Ph. D. THESIS

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

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CONTENTS

I. INTRODUCTION

I.1. *Alzheimer's disease:*

Alzheimer discovered the disorder that bears his name in 1906 when he reported the case of a 51-year-old woman (Auguste D.) with presenile dementia with characteristic changes in the cerebral cortex (Alzheimer, 1907, 1911). Alzheimer's disease (AD); the most common cause of dementia; is characterized by a slow, progressive decline of cortical functions, particularly cognition and memory. Terry and Davies (1980) pointed out that the presenile form - with onset before age 65 - is identical to the most common form of senile dementia. Thus, the terms AD-type dementia and AD are used for the designation of presenile and senile cases.

The pathological hallmarks of AD consist of a marked cortical atrophy, accumulation of senile plaques (known also as argyrophylic or neuritic plaques), neurofibrillary tangles and neuropil threads in the cerebral cortex. The occurrence of senile plaques was first reported by Blocq and Marinesco (1892) and the characteristic fibrillary changes of the neuronal cells were first described and documented by Alzheimer (1907). Recently, particularly from the use of a Gallyas silver technique, the accumulation of neuropil threads or curly fibers was recognized as a characteristic cortical lesion in AD (Gallyas, 1971).

Fibrillary amyloid substance accumulates in senile plaques, but also in leptomeningeal and cortical vessel walls (Glenner and Wrong, 1984:, Kang et al., 1987). The major subunit of the amyloid fibrils is the amyloid beta peptide (Aβ4), a small self-aggregating polypeptide. The 4.2-kD polypeptide was designated beta amyloid peptide

(Aβ) because of its partial beta-pleated sheet structure. Aβ is derived by proteolytic cleavage from a larger, transmembrane amyloid beta precursor protein (AβPP), which is expressed in a variety of tissues (Kang et al., 1987). AβPP contains features characteristic of glycosylated cell-surface receptors and revealed to be a proteoglycan core protein (Schubert, 1988).

Neurofibrillary tangles contain paired helical filaments (PHFs) composed of the microtubule-associated protein tau. Tau is hyperphosphorylated in PHFs, which abolishes its ability to bind microtubules and promote microtubule assembly. Lu et al. (1999) demonstrated that peptidyl-prolyl cis/trans isomerase, NIMA interacting, 1, (PIN1) binds hyperphosphorylated tau and copurifies with PHFs, resulting in depletion of soluble PIN1 in the brains in AD patients. PIN1 can restore the ability of phosphorylated tau to bind microtubules and promote microtubule assembly in vitro. Since depletion of PIN1 induces mitotic arrest and apoptotic cell death, sequestration of PIN1 into PHFs is one of many theories concerning PHF formation contributing to neuronal death.

The pathomechanism of Aβ and tangle formation still remains unclear.

I.2. *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 (McGeer and Rogers, 1992).

Activated microglia (the brain's representatives of the phagocytic cells that are 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 (McGeer et al., 1995; McGeer and McGeer, 2002).

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 (McGeer and Rogers, 1992; Webster et al., 1997). The conclusion that inflammation exacerbates AD pathology is now supported by more than 20 epidemiological studies showing that individuals are spared AD if they have been taking anti-inflammatory drugs or have suffered from unrelated conditions for which such drugs are routinely used (McGeer et al., 1996, Veld et al,.2000). This effect has been particularly evident in people using nonsteroidal anti-inflammatory drugs (NSAIDs), a group of drugs that inhibit cyclooxygenase, which catalyzes a step in prostaglandin synthesis, because these are the most widely used agents in various arthritic conditions. Three large epidemiological studies showed a reduction of risk of 55-80% for AD (Stewart et al., 1997; Zandi et al., 2000, Veld et al., 2000).

I.3. *Role of bacteria in inflammation and amyloid deposition*

Bacteria are powerful inflammatory stimulators. It has been known from almost a century that chronic bacterial infections (e.g. rheumatoid arthritis, leprosy, tuberculosis, osteomyelitis) may be associated with amyloid deposition (Khachaturian, 1985). It is also

generally accepted that experimental amyloidosis can be induced by injecting living, attenuated or killed bacteria to experimental animals (Picken, 2000).

In bacteria, the cell wall consists of peptidoglycan, a complex polysaccharide composed of two sugar derivatives, N-acetylglucosamine and N-acetylmuramic acid and a small group of amino acids. Peptidoglycan is present only in bacteria, and is found in the wall of virtually all Eubacteria. It is absent in the evolutionary higher plant and animal cells (Eukaryotes). Bacterial cell walls are highly resistant to degradation by mammalian enzymes and thus may provide a persisting inflammatory stimulus (Ohanian and Schwab 1967). Specific bacterial pathogens may infect a distant site, which on interaction with the immune-system, leads to a chronic inflammation (Lehman et al., 1983; Fox, 1990). It has been shown that human intestinal bowel contains soluble bacterial cell wall components that are arthropathic in an animal model [Stimpson et al., 1986]. In these models it was the bacterial cell wall peptidoglycan component which was found to be the arthritogenic factor in experimentally induced arthritides [Fleming et al., 1986]. The bacterial inflammatory surface molecule lipopolysaccharide (LPS) is another powerful inflammatory and amyloidogenic factor of Gram negative bacteria. LPS is widely used as inflammatory and amyloidogenic factor in experimental in vitro and in vivo models of inflammation and amyloidosis.

Poorly degradable "bacterial remnants" or alternatively, "dormant" fastidious bacteria may persist indefinitely in the affected organs acting as a chronic antigenic stimulus inducing chronic inflammation [Fox, 1990]. Bacteria or their synthetic or natural components such as bacterial peptidoglycan and LPS 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 (Fox, 1990). All these processes are involved in the pathogenesis of AD. Already in 1907, Fischer suggested that senile plaques may correspond to colonies of microorganisms.

I.4. *Chronic bacterial infection can cause dementia*

Noguchi and Moor (1913), using a simple silver method demonstrated the persistence of *Treponema pallidum* in the brain of patients with dementia paralytica and made one of the most important discoveries in the history of syphilis, as they established a direct pathogenic link between spirochetes and dementia. Based on their observation it is now generally accepted that *Treponema pallidum* may cause chronic neuropsychiatric disorders including general paresis. In the atrophic or "stationary"" form of general paresis known also as the long standing form of dementia paralytica, the bacterium - *Treponema pallidum* - is the causative agent for cortical atrophy, microgliosis and amyloid deposition

Intriguingly, the clinical and pathological hallmarks of AD are identical to those occurring in the atrophic form of general paresis. Notably, Alzheimer himself referred to the similarity of the clinical picture of his patient to dementia paralytica (Alzheimer, 1911). With respect to the histopathological changes, multiple authors have described *Treponema pallida* colonies confined to the cerebral cortex in patients with general paresis (e.g. Jahnel, 1916, 1917, 1920; Pacheco e Silva, 1926-27). The morphology and distribution of these colonies are identical to those of the senile plaques in AD (Fig. 1).

Neurofibrillary tangles have also been described in dementia paralytica (Bonfiglio, 1908; Perusini, 1910; Vinken and Bruyn, 1958) likewise amyloid deposition in the cerebral cortex Volland, 1978).

1.5. *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. *Treponema pallidum*, the causative agent of syphilis, is a tightly spiralled spirochete (about 0.1 μm x 20 μm) transmitted by sexual contact. *Treponema pallidum* has not yet been grown in synthetic media alone, although it has long been propagated in the testes of rabbits and cell monolayer systems as reviewed by Cox (1994).

Borrelia burgdorferi, which can be cultivated in a synthetic medium, is a larger (0.1-0.3 μm x 30 μm) spirochete, which is transmitted by tick bites to humans and causes Lyme disease. They both belong to the family Spirochaetaceae.

The similarity of the clinical and pathological manifestations of syphilis and Lyme disease, caused by the spirochete *Borrelia burgdorferi,* (Burgdorfer et al., 1982) is well established (Fallon and Nields, 1994). *Borrelia burgdorferi* may also persist in the infected host tissue and play a role in chronic neuro-psychiatric disorders. Dementia, including subacute presenile dementia, has been reported to occur not only in syphilis but also in Lyme disease (Dupuis, 1988).

1.6. *Previous contribution from our laboratory related to the basis of the thesis*

Helically shaped microorganisms were observed in the cerebrospinal fluid (CSF), blood and cerebral cortex in 14 AD cases that were absent in 13 controls (Miklossy, 1993, 1994a). Further taxonomical characterization using atomic force and scanning electron microscopy showed that the microorganisms taxonomically belong to the order Spirochaetales (Miklossy et al., 1994). In three of these 14 AD cases, spirochetes were grown in a medium selective for *Borrelia burgdorferi*. Based on these observations we proposed that AD may correspond to late stages of neurospirochetoses caused by several types of spirochetes, including *Borrelia burgdorferi*. As spirochetes showed immunoreactivity for AβPP (Miklossy 1993, 1994) we suggested that amyloidogenic proteins may be an integral part of these microorganisms and play a role in amyloidogenesis in AD. Further analyses using scanning electron microscopy and atomic force microscopy showed that the helically shaped microorganisms isolated and cultivated from the brains of AD patients possess axial filaments; indicating that taxonomically they belong to the order Spirochaetales (Miklossyet al., 1994).

If spirochetes play a role in the formation of senile plaques, neurofibrillary tangles and neuropil threads, we anticipated that they may contain DNA. The 4'6-diamidine-2'-phenylindole dihydrochloride (DAPI) binds selectively to DNA and forms strongly fluorescent DNA-DAPI complexes (Russel et al., 1975). DNA staining is the most popular method for the detection of cell culture Mycoplasma. Using this sensitive DNA stain, not only reference spirochetes may be visualized but also senile plaques, neurofibrillary tangles and neuropil threads. DNase pretreatment abolishes the DAPI staining of these structures indicating that they contain DNA (Miklossy et al., 1995).

Bacterial peptidoglycan, the potent inflammatory and amyloidogenic bacterial cell wall component had been detected immunohistochemically in senile plaques and neurofibrillary tangles of 17 AD cases analyzed and was absent in 7 control cases. In senile plaques bacterial peptidoglycan was co-localized with Aβ (Miklossy et al., 1996. Miklossy, 1998).

If AD may correspond to spirochetoses, the microorganism should spread by hematogen dissemination; therefore the choroid plexus, which represents a barrier between blood and CSF, and several other organs than the brain may be affected. The results of extensive studies (Miklossy et al., 1998, 1999) showed that "curly fiber" and "tangle" formation is not unique to the central nervous system (CNS). Pathological fibrils with histochemical properties of amyloid, similar to neurofibrillary tangles and neuropil threads, accumulate not only in the choroid plexus but also in several other organs than the brain.

Oral spirochetes may also invade the CNS via the olfactory system. If oral spirochetes may play a role in AD, as showed recently by Riviere et al., (2002) we anticipated that the olfactory system may be affected in early stages of AD. The analysis of 110 autopsy cases showed a close association between the cortical and olfactory AD-type changes and indicated that the involvement of the olfactory bulb and tract is one of the earliest events in the degenerative process in AD (Christen-Zaech et al., 2003).

Pyramidal involvement; as the denominations of the disease also indicates; is known to occur in general paresis. The primary motor cortex was thought to be spared in AD. The results of a morphometric analysis of 29 brains, including AD cases and

controls showed that the primary motor cortex is severely affected in late, terminal stages of AD (Suva et al., 1999; Suva, 2001; Miklossy et al., 2003).

Cerebral hypoperfusion occur in both, AD and neurospirochetoses (Hachinski and Munoz, 1997, Logigian, 1997). We analyzed whether vascular involvement secondary to cerebral hypoperfusion may occur in AD. The analysis of 184 brains of AD patients and controls showed that watershed cortical microinfarcts secondary to cerebral hypoperfusion occur and are significantly associated with AD (Suter et al., 2002). In addition to the direct parenchymal involvement in syphilis the occurrence of the meningovascular form is well established, where the parenchymal involvement is secondary to the inflammatory damage of the leptomeningeal vessels with consequent cerebral vascular infarcts. The meningovascular form of Lyme neuroborreliosis was also documented (Miklossy et al., 1990). These observations may give a comprehensive explanation why in neurospirochetoses and in AD the degenerative parenchymal changes may frequently be associated with vascular infarcts.

In the present study we continued investigations on the involvement of bacteria, namely of spirochetes in AD. We proposed that several types of spirochetes may be involved in AD, including oral, intestinal but also Borrelia spirochetes (Miklossy 1993, 1994, 1996, Miklossy et al., 1994, 1996). Recently Riviere et al., (2002) using species-specific PCR and monoclonal antibodies, detected oral Treponema in 14/16 AD cases and 4/18 non-AD controls.

