

Ph. D. THESIS

**CHRONIC INFLAMMATION AND AMYLOIDOGENESIS IN
ALZHEIMER'S DISEASE: THE ROLE OF SPIROCHETES**

Judith Miklossy



**UNIVERSITY OF DEBRECEN
MEDICAL AND HEALTH SCIENCE CENTER
MEDICAL SCHOOL
DEPARTMENT OF NEUROLOGY
DEPARTMENT OF PHYSIOLOGY
DEBRECEN, 2005**

Ph. D. THESIS

**CHRONIC INFLAMMATION AND AMYLOIDOGENESIS IN
ALZHEIMER'S DISEASE: THE ROLE OF SPIROCHETES**

Judith Miklossy



Supervisor: Dr. Patrick McGeer

**UNIVERSITY OF DEBRECEN
MEDICAL AND HEALTH SCIENCE CENTER
MEDICAL SCHOOL
DEPARTMENT OF NEUROLOGY
DEPARTMENT OF PHYSIOLOGY
DEBRECEN, 2005**

INTRODUCTION

Alzheimer's disease

Alzheimer's disease (AD), the most common cause of dementia, is characterized by a slow, progressive decline of cortical functions, particularly cognition and memory. The pathological hallmarks of AD consist of cortical atrophy with an accumulation of senile plaques and neurofibrillary tangles in the cerebral cortex. Fibrillary amyloid substance accumulates in senile plaques and in the walls of cerebral vessels. The major subunit of the amyloid fibrils is a small, 4.2-kD amyloid beta peptide ($A\beta$) which is derived by proteolytic cleavage from a larger (120 kDa) amyloid beta precursor protein (A β PP). Neurofibrillary tangles contain paired helical filaments (PHFs) composed of the microtubule-associated protein tau which is in a hyperphosphorylated state that impairs its ability to bind to microtubules. The patho-mechanism of $A\beta$ and tangle formation remains unclear.

Chronic inflammation in AD

Until recently, immune mechanisms in the pathogenesis of Alzheimer's disease have been largely overlooked. Following the pioneer work of McGeer and Rogers it is today generally accepted that cellular and molecular components of immune system reactions are associated with AD. Activated microglia designed to clean up debris and foreign bacteria surround senile plaques and extracellular neurofibrillary tangles. AD lesions are characterized by the presence of a series of inflammatory mediators, including cytokines, chemokines, proteases, adhesion molecules, free radicals, pentraxins, prostaglandins, anaphylatoxins, and activated complement proteins. It has been assumed that lymphocytic infiltration does not occur in AD. However, using specific immunohistochemical markers, both T-helper/inducer and T-cytotoxic/suppressor lymphocytes have been observed. Of particular importance is the association of the membrane attack complex (MAC, C5b-9) intended to lyse foreign cells, such as bacteria with dystrophic neurites.

Role of bacteria in inflammation and amyloid deposition

Bacteria are powerful inflammatory stimulators. Chronic bacterial infections are frequently associated with amyloid deposition. Furthermore, experimental amyloidosis can be induced by injecting bacteria into experimental animals. Bacteria have a variety of biological actions in mammals. They are inflammatory cytokine inducers, activate complement of the classic pathway, affect vascular permeability, generate nitric oxide, induce apoptosis and are amyloidogenic. All these processes are involved in the pathogenesis of AD. Poorly degradable bacterial debris may persist indefinitely in the affected organs and may be responsible for a persisting chronic inflammation.

Chronic bacterial infection can cause dementia

Noguchi and Moor (1913) by showing the persistence of *Treponema pallidum* in the brains of patients with dementia paralytica established a direct pathogenic link between chronic bacterial infection and dementia. Based on their observation, it is now generally accepted that *Treponema pallidum* may cause dementia and cortical atrophy associated with amyloid deposition. Intriguingly, the clinical and pathological hallmarks of AD also occur in the atrophic form of general paresis.

Spirochetes

Spirochetes are Gram negative free-living or host-associated helical bacteria, possessing periplasmic fibrils which are unique for these microorganisms. They are widespread in aquatic environments and are the causative agents of such important human diseases as syphilis, Lyme disease, periodontitis, ulcerative gingivitis, and leptospirosis. The similarity of the clinical and pathological manifestations of syphilis and Lyme disease is well established. Dementia has been reported to occur in both.

