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Borrelia burgdorferi persists in the brain in chronic lyme neuroborreliosis and may be associated with Alzheimer disease

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Abstract. 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. Positive identification of the agent as Borrelia burgdorferi sensu stricto was based on genetic and molecular analyses. Borrelia antigens and genes were co-localized with beta-amyloid deposits in these AD cases. The data indicate that *Borrelia burgdorferi* may persist in the brain and be associated with amyloid plaques in AD. They suggest that these spirochetes, perhaps in an analogous fashion to *Treponema pallidum*, may contribute to dementia, cortical atrophy and amyloid deposition. Further *in vitro* and *in vivo* studies may bring more insight into the potential role of spirochetes in AD.

Keywords: Alzheimer's disease, amyloid, bacteria, *Borrelia burgdorferi*, chronic inflammation, lyme disease, spirochetes, syphilis

1. Introduction

The patho-mechanism of amyloid formation in Alzheimer's disease (AD) remains unclear. A combination of genetic predisposition and environmental factors may contribute to changes in amyloid β protein precursor (AβPP) expression and amyloid beta peptide (Aβ) formation. Inflammatory processes play a crucial role in the development of AD [27]. Bacteria or bacterial components (e.g. bacterial lipopolysaccharide – LPS) are powerful activators of inflammatory processes and are known to stimulate amyloidogenesis.

Increasing recent data support the possibility that infectious agents may play a role in AD [2,7,17,18,20, 23,25,29–33,44]. Chronic bacterial infections, caused by spirochetes such as *Treponema pallidum* are known to be associated with chronic neuropsychiatric disorders including dementia. Spirochetes are Gram nega-

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tive 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 $\times$ 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 recently in cell monolayer systems [12], as reviewed previously [9].

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

In the tertiary form of syphilis known as general paresis, *Treponema pallidum* persists in the brain and can cause cortical atrophy, microgliosis, and amyloid deposition [38,39,46]. There is a similarity in the clinical and pathological manifestations of syphilis and Lyme disease which are both caused by spirochetes [11]. *Borrelia burgdorferi* may also persist in infected host tissue and play a role in chronic neuropsychiatric disorders. Dementia, including subacute presenile dementia, has been reported to occur, not only in syphilis, but also in Lyme disease [10].

Intriguingly, the clinical and pathological hallmarks of AD are also present in the atrophic form of general paresis [19,39]. Alzheimer himself referred to the similarity of the clinical manifestations of AD and general paresis, when he described one of his famous cases in 1911 [1]. In 1907, Fischer suggested that senile plaques may correspond to colonies of microorganisms [13].

Previously we reported helically shaped microorganisms in the cerebrospinal fluid (CSF), blood and cerebral cortex of 14 AD cases that were absent in 13 control cases [29]. An ultrastructural study indicated that the microorganisms taxonomically belong to the order Spirochaetales [30]. In three of these 14 AD cases spirochetes were grown in a medium selective for *Borrelia burgdorferi* [3]. We suggested that several types of spirochetes may be involved in AD, including *Borrelia burgdorferi*. Subsequently Riviere et al., using species-specific PCR and monoclonal antibodies, detected oral Treponema in 14/16 AD cases and 4/18 non-AD controls [44].

We analyzed the sequence of the16S 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. 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.

2. Material and methods

2.1. Patients, clinical data

Previously we reported spirochaets in the cerebrospinal fluid CSF, blood and cerebral cortex in 14 AD cases tested [29]. In 3 of these 14 AD cases spirochetes were cultivated from the brain in a synthetic BSK II medium 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 [36].

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.

A semiquantitative analysis of the density of senile plaques and neurofibrillary tangles in the AD cases was performed in the hippocampus and entorhinal cortex, as well as in the frontal and parietal associative areas, as previously described in detail [34]. The 3 AD cases with dementia fulfilled criteria for a definite diagnosis of AD. The neuropathological assessment of the severity of cortical involvement was also made following Braak and Braak criteria [4]. For the neuropathological diagnosis of AD, the criteria recommended by Khachaturian [22], CERAD [35] and the National Institute on

Aging (NIA) – Reagan Institute Working group were fulfilled [37].

