Title
Gram-negative bacterial molecules associate with Alzheimer disease pathology.

Permalink
https://escholarship.org/uc/item/9zr9t8j6

Journal
Neurology, 87(22)

ISSN
0028-3878

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Publication Date
2016-11-01

DOI
10.1212/wnl.0000000000003391

Peer reviewed
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ABSTRACT

Objective: We determined whether Gram-negative bacterial molecules are associated with Alzheimer disease (AD) neuropathology given that previous studies demonstrate Gram-negative Escherichia coli bacteria can form extracellular amyloid and Gram-negative bacteria have been reported as the predominant bacteria found in normal human brains.

Methods: Brain samples from gray and white matter were studied from patients with AD (n = 24) and age-matched controls (n = 18). Lipopolysaccharide (LPS) and E. coli K99 pili protein were evaluated by Western blots and immunocytochemistry. Human brain samples were assessed for E. coli DNA followed by DNA sequencing.

Results: LPS and E. coli K99 were detected immunocytochemically in brain parenchyma and vessels in all AD and control brains. K99 levels measured using Western blots were greater in AD compared to control brains (p < 0.01) and K99 was localized to neuron-like cells in AD but not control brains. LPS levels were also greater in AD compared to control brain. LPS colocalized with Aβ1-40/42 in amyloid plaques and with Aβ1-40/42 around vessels in AD brains. DNA sequencing confirmed E. coli DNA in human control and AD brains.

Conclusions: E. coli K99 and LPS levels were greater in AD compared to control brains. LPS colocalized with Aβ1-40/42 in amyloid plaques and around vessels in AD brain. The data show that Gram-negative bacterial molecules are associated with AD neuropathology. They are consistent with our LPS-ischemia-hypoxia rat model that produces myelin aggregates that colocalize with Aβ and resemble amyloid-like plaques.

GLOSSARY

AD = Alzheimer disease; CERAD = Consortium to Establish a Registry for Alzheimer’s Disease; DAPI = 4’,6-diamidino-2-phenylindole; GM = gray matter; IL = interleukin; LPS = lipopolysaccharide; NSAID = nonsteroidal anti-inflammatory drug; WM = white matter.

Although age is the primary risk factor for the late-onset sporadic form of Alzheimer disease (AD), infection has also been implicated. Infection increases the odds of developing AD by 2-fold over 5 years. Diphtheria, tetanus, and pertussis; polio; tetanus; and influenza vaccines reduce the risk of subsequent AD. Inflammatory molecules including C-reactive protein and interleukin-6 are elevated in blood years before dementia. Though clinical trials show that nonsteroidal anti-inflammatory drugs (NSAIDs) do not affect cognitive decline in AD, some studies suggest that NSAIDs decrease the risk of developing AD. Indeed, recent studies have demonstrated that sporadic late-onset AD is associated with infections. However, a consistent link between these agents and AD neuropathology has not been demonstrated.

Gram-negative bacteria like Escherichia coli can deposit amyloid. Moreover, we showed that administration of Gram-negative bacteria–derived lipopolysaccharide (LPS) followed by ischemia-hypoxia produce plaque-like aggregates of β-amylloid in rat brains. Though Gram-negative bacteria have been reported as the predominant bacteria found in normal human brains, these studies implicate Gram-negative bacterial molecules in AD neuropathology.
the study did not describe pathologic correlates. Because of our finding that LPS promoted formation of amyloid-like plaques in rat brain, we determined whether Gram-negative bacterial molecules were associated with human AD neuropathology.

METHODS Standard protocol approvals, registrations, and patient consents. The institutional review board approved this study. Informed consent was obtained from all participants who were enrolled in studies at the University of California at Davis Alzheimer’s Disease Center.

