Comparison of Flagellum and Sonicate Antigens for Serological Diagnosis of Lyme Borreliosis

M. Karlsson¹*, G. Stiernstedt¹, M. Granström³, E. Åsbrink⁴, B. Wretlind²

A sonicate antigen and two concentrations of a purified flagellum antigen of Borrelia burgdorferi were compared for serological diagnosis of Lyme borreliosis by an enzyme immunoassay (EIA). Generally, the higher concentration of flagellum antigen was found to be superior to the lower concentration, which was diluted eight times compared to the higher concentration. The diagnostic sensitivity for IgG antibody detection increased from 13% in the sonicate EIA to 31% in the best flagellum EIA assay (p = 0.01) in sera from patients with erythema migrans (n = 70), and from 34% to 55% (p = 0.01) in sera from patients with neuroborreliosis (n = 77). However, the sensitivity for IgG in sera from patients with acrodermatitis chronica atrophicans (n = 20) was high in both assays: 90% in the sonicate EIA compared to 95% in the flagellum EIA. Regarding IgM, there was no significant difference between the sensitivity of the assays in sera from any of the patient groups. The sensitivity values for IgM and IgG in cerebrospinal fluid (CSF) from patients with neuroborreliosis were also without significant differences. Sera and CSF from patients with menigitis/encephalitis of non-Borreliia etiology (n = 35), multiple sclerosis (n = 9) or syphilis (n = 24), served as controls. The flagellum EIA showed a significantly improved specificity for IgG in CSF from controls with syphilis (p < 0.01). It is concluded that purified Borrelia burgdorferi flagellum antigen is superior to a sonicate antigen, especially for serodiagnosis of the early stages of Lyme borreliosis.

Borrelia burgdorferi, the etiological agent of Lyme borreliosis, was first isolated from Ixodes dammini in the USA in 1982 (1), and later from Ixodes ricinus in Europe (2, 4) and from the skin (3, 4), blood (3, 5), cerebrospinal fluid (CSF) (3, 6) and joint fluid (7, 8) of patients. However, the culture of spirochetes from patients is a slow and, with the exception of culture from skin manifestations (9), a mostly low-yielding procedure (3, 7). Consequently, serological testing is the usual way to confirm the clinical diagnosis. The most commonly used tests have been indirect immunofluorescence assays and enzyme immunoassays (EIA), utilizing whole Borrelia burgdorferi cells or fractions of sonicated cells as antigens (10-14). Cross-reactivity with other spirochetes occurs (15), and Borrelia burgdorferi also shares immunogenic antigens with other bacteria (16).

The sensitivity and specificity of the current assays could probably be improved by the use of purified Borrelia burgdorferi antigens in EIA, thus avoiding some nonspecific antibody reactions. Several studies have shown an early and long-standing antibody response against a 41-kilodalton (kDa) flagellar protein in sera from most patients with Lyme borreliosis by immunoblotting (17-21). However, with varying frequency, sera from healthy controls have also been reported to react in Western blot with this antigen, raising doubts about the specificity of the 41-kDa antigen (17-21).

¹Department of Infectious Diseases, and ²Department of Bacteriology, Danderyd Hospital, S-182 88 Danderyd, Sweden.
³Department of Bacteriology, Karolinska Hospital, Stockholm, Sweden.
⁴Department of Dermatology, South Hospital, Stockholm, Sweden.
In recent years a few studies have evaluated the *Borrelia burgdorferi* flagellum antigen by EIA, either with a flagellum-enriched but not purified antigen preparation (20, 22), or by purified flagellum (23, 24). The flagellum-enriched antigen was shown to be equal to a sonicate antigen in one study (22) and better than a sonicate antigen in the other study (20). In the latter study, the 41-kDa flagellum antigen was also purified by elution from sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). This antigen was not found to be useful in EIA (20). The only studies so far on a purified flagellum antigen showed a markedly improved diagnostic sensitivity and specificity compared with a sonicate antigen (23, 24).

The aim of the present study was to evaluate the performance of a purified *Borrelia burgdorferi* flagellum antigen for the serological diagnosis of different stages of Lyme borreliosis in our own clinically defined patient material. Furthermore, since the flagellum antigen is difficult and expensive to produce in large quantities, we wanted to compare different coating concentrations of this antigen.

**Materials and Methods**

**Patients.** Seventy patients with erythema migrans without extracutaneous complications, 20 patients with acrodermatitis chronica atrophicans, and 77 patients with neuroborreliosis were included in the study. Only the first pretreatment serum and CSF samples from each patient with neuroborreliosis and only the first pretreatment serum sample from each patient with erythema migrans and acrodermatitis chronica atrophicans were analyzed. All patients were from the Stockholm area in Sweden.