Since the epsilon-4 allele of apolipoprotein E (Apo-E) is an important risk factor for AD, genotyping of Apo-E was performed in the three AD cases analyzed in this study. DNA was extracted from frontal cortical samples (about 25 mg) using a Quiagen DNA extraction Kit (Quiagen, 29304), following the instruction of the manufacturer. Amplification of the human Apo-E gene and restriction enzyme isotyping with Hha-I was performed as described by Hixson and Vernier [16]. Following cleavage with Hha-I, 1 μl samples were run on polyacrylamide gel (Phastgel Pharmacia, 8–25%, 17-0542-01) in Phastsystem electrophoresis (Pharmacia Biotech). DNA bands were revealed by silver impregnation using an automated program of Phastsytem (Pharmacia Biotech). The solution for silver impregnation and the developer were prepared following the instructions of the manufacturer. Cases with known Apo-E genotypes 3/3, 3/4, and 2/4 were analyzed in parallel and used as internal controls.

2.2. *Molecular characterization of the cultivated microorganisms*

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 [48], 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 [41]. As a negative control, buffer containing no amplifiable DNA was utilized. Cycling conditions were followed as previously described [41]. 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 15000 g and the pellet washed with 80% ethanol. The pellet was then dissolved in 35 ml of sterile water.

Sequencing and 16SrRNA data analysis followed those described by Fox et al. [14]. 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 80 ng of purified DNA from the PCR amplification was used for each sequencing reaction. Reaction products were run electrophoretically on 8% polyacrylamide-urea gels and were subsequently detected by exposure of the dried gels to X-ray film for 24 to 48 h.

2.3. *16S rRNA sequence analysis*

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 1000 sequences as determined in our laboratory [43]. 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 5000 sequences available from Ribosomal Database Project [42] 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 [8]. The similarity matrices were corrected for multiple base changes by the method of Jukes and Cantor [21]. Phylogenetic trees were constructed using the neighbor-joining method of Saitou and Nei [45].

2.4. Characterization of the cultured spirochetes using electron microscopy

For ultra-structural analysis using transmission electron microscopy, 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 10 mM 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.

2.5. Serological analysis

The serum of the healthy forester was analyzed using the Venereal Disease Research Laborartory (VDRL), Rapid Plasma Reagin (RPR) 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) were applied [6]. 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 were also tested.

2.6. Histochemical and immunohistochemical analysis

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 (G 9724, 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 (Fig. 1). For the detection of *Borrelia burgdorferi* specific antigens in the brain of the 3 AD cases, frozen sections were analysed. These were fixed in acetone for 10 minutes at 4°C, pretreated with 1% amylase at 37°C for 3–5 minutes, and washed 3×5 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 spirochetal 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.

2.7. In situ hybridization

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 2×3 min. On both frozen and paraffin sections, endogenous peroxidase was blocked by

Table 1
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	Apo-E	Phylogen	Serology	Antigens	ISH
AD1 +	3/4	*Borrelia burgdorferi ss*	+	+	+
AD2 +	3/4	*Borrelia burgdorferi ss*	−	+	+
AD3 +	3/3	0	+	+	+

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.