Brain samples. AD was rated using Consortium to Establish a Registry for Alzheimer’s Disease (CERAD) criteria and Braak stage on 24 AD and 18 age-matched controls. Brain tissue was provided by the Alzheimer’s Disease Center at the University of California Davis. Superior temporal gyrus gray matter (GM) was studied since it is commonly involved in AD and frontal lobe white matter (WM) was studied because it shows abnormalities in AD brains. Controls were age-matched individuals without cognitive deficits. Formalin-fixed brains used for immunostaining included 11 AD and 7 age-matched controls. Frozen brains used for Western blot analysis and PCR for E. coli DNA included 13 AD and 11 age-matched controls.

Frozen tissue (~ 1 cm³) from the superior temporal gyrus GM or frontal lobe WM was removed with sterile technique and frozen at ~70°C. The tissue surface was treated with 75% ethanol and removed with a sterile scalpel blade. Frozen tissue was cored with autoclaved trephine and plated in sterile, endotoxin-free tubes at ~70°C. Formalin-fixed (4%) brains were blocked, embedded in paraffin, and sectioned for immunostaining.

Immunofluorescence. Detailed methods are described in our previous studies. Briefly, after removing paraffin with xylene and rehydrating through graded alcohols, sections were treated with antigen retrieval buffer and autofluorescence elimination reagent (Millipore, Eporbicote, Canada). Primary antibodies were used in 1:200 concentrations unless stated otherwise. Secondary antibodies included either goat anti-mouse or goat anti-rabbit Alexa Fluor 488 or 594 (Invitrogen, Carlsbad, CA). Slide mounting medium included 4',6-diamidino-2-phenylindole (DAPI) to stain nuclei (Vector Laboratories, Burlingame, CA). For controls, primary antibody was deleted or immunodepleted with the target antigen of the antibody.

Western blot analysis. Tissue was homogenized in cold RIPA buffer containing a protease inhibitor mixture. After centrifuging homogenates for 30 minutes at 4°C (14,000 g), the pellet was discarded and proteins in the supernatant separated on 10% sodium dodecyl sulfate polyacrylamide gels. They were then transferred to nitrocellulose membranes and hybridized overnight at 4°C with primary antibodies listed below. Antibodies were stained using horseradish peroxidase conjugated anti-rabbit or anti-mouse immunoglobulin G (Bio-Rad, Hercules, CA) combined with electrochemiluminescence detection (Piers). β-actin staining served as the control. We used NIH Image J software to quantify band intensities.

Primary antibodies were obtained from Abbiotec (San Diego, CA) (β-actin, sc-69879), ThermoFisher (Walnham, MA) (GST-γ, PA526061; Synapsin 2, OOS00020W), and Wako Chemicals (Cape Charles, VA) (Iba1, 019–19741).

PCR for DNA and DNA sequencing. E. coli DNA was detected using PCR amplification of an E. coli glutamate decarboxylase B (gadB) DNA fragment. This DNA fragment was selected because it detects all E. coli strains, and does not detect the human gene. All reagents used for PCR were first tested for E. coli DNA contamination, which was indicated by detection of E. coli DNA in endotoxin free water. Only those reagents that were free of E. coli DNA were used for PCR amplification in human brains. Brain genomic DNA samples were isolated and purified using PureLink Genomic DNA Kits (K1820-01, Life Technologies, Carlsbad, CA). Approximately 20 μg of minced brain tissue from superior temporal gyrus was digested and DNA was eluted and frozen. E. coli DNA from the ATCC 8739 strain (ATCC) was used as a positive control. PCR amplification was performed in a 50 μL reaction mixture, containing 10 ng of DNA, 25 μL of 2 × GoTaq Colorless Master Mix (Promega, Madison, WI), and 0.5 μM primer mix. The reaction mixture were placed in a Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA) with the following settings: 5 minutes at 94°C, followed by 38 cycles of 40 seconds at 94°C, 45 seconds at 57°C, 30 seconds at 72°C, and a final extension time of 7 minutes at 72°C. Amplified products were analyzed in an Agilent (Santa Clara, CA) 2100 Bioanalyzer. Identification of the gadB gene was confirmed by a 3’ and 5’ Sanger DNA sequencing assay on 3 control and 3 AD samples at the UC Davis DNA Sequencing Facility. PCR products were sequenced with BigDye Terminator v 3.1 Cycle Sequencing Kit with Gel Company Better Buffer and postcyclye purification was achieved at a Beckman Coulter (Sharon Hill, PA) Biomek NxP laboratory automation workstation with Beckman-Coulter CleanSEQ magnetic bead purification. Data were analyzed with ABI Prism (Applied Biosystems) 3730 genetic analyzer, ABI Prism 3730 data collection software v 3.0, and ABI Prism DNA sequencing analysis software v 5.2. DNA primers used in this study for PCR amplification and DNA sequencing were forward sequence (5’→3’) CACGTTTTTGGAGAAGTCT and reverse sequence (5’→3’) TTTGTGCACATTCTTCCGTGC (Eurofins MWG, Huntsville, AL). Amplions were 175 bp with ampiclon accession number M84025.1.