As *Borrelia burgdorferi* was another candidate to play a role in amyloidogenesis in AD, in the present study we analyzed the sequence of the 16S rRNA gene of spirochetes cultivated from the brain of three of the 14 AD cases, grown in medium

selective for *Borrelia burgdorferi* (Miklossy, 1993) and carried out morphological characterization by transmission electron microscopy. Since diagnostic and serological tests are available for *Borrelia burgdorferi*, 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. The molecular analysis of spirochetes cultivated from the blood of a clinically asymptomatic forester who showed positive serology for Lyme disease was also performed. As a control, a previously characterized reference, B 31 strain of *Borrelia burgdorferi* was utilized for comparative genomic characterization.

In the second part of the work we further analyzed the amyloidogenic properties of spirochetes. Previous observations indicated that amyloidogenic protein may be integral part of spirochetes and play a role in amyloidogenesis in AD (Miklossy, 1993, 1994). This observation was recently reinforced by Ohnishi et al., (2000, 2001) who showed that the outer surface protein (OspA) of *Borrelia burgdorferi* forms amyloid fibrils in vitro, similar to human amyloid. Therefore, to test the hypothesis that bacteria might induce an amyloidogenic reaction in mammalian cells in culture, we exposed cultures of rat primary neurons and astrocytes to the spirochete *Borrelia burgdorferi*. We report here the results of such exposures.

II. THE GOALS OF THE STUDY

The principal aim of the present study was the continuation of previous investigations on the pathogenetic role of spirochetes in chronic inflammation and amyloidogenesis in Alzheimer's disease.

Spirochetes were cultivated from the brain of three AD cases in a medium selective for *Borrelia burgdorferi.* Phylogenetic characterization of these spirochetes was carried out following PCR and sequence analysis of the 16S rRNA gene. Morphological characterization of the spirochetes by transmission electron microscopy was also performed. Since diagnostic and serological tests are available for *Borrelia burgdorferi*, a post mortem serological analysis of blood and cerebrospinal fluid (CSF) and the detection of *Borrelia burgdorferi* antigens and genes in brain samples from the same patients where the spirochetes were cultivated were also made. The molecular analysis of spirochetes cultivated from the blood of a clinically asymptomatic forester who showed positive serology for Lyme disease was also performed. As a control, a previously characterized reference, B 31 strain of *Borrelia burgdorferi* was utilized for comparative genomic characterization (Miklossy et al, 2004).

In the second part of the study, we further analyzed the amyloidogenic properties of 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*. The in vitro induced beta amyloid was detected by immunohistochemistry and Western blot. Synchrotron InfraRed MicroSpectroscopy (SIRMS) was used to detect beta pleated sheet conformation of the in vitro induced amyloid. Cells not exposed to Borrelia were used as controls.

III. MATERIALS AND METHODS

III.1 Characterization of spirochetes cultivated from the brains of Alzheimer's patients

III.1.1 *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 absent in 13 control cases (Miklossy, 1993). An ultrastructural study showed that these microorganisms belong to the order Spirochaetales (Miklossy et al., 1994). In 3 of these 14 AD cases spirochetes were cultivated from the brain in a synthetic BSK II medium (Berger et al., 1985) using serial subcultures. According to clinical records, these patients suffered from AD type dementia. The age of the patients was 74, 78, and 86 years, and the cause of the death was rupture of an aortic aneurysm, cardiac failure, and bronchopneumonia, respectively. In case AD2 the clinical records mentioned traumatic brain injury 8 years before death. These three AD patients were living in the western (French-speaking) geographic area of Switzerland where Lyme borreliosis is endemic and is responsible for much systemic morbidity (Nahimana et al., 2000).

Spirochetes were also cultivated from the blood of a forester, a healthy blood donor (HF), whose serological tests were positive for *Borrelia burgdorferi*. Blood and serum samples as well as the cultivated spirochetes from this *latter* patient were also available for analysis.

At autopsy, from each case, about 5 ml cerebrospinal fluid (CSF) was removed by cisternal puncture and 10 ml blood by jugular vein or by direct cardiac puncture under sterile conditions. CSF and blood samples were examined by dark field microscopy, as previously described (Miklossy, 1993). About 5 ml blood samples were centrifuged at x1000 g at 4°C for 5 minutes and the serum was stored at - 80°C and used for the serological analyses. The remaining CSF and blood was stored at - 80°C until processing for further analyses. Post mortem blood and CSF samples from 3 control cases, without brain pathology and without AD were processed in the same manner.

From the unfixed brain, about 3x3x2 cm large blocks were taken from the frontal (Brodmann's areas 8, 9) and parietal (Brodmann's areas 39 and 40) cortical associative areas, as well as from the temporal cortex which included the hippocampus and the entorhinal cortex. From the frontal cortex 2 adjacent blocks were taken. One of them was processed, without freezing, for the cultivation of spirochetes and for the preparation of samples for dark field microscopy analysis. From unfixed tissue samples 10μm thick frozen sections were cut in cryostat and were stored at - 20°C for immunostaining and for in situ hybridization. Following 4-week formaldehyde fixation, additional samples were taken from cortical regions adjacent to the frozen samples, which were embedded in paraffin. Five μm thick paraffin sections were stained with Haematoxylin and eosin (H&E), Thioflavin S, Gallyas silver technique for neurofibrillary tangles and immunostained using a monoclonal antibody to β-amyloid protein (DAKO, M872, dilution 1:100). From each case paraffin sections of the frontal cortex and hippocampus were also stained using silver impregnation techniques described for the visualization of

spirochetes, namely the Levaditi, the Warthin-Starry and the microwave Bosma-Steiner techniques.

A semi-quantitative analysis of the density of senile plaques and neurofibrillary tangles was performed in all cortical areas as previously described in detail (Miklossy et al., 1998). The neuropathological assessment of the severity of cortical involvement was also made following Braak and Braak (1993). For the neuropathological diagnosis of AD consideration was given to the criteria proposed by Khachaturian (1985), CERAD (Mirra et al., 1993) and the National Institute on Aging (NIA) - Reagan Institute Working group (Newell et al., 1999). The 3 AD cases with dementia fulfilled criteria for the definite diagnosis of AD.

An additional blood sample of a forester, a healthy blood donor (HF), whose serological tests were positive for Lyme disease, was also available for analysis. As for the other cases whet preparations were analyzed by dark field microscopy and 100µl blood was inoculated in sterile condition into BSK II medium, than subcultures were prepared. The remaining blood was stored at - 80°C for further analyses.

III.1.2. *Cultivation of spirochetes and their immunohistochemical characterization*

From the fresh brain, about 3x3x2 cm large frontal samples were removed in sterile conditions. After rapidly passing through burner flame, 2mm thick sections were removed from each side of the brain sample using sterile razor blades. We always selected part of the remaining tissue, which was rich in cortical tissue, and discarded a large part of the white matter. The remaining sterile central part of the sample was cut into smaller pieces and put in 10ml BSK II medium containing Rimactan (Novartis, 25

µg/ml) and Fosfocin (Boehringer Mannheim, Mannheim, Germany, 60 µg/ml). After two weeks and 1 month of culture at 32°C, 1 ml culture medium was removed in order to prepare subcultures. Each two weeks, 30µl samples of the cultures and subcultures were checked for the presence of spirochetes using dark field microscopy and the results were recorded by photomicrographs. Smear preparations of cultures and subcultures were also stained with Warthin-Starry and Bosma-Steiner silver techniques for spirochetes and were immunostained with anti-*Borrelia burgdorferi* antibodies (listed below), using the avidin-biotin-peroxidase technique. Smear preparation of *Borrelia burgdorferi* (B31) cultivated in BSK II medium was used as positive control. The immunoreaction was also performed with the omission of the primary antibody or using an irrelevant antibody.

III.1.3. *Taxonomic characterization using transmission electron microscopy*

For electron microscopic analysis the cells of strains ADB1, ADB2 and those cultured from the blood of the healthy forester (strain HFB) were harvested by centrifugation and gently suspended in 10mM Tris-HCl buffer (pH 7.4) at a concentration of about 108 cells per µl. Samples were negatively stained with 1% (Wt/vol) phosphotungstic acid (pH 6.5) for 20 to 30 sec. Specimens were examined with a Jeol Model JEM-1200EX transmission electron microscope operating at 100 kV.

III.1.4. *Molecular characterization of the cultivated microorganisms*

Sequences for 16S rRNA genes were determined for 3 of the cultured spirochetes, two from AD patients and one from the healthy forester.

The comparative sequence analysis of the 16S rRNA gene sequences in the spirochetes isolated from the two AD brains and the healthy forester was carried out. Comparative sequence analysis of the 16S rRNA in the spirochetes isolated from the two AD brains and the healthy forester was carried out. 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. It exhibits functional constancy and its sequence has evolved slowly, that allow most phylogenetic relationships to be measured (Woese, 1987). Other conserved genes do not necessarily meet these criteria.

DNA was isolated from cultured spirochetal cells and PCR amplified using the universally conserved primers previously described (Paster et al., 1998). As a negative control, buffer containing no amplifiable DNA was utilized. Cycling conditions were followed as previously described (Paster et al., 1998). A spirochetal selective reverse primer C90 (5'-GTT ACG ACT TCA CCC TCC T-3') was used with a universal forward primer C75 (5' GAG AGT TTG CTG GCT CAG-3'). Three μl of the crude DNA and 1 μM of primers were added to the reaction mixture, which had a final volume of 82 μl. Ampliwax PCR Gem 100's was used in a hot-start protocol as suggested by the manufacturer. The following conditions were used for the amplification using primers C70 and B37: denaturation at 94°C for 45 sec, annealing at 50°C for 45 sec, and elongation at 72°C for 90 sec with 5 additional sec added for each cycle. A total of 30 cycles was performed followed by a final elongation step at 72°C for 15 min. Conditions for amplification using primers C90 and C75 were identical, except that the annealing temperature was 60°C. After removal of Ampliwax, 0.6 volumes of 20 % PEG 8000

(Sigma) in 2.5 M NaCl were added, and the mixture was incubated at 37°C for 10 minutes to precipitate the DNA. The sample was centrifuged for 15 minutes at 15 000 g and the pellet washed with 80% ethanol. The pellet was then dissolved in 35 ml of sterile water.

Sequencing and 16S rRNA data analysis followed those described by Fox et al. (1995). The DNA sample from PCR after purification was directly sequenced using cycle-sequencing kits (TAQuence Cycle Sequencing kit, USB, Cleveland, OH) or an fmol DNA Sequencing kit (Promega Corp.). Primers were end-labeled with 33P-ATP (NEN-Dupont) using the manufacturer's protocol. Twenty-five to 80ng of purified DNA from the PCR amplification was used for each sequencing reaction. Reaction products were run electrophoretically on 8 % polyacrilamide-urea gels and were subsequently detected by exposure of the dried gels to X-ray film for 24 to 48h.

Programs for data entry, editing, sequence alignment, secondary structure comparison, similarity matrix generation, and phylogenetic tree construction were written in Microsoft QuickBASIC for use on IBM PC-AT and compatible computers. Our sequence database contains approximately 1 000 sequences as determined in our laboratory (Paster and Dewhirst, 2000). The sequences of most of the cultivable species of oral bacteria, particularly Gram negative species, were present in our database. Other published sequences and about 5 000 sequences available from Ribosomal Database Project (Paster et al., 1988) and GenBank were also available for comparisons. Similarity matrices were constructed from the aligned sequences by using only those sequence positions for which 90% of strains have data (Choi et al., 1994). The similarity matrices were corrected for multiple base changes by the method of Jukes and Cantor (1969).

Phylogenetic trees were constructed using the neighbor-joining method of Saitou and Nei (1987).

III.1.5. *Serological analysis*

The blood of the healthy forester was analyzed using the Venereal Disease Research Laborartory (VDRL), Rapid Plasma Reagin (PRP) test, Fluorescent Treponemal Antibody Absorption (FTA-ABS), *Treponema Pallidum* Hemagglutination (TPHA), Indirect Immunofluorescent Antibody Test (IFAT) and the Enzyme-Linked Immunoabsorbent Assay (ELISA) tests. In addition, Western blot analysis was also performed (Immunosa, Nyon,CH; and BioGenex Lyme IgG/IgM, D601-Lyme) for the detection of specific anti-*Borrelia burgdorferi* IgG and IgM antibodies. A post mortem serological analysis of the blood and CSF of the AD cases was made using IFAT, ELISA and Western blot (BioGenex Lyme IgG/IgM, D601-Lyme). The serological analyses were made independently in two different laboratories.