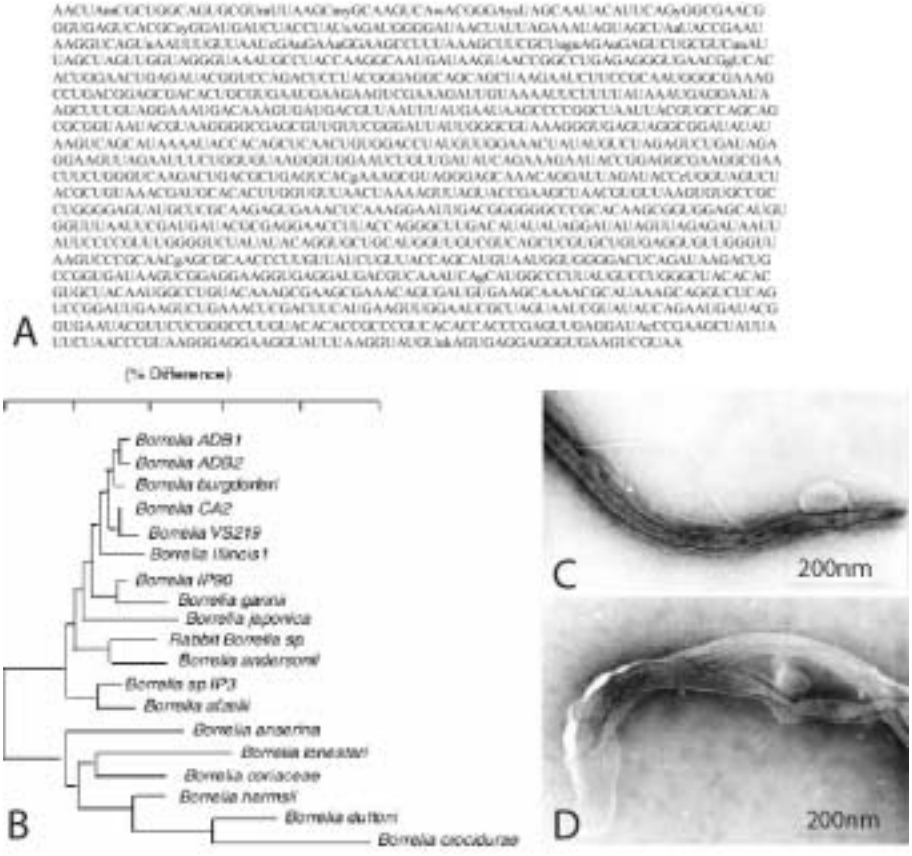

Fig. 1. Phylogenetic and ultrastructural characterization of the spirochetes cultivated from the brain. A and B: The phylogenetic analysis definitely identifies the cultured spirochetes (strains ADB1 and ADB2) cultivated from the AD brain as *Borrelia burgdorferi* sensu stricto. C and D: Strains ADB2 (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.

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 (10 μg/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 × 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 0.5 M Tris HCl, pH 7.4, 50 μl 20-X- SSC, 1 μl 0.05 M EDTA, 100 μl of 50% dextran sulfate, 250 μl formamide and 98 μl of pure water for a total volume of

Table 2
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).

	IFAT	ELISA	Western	Blot
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 (+/−)	+*	+*

500 μl) 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 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. The sections were coverslipped, 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 42°C for 2 × 20 min and in 0.1-X-SSC, 2 m,g $MgCl_2$, 0.1% Triton-X-100 at 60°C for 30 min. After a rinse in TBS 3 × 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.

3. Results

Table 1 summarizes the main results obtained in the present study. Of the three neuropathologically confirmed AD casees, Apo-E genotyping revealed that AD1 and AD2 were 3/4 while AD3 was 3/3.

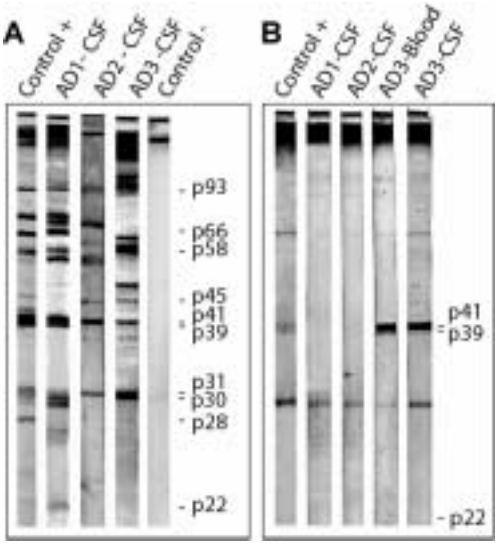

Fig. 2. Western blot analysis of specific anti-*Borrelia burgdorferi* antibodies in the cerebrospinal fluid (CSF) of the 3 AD cases (AD1, AD2 and AD3) using the BioGenex Lyme Western blot kit. Control + = positive control for IgG and IgM was provided by the manufacturer. Control − = control patient without 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).

3.1. Characterization of the cultivated microorganisms

For genomic characterization, the full sequences of the 16S rRNA 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 Fig. 1(A). 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 Fig. 1(B).

The ultrastructural analysis of the cultured spirochetes (strains ADB2 and HFB) demonstrated that they had ultrastructural characteristics of *Borrelia burgdorferi* species, i.e. thin helical cells with 10–15

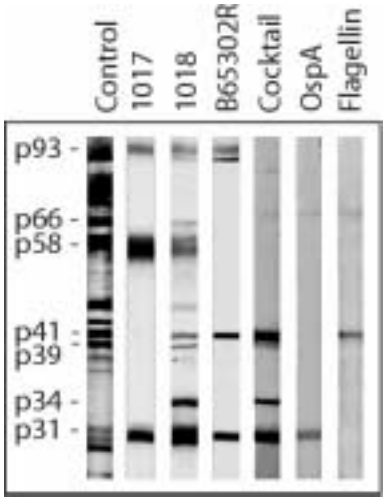

Fig. 3. Western blot analysis of the specificity of anti-*Borrelia burgdorferi* antibodies used in the present study. A: Western blot analysis of the polyclonal anti-Borrelia burgdorferi antibodies. Control = positive control provided by the manufacturer (Biogenex Lyme IgG Kit). Te rabbit anti-Borrelia 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.

periplasmic flagella inserted at each end of the cell (Fig. 1(C,D)).