Statistical analysis. Differences between groups were analyzed using a Student’s t test (continuous), Wilcoxon-Mann-Whitney test (ordinal), or Fisher exact test (categorical). Differences between multiple groups were analyzed using one-way analysis of variance with Student-Newman-Keuls post hoc test. A p < 0.05 was considered significant.

RESULTS Patient characteristics. There were no significant differences in age or sex between patients with AD and controls (table e-1 at Neurology.org). The differences in median Braak and Braak stage and CERAD plaque scores between AD and control brains were significant (table e-1).

Detection of E. coli K99 and Gram-negative bacterial LPS in AD and control brains. Control samples (n = 10) and AD samples (n = 13) including GM (figure 1A) and WM (figure 1B) were assessed for E. coli K99 pili protein. K99 was present in 9/13 AD GM compared to 1/10 control GM samples (p = 0.006).
and in 10/13 AD WM compared to 4/10 control WM samples ($p = 0.09$). Quantification showed significantly greater amounts of *E. coli* K99 protein in AD GM compared to controls and significantly more K99 in AD WM compared to controls (figure 1C).

In addition, we used Western blots to test the specificity of Gram-negative LPS antibody, which was used for the immunocytochemistry studies described below. The LPS Western blots using the Abcam antibody (figure 1D, upper panel) showed a similar pattern of expression as that seen for the K99 Western blots (figure 1D, lower panel). LPS (Abcam antibody) was detected by Western blots in 3/3 AD GM, 3/3 AD WM, and 0/3 control GM (figure 1D). As a control, we repeated the Western blots of AD brain using another antibody to LPS (RayBiotech) as well as doing a blot after immunoprecipitating the antibody with LPS. The RayBiotech anti-LPS antibody detected a large band at 37 kD and a smaller band at 150 kD (figure e-1), whereas the LPS Western blot analysis of *E. coli* K99 pili protein in gray matter (GM). *E. coli* K99 was detected in 9 out of 13 Alzheimer disease (AD) cases and 1 out of 10 control (C) cases. (B) Western blot analysis of *E. coli* K99 pili protein in white matter (WM). *E. coli* K99 was detected in 10 out of 13 AD cases and 4 out of 10 control cases. (C) Quantification of *E. coli* K99 protein. *E. coli* K99 protein levels in AD brains were greater than in control aged brains in both GM (*p < 0.05*) and WM (**p < 0.01**). (D) Western blot analysis of Gram-negative bacterial LPS. A total of 3/3 AD GM, 3/3 AD WM, and 0/3 control GM showed LPS. Error bars are standard errors of the mean. β-actin was used as a loading control.
immunodepleted antibody showed the 150 kD band only with complete loss of the 37 kD band (figure e-1). This is strong evidence the antibody detects LPS in the 37 kD band, and this is the same molecular weight as the 37 kD large band seen in figure 1D with the Abcam anti-LPS antibody (upper panel).

