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Molecular and immunological evidence of oral *Treponema* in the human brain and their association with Alzheimer's disease

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The purpose of this investigation was to use molecular and immunological techniques to determine whether oral *Treponema* infected the human brain. Pieces of frontal lobe cortex from 34 subjects were analyzed with species-specific PCR and monoclonal antibodies. PCR detected *Treponema* in 14/16 Alzheimer's disease (AD) and 4/18 non-AD donors ($P < 0.001$), and AD specimens had more *Treponema* species than controls ($P < 0.001$). PCR also detected *Treponema* in trigeminal ganglia from three AD and two control donors. Cortex from 15/16 AD subjects and 6/18 controls contained *Treponema pectinovorum* and/or *Treponema socranskii* species-specific antigens ($P < 0.01$). *T. pectinovorum* and/or *T. socranskii* antigens were also found in trigeminal ganglia and pons from four embalmed cadavers, and 2/4 cadavers also had *Treponema* in the hippocampus. These findings suggest that oral *Treponema* may infect the brain via branches of the trigeminal nerve.

Key words: Alzheimer's disease; immunohistochemistry; oral *Treponema*; PCR

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Miklossy observed spirochetes in blood, cerebral spinal fluid and brain from 14 people with Alzheimer's disease (AD), but not in material from 13 age-matched controls. She isolated spirochetes from the blood of four AD donors (8), and concluded that their fine structure was consistent with *Treponema* or *Borrelia* (9). McLaughlin et al. (7) detected unidentified spirochetes in the blood of 1/6 living patients with late-stage AD, but spirochetes were not observed in blood from 16 patients with early-stage AD or in brain from seven AD donors. It was suggested that spirochetes in blood might have derived from sites of periodontal disease (7). This suggestion raised the possibility that oral *Treponema* might infect the central nervous system of people with AD. The purpose of this investigation was to determine whether *Borrelia burgdorferi* or any of seven species of oral *Treponema* could be detected in human brain from donors who had AD and from non-AD controls. In addition, the possibility that oral *Treponema* might reach the brain through the trigeminal nerve was explored.

Materials and methods

Human tissues

Group 1

Frontal lobe cortexes from 16 donors with AD (55–87 years of age, eight males) and 18 controls (64–88 years, 10 males) were obtained from the Oregon Brain Bank (Oregon Health and Sciences University, Portland) through an approved protocol (IRB #5712 Exempt). Each donor was represented by 6–10 frozen pieces of cortex, and each piece weighed approximately 0.25 g. The precise anatomical origin of individual species was not known. Specimens were stored at −80°C.

Group 2

Frozen trigeminal ganglia (TG) from three AD donors (female, 48 years of age, with familial AD; female, 102 years of age, with severe AD; male, 86 years of age) and two controls (male, 44 years of age; female, 64 years of age) were obtained from the Oregon Brain Bank.

Group 3

TG, pons and hippocampus were removed from four human cadavers that had been embalmed with a solution containing (v/v) 33% methanol, 33% glycerin, 28% phenol and 6% formalin (IRB #6205 Exempt). One female (age 84 years) had AD and one female (age 89 years) died of unknown causes. Two males (ages 54 and 83 years) died of cancer.

Group 4

Whole unstimulated saliva was collected from 17 living patients with AD (ages 61–84 years, nine males) and from 16 non-AD control subjects (ages 52–88 years, 12 males) (IRB #5372).

Polymerase chain reaction (PCR)

Thawed pieces of cortex (group 1) and TG (group 2) were cut into smaller pieces of 25–50 mg and DNA was extracted from each piece (Qiagen #69506, Valencia, CA). DNA was also extracted from embalmed tissue (group 3) according to instructions for fixed tissues (Qiagen). Samples of AD and control specimens were extracted at the same time. Each extract was tested for *B. burgdorferi* and oral *Treponema* by amplifying species-specific 16S rRNA gene sequences (21), using spirochete-selective primers C75 and C90 (11) in the first reaction. Each PCR experiment included AD and control DNA, a positive DNA template, and a no-DNA control. PCR products were isolated by electrophoresis in 1% agarose gel, and authentic products were identified by comparison to positive controls and by reference to DNA standards. A subject was considered positive for a given species if at least one extract was positive in repeated assays.

