathic chronic diseases. In all cases, infectious etiology and/or involvement in chronic disease initiation have been difficult to establish and thus have not been accepted by either the research or clinical communities. In place of viral, bacterial, or mycological agents, many alternative mechanisms have been examined to explain chronic disease genesis. For example, several large-scale studies have explored the possible genetic bases of rheumatoid arthritis and other chronic clinical entities, including late-onset Alzheimer's disease (LOAD). Not surprisingly, most such studies have indicated that disease development is not attributable to one or a few mutations or gene polymorphisms, as is the case in familial Alzheimer's disease (FAD). Rather, research indicates that disease genesis appears to be multifactorial, resulting from

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complex interactions between environmental factors and genetic background [6, 7].

Many attempts have been made to define associations between infectious agents and LOAD, but none have proved to be either etiologic or exacerbating for LOAD-related neuropathology. Viral pathogens targeted and so far dismissed include measles virus, lentiviruses, adenovirus, and several others [8, 9]. Importantly, studies from a group in the UK have identified herpes simplex virus type 1 (HSV-1) infection as a risk factor for development of AD in people expressing *APOE* ε4 [10, 11] (see also other material in this publication). In addition, various bacterial pathogens, including *Coxiella burnettii* and *Chlamydia trachomatis* have been investigated, but no relationship with AD neuropathogenesis was identified [12]. In earlier reports, we described an association between infection with the intracellular respiratory bacterial pathogen *Chlamydophila* (*Chlamydia*) *pneumoniae* and the genesis of LOAD [13, 14]. In this review, we summarize that work, as well as newer studies published by our group and others that implicate chlamydial involvement in the genesis of LOAD.

THE AMYLOID CASCADE HYPOTHESIS

The single idea that has predominated for almost three decades in studies of the neuropathology of AD is the "amyloid cascade" hypothesis. This hypothesis contends that the generation and deposition of amyloid-β (Aβ) are the critical events underlying neuronal degeneration [15]. It is applicable to FAD, since it is well established that this type of AD is caused by genetic mutations which result in increased amyloid formation and deposition. However, many studies have demonstrated that the etiology of LOAD does not originate from a small number of identical, similar, or other genetic defects, thus calling into question the generality of Aβ as the universal causative factor in AD. Importantly, the neuropathology underlying both FAD and LOAD is essentially identical, indicating that factors other than the genetic lesions underlying the former must exist to explain the neuropathology of the latter. LOAD typically presents in older age; indeed age is the primary risk factor for its development. Further, other risk factors have been proposed, including: atherosclerosis [16], Type 2 diabetes [17], neurotrauma [18], and infection [10, 13, 19]. Thus, a likely scenario for development of LOAD centers on a poorly understood interplay between genetic risk, as exemplified by possession of the *APOE* ε4 allele (see below), and environmental factor(s), including infection.

CHLAMYDOPHILA (CHLAMYDIA) PNEUMONIAE

C. pneumoniae is an obligate intracellular bacterial pathogen of the human respiratory tract that is responsible for community-acquired pneumonia [20]. This organism, like all chlamydial species, infects mucosal surfaces, in this case the lung/pulmonary and nasal mucosa [20–22]. Systemic dissemination of the bacterium from the respiratory tract has been documented [23], and available evidence indicates that the major vehicle for dissemination is the monocyte [24]. Epidemiologic studies show that *C. pneumoniae* is ubiquitous [25]. The organism undergoes an unusual biphasic developmental cycle during normal growth. In the first phase, the elementary body (EB), the infectious extracellular form of the organism, attaches to a target eukaryotic host cell; these are most often epithelial cells, but other cell types can be infected, including astrocytes, microglia, and neurons [14, 26, 27]. The organism then is endocytosed into a cytoplasmic inclusion within which it reorganizes into the metabolically active growth form of the organism, the reticulate body (RB). RB undergo several rounds of cell division, after which most reorganize back to the EB form. Newly-formed EB are released from the host cell *via* lysis or exocytosis to continue propagation of the infection [26].

Many studies have shown that under certain conditions and/or within specific host cell types the organism can and often does alter its biologic state to generate persistent, long-term infections. Chlamydiae undergoing such infections are morphologically aberrant and display an unusual transcriptional profile [28–30]. Importantly, reports indicate that the mechanisms of pathogenesis differ between active and persistent chlamydial infection, and it is in the persistent state that these organisms elicit chronic disease [31, 32]. *C. pneumoniae* has been associated with several chronic pulmonary diseases [33]. Infection with this organism has been associated with a wide array of non-respiratory diseases, including atherosclerosis, inflammatory arthritis, multiple sclerosis, and others [34–37]. While some of these associations remain controversial, the role of this organism in atherogenesis has gained significant credence during the last fifteen years [36, 38, 39].