**Localization of LPS and K99 pili protein in cells in AD and control brains.** *E. coli* LPS (Abcam antibody) and K99 pili protein were detected immunocytochemically in all 11 AD and 7 control brains. LPS was detected in control (figure 2, A and C) and AD brains (figure 2, B and D) in GM (figure 2, A and B) and in periventricular WM (figure 2, C and D). For a control, coimmunoprecipitation of the anti-LPS antibody with LPS completely eliminated all tissue staining by immunofluorescence (figure e-2) or by immunohistochemistry (figure e-3), showing that LPS was being immunostained.

LPS was localized in neurons (figures e-4 and e-5), microglia (figure e-6), oligodendrocytes, and oligodendrocyte progenitor cells (figures e-7 and e-8). LPS colocalized with a nuclear marker, DAPI, in control GM (figure 2E) and WM (figure 2G) including ependymal cells (figure 2G, arrowheads). In AD GM there was LPS staining in what appeared to be amyloid plaques in cortex (figure 2, B and F, white arrow). Smaller LPS-stained structures appeared to be nuclei in GM (figure 2, B and F, yellow arrowheads) and WM (figure 2, D and H). Note that ependymal cells were missing in AD brain (figure 2H, arrowheads) and DAPI was absent in many nuclei that were stained with LPS in WM (figure 2H).

K99 pili protein was associated with neuron-like cells in AD cortex (figure 3B) but not in controls (figure 3A). K99 pili protein was localized to control ependymal cells (figure 3C) that were lost in AD brains (figure 3D, arrowheads). K99 pili protein immunostaining of fiber tracts in AD WM (figure 3D) was greater than in controls (figure 3C).

**Association of LPS with amyloid plaques in AD brains.** LPS positively stained clusters were frequently observed in AD brains (figure e-9, A1, arrow). Within these clusters, DNA stained with DAPI from coalesced cells and was indistinct (figure e-9, A2 and A3, arrows). Moreover, the coalesced DNA colocalized with Ab1-40/42 (figure e-9, B1-3, arrows).

LPS and Ab1-40/42 colocalized in 3 different patterns in AD brains. (1) Clusters composed mainly of LPS particles colocalized with Ab1-40/42 (figure 4A). (2) Ab1-40/42 deposits that colocalized with LPS were surrounded by LPS (figure 4, B and C). (3) The most common pattern showed confluent Ab1-40/42 stained amyloid plaques with scattered LPS particles in them (figure 4D).

LPS-stained plaques were surrounded by astrocytes (figure e-10, B1-4, arrows). In AD cortex, LPS was localized in neurons, microglia, oligodendrocytes, and oligodendrocyte progenitor cells (B1-B4, figures e-4 through e-8).

In contrast, K99 pili protein surrounded small Ab1-40/42 stained amyloid plaques (figure 4, E–G), but was absent around larger amyloid plaques (diameter > 50 μm) (figure 4H).

**Association of LPS and K99 pili protein with blood vessels.** In control brains, both LPS (figure 5A) and *E. coli* K99 (figure 5C) localized to blood vessels that did not stain for Ab1-40/42 (figure 5, A and C). In contrast, LPS and Ab1-40/42 colocalized in vessels walls of AD brains (yellow staining, figure 5B). Ab1-40/42 was sandwiched by but did not colocalize with K99 pili protein in vessels in AD brains (figure 5D, arrowheads).

**E. coli DNA in AD and control brains.** PCR with primers for the *E. coli* glutamate decarboxylase B gene (*gadB*) showed the predicted 175 bp DNA fragment, which was the same size in control and AD GM, as well as in the positive control ATCC 8739 *E. coli* strain (figure e-11). The 175 bp amplicon was detected in 9/10 normal control (figure e-11A) and 9/13 AD brains (figure e-11B). Of these, 1/10 control and 4/13 AD samples showed bands of different sizes (figure e-11) of unknown significance. All samples showed low molecular weight bands that likely represented primer interactions. A megablast (optimized for highly similar sequences; NCBI nonredundant nucleotide database) of the DNA sequences (table e-2) of the amplicons from the qPCR reactions showed that DNA from the 3 human controls (table e-2) was 100% identical to 115 *E. coli* strains/substrains/entries in the database, and to 5 *Shigella* strains. The DNA sequence from one of the AD samples (table e-2) was 100% identical to 7 strains/substrains/entries of *E. coli*, while the DNA from the other AD sample (table e-2) had one nucleotide mismatch from 116 *E. coli* strains/substrains/entries, and one mismatch (99.4% identity) from the same 5 *Shigella* strains as the other AD sample. One AD sample (AD1), negative on quantitative PCR, was also negative upon sequencing.