Previous observations from our laboratory related to this study

Previous observations suggested that AD may correspond to late stages of neurospirochetoses caused by several types of spirochetes and also that amyloidogenic proteins may be an integral part of these microorganisms and play a role in amyloidogenesis in AD. These observations were subsequently reinforced by others showing the presence of oral *Treponema* in the human brain and their association with

AD. Recently, it was also shown that the outer surface protein (OspA) of *Borrelia burgdorferi* is amyloidogenic and forms amyloid fibrils in vitro.

In the present study we analyzed the sequence of the 16S rRNA gene of spirochetes cultivated from the brain of three of 14 AD cases in medium selective for *Borrelia burgdorferi*. We carried out morphological characterization by electron microscopy. We correlated this with post mortem serological analysis of blood and cerebrospinal fluid (CSF) and were able to detect *Borrelia burgdorferi* antigens and genes in brain samples from the same patients where the spirochetes were cultivated. In addition we tested the hypothesis that bacteria might induce an amyloidogenic reaction in mammalian cells in culture. We report here the results of such exposures.

OBJECTIVES

The goal of the present study was to investigate further the pathogenetic role of spirochetes in amyloidogenesis in Alzheimer's disease.

I. Characterization of spirochetes cultivated from the brains of Alzheimer's patients

The characterization of spirochetes cultivated from the brains of AD patients was done by PCR and sequence analysis of the 16S rRNA gene, and by electron microscopy. We correlated this with post mortem serological analysis of blood and cerebrospinal fluid (CSF) and were able to detect *Borrelia burgdorferi* antigens and genes in brain samples from the same patients in which the spirochetes were cultivated.

II. In vitro induction of beta-amyloid by Borrelia spirochetes

In addition, we further analyzed the amyloidogenic properties of *Borrelia* spirochetes. We tested whether bacteria might induce amyloid deposition in mammalian cells in culture. We exposed cultures of rat primary neurons, astrocytes and rat CNS cell aggregates to *Borrelia burgdorferi* spirochetes.

MATERIALS AND METHODS

I. Characterization of spirochetes cultivated from the brains of Alzheimer's patients

Clinical data and tissue samples analyzed

Previously we reported helically shaped microorganisms in the cerebrospinal fluid CSF, blood and cerebral cortex in 14 AD cases that were not detected in controls. A taxonomical analysis showed that these microorganisms belonged to the order Spirochaetales. In 3 of these 14 AD cases spirochetes were cultivated from the brain in a synthetic medium specific for *Borrelia burgdorferi*

Cerebrospinal fluid, blood and unfixed and formalin fixed brain samples were available for analysis. A routine neuropathological examination was completed with a semiquantitative assessment of the AD-type cortical changes for the neuropathological confirmation of definite AD.

Cultivation of spirochetes and their immunohistochemical characterization

Small fresh cortical samples were inoculated into BSK II medium under sterile conditions. Serial subcultures were prepared. The cultures and subcultures were regularly analyzed by dark field microscopy. Immunohistochemical characterization of the helically shaped microorganisms was made using specific silver stains for spirochetes and using several antibodies specific for *Borrelia burgdorferi* antigens.

Taxonomic characterization using transmission electron microscopy

The helically shaped microorganisms were harvested by centrifugation and the negatively stained samples were examined with a transmission electron microscope.

Molecular characterization of the cultivated microorganisms

Comparative analysis of 16S rRNA gene sequences is presently considered to be the gold standard for bacterial identification. 16S rRNA is a highly conserved molecule that is present in all prokaryotic organisms. DNA was isolated from cultured spirochetal cells and PCR amplified using the universally conserved primers. A spirochetal selective reverse primer C90 was used with a universal forward primer C75.

The DNA sample from PCR after purification was directly sequenced using cycle-sequencing kits. Sequence alignment, secondary structure comparison, matrix generation similarity, and phylogenetic tree construction were performed. Similarity matrices were constructed from the aligned sequences, corrected for multiple base changes, then phylogenetic trees were constructed using the neighbor-joining method.

Serological analysis

Post mortem serological analysis of the blood and CSF of the AD cases was made using Indirect Immunofluorescent Antibody Test (IFAT), Enzyme-Linked Immunoabsorbent Assay (ELISA) and Western blot analysis for the detection of specific anti-*Borrelia* antibodies. The serological analyses were made independently in two different laboratories. For evaluation of the results, criteria of Centers for Disease Control and Prevention (CDC) were applied. Serum of three non-demented cases and the CSF of one non-demented subject were also analyzed. In addition, the blood and CSF of one AD case where *Borrelia burgdorferi* was not cultivated from the brain was also tested.