For the evaluation of Western blot analysis, criteria proposed by the Centers for Disease Control and Prevention (CDC) (1995) 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.

III.1.6. *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, the following anti-*Borrelia burgdorferi* antibodies were used at the indicated dilutions: monoclonal anti-OspA (H5332, H3T5, Symbicom, 1:10), Flagellin (G9724, H605,

Symbicom, 1:20), anti-*Borrelia burgdorferi monoclonal* (C63780M, Biodesign,1:30) and polyclonal (Biodesign, B65302R,1:30). Additionally, two rabbit anti-*Borrelia burgdorferi* antibodies prepared in the University Institute of Pathology, CHUV, Lausanne, Switzerland (BB-1017, 1:500 and BB-1018, 1:500) were tested. For the preparation of these polyclonal antibodies, two rabbits (weight 2.5 and 3 kg) were immunized weekly with 0.5 ml of cultured *Borrelia burgdorferi* (strain B31 in BSK II medium) in emulsion with an equal part of Freund's complete adjuvant. They were bled 1 week after receiving the third injection and the sera were used for immunostaining. The specificity of all these mono and polyclonal anti-*Borrelia burgdorferi* antibodies were tested by Western blot analysis (BioGenex Lyme IgG Kit; D601-Lyme), following the instructions of the manufacturer.

For the detection of *Borrelia burgdorferi* specific antigens in the brain of the 3 AD cases, frozen sections were analyzed. These were fixed in acetone for 10 minutes at 4°C, pretreated with 1% amylase at 37°C for 3-5 minutes, and washed 3 x 5 minutes with PBS before use. Two monoclonal antibodies for the detection of bacterial peptidoglycan (Biogenesis 7263-1006 and Chemicon MAB995, 1:100) were also used as previously described in detail (*S9, S10*). In order to determine if spirochete antigens, bacterial peptidoglycan, and Aβ are co-localized in senile plaques, serial sections, spaced at 14 μm were immunostained with anti-*Borrelia burgdorferi*, anti-bacterial peptidoglycan (Biogenesis 7263-1006 or Chemicon MAB995, 1:200) and anti-Aβ (DAKO, M872, 1:50) antibodies, respectively. For detection, the avidin-biotin-peroxidase technique was used. The sections were incubated with the primary antibody for 24, 48 or 72 hours at 4 °C. The immunoreaction was revealed by diaminobenzidine (DAB) alone, or with nickel-

ammonium sulfate enhancement. Smears of B31 were used as positive controls. Frozen sections immunostained in the absence of the primary antibody or with an irrelevant mono- or polyclonal antibody were used as controls. Brain sections of control cases without brain lesions were also used as negative controls.

III.1.7. *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 sections (5 µm) as well as frozen sections (10 or 20 µm) were utilized. The paraffin sections were dewaxed in xylene, hydrated in 99%, and 95% ethylene and rinsed in pure water 2 x 3 min. On both frozen and paraffin sections, endogenous peroxidase was blocked by treatment in methanol containing 3% H_2O_2. The sections were treated with 1% hot SDS (70°C) for 5 min, with Lysozyme (25 000 U/ml in PBS, pH 5.5 at 37°C) for 5 min and with Proteinase K (10µg/ml in 50 mM Tris-HCL, pH 7.6 at 37°C) for 30 min. Following each treatment, the sections were washed in pure water 3 x 10 minutes. The sections were post-fixed for 20 min with 1% paraformaldehyde in PBS containing 50 mM $MgCl_2$, rinsed with three changes of pure water, and dried in a series of ethanol washes. The sections were incubated with a prehybridization solution (1 µl 0.5M Tris HCl, pH 7.4, 50µl 20-X- SSC, 1µl 0.05 M EDTA, 100µl of 50% dextran sulfate, 250µl formamide and 98 µl of pure water for a total volume of 500µl) in the humidity chamber of the thermal cycler at 42°C for 1 hour. The prehybridization solution was then replaced by the hybridization solution containing 100ng 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. The sections were coversliped, and 10 min incubation at 100°C was followed by an overnight incubation at 42°C in the humidity chamber of the Hybaid cycler. Posthybridization washes were in an equal mixture of formamide and 2-X-SSC, pH 7 at 42ºC for 2 x 20 min and in 0.1-X-SSC, 2 mg $MgCl_2$, 0.1% Triton-X-100 at 60°C for 30 min. After a rinse in TBS 3 x 5 min, the sections were treated with a blocking solution containing normal rabbit serum diluted 1:5, 3% bovine serum albumin and 0.1% Triton-X-100 in TBS for 1 hour. For the detection of the hybridization products anti-digoxigenin alkaline phosphatase or peroxidase conjugates were used. The alkaline phosphatase substrate solution or DAB were used as chromogens for visualization of the reaction products. Control sections without specific probes and sections from patients without brain pathology were used as negative controls.

IV.2. In vitro induction of beta-amyloid by Borrelia spirochetes

III.2.1. *Cell cultures*

Primary astrocyte and microglial cell cultures from Sprague-Dawley rats were prepared as previously described (Janzer and Raff, 1987). The astrocytes were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, 12387-015). The microglial cells were cultured in RPMI medium (RPMI 1640, Sigma R8758), containing10% fetal calf serum (FCS). Microglia enriched astrocyte cultures (10^6 astrocytes and 10^5microglia)

were also prepared by combining astrocytic and microglia cultures prepared as described above. Neurons dissociated from the telencephalon of 21-day-old rats were cultured on collagen or poly-L-ornithine coated substrate surfaces (Fiumelli et al., 2000). The cells were cultured in 2-well chambers (177429 Lab-Tek, Christchurch, New Zealand), in six-well clusters (3506, Costar, Acton, Maryland), and in 75ml non-pyrogenic polystyrene flasks (3376, Costar, Acton, Maryland) in a humidified CO2 (6%) incubator at 37°C.

The organotypic culture consisted of aggregates of primary cells of fetal 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.

III.2.2. *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 $5x10^5$/ml. Before exposure to spirochetes, the cells were tested with 4',6-diamidine-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. The astrocyte and microglia enriched astrocyte cultures were infected for periods of 1, 2, 4 and 8 weeks, and the primary neuron and cell aggregate cultures for 1, 2 and 4 weeks. In addition, in each set of experiments, for all cell types and for each exposure time, the cells and cell aggregates were divided into six equal samples. Five of the samples were infected with spirochetes, while one uninfected sample was used as control. Immunohistochemical detection of Aβ was done at each time point in each set of experiments for all cell types including cell aggregates.

III.2.3. *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 for10 min with 4% paraformaldehyde. For Aβ immunostaining, 100% formic acid pretreatment for 20 min was used. Frozen sections (5μm thick) were cut from the brain cell aggregates for immunohistochemistry. Cultures not infected with spirochetes were used as controls.

The antibody types, as well as their source, dilution and structures recognized are given in Table 1. Astrocytes were identified with anti-glial fibrillary acidic protein (GFAP); rat microglial cells with ED1 and human microglia with anti CD68. To detect Aβ, antibodies recognizing amino acids 8-17 of Aβ and the C terminal part of Aβ 1-42 (21F12 - a gift from Dr Dale Schenk, Athena Neurosciences) were used. To detect AβPP, the 22C11antibody, which recognizes the N terminal part of all AβPP isoforms was used. Tau protein was detected with T-6402, Tau 2 and clone AT8. The AT8 antibody recognizes phosphorylated Ser 202/Thr205 residues of tau, and labels PHF in AD. Borrelia antigens were detected with monoclonal anti-OspA and anti-Flagellin antibodies, and also with rabbit anti-Borrelia antibodies. The anti-Borrelia antibodies were tested for their specificity to recognize Borrelia antigens by Western blotting using the BioGenex Lyme IgG Kit (D601-Lyme) following the instructions of the manufacturer.

III.2.4. *Western blot analysis*

For Western blots, whole cell extracts of infected and control primary astrocytes and microglia enriched astrocytes were prepared in lysis buffer containing 1% protease inhibitors (P-8340, Sigma, St. Louis, Missouri). Triplicate samples from experiments at 2, 4 and 8 week exposure times were analyzed. They were continuously rotated for 4 hours at 4°C and were exposed to alternate boiling (85°C) and cooling in dry ice, (3 times, 1 min). Extraction of Aβ was performed using formic acid as previously described in detail (Fonte et al., 2001). To 50 μg protein samples, Laemmli buffer was added followed by boiling for 5 min at 85°C. The samples were than electrophoresed on 14-20% Tris HCL and 4-20% TRIS HCl-urea polyacrylamide gels (BioRad). Transfer onto a

0.2 mm nitrocellulose membrane (BioRad) was performed at 350 mA for 1.5 hours at 4ºC. Immunoblotting was performed using the antibodies mentioned above for the detection of Aβ, AβPP, and tau proteins. For detection, ECL- Plus (Amersham, RPN2132) was used and the development of the film (Super RX, Fujifilm, 03G050) was performed in accordance with the manufacturer's protocol. Samples (1 µg or 500 ng) of synthetic Aβ1-42 peptide (H 1368, Bachem, Bubendorf, Switzerland) and 50 µg samples of whole cell extracts prepared from pCMV/AβPP695 transfected U87MG cells were used as positive controls. Antibodies to actin and Growth factor receptor bound protein 2 (Grb2) were used to verify loading conditions.

II.2.5. *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 AβPP 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 ($0.5x\ 10^6$) were co-cultured with $0.1x\ 10^6$ 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 $NaHCO_3$ 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).

III.2.6. *Synchrotron InfraRed MicroSpectroscopy (SIRMS)*

In order to analyze whether the β-sheet conformation typical of amyloid is present in the Borrelia induced amyloid deposits, Synchrotron InfraRed MicroSpectroscopy (SIRMS) analysis was performed. A Spectra Tech Continuμm infrared microscope coupled to a Nicolet Magna 860 FTIR was used, where the conventional infrared source was replaced by synchrotron light from Beamline U10B (National Synchrotron Light Source, Brookhaven National Laboratory). The infrared microscope was equipped with a light source and filters for fluorescence microscopy analysis (Choo et al., 1996, Dumas et al., 2004). For the SIRMS analysis, infected and uninfected cells were harvested by centrifugation, washed with PBS, placed on infrared-transparent BaF_2 slides and stained with Thioflavin S. Samples of infected and uninfected astrocyte cultures derived from three different experiments at 4 week exposure times were analyzed. Frozen sections from the frontal cortex of five AD and two control cases were also analyzed. Infrared microspectra were collected in transmission mode, 128 scans per point, 4 cm^{-1} resolution using Atlμs software (Thermo Electron Spectroscopy). 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}.

IV. RESULTS

IV.1 Characterization of spirochetes cultivated from the brains of Alzheimer's patients

Table 2 summarizes the main results obtained in the present study.

IV.1.1. *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 in their morphology, motility and proliferation rate. The initially stretched form was replaced by partially, then completely spiral forms (Fig.2 A-D) and following 4 months of culture the morphology of the cultured microorganisms was identical to the reference strain (B31) of *Borrelia burgdorferi* (Fig.2 E). No proliferation was detectable during the first 2-3 months. 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), when stained with specific silver techniques described for the visualization of spirochetes. The cultivated spirochetes showed a positive immunoreaction with 8 different anti-*Borrelia burgdorferi* antibodies, including a monoclonal antibody recognizing the Outer Surface protein A (OspA).

IV.1.2. *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 (Fig. 3C,D).

IV.1.3. *Genomic characterization of the cultivated microorganisms*

For genomic characterization, the full sequences of the 16 SrRNA gene for three of the cultivated spirochetes were determined: for strains ADB1 and ADB2 (cases AD1, AD2) and HFB (healthy forester). Although the spirochetal strain ADB1 was contaminated with an unknown bacterium, the use of spirochetal selective primers for PCR enabled genetic analysis of the spirochete to be determined. The sequence of the 16S rRNA gene was identical for the three spirochete strains analyzed as is illustrated in Figure 3A.

The phylogenetic analysis of the 16S rRNA gene sequence revealed that the cultured spirochetes (strains ADB1, ADB2 and HFB) correspond to *Borrelia burgdorferi sensu stricto (s. s.)*. The phylogenetic position of these spirochetes among other species of spirochetes and borrelial strains is shown in Figure 3 B.

