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Anti-neural antibody reactivity in patients with a history of Lyme borreliosis and persistent symptoms

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ABSTRACT

Some Lyme disease patients report debilitating chronic symptoms of pain, fatigue, and cognitive deficits despite recommended courses of antibiotic treatment. The mechanisms responsible for these symptoms, collectively referred to as post-Lyme disease syndrome (PLS) or chronic Lyme disease, remain unclear. We investigated the presence of immune system abnormalities in PLS by assessing the levels of antibodies to neural proteins in patients and controls. Serum samples from PLS patients, post-Lyme disease healthy individuals, patients with systemic lupus erythematosus, and normal healthy individuals were analyzed for anti-neural antibodies by immunoblotting and immunohistochemistry. Anti-neural antibody reactivity was found to be significantly higher in the PLS group than in the post-Lyme healthy ($p < 0.01$) and normal healthy ($p < 0.01$) groups. The observed heightened antibody reactivity in PLS patients could not be attributed solely to the presence of cross-reactive anti-borrelia antibodies, as the borrelial seronegative patients also exhibited elevated anti-neural antibody levels. Immunohistochemical analysis of PLS serum antibody activity demonstrated binding to cells in the central and peripheral nervous systems. The results provide evidence for the existence of a differential immune system response in PLS, offering new clues about the etiopathogenesis of the disease that may prove useful in devising more effective treatment strategies.

1. Introduction

Lyme disease is a multisystem infection, caused by bacteria of the *Borrelia burgdorferi* species complex and transmitted by *Ixodes* ticks (Stanek and Strle, 2003). It is the most commonly reported tick-borne disease in the northern hemisphere, widespread in Europe and endemic in more than 15 states in the United States (Stanek and Strle, 2008; Steere, 2001). The initial skin rash (erythema migrans) may be followed by complications affecting joints, heart, and the nervous system (Stanek and Strle, 2003; Wormser et al., 2006). The neurologic complications involve both the central and peripheral nervous systems. These include lymphocytic meningitis, encephalitis, cranial neuropathy, radiculopathy, and alterations of mental status, all of which usually respond well to antibiotic treatment (Halperin, 2008). However, some patients with Lyme disease continue to have persistent complaints despite treatment and in the absence of objective evidence of infection, as determined by currently available methods (Feder et al., 2007; Marques, 2008). The symptoms in these patients are generally accepted to include mild to severe musculokeletal pain, fatigue, and/or difficulties with concentration and memory (Feder et al., 2007; Marques, 2008). The condition, variably referred to as chronic Lyme disease, post-treatment Lyme disease syndrome (PTLDS), and post-Lyme disease syndrome (PLDS or PLS), is associated with considerable impairment in the health-related quality of life in some patients (Klempner et al., 2001).

Considering the lack of evidence for the presence of live spirochetes in PLS patients who have received recommended antibiotics, persistent infection is currently not thought to account for the symptoms of PLS by most investigators (Baker, 2008; Feder et al., 2007). However, despite several years of debate and a number of treatment clinical trials (Fallon et al., 2008; Klempner et al., 2001; Krupp et al., 2003), few clues to the causes of the symptoms have emerged. Lack of any biomarkers to aid in the diagnosis and follow-up has also compounded the problem of understanding the disease. Mechanisms other than active infection, including the possibility of involvement of adaptive or innate immune sys-

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tem abnormalities, have been suggested, but experimental evidence has been scarce (Marques, 2008; Sigal, 1997). The aim of this study was to characterize the level and specificity of antibody reactivity to neural antigens in PLS patients. Here, we show evidence of heightened anti-neural antibody levels in PLS, indicating the presence of objective immunologic abnormalities in affected patients that may be relevant to the pathogenic mechanism of the disease.

2. Methods

2.1. Subjects

Serum samples from 83 individuals with a history of Lyme borreliosis and persistent symptoms, recruited as part of a previous clinical trial (Klempner et al., 2001), were used in this study (37 female, 46 male; mean age 55.6 ± 12.0 y (SD); mean elapsed time since the original diagnosis of Lyme disease 5.0 ± 2.9 y (SD)). Selection of these specific specimens from the original cohort was based on limiting the elapsed time between diagnosis of acute Lyme disease and serum specimen collection to between 1 and 12 years. Patients had at least one of the following: a history of erythema migrans (EM) skin lesion, early neurologic or cardiac symptoms attributed to Lyme disease, radiculoneuropathy, or Lyme arthritis. Documentation by a physician of previous treatment of acute Lyme disease with a recommended antibiotic regimen was also required. Patients had one or more of the following symptoms at the time of enrollment: widespread musculoskeletal pain, cognitive impairment, radicular pain, paresthesias, or dysesthesias. Fatigue often accompanied one or more of these symptoms. The chronic symptoms had to have begun within 6 months after the infection with *B. burgdorferi*. Control subjects included 27 individuals who had been treated for early localized or disseminated Lyme disease associated with single ($n = 18$) or multiple ($n = 9$) EM, but had no post-Lyme symptoms after at least 2 years of follow-up (12 female, 15 male; mean age 54.4 ± 14.7 y (SD); mean elapsed time since the original diagnosis of Lyme disease 5.4 ± 3.8 y (SD)). The diagnosis of acute Lyme disease in control subjects was confirmed by recovery of *B. burgdorferi* in cultures of skin and/or blood sample. The elapsed time between diagnosis of acute Lyme disease and serum specimen collection was limited to between 1 and 12 years for post-Lyme healthy subjects. In addition to the above, serum samples from 15 patients with systemic lupus erythematosus (SLE) and 20 healthy individuals were analyzed in the study. All SLE patients met four or more of the American College of Rheumatology classification criteria for diagnosis (Tan et al., 1982). Serum specimens were stored at −80 °C prior to use. This study was approved by the Institutional Review Board of the Weill Medical College of Cornell University.