Detection of Borrelia antigens

For characterizing the spirochetes cultivated from the AD brains and from the blood of the healthy forester, as well as detecting spirochetal antigens in brain, antibodies to six different anti-*Borrelia burgdorferi* antibodies were utilized, including two rabbit antibodies prepared in our laboratory. The specificity of all these mono and polyclonal anti-*Borrelia burgdorferi* antibodies were tested by Western blot analysis. Monoclonal antibodies for the detection of bacterial peptidoglycan were also used. In order to analyze whether bacterial antibodies are co-localized with A β , serial sections were immunostained with anti-*Borrelia burgdorferi*, anti-bacterial peptidoglycan and anti-A β antibodies, respectively. For detection, the avidin-biotin-peroxidase technique was used. Frozen sections immunostained in the absence of the primary antibody or with an irrelevant mono- or polyclonal antibody were also used.

In situ hybridization for the detection of Borrelia genes

In situ hybridization (ISH) was performed using the Hybaid, OmniGene thermal cycler, equipped with a Satellite Module of In-Situ block. For ISH, paraffin and frozen

sections sections were utilized. The sections were post-fixed for 20 min with 1% paraformaldehyde, incubated with a prehybridization solution which was then replaced by the hybridization solution containing 100 ng of probe labeled by nick-translation with Digoxigenin (OspA gene BBB012, SN3, position 360-426); flagellin gene BBB032, WK3, position 396-425 purchased from GENSET). The nucleotide sequence of the probes was: 5'-CAA TGG ATC TGG AGT ACT TGA AGG GGT AAA AGC T-3' and 5'-AAT GCA CAT GTT ATC AAA CAA ATC TGC TTC-3', respectively. For the detection of the hybridization products anti-digoxigenin alkaline phosphatase or peroxidase conjugates were used. Control sections without specific probes and sections from patients without brain pathology were used as negative controls.

II. In vitro induction of beta-amyloid by *Borrelia* spirochetes

Primary cell cultures

Primary astrocyte and microglial cell cultures from Sprague-Dawley rats were prepared as previously described. Microglia enriched astrocyte cultures (10^6 astrocytes and 10^5 microglia) were also prepared by combining astrocytic and microglia cultures. Neurons dissociated from the telencephalon of 21-day-old rats were cultured on collagen or poly-L-ornithine coated substrate surfaces.

The organotypic culture consisted of aggregates of primary cells of foetal rat telencephalon (Monnet-Tschudi et al., 1996) that were kindly provided to us by P. Honegger. These brain cell cultures were obtained at an advanced maturational stage (20-21 days in vitro). They were exposed to 2-4 weeks of continuous rotation at 37°C which produced a large number of small identical spheres containing a mixture of CNS cells, including neuronal and glial cells. These were infected with *Borrelia* spirochetes.

Exposure of cells to *Borrelia* spirochetes

Reference spirochetes (*Borrelia burgdorferi*, B31 strain) maintained in BSK II medium were used to infect the cell cultures. The cells were infected for periods of 2, 4, and 8 weeks. In cell cultures exposed to spirochetes, a mixture of equal volumes of medium from the given cell and spirochetes (BSK II) culture was used. The final concentration of spirochetes in the infected cultures corresponded to 5×10^5 /ml. Before

exposure to spirochetes, the cells were tested with 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI) (236 276, Boehringer Mannheim, Germany), following the manufacturer's protocol, in order to verify Mycoplasma infection. After 2, 4 and 8-week exposure, the cell cultures in double chambers and six well clusters were washed with PBS prior to immunohistochemical analysis. Cells in flasks were harvested by centrifugation in preparation for immunohistochemistry and Western blotting.

Exposure of cells to *Borrelia* spirochetes was done ten times with primary astrocyte cultures, four times with microglia enriched astrocyte cultures, and three times with primary neurons and brain cell aggregates. Uninfected samples were always used as controls. Immunohistochemical detection of A β was done at each time point in each set of experiments for all cell types including cell aggregates.

Histochemical and immunohistochemical detection of amyloid

Smears prepared from the infected and uninfected cell cultures and frozen sections of the organotypic cultures were fixed in 4% paraformaldehyde and immunostained using the avidin-biotin-peroxidase technique. For immunostaining cells or frozen sections on glass slides, 15 min of acetone fixation was performed at 4°C before immunostaining with anti-*Borrelia* antibodies. Those in plastic chambers were fixed for 10 min with 4% paraformaldehyde. For A β immunostaining, 100% formic acid pretreatment for 20 min was used. Frozen sections (5 μ m thick) were cut from the brain cell aggregates for immunohistochemistry. Cultures not infected with spirochetes were used as controls.