A volume of 250 µl of each saliva sample (group 4) was centrifuged at $8944 \times g$ for 10 min at 4°C and DNA was extracted from each sediment. Another 250 µl was inoculated into synthetic media (15) and cultivated for 7 days to amplify viable *Treponema*. One ml from each culture was sedimented, DNA was extracted, and PCR was used to identify spirochetes.

PCR controls

To determine whether *Treponema* DNA was derived from the laboratory, extractions were performed without tissue or bacteria, and sham extracts were tested with PCR. In addition, PCR was performed using material from surfaces of instruments and devices, benches, the floor, walls, handles of refrigerators and freezers, tubes containing positive template, and tube racks. Reagents and disposable supplies were also examined as a source of DNA. Ambient air was sampled by leaving open PCR tubes containing all reagents but template. Research personnel were examined as a source of spurious DNA by sampling breath (over open PCR tubes), saliva, clothing and hair.

Sequencing of PCR products

The veracity of *Treponema* species-specific DNA amplification was tested by sequencing PCR products. Randomly selected PCR products representing each *Treponema* species and *B. burgdorferi* were purified (Concert, Gibco, Grand Island, NY, BRL #I1456-019) and cloned according to manufacturer's instructions (Topo-TA, Invitrogen, Carlsbad, CA #K4575-40). Inserts were isolated (QIA prep, Qiagen #27104) and sequenced (OHSU Vollum Institute Sequencing Core). Randomly selected clones from two subjects were chosen to represent each species-specific PCR product. Clone sequences were aligned using Sequencer 3.1 (Gene Codes Corporation, Ann Arbor, MI) and compared to known sequences in GenBank using the Basic Local Alignment Research Tool (National Center for Biotechnology Information, Bethesda, MA), and per cent homology was calculated.

Monoclonal antibodies (mAbs)

Molecular detection of *Treponema* DNA was verified by immunological detection of species-specific antigens, and the histological location of *Treponema* was determined by means of immunohistochemistry. Mouse monoclonal IgGs against *Treponema socranskii* subspecies *buccale, T. socranskii* subspecies *socranskii*, and *Treponema denticola* serovar antigens were provided by L. Simonson (Naval Dental Research Institute, Great Lakes, IL). These mAbs have proved to be sensitive and specific (14). Monoclonal antibodies against a species-specific surface protein on *Treponema pectinovorum* were produced in our laboratory. Adsorption with *T. pectinovorum*, but not *Treponema vincentii*, removed all activity against whole cells and tissues.

Immunohistochemistry

Thawed pieces of cortex (group 1) were fixed in formalin for 48 h. Fixed cortex and tissues from embalmed cadavers (group 3) were dehydrated and embedded in paraffin, and 10-µm-thick sections were de-waxed. Reactivity of mAbs was disclosed as previously described (16), except that avidin-peroxidase (Kirkegaard & Perry Laboratories (KPL) #71-00-38, Gaithersburg, MD) and TrueBlue chromagen (KPL #54-78-00, includes orcein which produces a red counterstain) were used to disclose immune complexes, and slides were mounted with Permount (Fisher #SP15-100, Fairlawn, NJ). *T. pectinovorum* mAbs were diluted 1 : 100.

Significance of differences between AD and control data

The proportions of AD and control donors who had *Treponema* in the brain were compared using χ^2. The significance of the difference between AD and controls, ranked by the number of species detected by PCR in individual pieces of brain cortex, was determined by the Mann–Whitney *U*-test. The percentage of extracts from each piece of cortex that were positive for any *Treponema* was calculated for each subject, and the significance of the differences in per cent positive extracts between AD and controls was determined by the Mann–Whitney *U*-test.

