Concurrent Neuroborreliosis and Alzheimer's Disease:

Analysis of the Evidence

M. A. PAPPOLLA, MD, R. OMAR, MD, PHD, B. SARAN, MD, A. ANDORN, MD, M. SUAREZ, MD, C. PAVIA, PHD, A. WEINSTEIN, MD, D. SHANK, MS, K. DAVIS, MD, AND W. BURGDORFER, PHD

Several recent reports have claimed a possible association between *Borrelia burgdorferi* infection and Alzheimer's disease (AD). Herein, we describe our search for additional evidence of neuroborreliosis in AD. Brain tissue from neuropathologically confirmed cases of AD was cultured for *B burgdorferi* using standard microbiologic methods. Material derived from culture was further examined using electron microscopy, direct immunofluorescence, and acridine orange fluorescence. Previous studies have shown high titers of antiborrelia antibodies in CSF in all cases of confirmed neuroborreliosis; therefore, we tested CSF from neuropathologically confirmed cases of AD by indirect immunofluorescence and enzyme-linked immunoassay. In addition, imprint preparations from AD and control brain tissues were studied by direct immunofluorescence using a monoclonal antiborrelia antibody. Finally, a Western blot method was used to analyze protein extracts from cultures and AD brain tissue for the presence of borrelia antigen. Contrary to previous studies, our results do not support an association between infection with *B burgdorferi* and AD. HUM PATHOL 20:753–757. This is a US government work. There are no restrictions on its use.

Alzheimer's disease (AD) is a neurologic disorder of unknown etiology characterized by progressive impairment of memory and intellectual functions.[1,2] Brains from patients with this disease develop intraneuron accumulations of abnormal filaments (neurofibrillary tangles) and discrete aggregates of degenerated neuron processes and microglial cells, frequently accompanied by deposition of amyloid (senile plaques).[3] AD occurs most often as a sporadic process, although a small subgroup is inherited with an autosomal dominant mode.[4] The infectious hypothesis of AD has the support of several research groups. This hypothesis is buttressed by some successful experimental transmissions of familial AD (Gerstmann-Straussler-Skinker syndrome) as a process resembling a spongiform encephalopathy and by the occurrence of neurofibrillary tangles in various neurologic disorders of known infectious etiology.[5-9] Unfortunately, efforts to transmit sporadic AD or to detect microbial antigens or nucleic acid sequences in AD brain tissue have been disappointing.[10]

Recently, several reports have claimed a possible association between *Borrelia burgdorferi* infection of the CNS and AD.[11-14] It was proposed that these spirochetes, as in tertiary neurosyphilis, may invade the brain, where they remain latent for many years.[11]

The vast significance of this hypothesis prompted us to search for additional evidence in support of the association between neuroborreliosis and AD.

MATERIALS AND METHODS

Microbiological Studies

Tissues from frontal lobes and hippocampi were selected from six autopsy cases of AD, one case of Pick's disease, one case of Creutzfeldt-Jakob disease, and four neurologically normal patients. All cases of AD met the National Institutes of Health consensus criteria for the autopsy diagnosis of AD.[15] All AD patients had a documented clinical history of chronic dementia; however, information needed to segregate the AD population into sporadic and familial subtypes was not available. Tissues were inoculated into modified Kelly's medium[16] and Spirolate broth (Becton-Dickinson, Cockeysville, MD). The ability of our systems to support the growth of borrelia and nonpathogenic treponemes was determined by using control *B burgdorferi* isolated from an infected Ixodes tick and a stock strain of *Treponema denticola*. Growth was monitored for up to 6 weeks by using direct acridine orange fluorescence, direct immunofluorescence, and transmission electron microscopy (TEM). Electron microscopy was used because it is of taxonomic value in spirochetal work.[17] TEM was performed on Formvar-coated grids negatively stained with 2% phosphotungstic acid. Some samples were processed by centrifuging culture vials and embedding the pellets in resin following conventional methods.

