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.

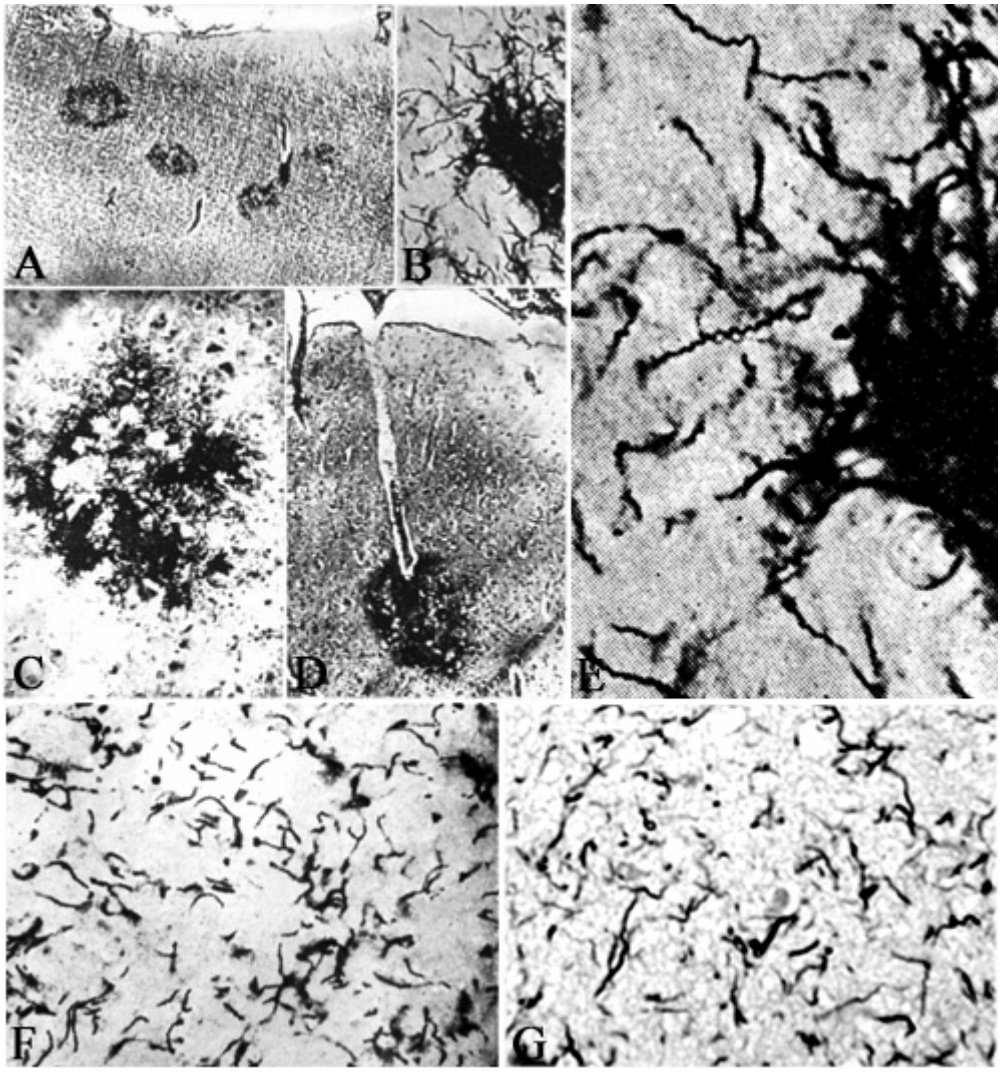
Table 3

	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).