The types, dilutions and origin of antibodies used for the characterization of cells in culture, for the detection of A β , A β PP and tau, as well as *Borrelia* antigens are illustrated in Table 1.

Western blot analysis

For Western blots, whole cell extracts of infected and control primary astrocytes and microglia enriched astrocytes were prepared. Triplicate samples from experiments at 2, 4 and 8 week exposure times were analyzed. Extraction of A β was performed using formic acid as previously described. To 50 μ g protein samples, Laemmli buffer was added followed by boiling for 5 min at 85°C. The samples were then electrophoresed on 14-20% Tris HCL and 4-20% TRIS HCL-urea polyacrylamide gels.

Following transfer onto a 0.2 mm nitrocellulose membrane, immunoblotting was performed using specific antibodies recognizing A β , A β PP, tau and Borrelia antigens. The immunoreactive bands were visualised by an ECL western blotting detection kit on light sensitive films. Samples of synthetic A β 1-42 peptide and samples of whole cell extracts prepared from pCMV/A β PP695 and pCMV/tau transfected U87MG cells were used as positive controls.

Western blot analysis of PC12/THP-1 cells exposed to LPS

In order to analyze whether the bacterial component alone may induce changes in the A β PP levels and in tau phosphorylation, bacterial lipopolysaccharide (LPS), an alternative powerful inflammatory stimulator was used to expose a mixture of PC12 cells (a pheochromocytoma cell line showing neuronal properties) and the monocytic THP-1 cells. PC12 cells (0.5×10^6) were co-cultured with 0.1×10^6 THP-1 cells in collagen coated Petri dishes (100 mm) for 24 hours in 10 ml of the following medium: 8 ml F12 Nutrient Mixture (HAM, 11765-054, Life Technologies, Gibco/BRL, Frederick, Maryland) and 2 ml VitaCell medium (ATCC, 30-2002, Manassas, Virginia). The medium was supplemented with 10 % horse serum, 2.5% FBS, 1 % Penicillin/Streptomycin and 870 mg NaHCO₃ per 500 ml medium. LPS (500ng, S1K4072, Sigma, St. Louis, Missouri) was then added. After 0h, 30min, 3h, 24h and 48h, the cells were harvested by centrifugation and whole cell extracts were analyzed by immunoblotting.

Densitometry analysis was performed using a Molecular Imager FX, equipped with Quantity one software (BioRad Laboratories, Hercules California).

Synchrotron InfraRed MicroSpectroscopy (SIRMS)

The β -sheet conformation of the in vitro induced amyloid deposits was analyzed with SIRMS. Infected and uninfected cells were placed on infrared-transparent BaF₂ slides and stained with Thioflavin S. Infrared microspectra were collected in transmission mode, 128 scans per point, 4 cm⁻¹ resolution using AtI μ s software. The final data format was absorbance, where the background was collected open beam. Protein secondary structure was determined by Amide I infrared absorption band (1600-1700 cm⁻¹) analysis. The frequency of Amide I band is sensitive to protein secondary structure, where β -sheet conformation absorbs near 1630 cm⁻¹.

RESULTS

I Characterization of spirochetes cultivated from the AD brains

Dark field microscopy, histochemistry and immunohistochemistry

Dark field microscopy analysis of the cultures and subcultures in the 3 cases showed that the initially stretched and immobile helically shaped spirochetes following 2-4 months culture undergo progressive changes and following 4 month of culture the morphology of the cultured microorganisms was identical to the reference strain (B31) of *Borrelia burgdorferi*. A slowly progressive growth was observed between 3-4 months, which was followed by rapid proliferation, identical to the reference *Borrelia* spirochetes.

The cultivated microorganism showed identical morphological and histochemical properties to the reference *Borrelia burgdorferi* spirochetes (B31 strain) and showed positive immunoreaction with 6 different anti-*Borrelia burgdorferi* antibodies, including with monoclonal antibody recognizing the Outer Surface protein A (OspA).

Taxonomic characterization using electron microscopy

The ultrastructural analysis of the cultured spirochetes (strains ADB2 and HFB) with transmission electronmicroscopy demonstrated that they had ultrastructural characteristics of *Borrelia burgdorferi* species, i.e. thin helical cells with 10-15 periplasmic flagella inserted at each end of the cell.

Genomic characterization of the cultivated microorganisms

The phylogenetic analysis of the 16S rRNA gene sequence and the phylogenetic position among other species of spirochetes and borrelial strains revealed that the cultured spirochetes (strains ADB1, ADB2 and HFB) correspond to *Borrelia burgdorferi sensu stricto (s. s.)*.