The primary antibody used in the indirect immunofluorescence procedure is a monoclonal antibody directed against a 30 kd peptide present in *B burgdorferi*. Characterization of this antibody has been published.[18]

Immunochemistry

Western blot method. The Western blot technique[19] was used to detect the possible presence of morphologically or microbiologically undetectable forms of *B burgdorferi* in cul-

From the Department of Pathology Montrose, VA Hospital, Montrose, NY; the Departments of Pathology, Psychiatry and Pharmacology, and the Lyme Disease Research Center, New York Medical College, Valhalla, NY; the Department of Immunology and Rheumatology, Westchester County Medical Center, Valhalla, NY; the Department of Immunology, Aultman Hospital and Northeastern Ohio Universities, Rootstown, OH; the Department of Psychiatry, Mount Sinai School of Medicine, New York; and the National Institutes of Health, Rocky Mountain Laboratories, Hamilton, MT. Accepted for publication February 6, 1989.

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Address correspondence and reprint requests to M. A. Pappolla, MD, Chief Laboratory Service, Montrose VA Hospital, Montrose, NY 10548.

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tures and brain tissue. Samples from culture vials from four cases of AD, one case of Pick's disease, one normal brain, and control cultures were electrophoresed in gradient polyacrylamide gels[20] and electroblotted to nitrocellulose sheets as described.[19] An additional control for the method consisted of a preparation of calf brain microtubules known to react with a monoclonal antibody to β-tubulin. Brain protein extracts from one case of AD were also studied by this method.

Indirect immunofluorescence of spinal fluids. Indirect immunofluorescence was performed on spinal fluids from 18 neuropathologically confirmed cases of AD (serum was unavailable) and from 35 patients with various neurological and nonneurological disorders (Table 1). Twenty microliters of appropriate dilutions of spinal fluids were applied to microtiter wells containing *B burgdorferi*, incubated for 30 minutes at room temperature, washed in PBS, incubated with fluorescein labeled antihuman γ-globulin, washed again, and mounted. Positive and negative control specimens were included in each multiwell slide. Samples testing positive were titered. All specimens were read by a technologist with 15 years of experience with the method. The absence or degree of fluorescence was graded 0 (no fluorescence), +/− (equivocal), or 1+ to 4+ intensity (weak to

TABLE 1. Reactivities of CSF Samples

	FBA-NON Abs[1:5]	FBA-Abs	ELISA	FTA	FTA-Abs
AD	2+ [1:30]	(−)	(−)	+/−	(−)
AD	(−)	(−)	ND	(−)	(−)
AD	(−)	(−)	(−)	(−)	(−)
AD	2+ [1:10]	(−)	ND	(−)	(−)
AD	(−)	(−)	(−)	(−)	(−)
AD	(−)	(−)	(−)	(−)	(−)
AD	(−)	(−)	(−)	(−)	(−)
AD	3+ [1:40]	2(+)	(+)	3+	2+ [1:5]
AD	3+ [1:10]	(−)	(−)	3-4+	3-4+ [1:5]
AD	4+ [1:20]	(−)	(+)	3-4+	3+ [ND]
AD	(−)	(−)	ND	(−)	(−)
AD	(−)	(−)	(−)	ND	ND
AD	(−)	(−)	(−)	ND	ND
AD	(−)	(−)	(−)	ND	ND
AD	(−)	(−)	(−)	ND	ND
AD	(−)	(−)	(−)	ND	ND
AD	(−)	(−)	(−)	ND	ND
AD	(−)	(−)	(−)	(−)	(−)
CLE	2+ [1:5]	(−)	(+)	1(+)	(−)
Stroke	(−)	(−)	(−)	(−)	(−)
Pick's disease	(−)	(−)	(−)	(−)	(−)
Neuronal dysplasia	(−)	(−)	(−)	(−)	(−)
Chronic renal failure	1(+)	2(+)	(−)	(+/−)	(−)
IE	+/−	(−)	(−)	1(+)	(−)
Lung tumor	3+ [1:40]	1(+)	(+)	2(+)	(−)
Multiple sclerosis	(−)	(−)	(−)	(−)	(−)
Transitional cell CA	(−)	(−)	(−)	(−)	(−)
Stroke	+/−	(−)	(−)	(−)	(−)
Head trauma	(−)	(−)	(−)	(−)	(−)
Multiple sclerosis	(−)	(−)	(−)	(−)	(−)
Pneumonia	(−)	(−)	(−)	(−)	(−)
Disc disease	(−)	(−)	(−)	(−)	(−)
Alcoholism	(−)	(−)	(−)	(−)	(−)
CHD	(−)	(−)	(−)	(−)	(−)
Meningitis	(−)	(−)	(−)	(−)	(−)
Ovarian carcinoma	+/−	(−)	(−)	(−)	(−)
Guillian-Barre	(−)	(−)	(−)	(−)	(−)
R/O meningitis	2+ [ND]	(−)	(−)	(−)	(−)
Bronchopneumonia	(−)	(−)	(−)	(−)	(−)
Headache	(−)	(−)	(−)	(−)	(−)
Disc disease	(−)	(−)	(−)	(−)	(−)
Gastroenteritis	(−)	(−)	(−)	(−)	(−)
Sleep disorder	(−)	(−)	(−)	(−)	(−)
Convulsion	(−)	(−)	(−)	(−)	(−)
Vertebral disease	(−)	(−)	(−)	(−)	(−)
Disc disease	(−)	(−)	(−)	(−)	(−)
Disc disease	(−)	(−)	(−)	(−)	(−)
Bladder carcinoma	(−)	(−)	(−)	(−)	(−)
Chronic renal failure	(−)	(−)	(−)	(−)	(−)
Neuronal dysplasia	(−)	(−)	ND	(−)	(−)