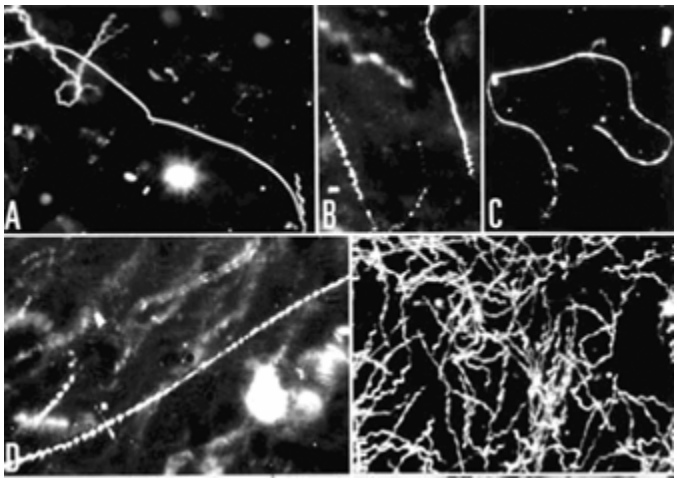
Figure 1



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

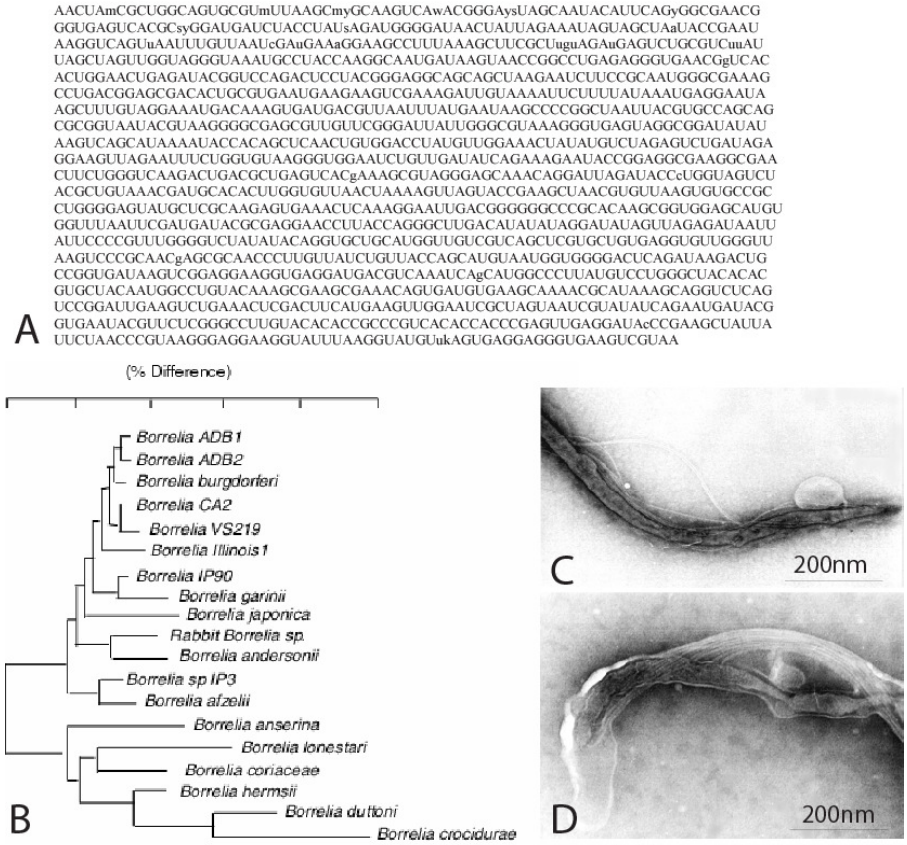
and excludes the possibility of a concurrent AD and general paresis. The regular spiral apearance of the individual Treponema spirochetes is clearly visible when B is further magnified (F) (C and D: Spirochetal colonies in the cerebral cortex morphologically identical with immature (C) and perivascular 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



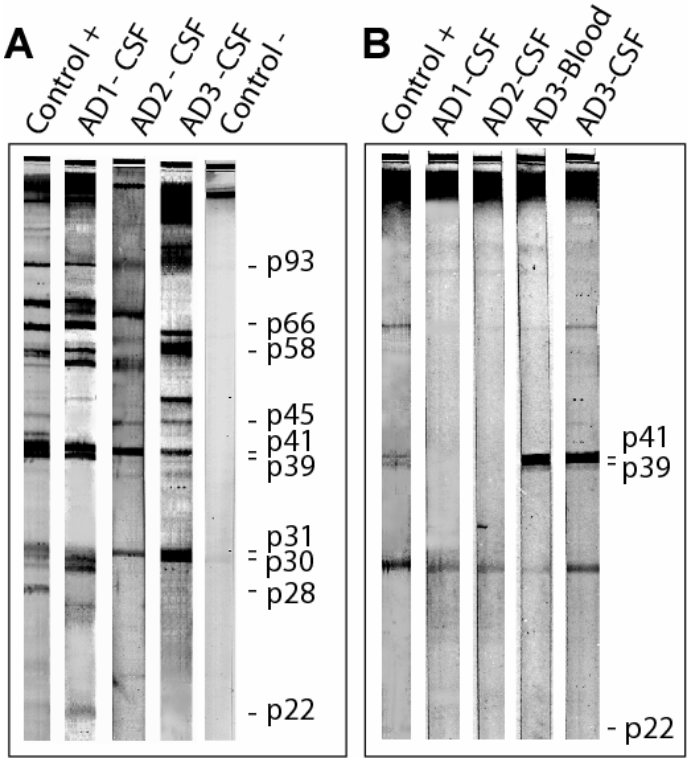
Analysis of the cultured spirochetes in the case AD1 by dark field microscopy and histochemistry. A-D shows the morphological changes of the spirochetes observed during cultivation of the spirochetes from the brain. The initially stretched spirochetes (A-C) became partially than completely spiral (see 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