Serological analysis

The analysis and the interpretation of the serological results were made following criteria of the Center for Disease Control (CDC). A positive serology for

Borrelia burgdorferi was detected in the blood and CSF of 2 AD cases (AD 1 and AD3) and in the blood of the healthy forester. In case AD3, in addition to a positive Lyme IgG, a positive IgM response was also observed by Western blot, a finding that is known to occur in some untreated patients with chronic Lyme disease. It is of interest to note that the *Borrelia burgdorferi* specific 31 kDa OspA band was present in all the 3 AD cases. The Western blot of the blood and CSF of the non-demented controls and of the AD subject where spirochetes were not cultured from the brain, were negative.

Detection of Borrelia antigens and genes in the brain

In the 3 AD cases, cortical atrophy, dissemination of microorganisms in the cerebral cortex in the form of scattered circumscribed colonies, and distribution of beta amyloid deposits were morphologically similar to previously described pathological changes in dementia paralytica caused by *Treponema pallidum*.

The colony-like masses showed positive immunoreactions with anti-*Borrelia burgdorferi* antibodies. The spirochete antigens showed the same pattern of distribution as amyloid beta peptide and on serial sections were co-localized with A β . *Borrelia burgdorferi* antigens were also detected in a number of neurofibrillary tangles in the wall of some blood vessels containing amyloid deposition. *Borrelia burgdorferi* OspA and flagellin genes were also detected in senile plaques and in a number of neurofibrillary tangles in all three AD cases by *in situ* hybridization. The pattern of distribution was similar to *Borrelia* antigens. The extranuclear localization of the ISH product excluded the possibility of unspecific DNA labelling. *Borrelia* antigens or genes were on control sections were not observed.

II. In vitro induction of beta-amyloid by *Borrelia* spirochetes

Immunohistochemical analysis of the infected and control cell cultures

Histochemical and immunohistochemical analysis showed the same results with respect to the A β deposition. A β deposition was observed in all cells and cell aggregates following 4 and 8 week of exposure to spirochetes. The plaque-like A β deposits were in a consolidated form as revealed by thioflavin-S staining. The majority of the amyloid deposits were large (100-150 μ m) extracellular aggregates. Their

numbers varied between five and twenty per well and between two and ten per spheroid in the cell aggregates.

In the infected neuronal and organotypic cultures, a few A β -42 and OspA-positive tangle-like formations were also observed. OspA positive intracytoplasmic granules in astrocytes, surrounded by a thin pale halo, similar to granulovacuolar degeneration of neurons in AD, were also seen. The uninfected control cultures did not show A β deposition, tangles- or granulovacuolar-like degeneration.

Western blot analysis of the infected and control cell cultures

A 4 kDa A β peptide band was detected by Western blot in all cell culture samples analyzed following 2-8 week exposure to *Borrelia* spirochetes. After 2-weeks exposure to *Borrelia* spirochetes, the A β deposition was weak in primary rat astrocytes but was more pronounced in microglia-enriched astrocyte cultures, suggesting that microglia enhance A β formation. Cell cultures infected for 8 weeks showed a higher level of A β than those infected for 2 weeks. A β was not observed in the uninfected control cultures.

In comparison to the uninfected control cultures, increased A β PP levels were also detected in *Borrelia*-infected cultures. A β PP levels were higher at 8-weeks than at 2-weeks following exposure to spirochetes. When we analyzed for tau phosphorylation in cell cultures exposed to *Borrelia* spirochetes, increased levels were observed when compared to the uninfected control cultures.

β -pleated sheet conformation of the in vitro induced amyloid as revealed by Synchrotron InfraRed MicroSpectroscopy (SIMRS)

The infrared absorption microspectrum of healthy brain tissue exhibits a protein (Amide I) absorbance maximum near 1655 cm⁻¹, representative of an average α -helical protein structure in the tissue. SIMRS analysis detected a second peak near 1630 cm⁻¹ in senile plaques in AD, representative of β -sheet protein structure. Most importantly, a second peak near 1630 cm⁻¹ was also observed in the Thioflavin S positive “plaques” in the cell cultures analyzed following 4-week exposure to spirochetes, which was absent in the uninfected control samples.

DISCUSSION

1 Characterization of spirochetes cultivated from the AD brains

Spirochetes were successfully cultured from the post mortem brains of 3 AD cases and from the blood of a clinically asymptomatic forester. In the present study, 16S rRNA gene sequence analysis identified the spirochetes cultivated from the brain of two AD cases and from the blood of the healthy forester as *Borrelia burgdorferi sensu stricto* (*s. s.*). The detection of *Borrelia burgdorferi* specific antigens and genes in the brains of these patients provided further evidence that they suffered from chronic Lyme neuroborreliosis. Consistent with the present findings, the genospecies *Borrelia garinii* and *Borrelia burgdorferi s. s.* have been reported to be predominantly involved in neuroborreliosis.