Results

Pcr

No source of extraneous DNA from the laboratory could be detected. No PCR activity was found using DNA extracted from embalmed tissue (group 3), even when spiked with positive template (data not shown). No positive PCR reactions were obtained using specific primers for *Actinobacillus actinomycetemcomitans*, *Chlamydia pneumoniae*, *Eikenella corrodens*, *Porphyromonas gingivalis* or *Prevotella intermedia* (data not shown).

Group 1

Table 1 summarizes PCR data using DNA from frozen brain cortex. 14/16

Table 1. Prevalence of spirochetes in human brain cortex determined by PCR and mAbs[1]

Group	*B. burgdorferi*	*T. amylovorum*	*T. denticola*	*T. maltophilum*	*T. medium*	*T. pectinovorum*	*T. socranskii*	*T. vincentii*	No. subjects
Control $N=18$	1	0	2	1	0	1 (3)	1 (3)	0	4 (7)
AD $N=16$	5	5	8	5	6	8 (10)[2]	7 (13)[2]	0	14 (15)[3]
Total $N=34$	6	5	10	6	6	9	8	0	18 (21)

[1]mAbs were not available for *T. amylovorum*, *T. maltophilum*, *T. medium* or *T. vincentii*. Tissues were not tested for *B. burgdorferi* antigens. *T. denticola* mAbs did not react with any tissue. Numbers in parentheses indicate total *T. pectinovorum* or *T. socranskii* detected by PCR and/or mAbs.

[2]Proportion of AD subjects with *Treponema* was significantly greater than that of control subjects for *T. pectinovorum* (10/16 vs. 3/18, $\chi^2 = 5.719$, $P < 0.02$) and *T. socranskii* (14/16 vs. 3/18, $\chi^2 = 11.71$, $P < 0.001$).

[3]Difference in proportion between control subjects (4/18) and AD subjects (14/16) with *Treponema* detected by PCR was significant ($\chi^2 = 11.99$, $P < 0.001$), including detection with PCR and/or mAbs (7/18 vs. 15/16, $\chi^2 = 8.89$, $P < 0.01$).

AD donors had one *Treponema* species compared to 4/18 controls ($\chi^2 = 11.99$, $P < 0.001$). AD subjects had a greater percentage of positive extracts from a single piece of brain than did controls ($U = 17$, $P < 0.001$). Neither *Treponema amylovorum* nor *Treponema medium* was found in control samples, and *T. vincentii* was not detected in any sample. *B. burgdorferi* was detected in five AD subjects and one control, all of which also had *Treponema*. Not every extract contained template, but positive PCR results were reproducible. Sequences of PCR products matched published sequences (homology range 98–99%).

Of the four control subjects with *Treponema*, one subject had two species and three subjects had only one species each. In contrast, one AD subject had six species, four had five species, one had four species, one had three species, and seven subjects had one species each ($U = 11$, $P < 0.001$).

Group 2

Treponema were detected by PCR in all five TG. All three AD subjects had *Treponema maltophilum*, two also had *T. medium*, and one also had *T. denticola* and *T. socranskii*. One control TG contained *T. medium* and the other control contained *T. denticola*.

Group 4

There were no meaningful differences in prevalence of oral *Treponema* in saliva from living AD patients and control subjects (Table 2).

Immunohistochemistry

Group 1

The reactivity of mAbs with cortex is summarized in Table 1. *T. denticola* mAbs did not react with any tested specimen, and tissues were not examined for *B. burgdorferi* antigens.

Controls

T. pectinovorum mAbs did not react with tissue from the one control that had been PCR-positive for *T. pectinovorum*, but they did react with tissue from two controls that had been PCR-negative for *T. pectinovorum* (one was PCR-positive for *T. maltophilum* and one was PCR-negative for any *Treponema*). *T. socranskii* mAbs reacted with antigen in cortex from the one control that had been PCR-positive and also reacted with cortex from two donors that had been PCR-negative for any *Treponema*. In total, mAbs against these two species identified three additional controls with *Treponema*, so that, considering both PCR and mAb data, 7/18 controls had *Treponema* in brain cortex.