C. PNEUMONIAE AND ALZHEIMER'S DISEASE

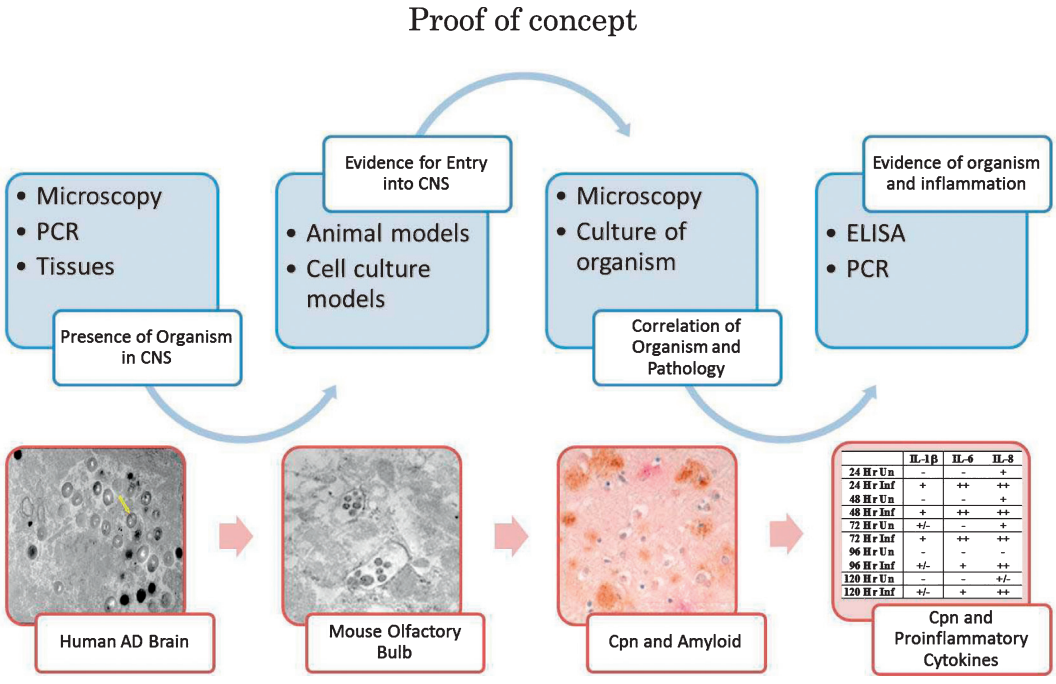
We first demonstrated DNA of *C. pneumoniae* in 90% of postmortem LOAD brain samples examined using specific polymerase chain reaction assays (PCR) [13, 14]; 5% of postmortem, age-matched, non-AD, control brain samples contained that DNA. Brain tissues from areas that typically display characteristic AD neuropathology were analyzed, including temporal cortex, hippocampus, parietal cortex, and pre-frontal cortex. Areas less often showing AD pathology, e.g., cerebellum, were included. In 17/19 LOAD brains, positive samples were obtained from at least one area with neuropathology, and in four cases, from the cerebellum. In the latter brains, severe neuropathology existed throughout, including the cerebella; in the two LOAD brains that were PCR-negative, mild pathology was observed [13]. Samples from PCR-positive brains were analyzed by immunohistochemistry and electron microscopy as well. LOAD samples contained *C. pneumoniae* antigens, particularly in the temporal cortex, hippocampus, parietal cortex, and pre-frontal cortex; perivascular macrophages, microglia, and astroglia all were immuno-positive for the organism. Electron microscopy of LOAD brain samples revealed chlamydial inclusions containing EB and RB. Immunoelectron microscopy demonstrated labeling of the organism with a monoclonal antibody to an outer membrane protein [13, 40]. Immunoelectron microscopy was negative in comparable PCR-negative control sections.

Frozen brain samples were analyzed by reverse transcriptase-PCR (RT-PCR) to determine if intact bacterial RNA was present. This analysis successfully targeted messenger RNA encoding the KDO transferase and a ∼376 kDa protein specific to *C. pneumoniae*. Homogenates of representative PCR and RT-PCR positive samples were prepared and incubated with THP-1 cells in culture. Recovery of viable bacteria was successful from two different AD brains and negative from two control brains [13]. Thus, *C. pneumoniae* DNA and antigens were present in areas of neuropathology and the organism remained viable within frozen AD brain tissues. Additional analyses revealed that 11 PCR-positive samples had at least one allele for the *APOE* ε4 isoform (64%), consistent with that allele type being a risk factor for development of LOAD [41; see also below]. Importantly, a separate study in individuals with reactive arthritis showed that in patients who had *C. pneumoniae* DNA in their synovial tissues, 68% had at least one copy of the *APOE* ε4 allele. These observations implicated a relationship between the *APOEε4* allelic genotype and *C. pneumoniae*, and that together both factors confer increased risk for chronic disease genesis [13, 42].