Antibodies to various spirochetes are highly prevalent in the population at large, and it is important to consider that spirochetes of the oral cavity as well as intestinal spirochetes could contain amyloidogenic proteins and play a role in AD. For the majority of these spirochetes, diagnostic and serological tests are not available. Recently, Riviere et al., using species-specific PCR and monoclonal antibodies, detected oral *Treponema* in 14/16 AD cases and 4/18 non-AD controls.

Based on previous analyses we also suggested that amyloidogenic protein may be an integral part of spirochetes. Recent observations showed that the outer surface protein (OspA) of *Borrelia burgdorferi* forms amyloid fibrils in vitro, similar to human amyloid.

Reports of associations between infection and AD are not confined to spirochetes. The presence of Herpes virus type 1 (HSV-1) in the AD brain has been reported. *Chlamydia pneumoniae* was also found to be associated with AD. Mice exposed to *Chlamydia* developed AD-like amyloid plaques.

The pathological findings observed in the 3 AD cases were reminiscent of those described in dementia paralytica caused by *Treponema pallidum*. They are consistent with primary parenchymatous involvement of tertiary Lyme neuroborreliosis. Our results show that similarly to *Treponema pallidum*, *Borrelia burgdorferi* may also persist in the brain and be associated with cortical atrophy, amyloid deposition, and

dementia. The present findings suggest that *Borrelia burgdorferi* may also be involved in the pathogenesis of several chronic neuro-psychiatric disorders.

An infectious origin of AD is not contradictory to the genetic defects shown to be present in AD. In chronic bacterial infections or in experimental amyloidosis, the bacterial infection or bacterial exposure always precedes the amyloid deposition. In patients with genetic defects which facilitate infection, the genetic problem will be the first step in the cascade of events, followed by infection then with amyloid deposition.

In vitro induction of beta-amyloid by *Borrelia* spirochetes

Exposure of mammalian neuronal and glial cells to *Borrelia* spirochetes induced the defining pathological hallmarks of AD, including A β deposition, increased A β PP levels, and hyperphosphorylation of tau. Thioflavin S positive and A β -immunoreactive “plaques”, as well as tangle- and granulovacuolar-like formations, were present in cell cultures exposed to *Borellia burgdorferi* spirochetes. In addition, Western blot analysis detected a 4kDa A β immunoreactive band in the infected cultures.

SIMRS is an extremely valuable tool for the analysis of chemical composition of biological and biomedical samples. The chemical spectra of protein solutions, single cells or well defined areas of tissue sections can be analyzed. Characteristic absorption bands observed in many biological materials include the ‘amide I’ band at 1650 cm⁻¹ resulting largely from the number of carbonyl (C=O) stretching vibrations of protein amide bonds. A second peak in the “amide I” band near 1630 cm⁻¹, is present in senile plaques representative of β -sheet protein structure. This peak near 1630 cm⁻¹ was observed not only in the senile plaques of the five AD cases tested, but also in the Thioflavin S positive “plaques” in infected cell cultures following 4 weeks exposure to *Borrelia*.

These observations indicate that exposure of mammalian cells to *Borrelia* spirochetes induces an AD-like host cell reaction which is in agreement with recent observations showing that several bacteria contain amyloidogenic proteins.

It was suggested that several types of spirochetes may be involved in AD, and also that amyloidogenic proteins may be an integral part of spirochetes. Indeed, recent observations showed that the OspA outer surface protein of *Borrelia burgdorferi* forms amyloid fibrils in vitro, similar to human amyloid. These observations are in line with the present findings showing that A β formation may be induced in vitro following

exposure of mammalian cells to *Borrelia burgdorferi*. Recently, the formation of amyloid deposits resembling AD plaques was induced in the brains of mice following intranasal infection with another bacterium, *Chlamydia pneumoniae*.

In vivo studies, with a longer exposure time would be useful to study efficiently the sequence of events and the cellular mechanisms involved in the bacteria induced beta amyloid deposition and “tangle” formation.

The increased A β PP level observed in cell cultures following exposure to *Borrelia* spirochetes or LPS reinforces the importance of host-derived A β PP in amyloidogenesis. A β PP is a proteoglycan core protein. It is well established that increased proteoglycan synthesis plays a significant role in amyloidogenesis, including the formation of A β in AD. An important role for proteoglycans in major histocompatibility complex (MHC)-mediated infections (e.g. viral, bacterial) is also well documented. The in vivo synthesis of proteoglycans by host cells in response to bacterial infections, including spirochetal infections, has been previously reported.