IV.1.4. *Serological analysis*

The results of the serological analyses are illustrated in Table 3 and Figure 4. The analysis and the interpretation of the serological results were made following criteria of

the Center for Disease Control (CDC) (1995). A positive serology for *Borrelia burgdorferi* was detected in 2 AD cases (AD 1 and AD3). 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 (Fig. 4B). It is of interest to note that the *Borrelia burgdorferi* specific 31 kDa OspA band was present in all the 3 AD cases, likewise the p39 band despite it being very weak in two cases, whereas the p34 OspB band was absent. Following CDC criteria, in case AD2 we concluded that the serology was negative, but that the detection of OspA and the weak p39 and p24-25 bands by Western blot was noteworthy. The serological tests of the healthy forester showed the following values: VDRL-; TPHA+ 320 (normal value > 80); FTA-Abs TP-; IFAT +/- 1/128 (normal value > 120) and ELISA +/- 121U (normal value > 120). The Western Blot was positive following the results obtained by Immunosa (Nyon, Switzerland) and also following the results obtained employing the BioGenex Lyme IgG Western blot Kit. The Western blot of the serum and CSF of the non-demented controls and of the AD subject where spirochetes were not cultured from the brain, were negative.

IV.1.5. *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 (Jahnel, 1916, 1917, 1920; Pacheco e Silva 1926-27, Lubarsch et al., 1958) caused by *Treponema pallidum* (Fig. 5; Fig. 6A). Thread-like

structures disseminated in the cortical neuropil, compatible with individual spirochetes, were also observed.

An immunohistochemical analysis was performed for the detection of *Borrelia burgdorferi* antigens in the brain of the patients from which Borrelia spirochetes were cultivated. Western blot analysis of 8 different antibodies showed their ability to recognize *Borrelia burgdorferi* antigens (Fig. 6). The colony-like masses (Fig. 7B) and part of the disseminated individual filaments (Fig. 7F) showed positive immunoreactions with anti-*Borrelia burgdorferi* antibodies, including the anti-OspA antibody. The spirochete antigens showed the same pattern of distribution as amyloid beta peptide (Aβ) (Fig. 7C). Although the immunoreaction was weaker for OspA, the labeling was consistent and was stronger in the center of the colony- or plaque-like structures. *Borrelia burgdorferi* antigens, including OspA were also detected in a number of neurofibrillary tangles (Fig. 7D,E) and in the wall of some blood vessels containing amyloid deposition (Fig.7G). On serial sections, Borrelia antigens, bacterial peptidoglycan and Aβ were co-localized in senile plaques and in blood vessels.

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 (ISH) (Fig.7H). The pattern of distribution was similar to Borrelia antigens. The extranuclear localization of the ISH product excluded the possibility of unspecific DNA labelling. Control sections where the specific Borrelia antibodies or probes were omitted were negative (Fig.7I).

IV.2. In vitro induction of beta-amyloid by Borrelia spirochetes

We assessed whether Aβ deposition could be induced in vitro by infecting primary mammalian neurons, astrocytes, microglial cells, and brain organotypic cell aggregates with spirochetes. We used *Borrelia burgdorferi* spirochetes, as they can be maintained in culture in synthetic medium (BSKII).

IY.2.1. *Immunohistochemical analysis of the infected and control cell cultures*

The histochemical and immunohistochemical analysis showed the same results, with respect to the Aβ deposition, in the form of plaque-like structures in all cell types and in the cell aggregates exposed to Borrelia. The results are illustrated in Figure 8.

Following one week exposure to spirochetes, Aβ was not detected in any infected cells or cell aggregate samples. At two week exposure to Borrelia, immunohistochemical detection of Aβ was observed in 4/10 of the astrocyte cultures, in 3/4 of the microglia enriched astrocytic cultures and in 2/3 of the neuronal cultures and in cell aggregates. Aβ deposition was observed in all cells and cell aggregates following 4 and 8 weeks of exposure to spirochetes. Plaque-like Aβ deposits following 4 week exposure to spirochetes as revealed by immunostaining is illustrated in Figure 8. The majority of the amyloid deposits were large (100-150μm) extracellular aggregates (Fig. 8a-e). They were in a consolidated form as revealed by thioflavin-S staining (Fig. 8c and d). Their numbers varied between five and twenty per well and between two and ten per spheroid in the cell aggregates. A weak intracellular Aβ immunoreactivity was also apparent in some cells in brain aggregates infected with Borrelia for 2-4- weeks (Fig. 8a, arrows). The in vitro

induced “plaques” were immunoreactive with the 6F/3D antibody recognizing residues 8-17 of Aβ (Fig. 8a, b, e) and also with the anti-Aβ 1-42 (21F12) antibody (Fig. 8g).

In the infected neuronal and organotypic cultures, a few Aβ-42 and OspA-positive tangle-like formations were also observed (Fig. 8g,h and i, respectively). OspA positive intracytoplasmic granules in astrocytes, surrounded by a thin pale halo, similar to granulovacuolar degeneration of neurons in AD, were also seen (Fig. 8j). Only rare, OspA positive solitary intracytoplasmic granules were seen in a few neurons in the infected neuronal cultures. The uninfected control cultures did not show Aβ deposition, or “granulovacuolar degeneration” (Fig. 8k).

IV.2.2. *Western blot analysis of the infected and control cell cultures*

We detected a 4 kDa Aβ peptide band by Western blot (Fig. 9a and b), in all cell culture samples analyzed, following 2-8 weeks exposure to *Borrelia* spirochetes. The presence of the 31 kDa outer surface protein (OspA) of *Borrelia burgdorferi* was utilized to monitor the presence of spirochetal infection. After 2-weeks exposure to Borrelia spirochetes, the Aβ deposition was absent or weak in primary rat astrocytes (Fig. 9a) but was more pronounced in microglia-enriched astrocyte cultures (Fig. 9b), suggesting that microglia enhance Aβ formation. Cell cultures infected for 8 weeks showed a higher level of Aβ than those infected for 2 weeks (Fig. 9b). Aβ was not observed in the uninfected control cultures.

To determine whether AβPP plays a role in Aβ deposition induced by Borrelia spirochetes in vitro, we analyzed AβPP levels in infected cell cultures using Western blot analysis. In comparison to the uninfected control cultures, increased AβPP levels were

detected in Borrelia-infected cultures (Fig. 9c). AβPP levels were higher at 8-weeks than at 2-weeks following exposure to spirochetes.

The microtubule associated protein tau is hyperphosphorylated in AD and is a component of paired helical filaments (PHF) in neurofibrillary tangles. When we analyzed the phosphorylation of tau in cell cultures exposed to Borrelia spirochetes, increased phosphorylated tau levels were observed when compared to the uninfected control cultures (Fig. 9c).

IV.2.3. *Western blot analysis of PC12/THP-1 cells exposed to LPS*

Natural or synthetic bacterial components alone may lead to chronic inflammation and amyloid deposition (Fox, 1990; Foyn Bruun et al., 1994). We analyzed, therefore, whether the bacterial cell wall peptidoglycan lipopolysaccharide (LPS) could alone induce increased AβPP and hyperphosphorylation of tau. As microglia enhanced Aβ formation in primary cell cultures, PC12 cells were co-cultured with THP-1 cells before treatment with LPS. By immunoblotting, a slight increase of AβPP was detected following 30 min and 6 h LPS exposure, followed by a strong increase at 24 and 48 hours (Fig. 10). In addition, we also observed hyperphosphorylation of tau following 24 and 48 hour LPS exposure (Fig. 10).

IV.2.4. *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 (Choo et al., 1996). 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 (Fig. 11 a-b and d), which was absent in the uninfected control samples (Fig. 11 c and e).

V. DISCUSSION

V.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 (Wilske et al., 1994).

Lyme disease is geographically confined and the incidence is low when compared to AD (Campbell et al., 1998). This coupled with the fact that our cases came from a geographic area known to be endemic for Lyme disease, may explain why previous investigators have failed to detect any association of Borrelia with AD (Gutacker et al., 1998; Marques et al., 2000; McLaughlin et al., 1999). In order to study the particular involvement of *Borrelia burgdorferi* in AD, it is important to analyze AD patients with a positive serology for *Borrelia burgdorferi*. Two other cases of concurrent neocortical neuroborreliosis and AD have been reported (McDonald and Miranda, 1987; McDonald, 1988): immunostaining showed *Borrelia burgdorferi* in brain tissue and the spirochetes were cultivated from the cerebral cortex.

Different types of spirochetes may be similarly involved in other AD cases (Miklossy, 1993: Riviere et al., 2002). 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 may induce chronic inflammation and amyloid deposition in the brain. For the majority of these spirochetes, diagnostic and serological tests are not available. In our initial analysis of the potential involvement of spirochetes in AD, we visualized by dark field microscopy helically shaped microorganisms in the CSF, blood and cerebral cortex in 14 AD cases that were absent in 13 control cases (Miklossy 1993, 1994). Further analyses using scanning electronmicroscopy and atomic force microscopy showed that they possess axial filaments; therefore taxonomically they belong to the order Spirochaetales (Miklossy et al., 1994). Subsequently Riviere et al., (2002) using species-specific PCR and monoclonal antibodies, detected oral Treponema in 14/16 AD cases and 4/18 non-AD controls. In endemic areas of Lyme disease, the wide distribution of other spirochetes (e.g. oral spirochetes), which were found to be associated with AD, may mask a clustering of an association of *Borrelia burgdorferi* with AD. Careful epidemiological studies will be necessary to analyze this point.

Based on previous analyses we also suggested that amyloidogenic protein may be an integral part of spirochetes (Miklossy, 1993, 1994; Miklossy et al., 1996). These observations were reinforced by Ohnishi et al., (2000, 2001) who showed that the outer surface protein (OspA) of *Borrelia burgdorferi* forms amyloid fibrils in vitro, similar to human amyloidosis.

The pathological findings observed in the 3 AD cases were reminiscent of those

described in dementia paralytica caused by *Treponema pallidum* (Jahnel, 1916, 1917, 1920, Pacheco e Silva, 1926, 1927). They are consistent with primary parenchymatous involvement of tertiary Lyme neuroborreliosis. Similar to the observations of Noguchi and Moore (1913) with respect to *Treponema pallidum*, our results show that *Borrelia burgdorferi* may also persist in the brain in chronic Lyme neuroborreliosis and be associated with dementia, cortical atrophy and amyloid deposition. The present findings reinforce the similarity between the clinical and pathological manifestations of syphilis and Lyme disease and suggest that *Borrelia burgdorferi* may also be involved in the pathogenesis of several chronic neuro-psychiatric disorders.

The case of the healthy forester, where the 16S rRNA analysis also defined the spirochetes cultivated from the blood as *Borrelia burgdorferi s. s.*, indicates that it could represent an acute, asymptomatic infection or may correspond to a more chronic latent stage of the disease. A clinical follow-up and repeated serological tests and cultures would be necessary to answer this question.

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 (Jamieson, 1991; Itzhaki, 1997, 2004). *Chlamydia pneumoniae* was also found to be associated with AD (Balin, 1998) and mice exposed to Chlamydia developed AD-like amyloid plaques (Little et al. 2004). The clinical and pathological hallmarks of Alzheimer's disease (AD) are present in the atrophic form of general paresis caused by spirochetes (Jahnel, 1916, 1917, 1920; Pacheco e Silva, 1926, 1927, Lubarsh et al., 1958) but they do not seem to be associated with Herpes simplex or Chlamydia infection of the

CNS. However, co-infection of spirochetes with other bacteria, including with Chlamydia but also viruses or even fungi is well known.

An infectious etiology of Alzheimer's disease would not be in controversy with the genetics defects shown to be present in AD. In chronic bacterial infections or in experimental amyloidosis the bacterial infection or bacterial exposure, always precede the amyloid deposition. In patients with a genetic defect that facilitates infection, the genetic problem would be the primary step in the cascade of events, followed by infection, then by amyloid deposition.

V.2. 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 Aβ deposition, increased AβPP levels, and hyperphosphorylation of tau.

Thioflavin S positive and Aβ-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 Aβ immunoreactive band in the infected cultures.

SIMRS is an extremely valuable tool for the analysis of chemical composition of biological and biomedical samples (Dumas et al., 2004). 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 (Choo et al., 1996). This peak near 1630 cm^{-1} was observed not only in the senile plaques of the 5 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. Recent observations show that several bacteria contain amyloidogenic proteins (Chapman et al., 2002; Jarrett and Lansbury, 1992; Miklossy, 1993, 1994, 1998; Miklossy et al., 1996; Ohnishi et al., 2000, 2001). Analysis of the periplasmic outer membrane lipoprotein - OsmB - of Escherichia coli showed a similarity in amino acid sequences to Aß peptide (Jarrett and Lansbury, 1992). Recent biochemical, biophysical, and imaging analyses revealed that fibers produced by Escherichia coli, termed "curly" were composed of amyloid (Chapman et al., 2002). 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, which may therefore play a role in amyloidogenesis in AD (Miklossy, 1993, 1994, Miklossy et al., 1996, 1998). Furthermore, it was shown that the OspA outer surface protein of *Borrelia burgdorferi* forms amyloid fibrils in vitro, similar to human amyloid (Ohnishi et al., 2000, 2001). These observations are in line with the present findings showing that Aβ 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* (Little et al., 2004).