AD donors

T. pectinovorum mAbs reacted with antigen in cortex from 4/8 AD subjects that were PCR-positive for *T. pectinovorum*, and with cortex from 2/8 AD donors that had been PCR-negative for *T. pectinovorum*. Thus, a total of 10/16 AD subjects had *T. pectinovorum* in brain cortex. *T. socranskii* was confirmed in cortex from 3/7 AD donors that were PCR-positive for *T. socranskii*. *T. socranskii* mAbs also reacted with tissue from 6/9 AD donors that had been PCR-negative for *T. socranskii*, making a total of 13/16 AD donors positive for *T. socranskii*. Both *T. pectinovorum* and *T. socranskii* mAbs reacted with antigen in cortex from one AD subject that was PCR-negative for all spirochetes, raising the total of AD donors with *Treponema* to 15/16.

Considering both PCR and mAbs, there were significantly more AD subjects than controls with *T. pectinovorum* (10/16 vs. 3/18, $\chi^2 = 5.72$, $P < 0.02$) and *T. socranskii* (13/16 vs. 3/18, $\chi^2 = 11.71$, $P < 0.001$). Proportions of AD subjects with *T. pectinovorum* and/or *T. socranskii* were significantly greater than those of controls (15/16 vs. 6/18, $\chi^2 = 10.658$,

Table 2. Treponema species detected with PCR in human saliva before and after cultivation[1]

Group	*T. amylovorum*	*T. denticola*	*T. maltophilum*	*T. medium*	*T. pectinovorum*	*T. socranskii*	*T. vincentii*
Control $N=16$	1 (2)	7 (7)	11 (12)	4 (6)	9 (10)	13 (13)	1 (1)
AD $N=17$	4 (4)	9 (9)	11 (12)	8 (10)	1 (8)	15 (15)	0 (1)

[1]*Treponema* species detected following cultivation of saliva are given in parentheses. There were no meaningful differences in prevalence between AD and control subjects.

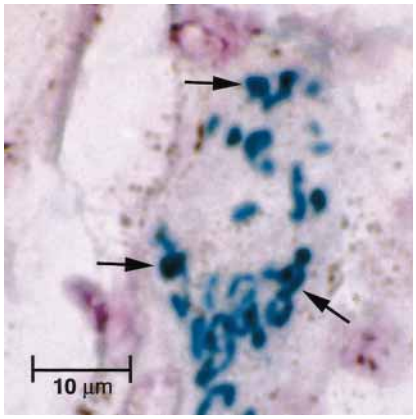

Fig. 1. Trigeminal ganglia from an 84-year-old woman with Alzheimer's disease. The section was incubated with mAbs to *T. pectinovorum*, and binding was disclosed using biotinylated antimouse antibodies and avidin-peroxidase. *Treponema* were stained dark blue (arrows). The section was counterstained red. The photomicrograph was taken at 1000× (bar = 10 μm).

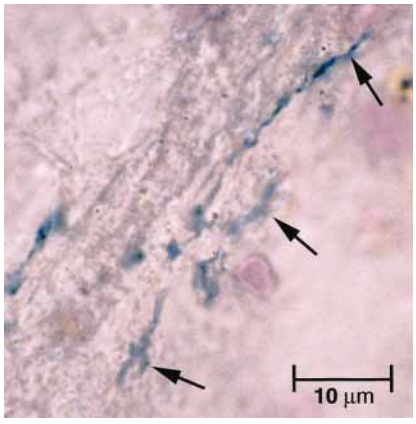

Fig. 2. A section of pons from an 84-year-old woman with Alzheimer's disease. The section was treated as outlined in Fig. 1. Extracellular *Treponema* were stained dark blue (arrows show representative shapes), and appear to be aligned along a structure that could have been a small vessel or axon. The photomicrograph was taken at 1000× (bar = 10 μm).

$P < 0.01$). The total number of AD subjects with any spirochete (including *B. burgdorferi*) was significantly greater than that of controls (15/16 vs. 7/18, $\chi^2 = 8.89$, $P < 0.01$).