Our initial studies and the implications of bacterial infection in the genesis of LOAD (see Fig. 1. Proof of Concept) led other groups to attempt identification of *C. pneumoniae* in tissue and other samples from patients with LOAD. Those studies provided mixed results, with some reports giving positive identification [43, 44], and others failing to find DNA or antigens from the organism in relevant samples [45–48]; many different techniques were used in these studies, with no other study using identical methodology to our own. In a previous review of the literature from other areas in which *C. pneumoniae* was implicated as a factor in disease genesis, discrepancies in analytical methods used among laboratories, and the variable data resulting from them, were pointed out [49]. It is clear that multiple reasons for these discrepancies are apparent.

We later extended our studies using new tissues from LOAD and non-LOAD control brains [14]. PCR analysis in multiple assays targeting two *C. pneumoniae* genes revealed that tissues from 20/25 LOAD brains, and from 3/27 non-LOAD control brains, were PCR-positive [14]. The organism was cultured from LOAD brains, and various chlamydial transcripts from additional LOAD brains, demonstrated the viability and metabolic activity of the organisms in those samples. Immunohistochemical analyses revealed that astrocytes, microglia, and ∼20% of neurons were infected by *C. pneumoniae*. The finding of a large proportion of neurons PCR-positive for the organism in this later study was unique [14]. As in our initial study, infected cells were located in close proximity to both neuritic senile plaques and neurofibrillary tangle-containing neurons in the brain [13, 14]. These observations suggest a direct effect of *C. pneumoniae* on neuronal cell injury/death, as well as on the potential for the organism to act as a perpetrator/initiator of granulovacuolar degeneration in the LOAD brain.

Further analyses showed that intracellular and extracellular labeling for *C. pneumoniae* was present in the entorhinal cortex, the hippocampal formation, and the frontal cortex of all AD brains [50]. Serial sections from these areas exhibited both amyloid pathology and *Chlamydia* immunoreactivity in apposition to one another. Staining with



	IL-1β	IL-6	IL-8
24 Hr Un	-	-	+
24 Hr Inf	+	++	++
48 Hr Un	-	-	+
48 Hr Inf	+	++	++
72 Hr Un	+/-	-	+
72 Hr Inf	+	++	++
96 Hr Un	-	-	-
96 Hr Inf	+/-	+	++
120 Hr Un	-	-	+/-
120 Hr Inf	+/-	+	++

Fig. 1. Experimental protocols for proof of concept studies of *Chlamydia pneumoniae* in Alzheimer's disease. Using light and electron microscopy, molecular biology techniques (PCR and RT-PCR), and culturing of human brain tissues, *Chlamydia pneumoniae* was identified from Alzheimer brain tissues. Evaluation of the entry of *Chlamydia pneumoniae* into the CNS utilized animal models and *in vitro cell* culture models to demonstrate that olfaction and leukocyte infection resulted in CNS infection. Further analysis of both human AD brains and animal brains using a variety of microscopic and culture methods revealed the relationship between the presence of the organism and pathology characteristic of AD. Inflammation was determined to be one mechanistic process leading to damage in the CNS following infection using protein (ELISA) and molecular (mRNA microarrays) techniques. AD, Alzheimer's disease, PCR, polymerase chain reaction, RT-PCR, reverse transcriptase-polymerase chain reaction, CNS, central nervous system, ELISA, enzyme linked immunosorbent assay, mRNA, messenger ribonucleic acid.

Thioflavin S and anti-*C. pneumoniae* antibodies on the same sections revealed fibrillary amyloid and chlamydial immunoreactivity, respectively. Two extracellular patterns of chlamydial immunoreactivity were observed: a punctate pattern and an amorphous foci pattern. These likely represent extrusion of whole organism (punctate) or secreted chlamydial products, e.g., lipopolysaccharide (amorphous foci) [51, 52]. Amyloid has been shown to have anti-microbial properties, possibly allowing it to act as an anionic defensin [53–55]. These observations suggest that *C. pneumoniae* has a tropism for these brain regions, and that infection at these sites may be a precursor or trigger for development of damage. The olfactory structures, the entorhinal cortex, and the hippocampal formation are regions demonstrating the earliest damage in AD [56, 57]. The organism has been demonstrated in both human and animal olfactory bulbs [13, 58, 59]; in animals, the organism appeared to spread centrifugally from the olfactory bulbs into the brain proper [58–60]. Together, these and other observations given below support the idea that infection by this pathogen is an early event involved in the triggering of pathogenesis, and not a consequence of prior damage providing access of infection to the CNS.

C. pneumoniae is a respiratory pathogen, and this route for CNS infection is supported by studies in which the organism isolated from an AD brain was more closely related to respiratory than to atherosclerotic strains. As indicated, we prepared cultures of *C. pneumoniae* from LOAD brain tissues and determined molecular genetic and cell biological characteristics for two of them. Both isolates were genetically diverse (i.e., not clonal), as with most respiratory isolates [61]. Analyses for single nucleotide polymorphisms (SNPs) around the chromosome indicated several differences from standard respiratory isolates and strains, but we did not identify genetic attributes indicating a neurotropism. Recent full genome sequencing on one of the isolates confirmed this initial observation [62]. Cell biological

studies demonstrated standard inclusion morphology and chlamydial morphology of organisms of both isolates in human epithelial cells (HEp-2), astrocytes (U-87 MG), and microglial cells (CHME-5), as in our previously published studies [27].