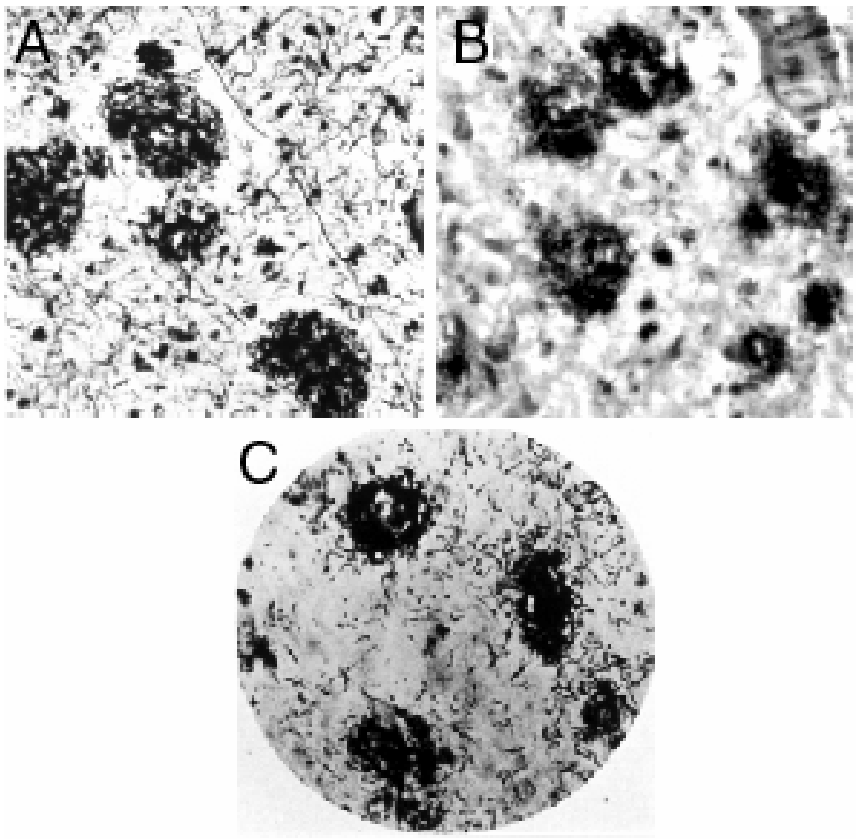
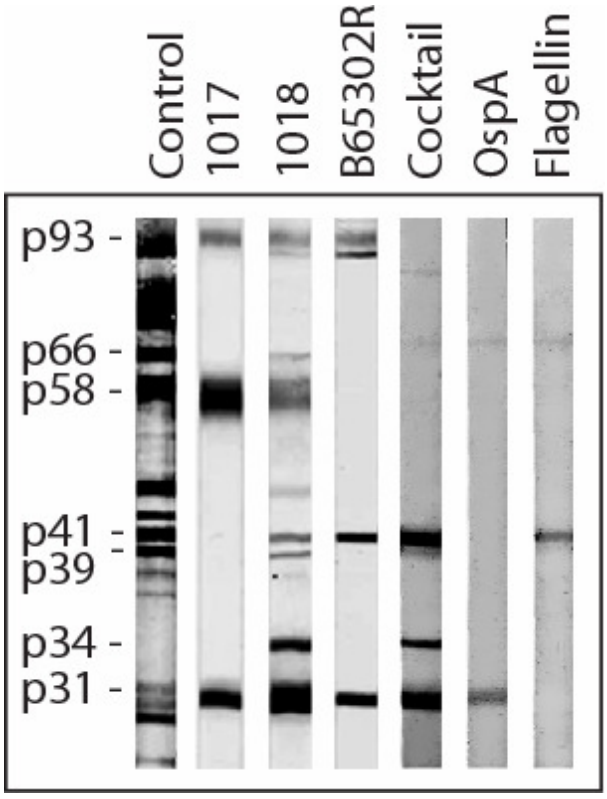