**DISCUSSION** We demonstrate Gram-negative bacterial LPS, *E. coli* K99 pili protein, and DNA in control and AD brains. K99 and LPS levels were greater in AD compared to control brains. Moreover, LPS colocalized with Ab1-40/42 in amyloid plaques and with Ab1-40/42 around blood vessels in AD brains. These data suggest that Gram-negative bacterial molecules are associated with AD pathology.
Though the monoclonal antibodies were raised to \textit{E. coli} molecules, they may not be specific for \textit{E. coli}. PCR showed appropriate size bands for \textit{E. coli} DNA in the majority of AD and control brains. Sequencing the bands showed that though most 100% identical hits were for \textit{E. coli}, there were 100% identical hits for

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**Figure 2** Immunofluorescence of lipopolysaccharide (LPS) in human brains

Immunocytochemistry for LPS showed staining in both control (A, C) and Alzheimer disease (AD) brains (B, D) in gray matter (GM) (A, B) and in periventricular white matter (PVWM, C, D). LPS colocalized with 4',6-diamidino-2-phenylindole (DAPI)-stained nuclei in control GM (E) and WM (G) including ependymal cells (G, arrowheads). The patterns of control LPS staining in the DAPI-stained nuclei varied (E; yellow, green, and white arrowheads). In AD GM, there were large foci of LPS staining that appeared to be plaques in cortex (Ctx) (B, F; white arrow), and other LPS-stained structures that appeared to be the size of nuclei in GM (B, F; yellow arrowheads). LPS staining was detected in control (C, G) and AD (D, H) WM. DAPI staining of nuclei was decreased in AD Ctx (F) and in WM (H) including ventricular ependymal cells (H, white arrowheads), likely indicating cell loss. LPS was detected in all 11 AD brains and all 7 control brains by immunofluorescence. Bar = 25 μm.
a related Gram-negative family member, *Shigella*, which is due to the sequence homology between the 2 species. Thus, the data support the presence of proteins and DNA from *E. coli* or Gram-negative bacteria related to *E. coli*.

Our findings complement a recent RNAseq study that showed bacterially encoded 16S RNA sequences in all human brains with Gram-negative *Proteobacteria* representing over 70% of bacterial sequences. The other 30% of bacterial classes varied widely, with *γ-Proteobacteria*, including *E. coli*, being present at <5% of total bacterial transcripts found. Thus our data support this previous study.

A major question arises as to how Gram-negative bacterial LPS, proteins and DNA in this study, and *α-Proteobacteria* molecules or Gram-positive bacterial peptidoglycan in other studies enter brain. In our LPS/ischemia/hypoxia animal model, we showed that LPS increased in rat brain over a period of 3 months after a single intraperitoneal injection that was associated with a parallel increase in IL-1β and granzyme B in brain. We postulated that IL-1β-producing monocytes and granzyme B–producing cytotoxic T cells or natural killer cells phagocytized the LPS in the periphery via Toll 4 receptors and carried LPS into brain. Gamma *Proteobacteria enterobacteriaceae* like *E. coli*, and related family members like *Salmonella* and *Shigella*, cause diarrheal illness in humans, and some strains of *E. coli* are resident in the gastrointestinal tract. The virulence of enterotoxigenic *E. coli* that cause diarrhea are dependent on production of adhesins and enterotoxins, and the *E. coli* K99 pili protein found in control and AD brains is one of the surface antigens in enterotoxigenic (diarrhea) *E. coli* adhesive pili. It is tempting to speculate that this study provides another example of a gut-to-brain connection with gut being one possible source of brain *E. coli* supporting the concept of AD being a systemic disease.