3.2. Serological analysis

The results of the serological analyses are illustrated in Table 2 and Fig. 2. The analysis and the interpretation of the serological results were made following criteria of the Centers for Disease Control (CDC) [6]. 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. 2(B)). 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 p.39 and p.24–25 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.

3.3. 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 [19,24,39] caused by *Treponema pallidum* (Fig. 4). 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. 3).The colony-like masses and part of the disseminated individual filaments showed positive immunoreactions with anti-*Borrelia burgdorferi* antibodies (Fig. 5(B)), including the anti-OspA antibody. The spirochete antigens showed the same pattern of distribution as Aβ. 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. 5 (D,E)) and in the wall of some blood vessels containing amyloid deposition (Fig. 5(F)). 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. 5(H)). 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. 5(I)).

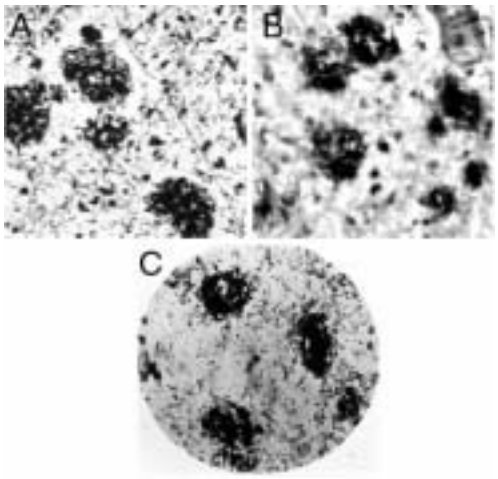

Fig. 4. Illustration of the striking similarity of the agglomeration of spirochetes in the cerebral cortex in case AD1 with positive Lyme serology and in general paresis. Compare the similarity of the silver impregnation pattern when using a modified Bielschowsky stain for senile plaques (A) or using 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 Fig. 4 of Jahnel (Abb. 4. 1929)[28].

4. Discussion

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 [47].

Lyme disease is geographically confined and the incidence is low when compared to AD [5]. 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 [15,26,28]. 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*. Different types of spirochetes may be similarly involved in other AD cases [29,44]. 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 chronic neuroinflammation. 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 [29]. Further analyses using scanning electron microscopy and atomic force microscopy showed that they possess axial filaments, therefore taxonomically they belong to the order Spirochaetales [30]. Subsequently Riviere et al., using species-specific PCR and monoclonal antibodies, detected oral Treponema in 14/16 AD cases and 4/18 non-AD controls [44]. 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 [29,33]. These observations were reinforced by Ohnishi et al., [40] who showed that the outer surface protein (OspA) of *Borrelia burgdorferi* forms amyloid fibrils *in vitro*, similar to human amyloidosis.

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 [17,18,20]. *Chlamydia pneumoniae* was also found to be associated with AD. Mice exposed to Chlamydia developed AD-like amyloid plaques [2,23].

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. Similar to the observations of Noguchi and Moore with respect to *Treponema pallidum* [38], our results show that *Borrelia burgdorferi* may also persist in the brain in chronic Lyme neuroborreliosis and be associated with cortical atrophy, amyloid deposition, and clinical dementia. The present findings reinforce the similarity of 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.*, in-