Borrelia and LPS induced tau phosphorylation represents another experimental evidence of the ability of bacteria to induce an AD-like host cell reaction, but further analysis will be required to identify in detail the specific tau phosphorylation sites.

Bacteria and natural or synthetic bacterial components (e.g. cell wall peptidoglycan and LPS) may induce chronic inflammation and amyloidosis. They are inflammatory cytokine stimulators, they activate complement, they affect vascular permeability, they generate nitric oxide, and they induce proteoglycan synthesis and apoptosis. All of these processes are implicated in the pathogenesis of AD.

Mammals are constantly exposed to bacteria. Bacterial cell components are highly resistant to degradation by mammalian enzymes and, thus may provide persisting inflammatory and amyloidogenic stimulus and trigger a cascade of events leading to chronic inflammation and amyloid deposition.

The results indicate that *Borrelia burgdorferi* may persist in the brain and is associated with amyloid plaques in AD. The data suggest that *Borrelia burgdorferi*, perhaps in an analogous fashion to *Treponema pallidum*, may contribute to dementia, cortical atrophy and amyloid deposition. In addition, the present results reinforce previous observations that spirochetes can induce a host reaction with similarities to that seen in AD. The results indicate that bacteria and/or their degradation products may enhance a cascade of events leading to amyloid deposition in AD.

Published data related to the results of the present thesis (*in extenso* publications):

Miklossy J, Molnar L. Prognostic value of non-protein nitrogen (NPN) content in serum and cerebrospinal fluid. *Ideggyógyászati Szemle* 1975;28:347-352.

Miklossy J, Molnar L, Rochlitz Sz. The prognostic value of the non-protein nitrogen (CPN) content in serum and the cerebrospinal fluid. *Arch Psychiat Nervenkr* 1976;222:77-85.

Miklossy J, Kuntzer T, Bogousslavsky J, Regli F, Janzer RC. Meningovascular form of neuroborreliosis: Similarities between neuropathological findings in a case of Lyme disease and those occurring in tertiary neurosyphilis. *Acta Neuropathol* 1990;80:568-572. **IF: 2.126**

Miklossy J. Alzheimer's disease - A spirochetosis? *Neuroreport* 1993; 4: 841-848. Paper selected for press release **IF : 2.374**

Miklossy J, Kasas S, Janzer RC, Ardizzoni F, Van der Loos H. Further morphological evidence for a spirochetal etiology of Alzheimer's Disease. *NeuroReport* 1994; 5 1201-1204. **IF : 2.374**

Miklossy J. The spirochetal etiology of Alzheimer's disease: A putative therapeutic approach. In: *Alzheimer Disease: Therapeutic Strategies. Proceedings of the Third International Springfield Alzheimer Symposium.* Giacobini E, Becker R (Eds.) Birkhauser Boston Inc., 1994, Part I, pp.41-48.

Miklossy J, Gern L, Darekar P, Janzer RC, Van der Loos H. Senile plaques, neurofibrillary tangles and neuropil threads contain DNA? *J. Spirochetal and Tick-borne Diseases (JSTD)*, 1995;2:1-5.

Miklossy J, Darekar P, Gern L, Janzer RC, Bosman FT. Bacterial peptidoglycan in neuritic plaques in Alzheimer's disease. *Alzheimer's Research* 1996; 2: 95-100.

Miklossy J. Chronic inflammation and amyloidogenesis in Alzheimer's disease: Putative role of bacterial peptidoglycan, a potent inflammatory and amyloidogenic factor. *Alzheimer's Rev* 1998;3:45-51.

Miklossy J, Kraftsik R, Pillevuit O, Lepori D, Genton C, Bosman FT. Curly fiber and tangle-like inclusions in the ependyma and the choroid plexus - A pathogenetic relationship with the cortical Alzheimer-type changes? *J Neuropathol Exp Neurol* 1998;57:1202-1212. **IF: 5.533**

Miklossy J., Taddei K, Martins R, Escher G, Kraftsik R, Pillevuit O, Lepori D, Campiche M Alzheimer's disease: curly fibers and tangles in organs other than brain. *J Neuropathol Exp Neurol* 1999; 58:803-814. **IF: 5.533**

Suvà D, Favre I, Kraftsik R, Esteban M, Lobrinus A, **Miklossy J**. Involvement of primary motor cortex in Alzheimer's disease. *J Neuropathol Exp Neurol* 1999; 58:1125-1134. **IF: 5.533**