C. PNEUMONIAE, *APOE*, AND LOAD

Several chronic diseases with which *C. pneumoniae* infection has been associated also are associated with possession of the ε4 allele type at the *APOE* locus on human chromosome 19 [5]. We provided evidence of a relationship between possession of that allele and infection with *C. pneumoniae* in LOAD. *In situ* hybridization analyses indicated that the number of *C. pneumoniae*-infected cells in affected brain regions of ε4-bearing LOAD patients was higher than that in congruent brain regions from LOAD patients lacking that allele [63]. Real time PCR analyses of brain tissues targeting DNA sequences from *C. pneumoniae* showed that the bacterial burden in samples lacking the ε4 allele varied widely, but that samples from ε4-bearing patients had significantly higher bacterial loads than did congruent samples from patients without the allele [63]. Other studies have elucidated the relationship between the *APOE* ε4 gene product, infection by *C. pneumoniae*, and disease genesis in several contexts. In all its forms, apoE is a secreted glycoprotein. Unlike apoE2 and apoE3, apoE4 enhances attachment of *C. pneumoniae* EB to host cells, including astrocytes and microglial cells, by about 3-fold over levels observed in the absence of that allelic product [64]. apoE4 adherent to the chlamydial EB retains its ability to attach to its normal receptor on the surface of host eukaryotic cells, i.e., the LDL receptor and other members of that receptor family [65;APH unpublished observations]. Thus, while we do not understand all details concerning how apoE4-enhanced host cell attachment is accomplished for *C. pneumoniae*, these observations provide a link between infection, apoE4, and clinical entities associated with both, including LOAD.

C. PNEUMONIAE, NEUROINFLAMMATION, AND LOAD

Chlamydia-induced disease is largely a result of immunopathogenesis. Chlamydial infection promotes secretion of proinflammatory cytokines [66]; strong inflammatory responses are engendered by chlamydial lipopolysaccharide (LPS), heat shock proteins, and outer membrane proteins. LPS alone could account for many aspects of LOAD pathology, as studies have shown that *E. coli* LPS, when injected at low dose into the brains of rats, results in inflammation characterized by increased cytokine production and microglial activation [67]. Comparable damage to that found in LOAD was observed in the rat temporal lobe (induction of the amyloid-β protein precursor) suggesting that products of infection produced by an organism, or by the host in response to it, stimulates inflammation leading to LOAD-related neurodegeneration.

In the LOAD brain, inflammation is thought to result from Aβ deposition, which has been advanced as the primary mechanism in LOAD pathogenesis [68]. Trials investigating the effects of non-steroidal anti-inflammatory drugs (NSAIDs) also implicate inflammation as a factor, since some have shown that these drugs can delay onset of LOAD [69]; however, they appear to be ineffective as a therapeutic for the disease. The resident cells in the brain responsible for inflammation are typically microglia and astroglia. Both are activated in the LOAD brain and often are identified in and around amyloid plaques [70]. Microglia and astroglia respond to insult by producing proinflammatory cytokines and reactive oxygen species (ROS). Identification of *C. pneumoniae* in the CNS in both cell types suggests that infection-initiated inflammation may be involved in LOAD neuropathology [13, 14]. We observed infected microglia, astroglia, perivascular macrophages, and neurons in areas of amyloid deposition. Activation of microglia and astroglia in response to infected, activated monocytes could promote increased production of a variety of cytokines and chemokines [71, 72]. Likewise, proinflammatory molecules were significantly higher in supernatant fluids of *C. pneumoniae*-infected murine microglial cells compared with controls [73]. Infected murine astrocytes showed higher levels of MCP-1 and IL-6 compared to controls. Neurons exposed to conditioned supernatant from infected murine microglial cells showed increased cell death compared with mock-infected supernatants; addition of neutralizing antibodies to IL-6 and TNFα to the conditioned supernatant reduced neuronal cell death by ∼50%.

Monocytes are altered with regard to expression of cytokines, apoptosis, and β−amyloid clearance in AD [74–78]. Our observations suggest that transcription of genes encoding inflammatory products changes significantly at 48 hr post-infection by *C. pneumoniae*, and that infected cells maintain

pro-inflammatory cytokine secretion over 5 days, including IL-1β, IL-6, and IL-8 [78]. High levels of IL-1β are correlated with neuroinflammation in the AD brain [79–82]. This cytokine activates nitric oxide synthase that has been implicated in hippocampal neuronal cell death [83, 84]. Further evidence has implicated IL-1β in promotion of the neuronal synthesis of the β-amyloid precursor protein [82]. These observations provide a rationale for triggering events by which Aβ would be a resultant factor in neuropathogenesis, not an initializing event.