Abbreviations: ND, not done; FBA-Non Abs, fluorescent borrelia antibody test without absorption; FBA-Abs, fluorescent borrelia antibody test absorbed with *Treponema phagedenis* as described in Methods section; ELISA, enzyme-linked immunosorbant assay; FTA, fluorescent Treponema antibody test; FTA-Abs, fluorescent Treponema antibody test absorbed with *T phagedenis* filtrates; CLE, chronic lymphocytic encephalitis; IE, infectious endocarditis; R/O, rule out; CHD, coronary heart disease; CA, carcinoma.

* Titers are indicated between brackets.

strong fluorescence). The samples were also absorbed with filtrates of *Treponema phagedenis* (Reiter strain) as standardized for syphilis serology,[21] to remove low titered nonspecific group antibodies, which are highly prevalent in the general population.[22] Minimally positive control (end-point dilution) serum samples from cases of confirmed Lyme disease remained positive after this step (unpublished observation).

Enzyme-linked immunosorbant assay of spinal fluids. The enzyme-linked immunosorbant assay (ELISA) was performed by standard methods[23] using an alkaline phosphatase p-nitrophenzyl system. Briefly, *B burgdorferi* were harvested from cultures at logarithmic growth phase. A soluble fraction antigen was obtained by sonication and ultracentrifugation of the organisms. Plates were coated with 0.1 mL of spirochetal antigen at a protein concentration of 5 μg/mL. Results were considered significant when the optical densities were above 3 SD of the mean of 50 normal sera.

Direct immunofluorescence. Direct immunofluorescence was performed on imprint preparations from brain tissue (frontal cortex and hippocampi) following conventional methods. We used a fluorescein labeled goat-antimouse IgG (1:50 dilution) and the monoclonal antibody referred to above.

RESULTS

Microbiological Studies

Growth of *B burgdorferi* or *T denticola* was consistently obtained in all control vials. In the control cultures, spirochetes were clearly visualized by all the methods used, and in the case of *B burgdorferi,* it was positively confirmed by electron microscopy (Fig 1). No growth of spirochetes was detected in any of the brains obtained for this study.