The diagnosis of erythema migrans and acrodermatitis chronica atrophicans was based on clinical evidence, and the clinical diagnosis of acrodermatitis was confirmed by histopathology. The study included 20 men and 50 women with erythema migrans, aged 12 to 83 years (median age 54). The time of onset for erythema migrans until blood sampling ranged from less than one week to 12 months (median duration 6 weeks). Of the 20 patients with acrodermatitis chronica atrophicans, six were men and 14 women, aged 33 to 85 years (median age 47). The duration of acrodermatitis ranged from six months to 20 years (median duration 2.5 years).

Neuroborreliosis was defined as a) pleocytosis (> 5 leukocytes x 10^6/l) with lymphocytic predominance in CSF together with neurological signs and symptoms and/or general symptoms compatible with neuroborreliosis, or b) neurological signs and symptoms within one month after the onset of erythema migrans. Neurological signs and symptoms were defined as sensory or motor neuroarachnoiditis, myelopathy, or cranial nerve neuritis. General symptoms compatible with neuroborreliosis were those of a subacute or chronic meningitis as described previously (25, 26). In many cases fulfilling criteria "a", the diagnosis was strengthened by a history of a recent tick-bite and/or erythema migrans. Thirty men and 47 women, ranging in age from 6 to 87 years (median age 47), had neuroborreliosis. Ninety-four percent (72/77) of patients with neuroborreliosis had pleocytosis of the CSF (range 6-920 x 10^6/l leukocytes, median 128), 57% (44/77) reported a recent tick-bite, and 39% (30/77) had a recent or current erythema migrans. The duration of neurological symptoms before blood and CSF sampling ranged from one day to 11 months (median duration 22 days).

**Controls.** Sera from patients admitted to hospital due to disorders not related to Lyme borreliosis (n = 130) were used for determination of diagnostic cut-off levels in the EIA. These controls were between 1 and 80 years old (median age 38). Furthermore, sera and CSF from patients with different infectious diseases of the central nervous system (CNS) (n = 35), multiple sclerosis (n = 9), or syphilis (n = 24) were investigated. The CNS infections included tick-borne virus encephalitis, varicella-zoster virus encephalitis, herpes simplex type 1 virus encephalitis, herpes simplex type 2 virus meningitis, Epstein-Barr virus encephalitis, morbilli encephalitis, mycoplasma encephalitis, influenza A encephalitis, mumps virus meningitis, enterovirus meningitis, and tuberculous, meningococcal and cryptococcal meningitis. Twenty-four men and 3 women with CNS infections, multiple sclerosis or syphilis, aged 14 to 83 years (median age 33). All controls were from the Stockholm area in Sweden.

**Sonicate Antigen.** The method for preparation of sonicate antigen has been described previously (12). Briefly, spirochetes isolated from an *Ixodes ricinus* tick in Sweden (strain STG132) were grown in modified Kelly's medium (3) for five days, pelleted by centrifugation at 10,000 x g for 30 min (Sorvall RC-BS, Du Pont Instruments, USA) and washed four times in PBS with 5 mM MgCl₂. The final pellet was suspended in PBS without MgCl₂ and sonicated on ice four times for 30 sec each time (Sonifier B 30, Branson Sonic Power Company, Switzerland). The sonic extract was centrifuged at 10,000 x g for 30 min and the supernatant was used as antigen. Protein content was determined by BCA protein assay (Pierce, USA).

**Flagellum Antigen.** The flagellum antigen was purified by Klaus Hansen, Statens Seruminstitution, Copenhagen, Denmark. A Danish *Borrelia burgdorferi* strain, DK 1, isolated from the skin of a patient with erythema migrans was used for the antigen preparation. The purification procedure has been described in detail previously (23). Briefly, the spirochetes were subjected to a mild ionic detergent for removal of the outer envelope. The detergent-insoluble material containing the protoplasmatic cylinders with the periplasmatic flagella attached was sheared in a blender to achieve a mechanical detachment of the flagella from the cell bodies. The sheared material was then subjected to several differential centrifugation steps and finally purified on a CsCl density gradient. Protein content was determined by a modified Bradford protein assay (Bradford, UK). The flagellum antigen prepared in this way proved to be highly pure by Western blot (Figure 1). Figure 2 shows the sonicate and flagellum antigens after SDS-PAGE.

**Enzyme Immunoassay.** The EIA used has been described in detail previously (12). Briefly, microtiter plates (Dynatech, USA) were coated overnight at room temperature (22 °C) with 100 µl of either sonicate or flagellum antigen diluted in PBS. The optimal coating concentration was determined by testing positive and negative serum samples (Figure 3 a, b). A coating concentration of 12 µg protein/ml was judged optimal in the sonicate EIA. For the flagellum EIA a coating concentration of 5 µg protein/ml was found optimal, corresponding to an antigen dilution of 1/100. Furthermore, all samples were analyzed with a lower co-