Expression of four genes was significantly up-regulated after 48 hr infection in our studies; all encoded products are involved with host defense against bacteria [78]. *DEFB4* encodes a defensin protein with anti-microbial activity which links the innate and adaptive immune responses [85]. Interestingly, the β−amyloid 1–42 peptide in AD has been shown to act as an anionic defensin with antibacterial properties. Another important transcript up-regulated was that encoding inflammasomes. These are associated with toll-like receptors and mediate the response to both extracellular and intracellular pathogens [86]. Independent infection of monocytes with two strains of *C. pneumoniae* resulted in up-regulation of transcripts for two different inflammasome complexes, NLRC4 (IPAF) and AIM2. The former can be activated by type III secretion systems characteristic of *C. pneumoniae* and other gram negative bacteria [87]; this system acts to transfer effector proteins from the bacteria into the cytosol of the host cell, resulting in generation of ROS. ROS are thought to result in assemblage of another inflammasome complex, NLRP3 [88, 89] which also is activated by chlamydial infections [90, 91]. Furthermore, we observed up-regulation of the AIM2 inflammasome transcript, which would result in a complex activated by detection of double-stranded DNA in the cytosol [92, 93].

The transcript encoding MCP1/CCL2, a key chemokine for recruiting monocytes and macrophages, was increased up to 1000 fold following infection of monocytes with *C. pneumoniae;* [78, 94]; CCL2 was the most dramatically altered. This gene product is an important contributor to the neuroinflammatory process observed in AD and is increased in both CSF and plasma from individuals with mild cognitive impairment and AD [95, 96]. CCL2 may alter the blood brain barrier to allow increased monocyte migration into brain tissues, as well as affecting production and clearance of Aβ from the brain [95–97]. Thus, our studies demonstrating increased CCL2 production during *C. pneumoniae* infection in monocytes has implications for AD, since *C. pneumoniae* has been found in both the brain proper and in perivascular monocytes and macrophages [13, 14, 50, 98].

ANTIBIOTIC TREATMENT STUDIES

If infection by *C. pneumoniae* is involved in the genesis of LOAD, then antimicrobial treatment might be a therapeutic approach. A clinical trial has been reported that used a combination approach for treatment of LOAD [99]. The primary outcome was a change in Standardized AD Assessment Scale cognitive subscale (SADAScog) at 6 months. Secondary outcomes included changes in the SADAScog at 12 months and analysis of dysfunctional behavior, depression, and functional status. Results showed less decline in SADAScog score at 6 months in the antibiotic group compared to the placebo group; the SADAScog score at 12 months in the antibiotic and placebo groups was not significantly different. However, the antibiotic group showed significantly less dysfunctional behavior at 3 months, and at 12 months the antibiotic group showed reduced decline in mini-mental status scores. No correlations to change in criteria for *C. pneumoniae* infection were apparent as determined by analysis of serum antibody titers and PCR of blood samples. As with antibiotic trials to assess efficacy in obviating aspects of atherogenesis and cardiovascular disease, the outcome of the LOAD-related antibiotic trial indicated no meaningful efficacy in amelioration of relevant pathogenesis. These failures have been understood to mean that simple, straightforward antibiotic treatment of complex disease entities is not a viable strategy. It remains to be determined whether an antibiotic/anti-inflammatory regimen in at-risk patients or following early diagnosis could be effective.

Approaches other than antibiotic therapy may be helpful in treating AD. *C. pneumoniae* is known to persist in various contexts, and this persistent form is implicated in chronic diseases. One approach is to activate an immune response to eliminate intracellular infection. We used a synthetic peptide, acALY18 derived from an 18-mer sequence of the transient receptor channel protein 1(TRPC1) to treat *C. pneumoniae* infections of monocytes *in vitro* [100]; this peptide activates in part the NLRP3 inflammasome [101]. Using only a low dose of acALY18, only 12% of the cells remained infected at 24 h post-treatment, compared to 90% of cells left untreated

with the peptide [101]. At 48 h post-infection, 26 innate and adaptive immune transcripts were up-regulated in the infected/treated cells compared to infected/untreated cells. These transcripts occurred in four functional groups: (1) cytokines, chemokines, receptors, and signaling molecules; (2) host defense; (3) anti-bacterial response; and (4) modulators of the tissue response to inflammation. Future studies will address specific transcript up-regulation and protein expression leading to eradication of *C. pneumoniae* infection.