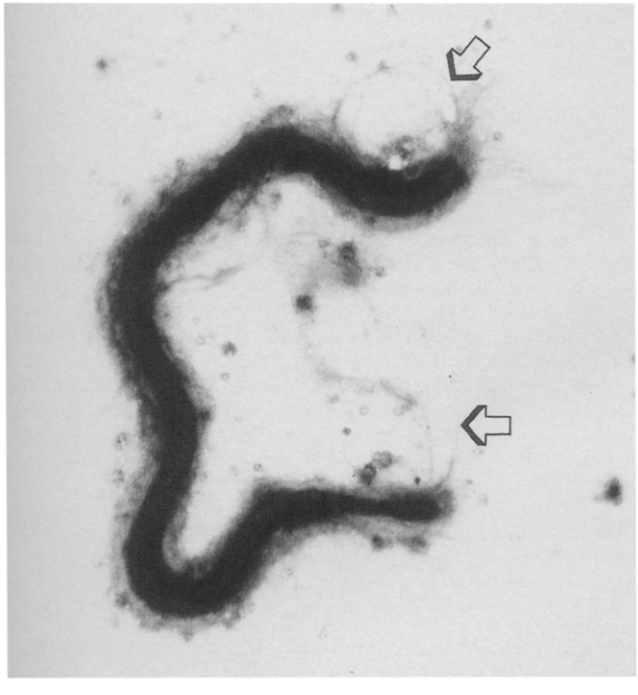

FIGURE 1. Electron microphotograph of borrelia organisms showing typical ends and flagellae (arrows).

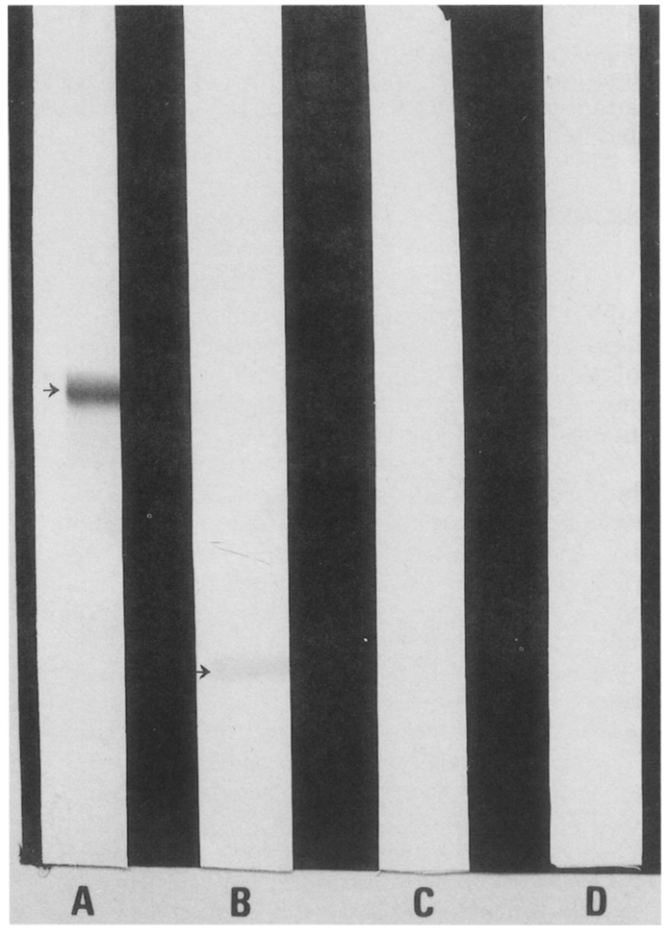

FIGURE 2. Photograph of nitrocellulose strips after electroblotting the following preparations: (A) microtubule preparation reacted with a monoclonal antibody directed against β-tubulin (control for technique); (B) homogenate of lysed *B burgdorferi* organisms reacted with a monoclonal antibody against the 30 kd peptide as described in text; (C) material from brain cultures of AD brain reacted with same antibody as B above; (D) Immunoblotting of brain protein extracts from an AD case reacted against the same monoclonal antibody as B above. The remaining specimens also failed to show any reactive bands in a similar manner to C and D. The color reaction was developed using an alkaline phosphatase system, nitrotetrazolium blue was used as the chromogen. Specific bands are detected in A (β-tubulin) and B (30 Kd borrelia peptide) (arrows).

Immunoblotting

A specific *B burgdorferi* peptide was identified as a single band in immunoblots from cultures of control spirochetes (Fig 2). Immunoblots from AD and Pick's disease brain cultures, or AD brain tissue, yielded no reactive bands.

Indirect Immunofluorescence Assay and ELISA

There were no statistically significant differences between test results on the spinal fluid samples from AD and the control groups tested by both methods (Table 1).