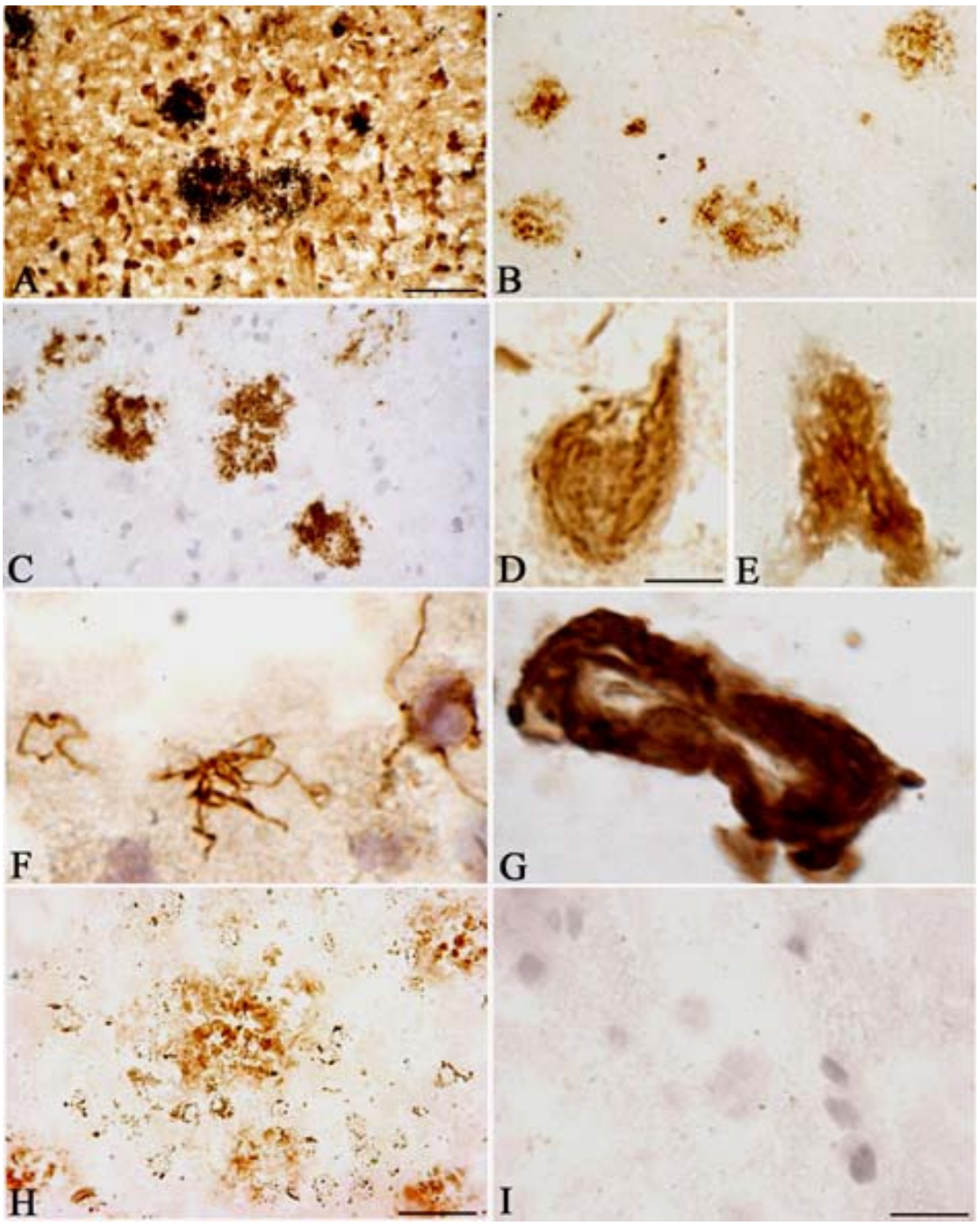
Illustration of the striking similarity of the agglomeration of spirochetes in the cerebral cortex in case AD1 with positive Lyme serology and in general paresis. Compare the similarity of the silver impregnation pattern when using a modified Bielschowsky stain for senile plaques (A) or using a silver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