2.2. Total IgG

Total IgG concentration of serum specimens was measured with an ELISA kit (ICL), according to the manufacturer's instructions.

2.3. Anti-borrelia antibodies

IgG anti-borrelia antibody levels were determined by ELISA. 96-well polystyrene plates (BD Biosciences) were incubated overnight with 0.5 μg/well of *B. burgdorferi* B31 antigen (Meridian) in 0.1 M carbonate buffer (pH 9.6). Blocking of wells was done with 1% BSA in phosphate-buffered saline containing 0.05% Tween-20 (PBST) for 1.5 h. Incubation with diluted serum samples (50 μL/well at 1:800 in blocking buffer) was done for 1 h. Each plate contained one negative and two positive controls. Incubation with HRP-conjugated sheep anti-human IgG (Amersham) secondary antibody was for 1 h. Incubation with developing solution, comprising 27 mM citric acid, 50 mM Na_2HPO_4, 5.5 mM *o*-phenylenediamine, and 0.01% H_2O_2 (pH 5), was for 20 min. Absorbance was measured at 450 nm and corrected for non-specific binding by subtraction of the mean absorbance of corresponding wells not coated with the borrelia antigen. Absorbance values were normalized based on the mean for the positive controls on each plate. Cutoff for positivity was assigned as two standard deviations above the mean for the healthy control group results.

2.4. Anti-neural antibodies

2.4.1. Immunoblotting

Antibodies to brain proteins were detected by immunoblotting for all specimens as follows. Mouse brain was utilized in order to avoid artifactual bands that result from the binding of secondary anti-human antibodies to endogenous immunoglobulins when using the sensitive chemiluminescence method of detection. Mouse tissue was specifically chosen among non-primate sources due to the high level of known homology and orthology between human and mouse proteomes (Southan, 2004), a strategy that has been used in other studies as well (Maruyama et al., 2004; Shoenfeld et al., 2003; Tin et al., 2005). Mouse (C57BL/6J strain) brain lysate was prepared as previously described (Alaedini et al., 2007). SDS–PAGE (4–15% pre-cast 2D-prep gel from Bio-Rad) was carried out on 400 μg protein aliquots of lysate at 200 V in Tris–glycine–SDS buffer for 35 min, followed by transfer to nitrocellulose membrane at 33 V in Tris–glycine buffer containing 20% methanol for 16 h. Each gel contained the Precision Plus molecular weight marker mix (Bio-Rad) in one lane. The membrane was incubated in blocking buffer, containing 5% milk and 0.5% BSA in Tris-buffered saline containing 0.05% Tween-20 (TBST) for 2 h. Incubation with patient serum (1:2000 in dilution buffer containing 10% blocking buffer and 10% fetal bovine serum in TBST) was carried out for 1 h in a Mini-PROTEAN II Multiscreen apparatus (Bio-Rad). A positive control sample was included on every membrane. HRP-conjugated sheep anti-human IgG (Amersham) was used as the secondary antibody. Detection of bound antibodies was by the ECL system (Millipore) and BioMax MR film (Kodak) after 10 s exposure. Each membrane was treated with stripping buffer (Pierce) at 58 °C for 30 min, and reblotted with HRP-conjugated rabbit anti-β tubulin antibody (Novus). Detection of bound antibodies was as before. Conversion of immunoblots to line graph, density analysis, and subtraction of background were performed by the Unscan-It program (Silk Scientific). Measurement of total antibody reactivity towards neural proteins in each sample was done by calculating the sum of gray-level intensities for all software-assigned and background-subtracted reactive bands. Total gray-level intensity for each specimen was corrected for (1) inconsistencies within each membrane (e.g., for variation in sample loading and efficiency of protein transfer) according to the gray-level intensity of the tubulin band for each lane, and (2) inconsistencies in experimental conditions between membranes (e.g., for variation in sample loading, efficiency of protein transfer, and autoradiography exposure time) according to the total gray-level intensity for the positive control on each membrane.

2.4.2. Immunohistochemistry

Immunohistochemical analysis was similar to previously described procedure (Alaedini et al., 2008). Formaldehyde-fixed and paraffin-embedded sections of human cerebral cortex and dorsal root ganglia (DRG), obtained at post mortem, were cut (10 μm thickness) and placed on slides. Sections were deparaffinized and rehydrated by sequential incubation in xylene, ethanol (100%, 90%, 80%, and 70%), and PBS. Antigen retrieval was done by incubation in 0.05% citraconic anhydride buffer (pH 6.0) for 20 min at