ANIMAL MODELS FOR *C. PNEUMONIAE* INFECTION

Previous models of AD have utilized transgenic mice that over-express mutants of presenilin and that for amyloid-β protein precursor [102]. Over-expression of amyloid results in development of amyloid plaques in the brain, paralleling the pathology observed in familial AD. However, these systems do not address the initiating events of LOAD, in which mutations of the amyloid-β protein precursor and presenilin are not present. We developed a non-transgenic animal model to address how infection might play a role in the pathogenesis of LOAD, independent of predisposing genetic factors [58]. This work utilized *C. pneumoniae* (AD brain isolate [13]) infection of naïve BALB/c mice to determine whether it would promote damage in the brain similar to that identified in sporadic LOAD. BALB/c mice are susceptible to a respiratory infection with *C. pneumoniae* (strain AR-39), and can maintain a persistent respiratory infection [103]. We tested the hypothesis that *C. pneumoniae* infection in BALB/c mice could initiate processes that result in the development of AD-like pathology in the brain [58].

Following intranasal infection, *C. pneumoniae* was identified in the olfactory epithelia (chlamydial antigens) and the olfactory bulbs (chlamydial antigens and typical morphology) by both light and electron microscopy [58]. Analysis of pathology in the brain revealed Aβ1–42 deposits that resembled amyloid plaques found in human AD. Activation of astrocytes and co-localization of some of these reactive astrocytes with the amyloid deposits suggested that a cellular inflammatory response was initiated. This response may be due to *C. pneumoniae* or directed against amyloid deposits or soluble amyloid induced by *C. pneumoniae* infection. These findings suggest that Aβ generation is a response to the infectious insult and lend support to the hypothesis that Aβ can act as a "bioflocculant" [104]. Induction of amyloid deposits in the brains of non-transgenic BALB/c mice supports the hypothesis that infection with *C. pneumoniae* can accelerate or induce AD-like pathology and may be a trigger in LOAD pathogenesis.

Further study was initiated to determine if antibiotic intervention following intranasal infection could treat or limit the pathology induced by infection in the CNS [105]. Following intranasal infection with *C. pneumoniae* (strain AR-39), mice were treated with moxifloxacin hydrochloride (Avelox) at days 7–21, 28–42, 56–70, or 84–98 post-infection; sacrifice was at 6 months post-infection with brains analyzed for *C. pneumoniae*, Aβ1–42 deposition (plaques), and astrocyte (GFAP) cellular reactivity. Immunohistochemistry analysis indicated that the organism was still present at 6 months post-infection in olfactory tissues and in the brain. At the earliest time of antibiotic treatment, the number of Aβ1–42 reactive amyloid plaques was equivalent to the level observed in uninfected mice. In the infected mice in which treatment was delayed until 56 days post-infection, the number of amyloid plaques was 8–9-fold higher than baseline; this was comparable to the number found in the brains of infected animals that received *no* antibiotics. These data suggest that early antibiotic intervention after infection is effective in limiting the amyloid plaques that arise as a result of infection, even though complete eradication may not be achieved. While more studies of early antibiotic treatment are underway, these results suggest that early intervention may be the most effective in limiting amyloid deposition.

RECENT ANIMAL MODEL STUDIES

Our recent studies used the AR-39 strain for infection, rather than *C. pneumoniae* isolates from the human brain. Brains were analyzed at 1–4 months post-infection by immunohistochemistry with *Chlamydia*-specific antibodies and antibodies specific for Aβ-amyloid 1–42 [106]. As in our report utilizing the brain isolate, no substantial amyloid deposits were observed at 1 month, and only limited AD-like pathology was identified at 2 months, post-infection. In contrast to the original study, at 4 months AD-like pathology was diminished; brains resembled those from mock-infected mice, suggesting that pathology had decreased 2–4 months post-infection. Interestingly, analyses

indicated that peak chlamydial burden preceded peak amyloid deposition by 1 month. These data suggest that *C. pneumoniae* infection serves as a primary stimulus for β-amyloid processing and subsequent deposition in brain tissues.

Precedents for infection in the exacerbation of AD-like pathology have been reported for other pathogens in other animal models [107, 108]. Once infection has been controlled, levels of soluble amyloid apparently decrease, resulting in fewer deposits at 3-4 months [109]. In mice infected with the brain isolate, our studies identified β-amyloid deposits as early as 2 months post-infection, with the greatest number of deposits identified at 3 months. AD-like pathology developed progressively as the number and size of amyloid deposits increased. Animal models that mimic sporadic LOAD have been hampered by the lack of understanding of primary factors that promote the early deposition of β-amyloid. However, models utilizing direct injection of microbial products have shown induction of transient amyloid production and deposition [110, 111]. One previous study did not identify substantial AD-like pathology in the brain following infection with a respiratory isolate/laboratory strain of *C. pneumoniae* [112]. The authors of that report noted that discrepancies could be due to the fact that the laboratory *C. pneumoniae* strain used may have different virulence properties than the human AD-brain isolate.