*E. coli* from urinary tract and other infections could also be a source for brain bacterial molecules. Finally, since LPS derived from Gram-negative bacteria injures the blood–brain barrier, this could promote entry of LPS and other bacterial molecules into the brain.

Previous studies show that sporadic late-onset AD can be associated with infection. Infectious agents previously associated with AD include *Spirochetes*, *P. gingivalis*, *Borrelia burgdorferi*, *Chlamydia pneumoniae*, *Helicobacter pylori*, *C. glabrata*, various fungi, herpesviruses, and cytomegalovirus. A major difference between previous studies and this
one is that LPS colocalized with amyloid plaque and with perivascular amyloid in every AD brain.

LPS is the major component of the outer membrane of Gram-negative bacteria. Gram-negative bacteria include α-Proteobacteria found in human brain and include γ-Proteobacteria like E coli, molecules of which were found in brain in this study. LPS was colocalized with Aβ1-40/42 in amyloid plaques.
and with perivascular \( \text{A} \beta_1-40/42 \) in all AD brains. In our recent animal study, systemic LPS combined with cerebral ischemia/hypoxia produced aggregates of myelin that colocalized with \( \text{A} \beta_1-40/42 \) in adult rat brains and the aggregates had features of amyloid plaques. These animal data, combined with the current human brain data, suggest the possibility that LPS in combination with other factors could cause AD neuropathology.

LPS and \( E.\text{coli} \) K99 pili protein in vessels and ependymal cells could contribute to vessel injury and ependymal injury and WM injury observed in AD brains. There was more K99 pili protein in GM and WM of AD brains, and both WM and GM are consistently damaged in AD. However, the current human data cannot determine if the bacterial molecules are a cause or consequence of the injury to AD brain.

There are limitations to this study. \( E.\text{coli} \) could contaminate tissue samples. We used sterile techniques, sterile solutions, and sampled the core of frozen tissue blocks to reduce contamination. Evidence against \( E.\text{coli} \) contamination includes the finding that LPS and \( E.\text{coli} \) K99 staining patterns in control and AD brains were different, the staining patterns were consistent in all brains, and levels of K99 and LPS were higher in AD compared to control brains. Studies of brain biopsies obtained during life would help address the possibility of contamination during autopsy, however.

The current study did not test whether there are live bacteria in human brain. However, a recent study found evidence for live bacteria in brain and supports the idea that every organ, including brain, has its own microbiome. Since this study focused on \( E.\text{coli} \) proteins and DNA, future studies will be needed to address the potential for other bacteria in AD pathogenesis.

**AUTHOR CONTRIBUTIONS**

Xinhua Zhan designed the studies, collected data, and wrote the manuscript. Boryana Stamova designed the studies, performed statistical analysis, and made changes to the manuscript. Lee-Way Jin provided brain samples, reviewed samples for correct diagnosis, and made changes to the manuscript. Charles DeCarli provided brain samples, reviewed samples for correct diagnosis, and made changes to the manuscript. Brett Phinney analyzed data and made changes to the manuscript. Frank R. Sharp designed the studies, wrote the manuscript, and made changes to the manuscript.

**ACKNOWLEDGMENT**

The authors thank the Alzheimer’s Disease Center of University of California for providing brain samples.

**STUDY FUNDING**

This study was supported by NIH/NINDS grant RO1 AG042292 (F.R.S., C.D.), ADC grant AG P30 10129 (C.D.), and grants from The Rotary...
Neurology 87 November 29, 2016

DISCLOSURE
The authors report no disclosures relevant to the manuscript. Go to Neurology.org for full disclosures.

Received March 8, 2016. Accepted in final form August 24, 2016.

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