Group 3

Figures 1–3 illustrate the reactivity of *T. pectinovorum* mAbs in TG, pons and hippocampus from the embalmed AD cadaver. *T. socranskii* mAbs produced similar findings. Antibody reactivity in TG was most often intraneuronal (Fig. 1), but reactivity in pons (Fig. 2) and hippocampus (Fig. 3) was generally detected within interstitial tissues. Similar reactivity was observed in TG and pons from the other three cadavers.

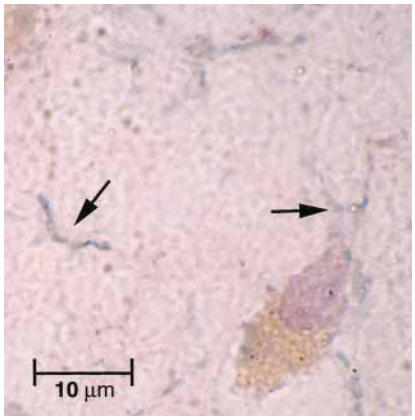

Fig. 3. A section of hippocampus from an 84-year-old woman with Alzheimer's disease. The section was treated as outlined in Fig. 1. Interstitial *Treponema* were stained dark blue (arrows). The photomicrograph was taken at 1000× (bar = 10 μm).

Discussion

The etiology and the pathogenesis of AD are not well defined. Immune and inflammatory mechanisms are involved, but the stimulus for these protective responses has not been identified (20). Although viruses or bacteria could elicit such reactions, it has been difficult to verify associations between microorganisms and AD. For example, herpes virus was detected with *in situ* hybridization more often in trigeminal ganglia of individuals with AD than in those of controls, but no viral RNA was found within the central nervous system of infected individuals, including areas of AD brains that contained extensive neuropathological changes (4). Molecular, immunological, microscopic and culture assays were used to demonstrate the presence of *Chlamydia pneumoniae* in brain from individuals with AD but not controls (1), but this work could not be reproduced (5, 13). Miklossy (8) reported the presence of viable spirochetes in blood, and observed spirochetes in cerebral spinal fluid and brain of AD subjects but not controls. Electron microscopic analysis of isolates revealed structures consistent with *Borrelia* or *Treponema* (9), but Gutacker et al. (6) could find no molecular or immunological evidence of *B. burgdorferi* in blood or brain of subjects with AD. Although McLaughlin et al. (7) could find no microscopic evidence of spirochetes in blood from 21/22 live AD donors, or in brain samples from seven AD donors, they speculated that oral spirochetes might disseminate systemically from sites of periodontal disease.

Some oral *Treponema* are invasive (12, 16, 17), and it is possible that, like other invasive spirochetes, oral spirochetes could disseminate by neurological pathways. For example, both *B. burgdorferi* and *Treponema pallidum* were identified within axons of peripheral nerves in experimental animals (3, 19), and both spirochetes invade the central nervous system (10). Although there is no published evidence that oral *Treponema* invade neurological tissues, *T. denticola* has been found within pulp chambers of teeth associated with periodontal infections (18). Furthermore, herpes virus infected the brains of mice following intrapulpal inoculation (2). These observations raise the possibility that oral *Treponema* might invade trigeminal nerves. If so, oral *Treponema* ought to be detected in TG and brain. If they are associated with AD, then they ought to be more prevalent in brain from AD donors than in that from controls.

In the current investigation, *Treponema* species commonly found in the oral cavity were detected in TG using both PCR and mAbs, but other common nonspirochete plaque bacteria were not detected. *Treponema* were detected in ganglia from both AD and control subjects, and 6/7 *Treponema* species were identified using PCR. Monoclonal antibodies disclosed the presence of *T. pectinovorum* and *T. socranskii* within neuron cell bodies in TG, suggesting that they may have disseminated within axons. *Treponema* species-specific mAbs also identified *T. pectinovorum* and *T. socranskii* within the pons, the area of the brainstem that connects TG with the CNS, and within hippocampus and frontal lobe cortex, regions of the brain that are commonly affected in AD. Although the point of origin cannot be determined from these studies, outcomes suggest that most species of oral *Treponema* have the ability to invade both peripheral and central nervous systems.