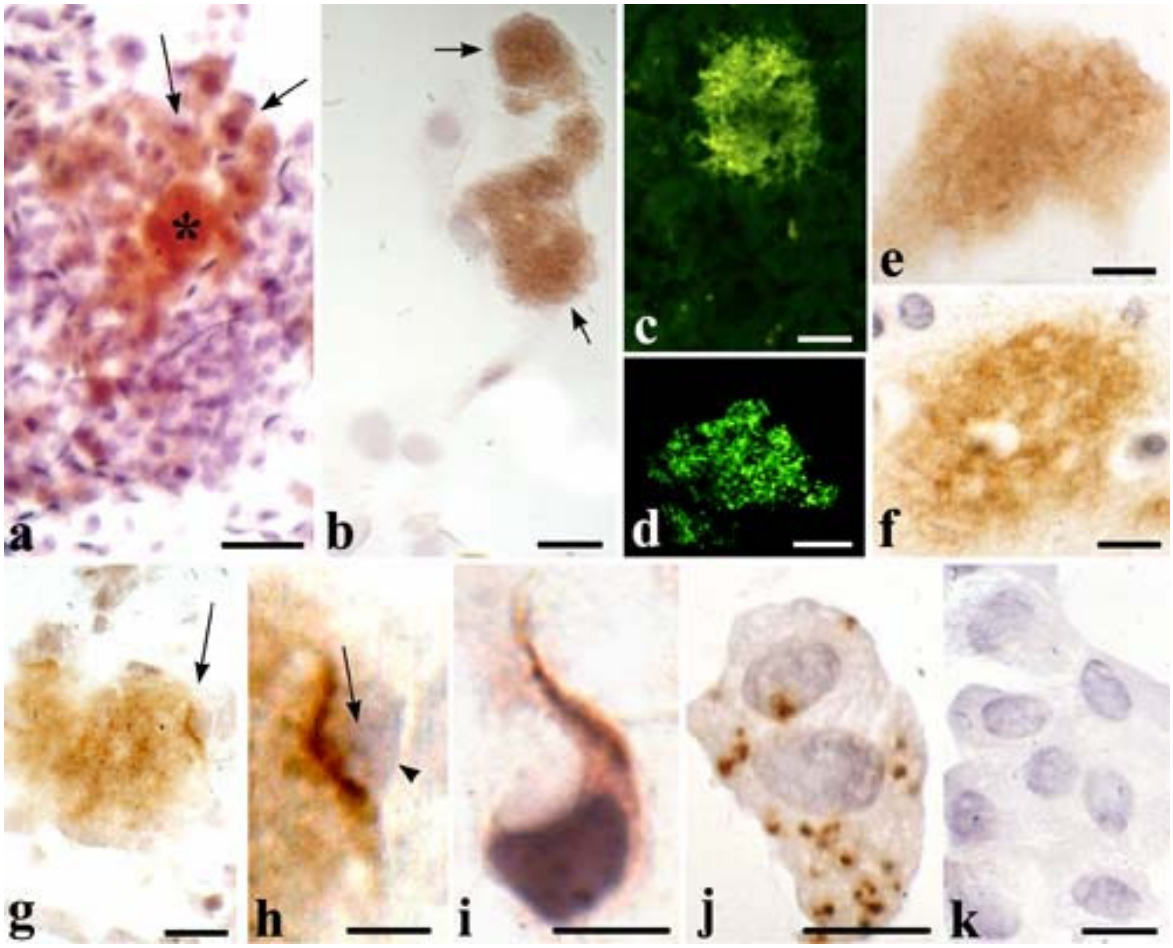
Western blot analysis of the specificity of anti-*Borrelia burgdorferi* antibodies used in the present study. A: Western blot analysis of the polyclonal anti-*Borrelia burgdorferi* antibodies. Control = positive control provided by the manufacturer (Biogenex Lyme IgG Kit). Te rabbit anti-Borrelia antibodies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