In addition to the bacteria induced Aβ "plaques" tangle-like structures were also observed. They were immunoreactives with anti-Aβ antibody (21F12) sprcific for Aβ-42. There are evidences that AβPP and Aβ immunoreactivities are both associated with neurofibrillary tangles (Perry et al., 1993; Schwab et al., 2000). Bacteria induced Aβ positive "plaques" and "tangles" were also immunoreactive to Borrelia antigens. One might hypothesize that the anti-OspA "plaque" and "tangle" formed first, and then had Aβ deposited on them. This would also suggest that thread-like helically shaped microorganisms would be important for the formation of bacteria induced "tangles". This would also be in harmony with recent observations that in AD patients with neuroborreliosis, spirochetal antigens were co-localized with Aβ and Borrelia antigens were also present in neurofibrillary tangles (Miklossy et al., 2004). Detailed immunohistochemical and electron microscopical analysis of the Borrelia induced "plaques" and "tangles" would be important. However, in vivo studies, with a longer exposure time would be necessary to study efficiently the sequence of events and the cellular mechanisms involved in the bacteria induced beta amyloid deposition and "tangle" formation. Similarly, it is difficult to determine from the present in vitro studies, whether Aβ deposits are initially intracellular then become extracellular, or whether they are from the beginning extracellular. To answer this question, further in vitro and in vivo studies will be necessary.

The occurrence of intracytoplasmic granular changes in astrocytes and microglia in AD was previously reported (Perusini, 1910). A recent report showed the presence of granular structures recognized by the C-terminal part, but not the N-terminal part of the Aβ peptide in a subset of astrocytes and microglia (Akiyama et al., 1996). Whether the

bacteria induced OspA positive intracytoplasmic granules in astrocytes may be related to these glial changes or whether they may represent changes similar to neuronal granulovacuolar degeneration will require further in vitro and in vivo studies.

The increased AβPP level observed in cell cultures following exposure to Borrelia spirochetes or LPS reinforces the importance of host-derived AβPP in amyloidogenesis. AβPP is a proteoglycan core protein (Schubert et al., 1988; Wu et al., 1997). It is well established that increased proteoglycan synthesis plays a significant role in amyloidogenesis, including the formation of Aβ in AD (Castillo et al., 1996). Proteoglycans are present in early stages of amyloid deposition, but their exact role is yet to be determined. 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 (Strugnell et al., 1988), has been previously reported.

The microtubule associated protein tau is hyperphosphorylated in AD and is a component of the paired helical filaments of neurofibrillary tangles. When we analyzed the phosphorylation of tau in cell cultures exposed to Borrelia spirochetes, increased phosphorylated tau levels were observed. Although further analysis will be required to identify in detail the specific tau phosphorylation sites, the Borrelia and LPS induced tau phosphorylation represents additional experimental evidence of the ability of bacteria to induce an AD-like host cell reaction.

We observed increased AβPP and hyperphosphorylation of tau not only in cell cultures exposed to Borrelia spirochetes but also in PC12/THP-I cell cultures exposed to LPS. These findings indicate that increased AβPP together with hyperphosphorylation of

tau may be induced not only by living bacteria, but also by biologically active bacterial components alone. In agreement with the present observations, increased AβPP mRNA was also reported to occur in the basal forebrain region and the hippocampus in the rat in response to LPS. In addition, Aβ deposition was observed in the brains of rats chronically infused with lipopolysaccharide (Hauss-Wegrzyniak et al., 2000 Hauss-Wegrzyniak and Wenk, 2002).

Natural or synthetic bacterial components, such as the bacterial cell wall peptidoglycan and LPS, may induce chronic inflammation and amyloidosis (Fox, 1990; Foyn Bruun et al.,, 1994). They are inflammatory cytokine stimulators, they activate complement, they affect vascular permeability, they generate nitric oxide, and they induce proteoglycan synthesis and apoptosis (Fox, 1990; Foyn Bruun et al., 1994; Hauss-Wegrzyniak et al., 2000 Hauss-Wegrzyniak and Wenk, 2002). 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 stimuli (Fox, 1990). During chronic exposure, bacteria or bacterial debris may accumulate and persist in host tissues and trigger a cascade of events leading to chronic inflammation and amyloid deposition.

VI. SUMMARY

The pathological hallmarks of Alzheimer's disease (AD) consist of β-amyloid plaques and neurofibrillary tangles in affected brain areas. The processes which drive this host reaction are unknown. The cause, or causes, of the vast majority of Alzheimer's disease cases are unknown. A number of contributing factors have been postulated, including infection.

It has long been known that the spirochete *Treponema pallidum*, which is the infective agent for syphilis, can in its late stages cause dementia, chronic inflammation, cortical atrophy and amyloid deposition. Spirochetes of unidentified types and strains have previously been observed in the blood, CSF and brain of 14 AD patients tested and absent in 13 controls. In three of these AD cases spirochetes were grown in a medium selective for *Borrelia burgdorferi*.

In the present study, the phylogenetic analysis of these spirochetes was made. We analyzed the sequence of the 16S rRNA gene of the spirochetes grown in medium selective for *Borrelia burgdorferi* and carried out morphological characterization by transmission electron microscopy. Since diagnostic and serological tests are available for *Borrelia burgdorferi*, 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 cases where the spirochetes were cultivated.

Positive identification of the agent as *Borrelia burgdorferi s. s.* was based on genetic and molecular analyses. Borrelia antigens and genes were co-localized with beta-amyloid deposits in these AD cases.

To determine whether an analogous host reaction to that occurring in AD could be induced by the same infectious agent, we exposed mammalian glial and neuronal cells in vitro to *Borrelia burgdorferi* spirochetes. Morphological changes analogous to the amyloid deposits of AD brain were observed following 2-8 weeks of exposure to the spirochetes. Increased levels of β-amyloid presursor protein (AβPP) and hyperphosphorylated tau were also detected by Western blots of extracts of cultured cells that had been treated with spirochetes or LPS. These observations indicate that, by exposure to bacteria or to their toxic products, host responses similar in nature to those observed in AD may be induced.

The results of this multifaceted study allow us to conclude that *Borrelia burgdorferi* like *Treponema pallidum* in syphilis, may persist in the brain and be associated with AD amyloid plaques. The data suggest that *Borrelia burgdorferi*, perhaps in an analogous fashion to *Treponema pallidum*, may contribute to dementia, cortical atrophy and amyloid deposition. Furthermore, 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.

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TABLES AND FIGURES

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

Table 1

Antigen	Antibody [ref]	Source	Type	Dilution
GFAP	Anti-GFAP Z334	Dakopatts	Rabbit, IgG	1: 1000
ED1	MCA341	Dakopatts	Rabbit, IgG	1: 1000
CD68	M 814	Dakopatts	Mouse, IgG	1 : 2000
B-amyloid	M 872	Dakopatts	Mouse, IgG	1: 200
Aβ 1-42	21F12	Dr Dale Schenk	Mouse, IgG	1: 100
AβPP	22C11, 1285262	Boehringer	Mouse, IgG	1: 100
Tau	T-6402	Sigma	Rabbit, IgG	1: 1000
Tau 2	T-5530	Sigma	Mouse, ascites	1: 20
AT8	AT8-PHF-Tau	Endothelin	Mouse, IgG	1:1000
AT8	BR-030	Innogenetics	Mouse, IgG	1:1000
OspA	H3T5	Symbicom	Supernatant	1: 1000
OspA	H-5332	Symbicom	Supernatant	1: 1000
Flagellin	H-9724	Symbicom	Supernatant	1: 100
Flagellin	H-605	Symbicom	Supernatant	1: 100
Borrelia burg.	B65302R	Biodesign	Rabbit, IgG	1: 200
Borrelia burg	1017	J. Hurliman	Rabbit, IgG	1: 500
Borrelia burg	1018	J. Hurliman	Rabbit, IgG	1: 500
Grb2	clone 81	BD Transd Lab	Mouse IgG	1: 1000
Actin, C-11	sc-1615	Santa Cruz	Goat IgG	1: 2000

Primary antibodies employed in the analysis of in vitro amyloid formation induced by Borrelia spirochetes. The source, type and the dilutions are given for each antibody.

Table 2

	BSK-II	Apo-E	Phylogen	Serology	Antigens	ISH
AD1	+	3/4	*Borrelia burgdorferi ss*	+	+	+
AD2	+	3/4	*Borrelia burgdorferi ss*	-	+	+
AD3	+	3/3	0	+	+	+

Results of the analysis of the involvement of *Borrelia burgdorferi* in the 3 Alzheimer's cases (AD1, AD2, AD3) where spirochetes were cultivated from the brain.

BSK-II : + = successful cultivation of spirochetes from the brain in BSK-II medium; Apo-E: results of the Apo-E genotyping. Phylogen: results of the phylogenetic analysis of the spirochetes cultivated from the brain. Antigens : + = presence of *Borrelia burgdorferi* antigens in the brain ; ISH = In situ hybridization. + = positive ; - = negative ; 0 = not done.

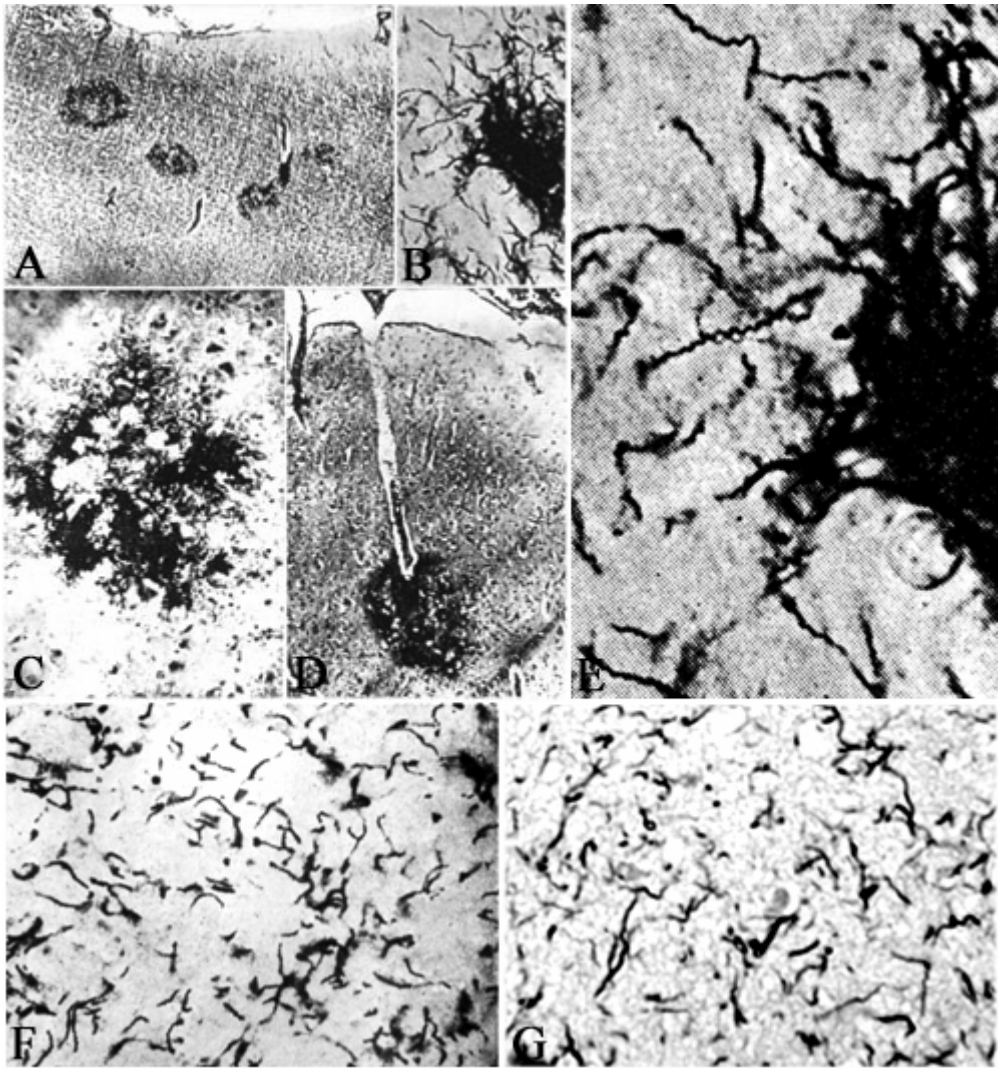
Table 3

	IFAT	ELISA	Western Blot	
			IgG	IgM
AD1 Blood	1/2048 (+)	200U (+)	+	-
AD1 CSF	1/2600 (+)	236U (+)	+	-
AD2 Blood	1/16 (-)	83U (-)	-	-
AD2 CSF	1/16 (-)	84U (-)	-	-
AD3 Blood	0	0	+	+
AD3 CSF	0	224U(+)	+	+
HFB Blood	1/128 (+/-)	121 (+/-)	+*	+*

Results of the serological analysis of blood and cerebrospinal fluid (CSF) of the 3 AD cases and of the healthy forester. IFAT = Indirect Immunofluorescent Antibody Test, ELISA = Enzyme-Linked Immunoabsorbent Assay: + = positive; - = negative; 0 = the analysis was not performed.