PCR studies indicated that there was a strong statistical association between

the presence of oral *Treponema* and AD. Not only was the prevalence of *Treponema* DNA in brain cortex much higher among AD samples than in controls, but also AD subjects had more *Treponema* species present. It is likely that PCR underestimated the presence of *Treponema* because PCR requires DNA from at least 10 target cells per reaction while a single *Treponema* cell can be detected by immunohistochemistry. The increased sensitivity of immunological detection is illustrated by the fact that *T. pectinovorum* and *T. socranskii* mAbs identified three additional infected controls and one more infected AD subject. Application of additional species-specific mAbs might increase the prevalence of *Treponema* in brain. Nevertheless, PCR studies indicated that the extent of infection (percentage of DNA extracts amplified), the number of *Treponema* present (at or above threshold detection levels), and the diversity of infection (number of species identified) were greater for AD subjects than for controls. These results demonstrated that AD subjects were more susceptible to *Treponema* infection within the CNS than were controls, but no cause-and-effect relationship can be derived from these studies.

It seems unlikely that differences in prevalence of *Treponema* between AD and controls could have been due to postmortem contamination of tissues or assays. We found that AD and age-matched control patients had similar profiles of bacteria in saliva, and the risk of contaminating brain or ganglia with saliva during autopsy should have been the same for both groups. Contamination of tissues with spirochetes from blood is improbable because McLaughlin et al. (7) demonstrated that spirochetes were rarely observed in blood of AD subjects, and it may be assumed that the same is true of subjects without AD. It is also unlikely that spurious DNA was introduced in the laboratory. Samples of AD and control tissues were always processed together and accidental contamination during DNA extraction or PCR amplification should have occurred with equal frequency in both groups. Furthermore, all *Treponema* species were processed in the laboratory to generate PCR and immunochemistry controls, and each was as likely as another to be distributed into the environment. Nevertheless, repeated attempts failed to identify a source of extraneous template.

Immunohistochemical analyses not only confirmed PCR detection of *Treponema*, but also demonstrated that *Treponema* were concentrated in foci, identified their histological location, and revealed their unique morphology. Because chance alone would dictate whether a piece of brain contained sufficient DNA to produce a positive PCR, or whether a particular section would contain antigen to react with mAbs, it was necessary to examine multiple DNA extracts and multiple tissue sections before conclusions were drawn about the presence of *Treponema*. Perhaps the uneven distribution of *Treponema* was responsible for the disparate microscopic observations of spirochetes in brain reported by Miklossy (8) and McLaughlin et al. (7). Furthermore, the failure of Gutacker et al. (6) to find *B. burgdorferi* in 4–6-mm^3 samples of brain from 10 AD patients may also be explained by the low prevalence of infection (5/16 AD and 1/18 controls) and by the focal distribution of spirochetes, although it is possible that they might have detected *B. burgdorferi* if more samples from each subject had been tested. However, *B. burgdorferi*-specific PCR would not have detected *Treponema* in brain (6).

Although it was expected that similar findings would derive from both molecular and antibody studies, this confirmation could only be achieved for *T. pectinovorum* and *T. socranskii* because mAbs to *T. amylovorum*, *T. maltophilum*, *T. medium* and *T. vincentii* were not available. Although PCR detected *T. denticola* DNA in cortex and trigeminal ganglia from AD subjects and controls, no reactivity was detected using mAbs to *T. denticola*. These antibodies were directed against carbohydrate antigens, and it is likely that they were lost during tissue dehydration using xylene and alcohols.

In conclusion, outcomes of experiments using both molecular and immunological techniques support the presence of *Treponema* in TG, the brainstem, and the cortex of human subjects. AD donors were more likely to have *Treponema* than controls, and they had more *Treponema* species in brain than controls. These experiments support the hypothesis that oral *Treponema* reached the brain via the trigeminal nerve, but further experiments are needed to determine what role, if any, oral *Treponema* play in the pathogenesis of AD.

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