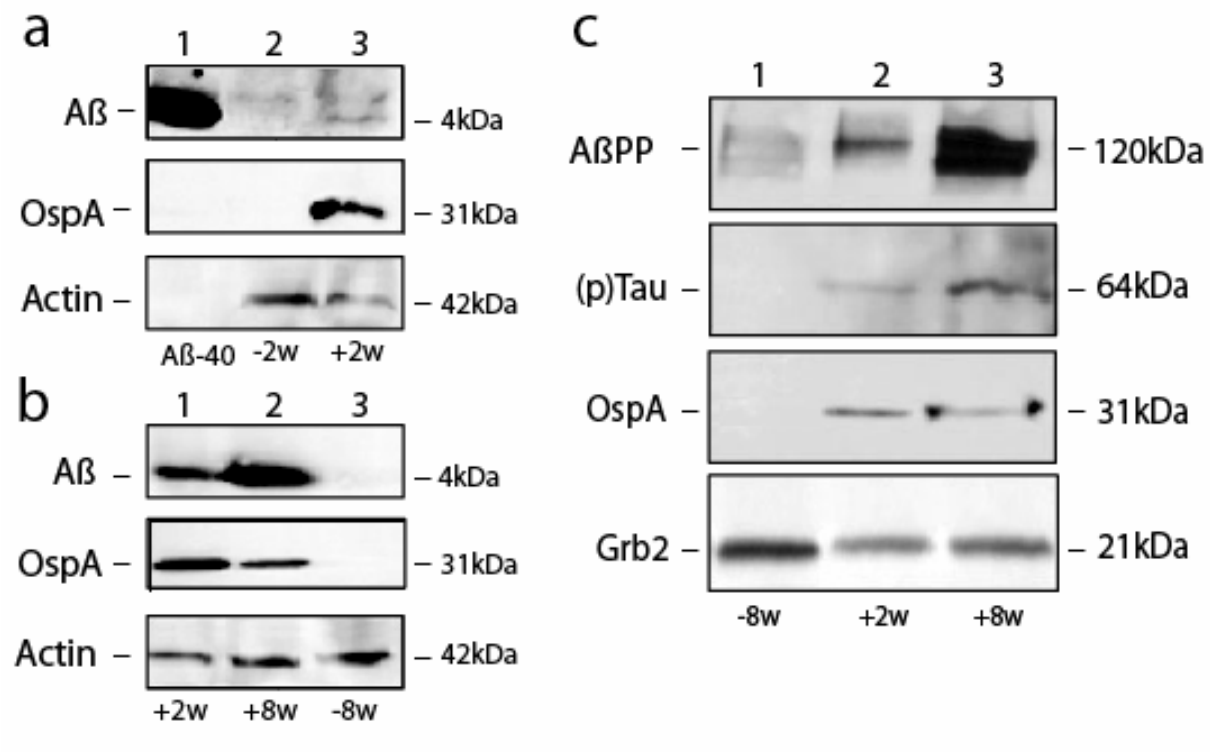
Figure 8



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).

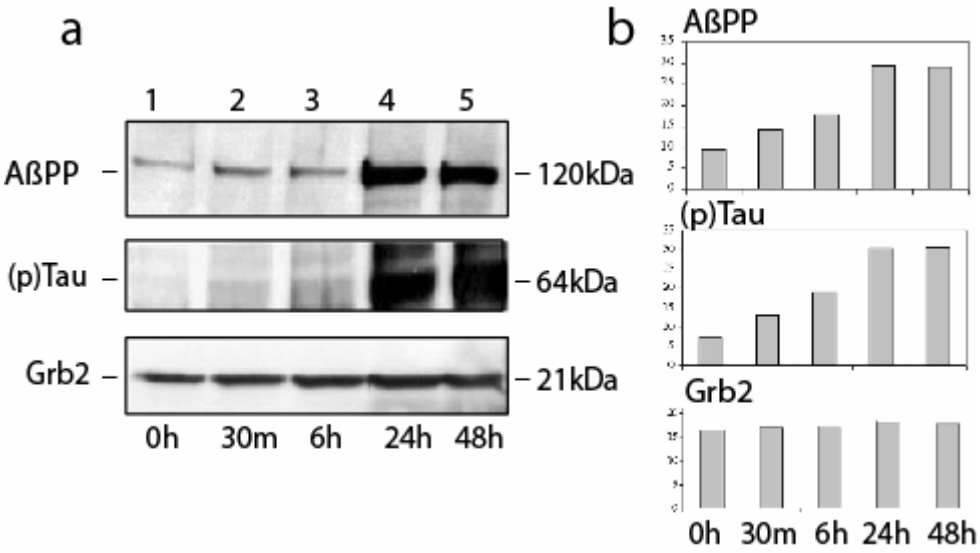
Figure 9



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.