Our data indicate that isolates of *C. pneumoniae* differ in the ability to establish persistent infection and promote progressive neuropathology. A critical issue in development of sporadic LOAD is age, and by extension the age at which *C. pneumoniae* infection occurs. An earlier study from our group suggests that infection in older animals promotes establishment of a brain infection [59]. In other studies, aged C57BL/6 mice as compared to young counterparts had a greater propensity to develop chronic and/or progressive respiratory infections following intranasal infection with *C. pneumoniae* [113]. A heptavalent CTL epitope minigene vaccine conferred equal protection in the lungs of both aged and young mice. The vaccine partially protected against infection spread to the cardiovascular system of young animals but failed to provide protection in aged animals [113]. Our data suggest that vaccine strategies targeting the *C. pneumoniae*-specific CTL response are protective for respiratory infection in both young and old animals; however, the vaccine used was ineffective in preventing dissemination to the cardiosvascular system in aged mice or controlling replication of organism in these tissues [113]. On a related issue, we inoculated a small group of BALB/c mice with strain AR-39 either twice or three times at 30 day intervals, then sacrificed at day 90. Animals inoculated twice displayed 68, and those inoculated 3 times had 177, amyloid deposits (unpublished observations); mice receiving only a single intranasal inoculation showed an average of 17-18 deposits at 3 months post-infection. These preliminary observations suggest that multiple inocula of *C. pneumoniae* exacerbate pathology in the brain, which has implications for multiple exposures increasing risk for generating increased pathology in *C. pneumoniae*-infected AD patients.

CONCLUSIONS, FUTURE DIRECTIONS

It is clear from our observations and those of others summarized here that much remains to be elucidated regarding the fundamental biochemical, cellular, and molecular genetic underpinnings supporting the initiation and development of neuropathology of LOAD, and the possible involvement of *C. pneumoniae* in those processes (see Fig. 2. Cpn Infection Paradigm) Regarding the latter issue, host immune responses that limit or reduce *C. pneumoniae* replication and antigen burden may effectively decrease the organism as a primary stimulus for long-term production of β-amyloid. We suggest that the difference between progressive and non-progressive AD-like pathology is due to as yet uncharacterized differences between/among *C. pneumoniae* strains and the host genetic background in which those infecting strains operate. This implies that there are critically important different virulence factors including tissue tropism among *C. pneumoniae* isolates and strains. Thus, the ability of the organism to enter and persist in the CNS and potentiate a chronic inflammatory response will be critical to its role in the initiation and maintenance of AD pathogenesis. Future studies concerning infectious involvement in elicitation of AD pathology of course must include brains from mild cognitively impaired individuals, as well as additional non-AD cases, to assess when and where *C. pneumoniae* and/or other pathogens enter the brain. These investigations are important, since some control non-AD brains in our studies have shown chlamydial immunoreactivity associated with diffuse amyloid deposition, perhaps suggesting that CNS infection with *C. pneumoniae* is a prodromal event leading to neuronal damage prior to onset of AD.