In the case indicated by asterisk the Western blot was performed in parallel by the BioGenex Lyme IgG Western blot Kit and by Immunosa (Nyon, Switzerland).

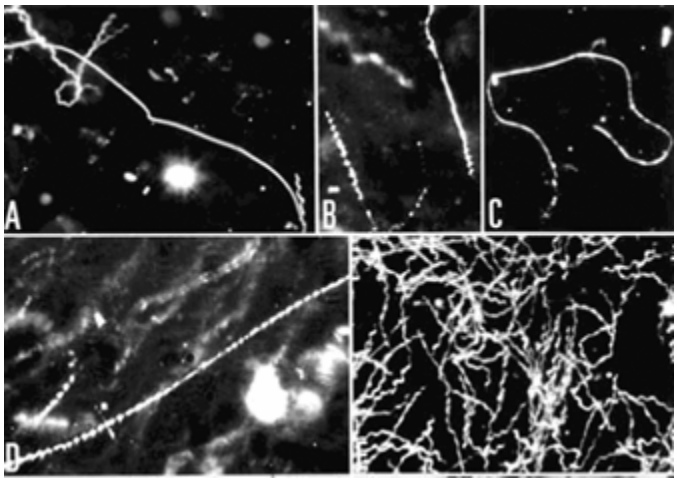
Figure 1



A-E: Reproduction of the illustrations of Pacheco e Silva (1926-27) showing spirochetal colonies in the form of plaques in the cortex of a general paretic case. A: The legend used by the author himself: "Colonias de espirochetas em torno dos capillares periphericos do cerebro. Lobo parietal. Caso de paralysia geral. Meth. Jahnel. Pequeno augmento." The morphology and the distribution of spirochetal colonies are identical to those of senile plaques in Alzheimer's disease. B: At high magnification the typical spiral appearance of several spirochetes clearly indicates that the plaques are indeed made up by spirochetes

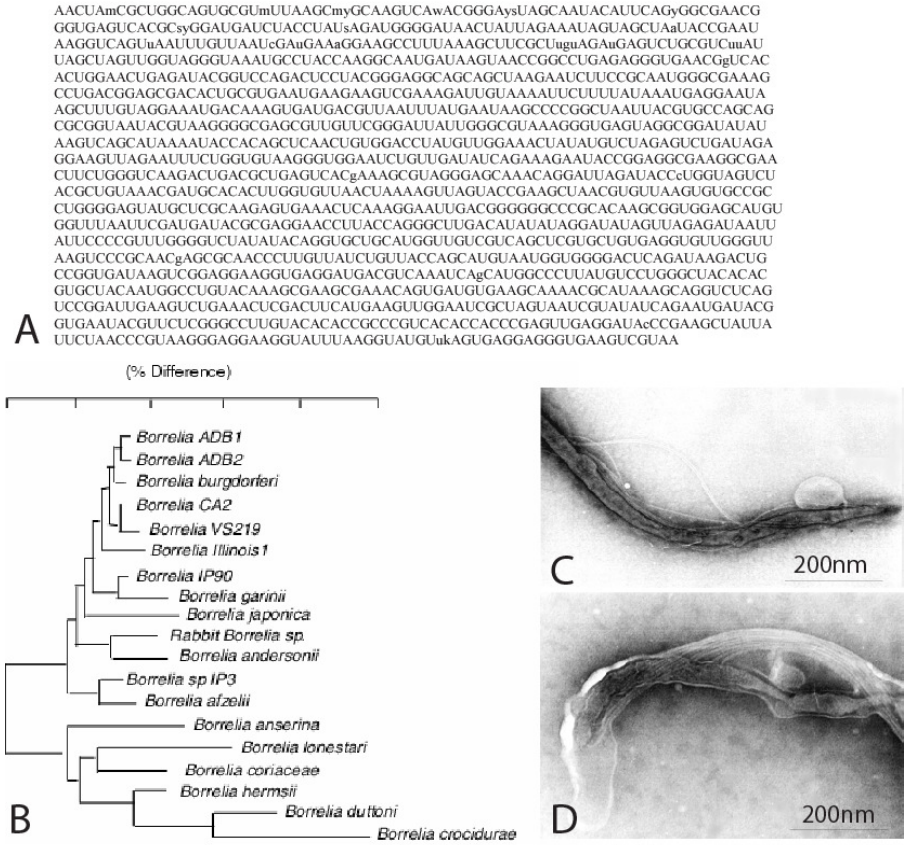
and excludes the possibility of a concurrent AD and general paresis. The regular spiral apearance of the individual Treponema spirochetes is clearly visible when B is further magnified (F) (C and D: Spirochetal colonies in the cerebral cortex morphologically identical with immature (C) and perivascular plaques (D). F and G: Morphological similarity of disseminated form of spirochetosis in general paresis and curly fibers in Alzheimer's disease. Compare A (disseminated spirochetes in the cerebral cortex of a patient with progressive paralysis) with B (Gallyas silver impregnated section showing disseminated curly fibers in the frontal cortex of a sporadic AD case.

Figure 2



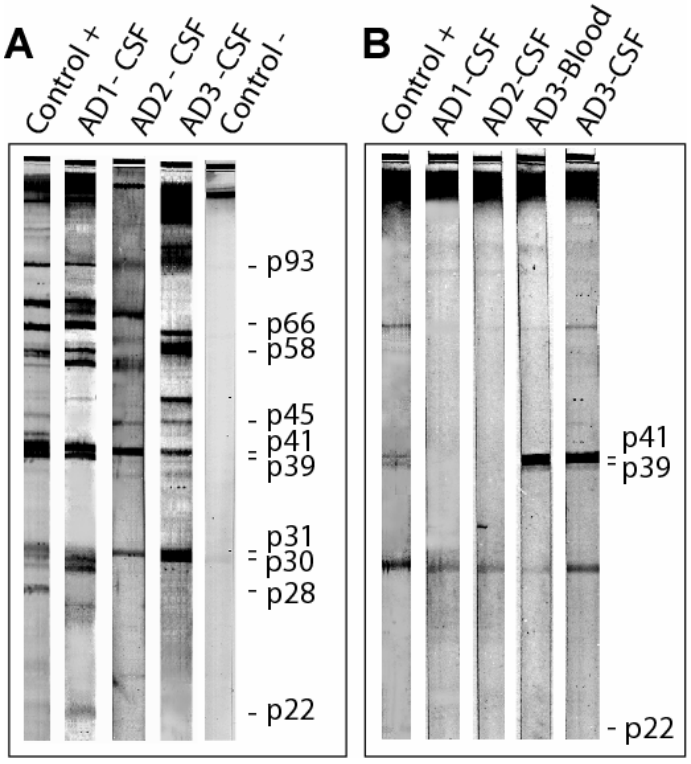
Analysis of the cultured spirochetes in the case AD1 by dark field microscopy and histochemistry. A-D shows the morphological changes of the spirochetes observed during cultivation of the spirochetes from the brain. The initially stretched spirochetes (A-C) became partially than completely spiral (see D and E). Finally, after 4 months of culture (E) they showed the loosely spiral morphology typical for Borrelia spirochetes.

Figure 3



Phylogenetic and ultrastructural characterization of the spirochetes cultivated from the brain. A and B: The phylogenetic analysis definitely identifies the cultured spirochetes (strains ADB1 and ADB2) cultivated from the AD brain as Borrelia burgdorferi sensu stricto. C and D: Strains ADB2 (C) and HFB (healthy forester, D) show the presence of 10-15 periplasmic flagella inserted at each end of the cell, which taxonomically identify them as Borrelia species.

Figure 4



Western blot analysis of specific anti-*Borrelia burgdorferi* antibodies in the cerebrospinal fluid (CSF) of the 3 AD cases (AD1, AD2 and AD3) using the BioGenex Lyme Western blot kit. Control + = positive control for IgG and IgM was provided by the manufacturer. Control - = control patient without dementia. A: Western blot analysis of specific anti-Borrelia burgdorferi IgG: AD1: p93, p66, p58, p41, p39, p31/30, p22 (positive); AD2: p93, p45, p41, p 31, p25 (negative). Notice a very weak p 39. AD3: p93, p58, p45, p41, p39, p31/30 (positive); Control -: weak p93 and p30 bands (negative). B: Western blot analysis of specific anti-Borrelia burgdorferi IgM: AD1: negative, AD2: negative, AD3: p39, p41 (positive).

Figure 5

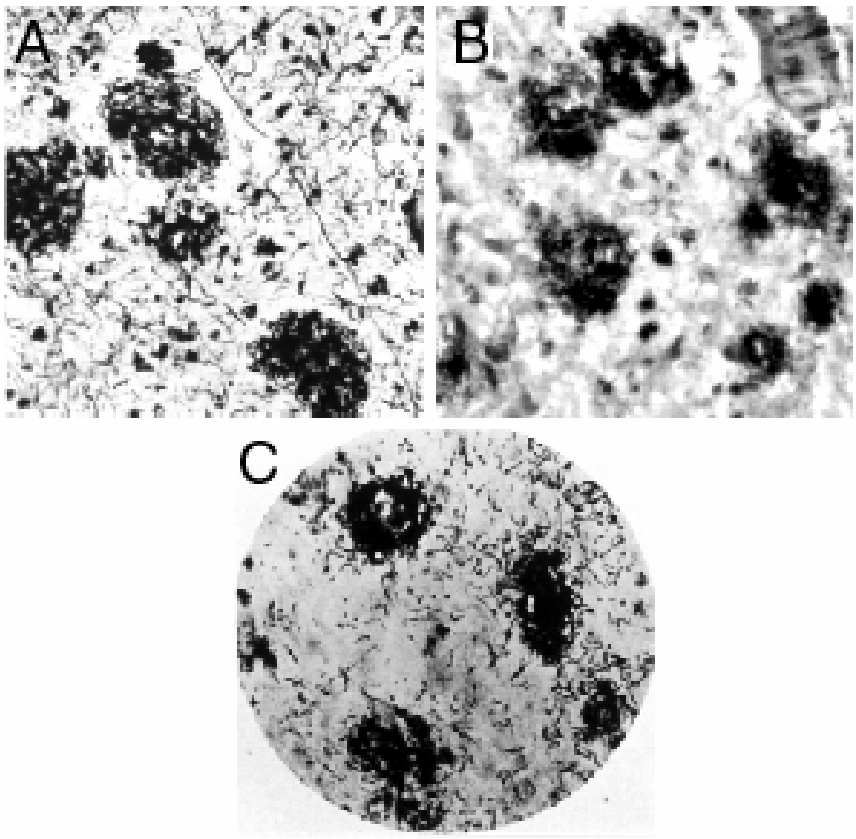
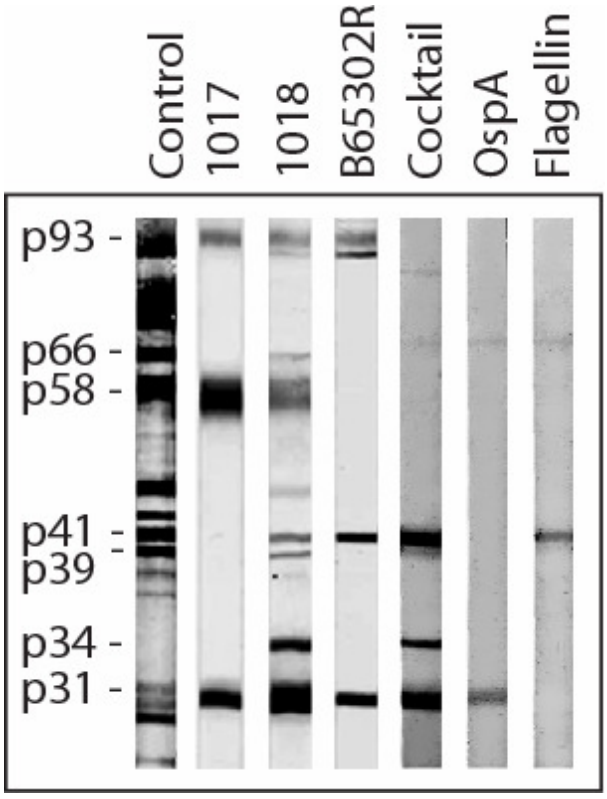