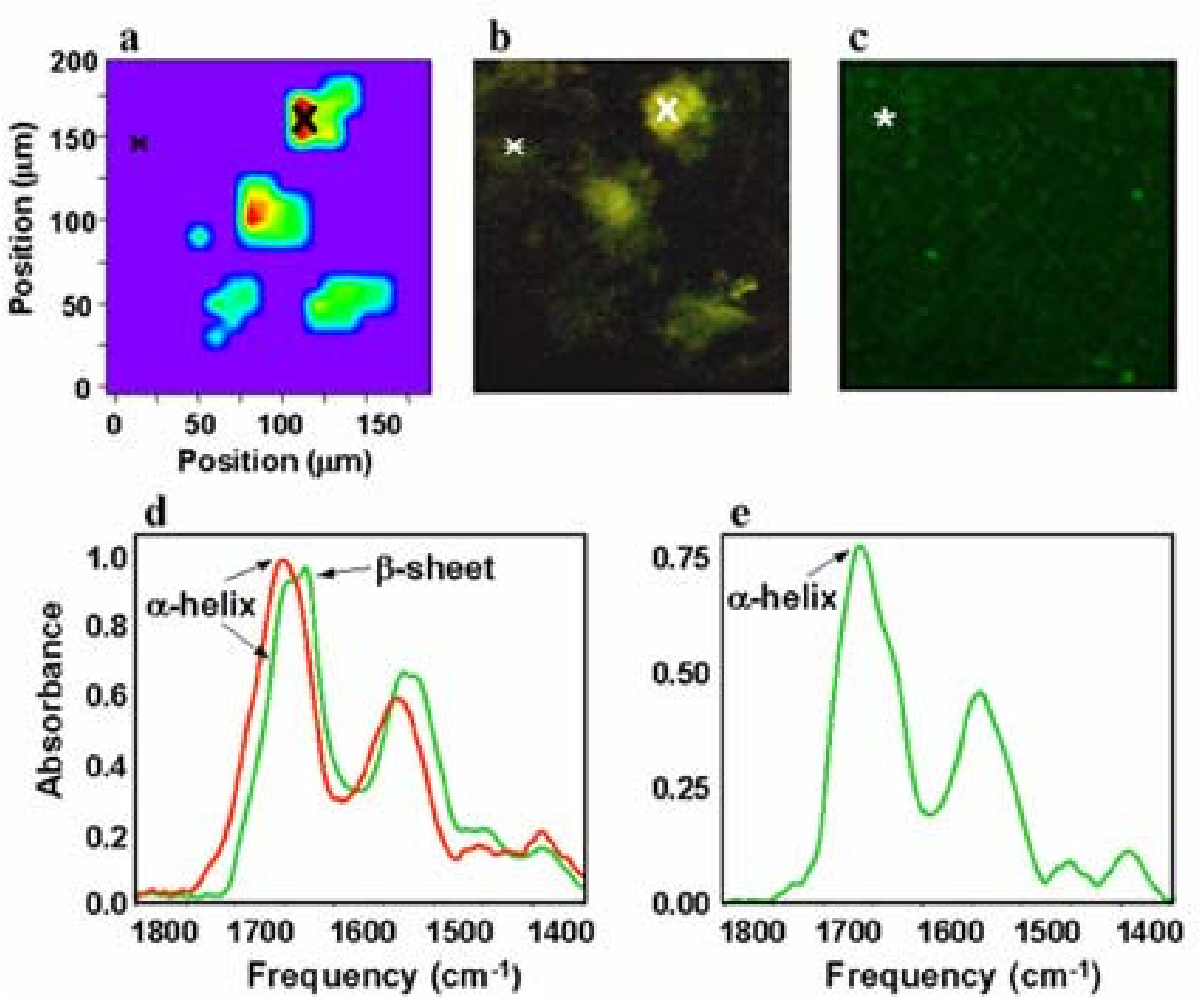
b: lane 1: microglia enriched culture infected for 2 weeks (+2w); lane 2: infected for 8 weeks (+8w); lane 3: uninfected culture maintained uninfected for 8 weeks (-8w). In (b) a stronger band was observed in microglia enriched cultures exposed to bacteria (b, +2w). Following 8-week exposure to spirochetes (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).