Direct Immunofluorescence

Numerous wavy filamentous structures were identified in touch imprints from AD brains and in

preparations obtained from three normal control brains. These forms resembled spirochetes only slightly and were interpreted as artifacts, probably caused by nonspecific binding of IgG to small myelinated nerve cell endings.

DISCUSSION

The cause of AD remains unknown. Yet, substantial work needs to be done to completely exclude the possibility of an infectious agent that may be responsible for this disorder. Some experimental and naturally occurring lines of evidence for an infectious etiology are as follows:

1. Cases of familial AD can be transmitted to chimpanzees as encephalopathies, histologically resembling Cruetzfeld-Jakob disease (CJ), and the CJ virus can be isolated from some cases of familial AD.[24] However, the characteristic neuropathologic lesions of AD have not been observed in infected animals.

2. Neurofibrillary tangles and plaques that closely resemble those found in AD occur in several neurologic conditions of proven infectious nature.[5-9]

3. Amyloid plaques similar to those found in AD can be induced in recipient experimental animals inoculated with the scrapie agent.[25] However, the biochemical composition of scrapie plaque amyloid differs from the AD amyloid.[26]

The spirochetal hypothesis of AD was proposed by MacDonald and Miranda,[11] who suggested that *B burgdorferi* may cause AD. The hypothesis is interesting for several reasons. For one, spirochetes can survive latent in host tissue for many years. We know from the late neurosyphilis model that spirochetes are exceedingly difficult to find in the nervous tissue by histologic methods. This has been claimed as the reason for not finding this newly discovered spirochete in AD.[14] In addition, direct infection of the nervous system by *B burgdorferi* can and does occur. In fact, involvement of the nervous system in Lyme disease is seen in approximately 11% of cases. The neurologic manifestations of Lyme disease may mimic various inflammatory and demyelinating disorders, including multiple sclerosis-like syndromes and dementia.[27-38]

Results of our study do not support an association between *B burgdorferi* and AD. Virtually all previously documented cases of neuroborreliosis have shown increased immunoglobulin (IgG) titers against *B burgdorferi* in spinal fluid with titers usually higher than 1:160.[27,28,34,39] In contrast, most cases of AD included in this study were unreactive. Few CSF specimens from AD patients were reactive at low titers in the nonabsorbed fluorescent borrelia antibody test (Table 1). However, this reactivity was most likely due to the presence of spirochetal group antibodies as indicated by the results obtained following absorption with filtrates of *T phagedenis* (Reiter strain) (Table 1). Spirochetal group antibodies are highly prevalent in the population at large[22] and have also been a source of nonspecificity in syphillis serology. Spirochetal group antibodies might gain access to the CSF through a disrupted blood brain barrier, known to occur in AD.[39] In any case, the number of positive cases in the AD group was very small and not different from that of the general population.

Although our microbiologic culture system consistently supported growth of *B burgdorferi* and *T denticola,* neither treponemas nor spirochetes could be detected by direct immunofluorescence, electron microscopy, or Western blots of culture material of brain tissue. All the above methods were successful in detecting spirochetes or their products in control cultures. The rationale to include *T denticola* in our studies was to exclude the possibility of commensal treponemas misclassified as *B burgdorferi* in previous reports of concurrent borreliosis and AD. Our findings do not support those of MacDonald[12,13] and MacDonald and Miranda,[11] who reported growth in three of three AD brains.

The role of amyloid deposition in AD has been the focus of intense research, especially since the genetic characterization of the sequences encoding the amyloid β-protein.[40-44] However, mechanisms leading to amyloid accumulation are still controversial and new evidence has shown lack of allelic association for restriction fragment length polymorphisms at certain genes and in AD.[44] These data may emphasize the importance of environmental (toxic, infectious) factors. Therefore, the possibility of a different spirochete in AD not detectable by the methods used herein should remain open to further work. Because some forms of amyloid accompany chronic infections and other reactive processes, additional work is needed to totally exclude the role of an infectious organism in this disease. This study was directed to the detection of the Lyme disease agent, ie, *B burgdorferi,* and comprised a relatively small sample of AD patients.

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