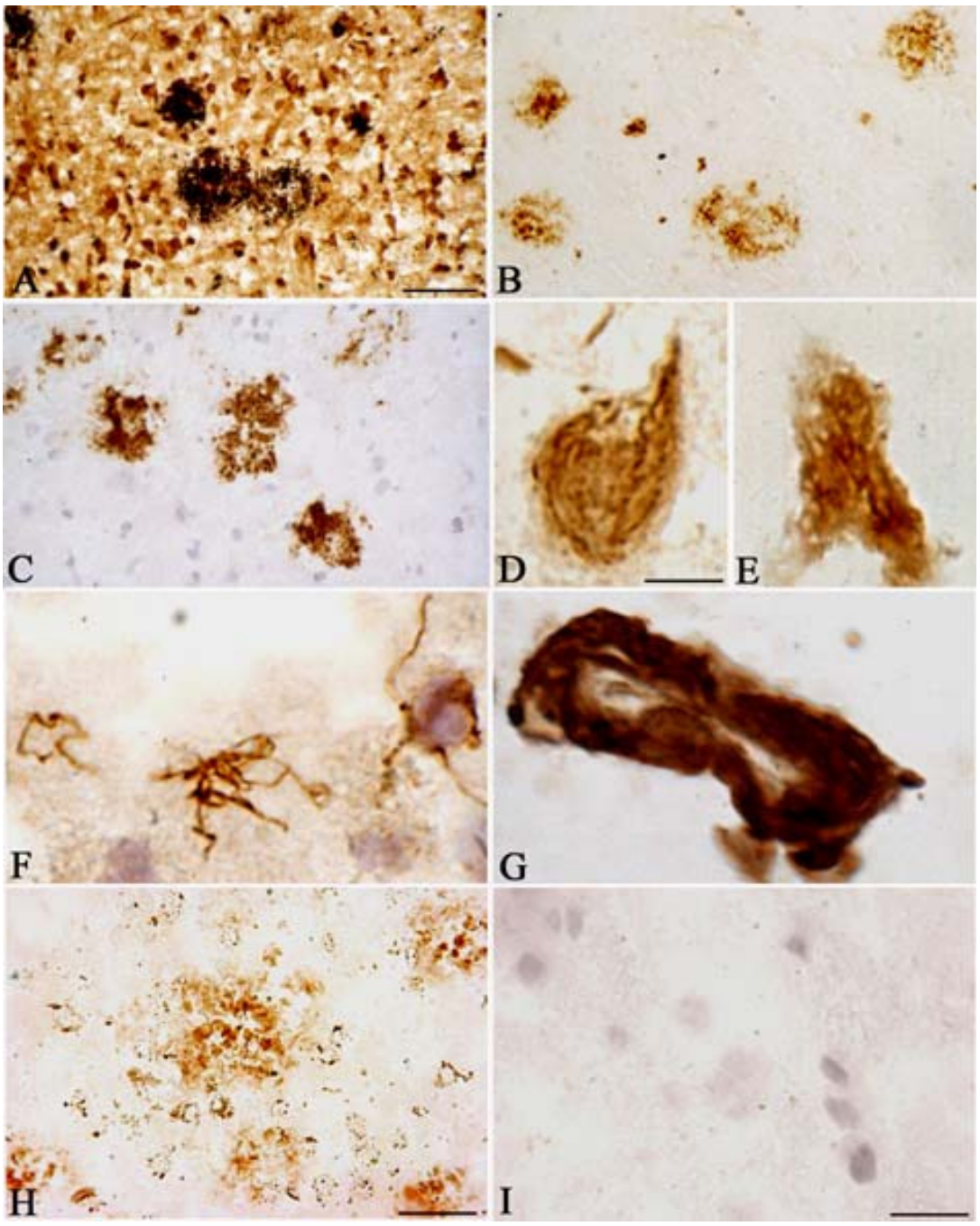
Illustration of the striking similarity of the agglomeration of spirochetes in the cerebral cortex in case AD1 with positive Lyme serology and in general paresis. Compare the similarity of the silver impregnation pattern when using a modified Bielschowsky stain for senile plaques (A) or using a silver impregnation technique for spirochetes (Warthin and Starry) (B) in the cerebral cortex of case AD1 and in a case of general paresis (C). The permission for reproduction of figure C was kindly provided by Springer-Verlag publisher and corresponds to Figure 4 of Jahnel (Abb. 4. 1929) (Lubarsch et al., 1958).

Figure 6



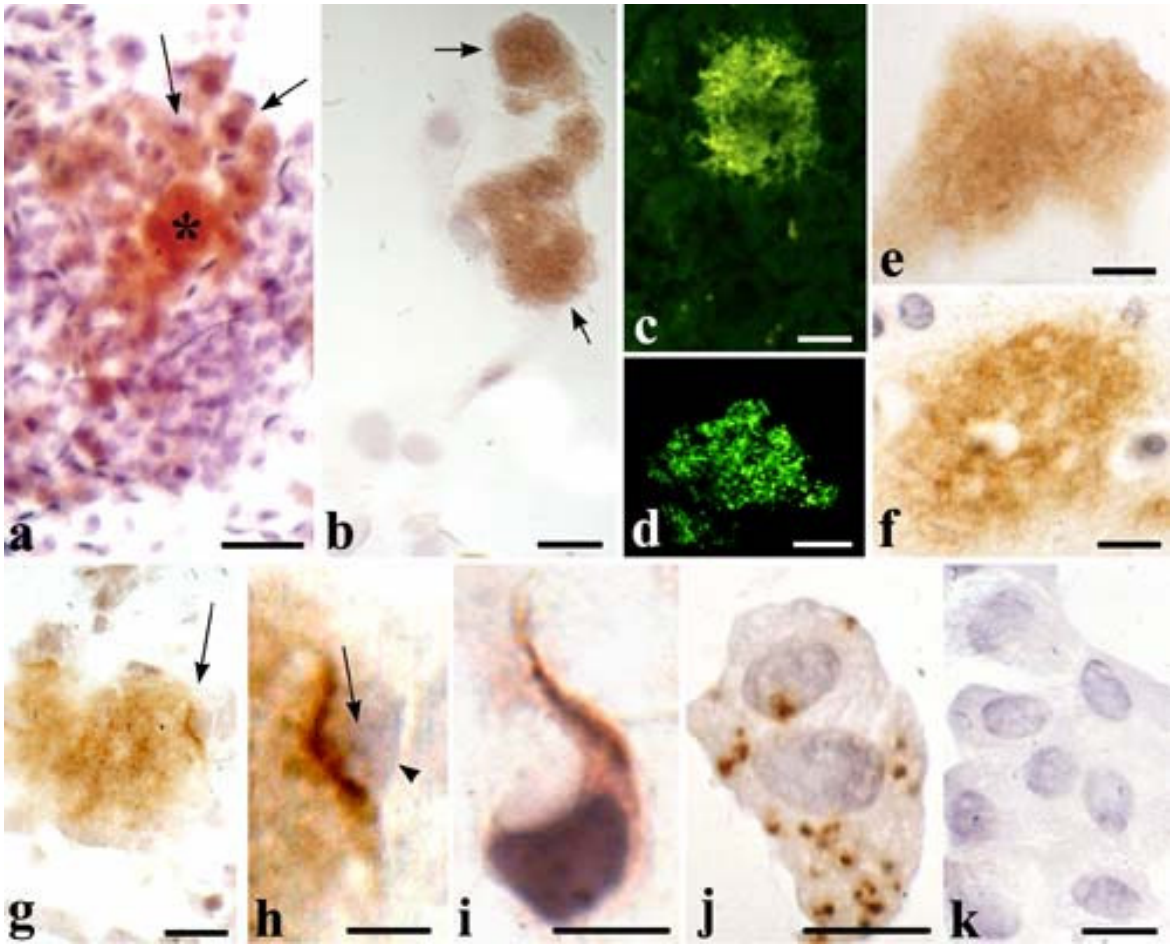
Western blot analysis of the specificity of anti-*Borrelia burgdorferi* antibodies used in the present study. A: Western blot analysis of the polyclonal anti-*Borrelia burgdorferi* antibodies. Control = positive control provided by the manufacturer (Biogenex Lyme IgG Kit). Te rabbit anti-Borrelia antibodies 1017 and 1018 were prepared in the University Institute of Pathology, Lausanne, Switzerland, B65302 antibody is from (Biodesign). A monoclonal antibody against OspA and Flagellin but also a cocktail of OspA, OspB and Flagellin monoclonal antibodies were also used in the present study.

Figure 7



Presence of Borrelia burgdorferi antigens in the brains of the AD cases in which spirochetes were cultivated in BSK II medium. A: The Warthin & Starry silver impregnation technique for spirochetes shows colony like masses of spirochetes in the frontal cortex. B: A similar distribution of Borrelia antigens on adjacent sections as revealed by a rabbit anti-Borrelia burgdorferi antibody B65302R, Biogenesis). C: Note the identical distribution of Aβ immunostained senile plaques in the frontal cortex of the same patient. Neurofibrillary tangles were immuno-labeled with rabbit anti Borrelia antibody 1017 (D) and anti-OspA antibody (E). F: Individual spirochetes showing immunoreactivity to anti-Borrelia antibody in the cerebral cortex. G: Leptomeningeal vessel showing positive immunoreaction to bacterial peptidoglycan. H: The pattern of distribution of Borrelia burgdorferi genes as detected by in situ hybridization was identical to those of Borrelia antigens and Aβ. Control sections in which the primary antibody (I) or the specific probes were omitted (not shown here) were negative. Scale bar in A is the same for B-C and G = 100 μm ; bar in D is the same for E and F = 10 μm ; H = 50 μm ; I=20 μm.

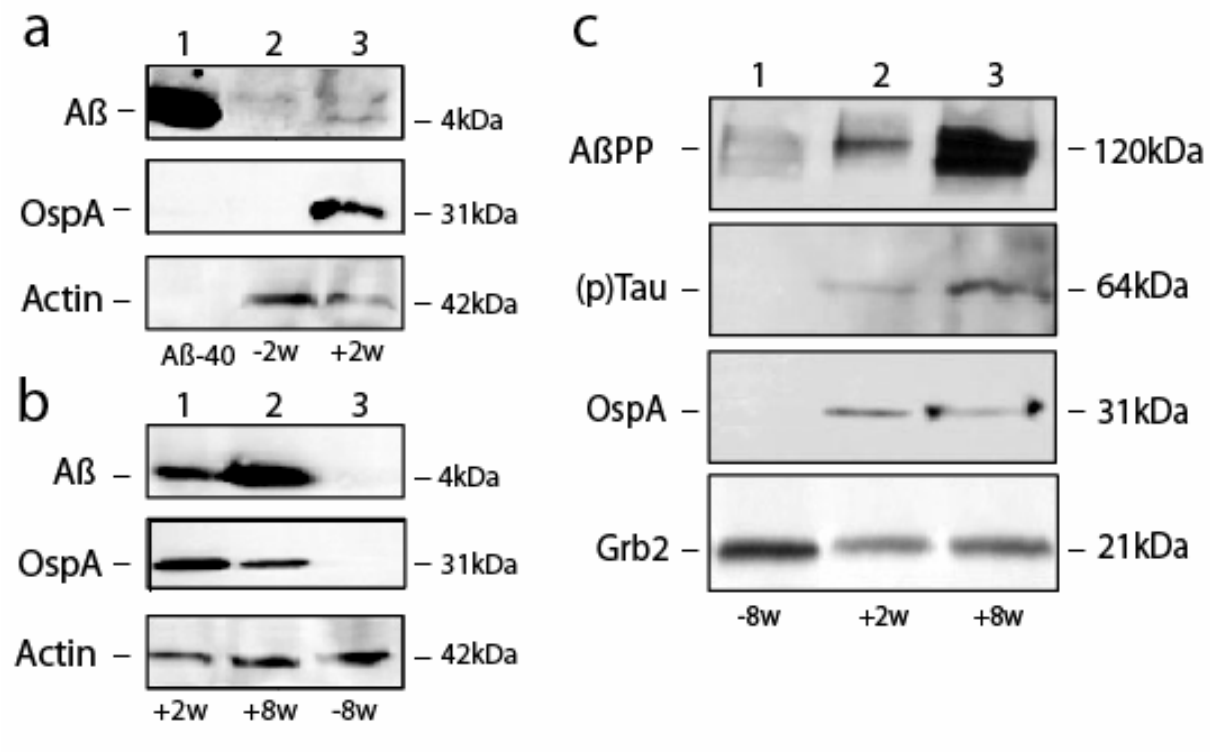
Figure 8



In vitro Aβ deposition induced by exposure to *Borrelia burgdorferi* for 4 weeks. a-e: Aβ immunoreactive and thioflavin S positive plaque-like formations illustrated in brain cell aggregates (a), in primary rat astrocytic culture (b, c and e) and in neuronal culture (d). For immunostaining, a monoclonal antibody which recognizes residues 8-17 of Aβ was used and the sections were counterstained with hematoxylin. a: Frozen section of a spheroid from infected brain cell aggregates. The section represents half portion of a spheroid of aggregated brain cells. A large extracellular deposit labeled with an asterisk. Arrows point to apparently intracellular deposits showing weak Aβ reactivity in some