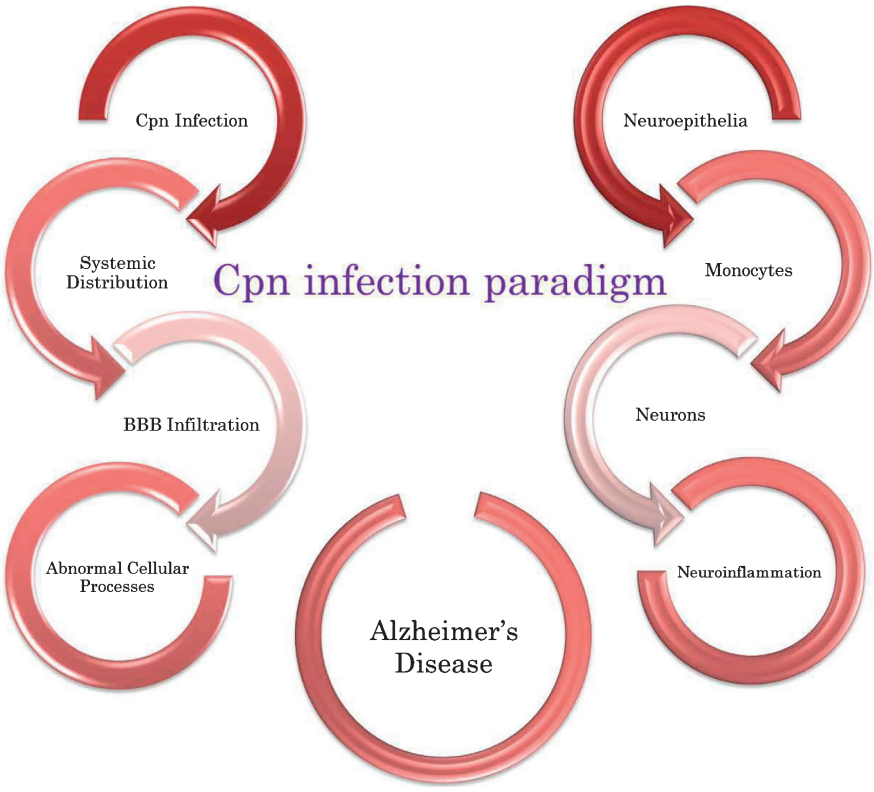


Fig. 2. *Chlamydia pneumoniae* infection paradigm for the etiology of late onset Alzheimer's disease. As a respiratory intracellular bacteria ubiquitous in nature, entry into the human respiratory tract begins following inhalation into the nose and lungs. Olfactory neuroepithelia in the upper nasal passages provides ready access of the infection into the olfactory pathways into the brain highlighting the selective vulnerability of this area. Organism inhalation into the lungs provides access to surveilling monocytes which engulf the organism and traffic back into the systemic circulation. Infection of both olfactory cells and monocytes results in eventual infiltration deeper into the brain where upon endothelial cells, astrocytes, microglia and neurons become infected. Presence of the infection in these cells and in the brain proper results in neuroinflammation and in abnormal cellular processes including the abnormal processing of the β amyloid precursor protein into Aβ amyloid.

We further suggest that, at this point in our understanding of AD, disease definitions usefully can be reconsidered in light of new observations from our group and many other sources. The most obvious neuropathologic aspects, and the disease phenotype, of LOAD are similar to, indeed apparently functionally identical to, those of early-onset disease. However, all studies to date consistently demonstrate that the former does not result from lesions in any of the three genes associated with the latter, and extensive research from many groups over the last thirty years has failed to identify any convincing mechanism by which the plaques and tangles are produced specifically in LOAD patients. Thus we have an etiologic conundrum – if the neuritic senile plaques and neurofibrillary tangles indeed are responsible in a direct manner for the neuronal death and consequent cognitive dysfunction that characterize both forms of the disease, it is not obvious how we can explain the genetic etiology of one but not the other form. One can argue that the plaques and tangles seen in LOAD are not caused directly by overtly pathologic mechanisms, such as those provided by lesions in *APP, PSEN1,* and/or *PSEN2*, but rather result from a gradual accumulation to neuropathologic levels as a function of normal biological/biochemical processes. However, those processes have not been identified to date despite an intense search for them, and in any case it has never been completely clear precisely how the neuritic senile plaques and neurofibrillary tangles underlie the neurotoxicity causing neuronal cell death in either early- or late-onset disease.

Current diagnostic criteria for LOAD add to the etiologic problem. At present, firm diagnosis results only from *post-mortem* examination of brain samples from candidate patients – disease definition is a function of the relative densities of neuritic senile

plaques and neurofibrillary tangles in relevant areas of the brain. The conundrum is made more profound by the fact that many individuals of advanced age show no signs or symptoms of dementia but possess plaque and tangle densities in excess of those accepted as characteristic of late-onset disease; conversely, other individuals tentatively diagnosed with LOAD on the basis of lack of evidence for Lewy-body involvement, vascular causation, or other problems underlying senile dementia can show low plaque and tangle densities [114]. In our view, these and other aspects of characterization and definition for each disease form require a rethinking of how causation is understood for each, and this is particularly imperative in the case of the increasingly prevalent late-onset clinical entity.

We therefore contend, in summary, that current evidence suggests that the early-onset form and the phenotypically similar, non-genetically-based late-onset form of dementia are in fact unrelated diseases. Clearly, early-onset disease has a genetic etiology, although the gene products of the affected genes may not elicit neuropathology exclusively by generation of the well-described plaques and tangles of the amyloid cascade hypothesis, but rather by other more subtle long-term developmental means. We suggest that the plaques and tangles may be an epiphenomenon in both early- and late-onset disease, and that they bear little or no responsibility for neuronal cell death that underlies the cognitive dysfunction. We suggest that, for both historical and biological reasons, early-onset disease can legitimately be designated Alzheimer's disease, but that the designation is not applicable or appropriate to the unrelated LOAD. The etiology of the latter not caused by vascular problems, Lewy body involvement, or other demonstrable origins remains to be elucidated, although it probably is a result of complex interactions between aspects of the genetic background of individuals at issue and any/several of a large number of possible environmental factors to which that individual has been exposed. Perhaps late-onset dementia should simply be referred to by that designation until a more detailed understanding of its causation is provided.

This proposed differentiation of early-onset dementia designated as Alzheimer's disease from late-onset disease is not, in our view, merely a superficial issue of formal categorization. Interestingly, late-onset Alzheimer's disease was classified originally as "senile dementia of the Alzheimer's type", based on endstage pathological findings of plaques and tangles without much understanding of mechanism or associated risk factors. Furthermore, as numerous insults to the brain can result in senile plaques, tangles, and/or other pathologies, to designate late-onset dementia as Alzheimer's disease is clearly inaccurate and thus untenable. Rather, we contend that the etiologies of early onset Alzheimer's disease and that of late-onset dementia are fundamentally different, and that progress in prevention and treatment of the latter, increasingly prevalent disease will be promoted by that distinction.

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