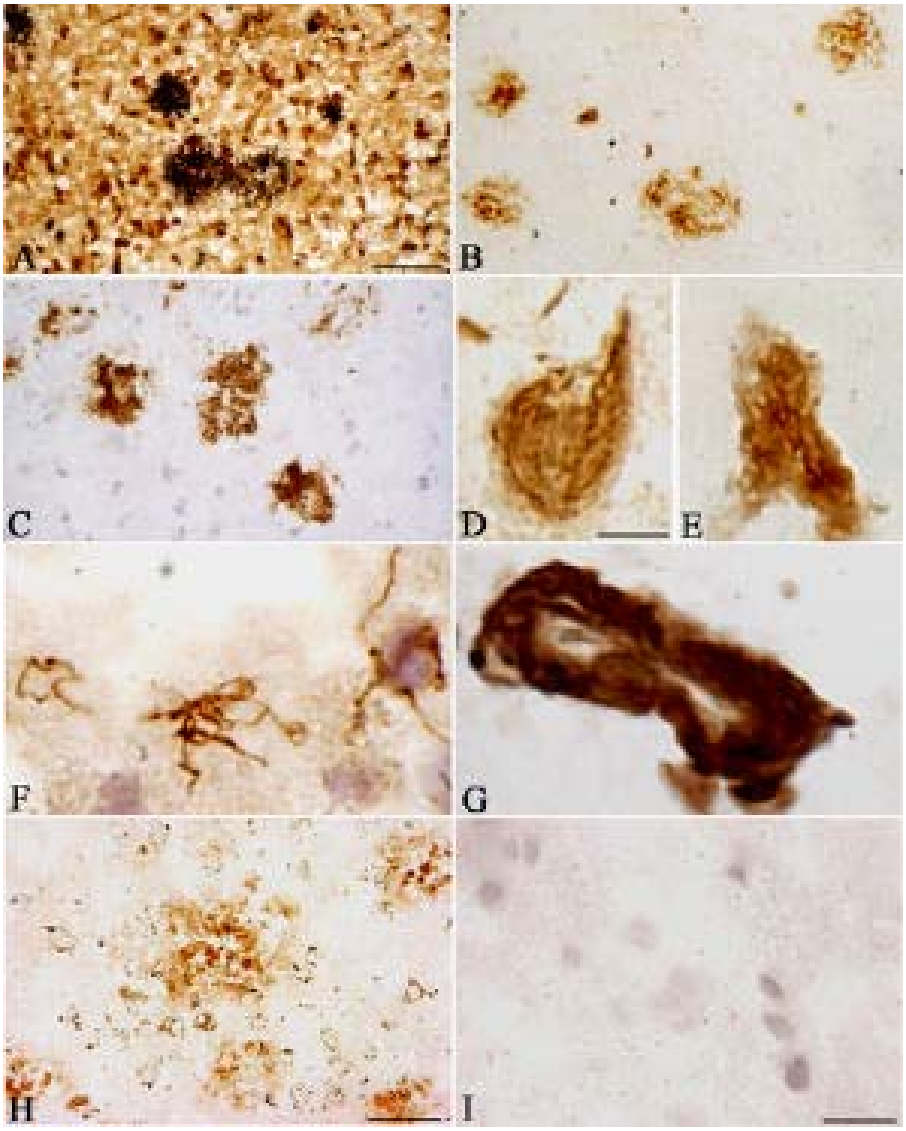

Fig. 5. Presence of Borrelia burgdorferi antigens in the brains of the AD cases in which spirochetes were cultivated in BSK II medium. A: The Warthin & Starry silver impregnation technique for spirochetes shows colony like masses of spirochetes in the frontal cortex. B: A similar distribution of Borrelia antigens on adjacent sections as revealed by a rabbit anti-*Borrelia burgdorferi* antibodyl 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.

dicates 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.

A well-defined risk factor for late onset AD is the epsilon-4 variant of the apolipoprotein E gene. The Apo-E genotyping of the three AD cases suffering from Lyme neuroborreliosis, showed that two of them possessed the epsilon-4 allele. The low number of cases does not allow any conclusive evidence; however this result may suggest that in patients with genetic risk factors, such as carriers of the epsilon-4 allele of Apo-E, or promoter polymorphisms in pro-inflammatory cy-

tokines, infection may result in a more severe phenotype, which includes enhanced Aβ accumulation when compared to non-carriers.

The clinical and pathological hallmarks of Alzheimer's disease (AD) are present in the atrophic form of general paresis [19,24,39]. In general paresis the accumulation of "plaques" in the cerebral cortex, the cortical atrophy and the amyloid deposition, as generally accepted, are secondary to the spirochetal infection. Similarly, in several other chronic bacterial infections or in experimental amyloidosis, the bacterial infection or bacterial exposure always precedes the amyloid deposition. In patients with a genetic defect which facilitate infection, the genetic problem will be the first step in the cascade of events, followed by infection, than with amyloid deposition.

The results of this multifaceted study allow us to conclude that *Borrelia burgdorferi*, like *Treponema pallidum* in syphilis, 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 vitro and *in vivo* analyses exposing mammalian CNS cells or experimental animals to *Borrrelia burgdorfgeri* or other cultivatable spirochetes will bring further information about a potential causal role of spirochetes in amyloidogenesis. The AβPP transgenic mouse expressing mutant human AβPP is a well recognized model for AD, albeit incomplete. Future studies, where such animals are infected with spirochetes, may also give further insight into their significance in contributing to the pathogenesis of AD.

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