unidentified cells. b: Arrows point to large plaque-like extracellular deposits. c and d: Thioflavin S positive "plaques in astrocytic (c) and neuronal (d) culture. e and f: Aβ-immunoreactive "plaque" induced in vitro (e) and Aβ-immunoreactive senile plaques in the cerebral cortex of a sporadic AD case (f) where a paraffin section from the frontal cortex was immunostained using the same anti-Aβ antibody as used for e. Notice the similarity in morphology and Aβ-immunoreactivity of the in vitro induced deposits and that of the senile plaque. (i.e. compare e with f). g: At the periphery of a Aβ-immunoreactive "plaque", a structure reminiscent of a neurofibrillary tangle (arrow) in the organotypic culture exposed to *Borrelia burgdorferi*. h: At higher magnification the large nucleus (arrowhead) and nucleolus (arrow) of the cell indicates that the cell morphologically corresponds to a neuron. A frozen section was cut from cell aggregates following 2 week exposure to *Borrelia burgdorferi* and immunostained with anti-Aβ1-42 (21F12) antibody followed by counterstaining with hematoxylin. i: Neuron showing morphological changes reminiscent of neurofibrillary tangle formation. A rat primary neuronal culture was exposed to spirochetes for two weeks. Immunostaining was performed with an antibody, which recognizes the outer surface protein A (OspA) of *Borrelia burgdorferi*. The section was counterstained with hematoxylin. j: OspA immunoreactive intracytoplasmic granules (arrows), surrounded with a pale halo, reminiscent to the morphology of granulovacuolar degeneration. A primary rat astrocyte culture was infected with Borrelia spirochetes. In control cultures (k), not exposed to spirochetes (primary rat astrocyte culture), these morphological changes were not observed. Bars: 120μm (a), 50μm (b, c, d, g), 20μm (e, f, g, h, i, j, k).

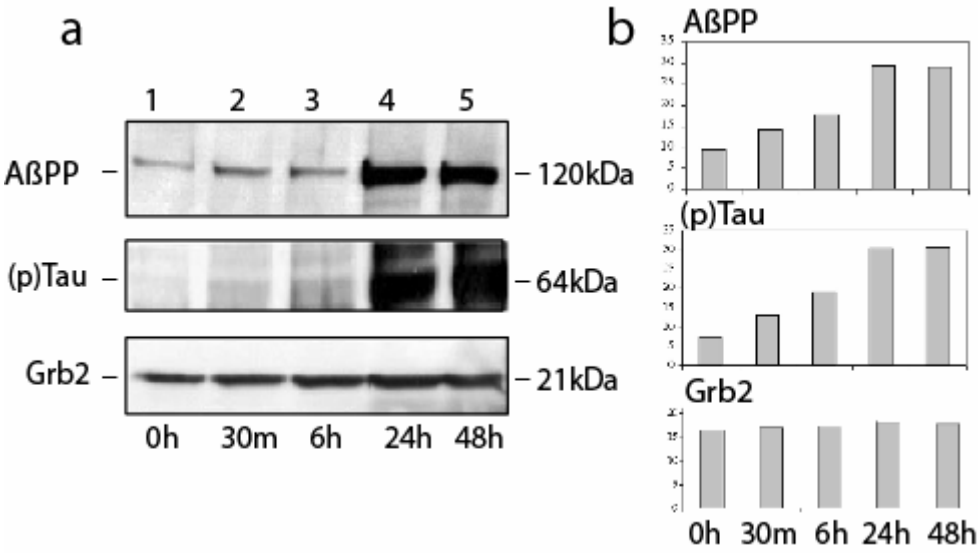
Figure 9



Western blot analysis of Aβ, AβPP and tau following exposure to *Borrelia burgdorferi* and LPS. The number of weeks after bacterial infection was entered "+ (number) of weeks (w)", and the number of weeks of the uninfected control maintained in parallel in culture "– (number) of weeks (w)". a: lane 1 = synthetic "Aβ-40"; lane 2 = control primary astrocye culture maintained for 2 weeks (-2w), lane 3: infected primary astrocytic culture exposed to Borrelia for 2 weeks (lane 3, +2w). In (a), a very weak 4kDa Aβ reactive band is seen in primary rat astrocytes exposed to Borrelia spirochetes for 2 weeks (a, +2w), which is absent in the control culture. OspA is absent in the uninfected and present in the infected culture. Lane 1 shows that the purified Aβ-40 does not react with OspA and actin antibodies.

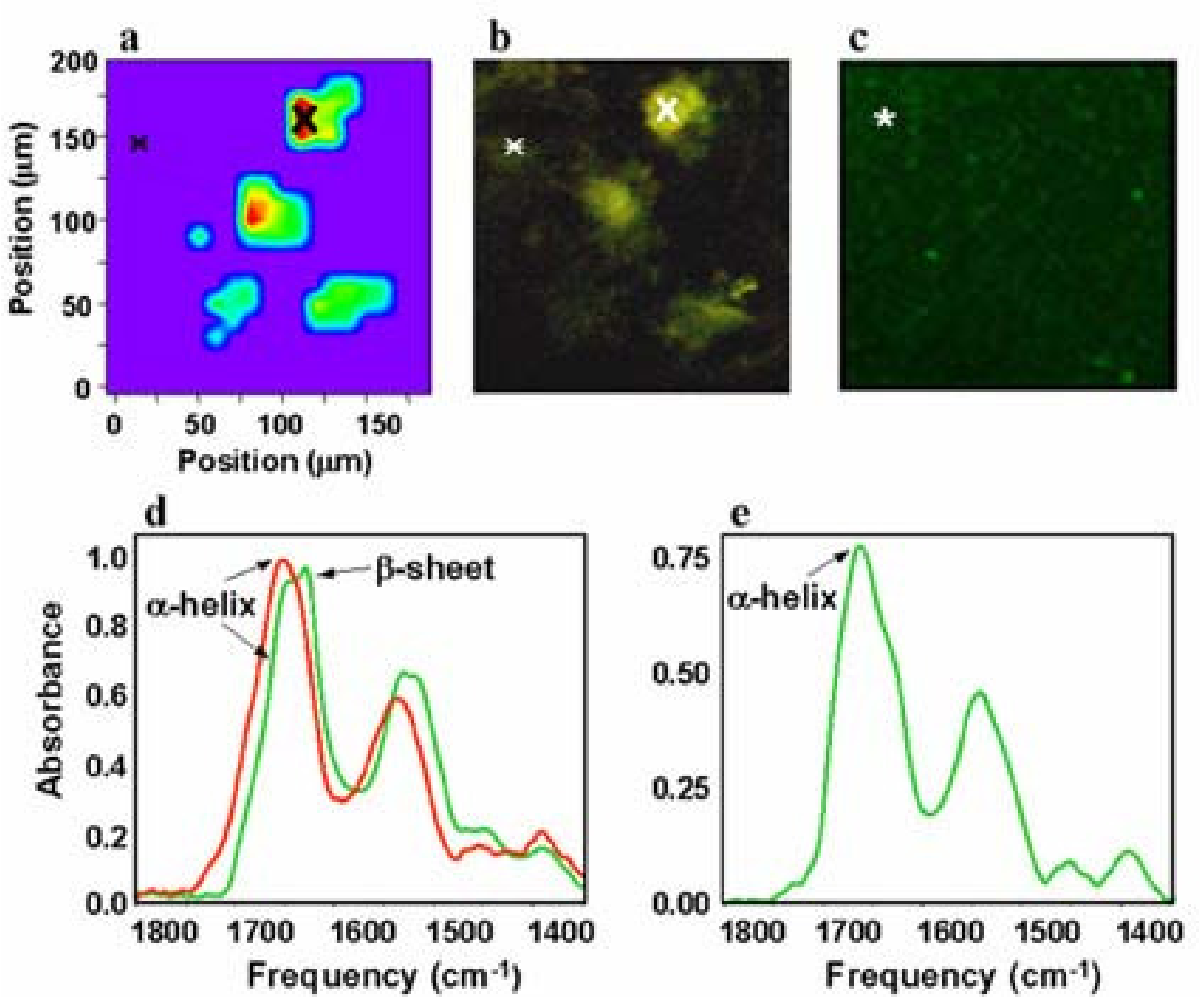
b: lane 1: microglia enriched culture infected for 2 weeks (+2w); lane 2: infected for 8 weeks (+8w); lane 3: uninfected culture maintained uninfected for 8 weeks (-8w). In (b) a stronger band was observed in microglia enriched cultures exposed to bacteria (b, +2w). Following 8-week exposure to spirochetes (b, +8w), the Aβ level was much higher than at 2 weeks (b, +2w). The 4kDa Aβ band was reactive with antibodies recognizing amino acids 7-15 of Aβ or the C terminal part of Aβ 1-42. An antibody to the 31 kDa outer surface protein (OspA) of *Borrelia burgdorferi* was utilized to monitor the presence of spirochetal infection, and an antibody to the 42 kDa protein actin was used to verify loading conditions. In uninfected cells cultured for 2 (a, -2w) and 8 weeks (b, -8w) Aβ was not detected. c: AβPP expression: lane 1: uninfected primary rat astrocytes maintained for 8 weeks (-8w); lane 2: primary rat astrocytes exposed to Borrelia for 2 weeks (+2w) and lane 3: for 8 weeks (+8w). Compared to the uninfected control culture (c, -8w), primary rat astrocytes exposed to Borrelia spirochetes for 2 (c, +2w) and 8 (c, +8w) weeks showed increased AβPP levels and hyperphosphorylation of tau. For immunoblotting, antibody 22C11 which recognizes the N terminal part of all AβPP isoforms, and AT8, which recognizes the phosphorylated Ser 202/Thr205 epitope of tau, were used. The AβPP and (p)tau levels were higher at 8 weeks than at 2 weeks exposure. The 31 kDa Borrelia OspA was used to monitor infection and the growth factor receptor-bound protein 2 (Grb2) to verify loading conditions.

Figure 10



LPS induced changes of AβPP and tau. a: lanes 1-5: PC12/THP-1 cells exposed to LPS for 0 hour, 30minutes, 6hours, 24 hours and 48 hours. Increased AβPP and (p)tau levels were observed in PC12/THP-1 cells following 24 and 48 hours of LPS (500ng) exposure. Densitometry analysis (b) clearly showed the increased AβPP expression and (p)tau levels when compared to the growth factor receptor-bound protein 2 (Grb2), used to verify loading conditions. Each bar represents the mean density from 3 different gels.

Figure 11



Synchrotron Infrared Microspectroscopy analysis of the in vitro induced Aβ deposition in cell cultures exposed to Borrelia spirochetes for 4 weeks. a: Chemical map based on infrared microspectroscopy data showing the relative concentration of β-sheets in the Borrelia infected culture. Red corresponds to an increased concentration and purple to normal levels. The areas of increased β-sheet formation of the chemical map correspond to the Thioflavin S positive plaque-like formations induced in vitro (compare b with c).

d: The green infrared Amid spectrum taken from the center of the Thioflavins S positive amyloid "plaque" labeled with X in a and b, showed a peak near 1630 cm^{-1} which is indicative of β-sheet. The red spectrum in d, was taken from a Thioflavin S negative area of the same infected culture (marked with an asterisk in a and b) showed a peak near 1650 cm^{-1} which is consistent with α sheet formation. c: In Thioflavin S stained control cultures (c), not exposed to spirochetes, plaque-like formation was not observed and the Amid spectrum corresponded to α sheet formation. e: shows the amid spectrum of the area labeled with an asterisk in (c).