Miklossy J, Taddei K, Suva D, Verdile G, Fonte J, Fisher C, Gnjec A, Ghika J, Suard F, Mehta PD, McLean CA, Masters CL, Brooks WS, Martins RN. Two novel presenilin-1 mutations (Y256S and Q222H) are associated with early-onset Alzheimer's disease. *Neurobiol Aging* 2003;24:655-662. **IF: 5.552**

Suter OC, Sunthorn T, Kraftsik R, Straubel J, Darekar P, Khalili K, **Miklossy J**. Cerebral hypoperfusion generates cortical watershed microinfarcts in Alzheimer disease. *Stroke* 2002;33:1986-1992. **IF:5.330**

Miklossy J. Cerebral hypoperfusion induces cortical watershed microinfarcts which may further aggravate cognitive decline in Alzheimer's disease. *Neurological Research*. Review **IF: 1.676**.

Christen-Zaech S, Kraftsik R, Pillecuit O, Martins RN, **Miklossy J**. Early appearance of degenerative changes in the olfactory system in Alzheimer's disease. *Can J Neurol Sci* 2003; 30: 20-25. (*Paper chosen for MCE*) **IF: 1.317**

Miklossy J, Khalili K, Gern L, Ericson RL, Darekar P, Bolle L, Hurlimann J, Paster BJ. *Borrelia burgdorferi* persists in the brain in chronic Lyme neuroborreliosis and may be associated with Alzheimer disease. *J Alzheimer's Dis* 2004; 6: 639-649.

Miklossy J, Kis A, Radenovic A, Miller L, Darekar P, Martins RN, Mihaly L, Reiss K, Darbinian N, Forro L. Beta amyloid deposition and morphological changes reminiscent of senile plaques and tangles induced by *Borrelia* spirochetes in vitro. *Neurobiology of Aging*. In the Press. (**IF: 5.552**)

Abstracts.

Miklossy J, Darekar P, Janzer RC, Bosman FT. Amyloidogenesis in Alzheimer's disease: Bacterial cell wall peptidoglycan, a potent inflammatory and amyloidogenic factor is co-localized with β -amyloid in senile plaques. *Brain Pathol*. 1997; 7: p1370

Miklossy J, Kraftsik R, Lepori D, Genton C, Pillecuit O, Campiche M. Curly fiber and tangle-like structures in the ependyma and the choroid plexus - A pathogenetic relation with Alzheimer's type cortical changes? *European Journal of Neuroscience* 1998;10: p 93.

Suter OC, **Miklossy J**. Ischaemic cortical involvement of boundary zones induced by cerebral hypoperfusion in Alzheimer's disease. *Neuropathol Appl Neurobiol.*; 1999;25 (S1):95-96.

Miklossy J, Lepori D, Kraftsik R, Pillevuit O, Genton C, Campiche M. Curly fibers and tangles in other organs than brain in Alzheimer's disease. *Neuropathol Appl Neurobiol.*; 1999;25 (S1):75-76.

Miklossy J, Lepori D, Genton C, Kraftsik R, Pillevuit O, Bosman F. Curly fiber and tangle-like inclusions in the ependyma and choroid plexus - a pathogenic relationship with the cortical Alzheimer's type changes? *Neuropathol Appl Neurobiol.*; 1999;25 (S1): p.76.

Suter OC, Sunthorn T, Kraftsik R, Darekar P, **Miklossy J**. Alzheimer's disease: Boundary zone cortical infarcts caused by cerebral hypoperfusion. *Alzheimer's Reports*. 2000;3(S): p38.

Christen-Zaech S, Kraftsik R, Pillevuit O, **Miklossy J**. Early appearance of degenerative changes in the olfactory system in Alzheimer's disease. *Neurobiology of Ageing*, p. 2000 ; 21(S): p193.

Miklossy J, Zaech S, Kraftsik R. Early degenerative changes in the olfactory system in Alzheimer's disease. *Brain Pathology* 2000 ; 10 :p.509.

Miklossy J, Gern L, Khalili K, Paster B. Molecular gene analysis identifies *Borrelia burgdorferi* as one of the spirochetes involved in the pathogenesis of Alzheimer's disease. *J NeuroVirology*, 2002;8: 72-73.

Miklossy J, Kiss A, Radenovic A, Miller L, Mihaly L, Khalili K, Forro L. Beta amyloid and Alzheimer's type changes induced in vitro by *Borrelia burgdorferi*. *J NeuroVirology* 2002;8:74-75.