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Identification of *Borrelia burgdorferi* Ribosomal Protein L25 by the Phage Surface Display Method and Evaluation of the Protein's Value for Serodiagnosis

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The phage surface display technique was used to identify *Borrelia burgdorferi* antigens. By affinity selection with immunoglobulin G from pooled sera of six Lyme borreliosis (LB) patients, the ribosomal protein L25 was identified. The diagnostic value of L25 was investigated by an enzyme-linked immunosorbent assay, using sera from 80 LB patients and 75 controls, and the use of the protein resulted in a specificity of 99% and a 23% sensitivity, which qualify L25 as a useful antigen when combined with others.

Lyme borreliosis (LB) is increasingly recognized to cause chronic manifestations, such as arthritis, neurological disorders, skin manifestations, and arrhythmia (25, 26). Commonly, LB diagnosis is based on clinical signs and confirmed by serological findings. For serodiagnosis, a two-step process, including an enzyme-linked immunosorbent assay (ELISA) followed by Western immunoblot analysis, is recommended in Europe and the United States. Serological tests are widely used despite several shortcomings, such as the heterogeneity of antigen preparations and a lack of standardization causing interlaboratory variation (3, 23). Furthermore, serological tests show insensitivity in the early stages of LB (1, 13). It is well established that *Borrelia* species express different surface components depending on the temperature (21, 27), pH (4, 21) and cell density (30). Through differential gene expression, the pathogen is able to adapt to different hosts and to evade immune responses (14, 15). Therefore, assays using antigens derived from *Borrelia* cultures might not represent antigens which are selectively expressed in the human host. Recent studies with the recombinant proteins BBK32 and VlsE as well as two synthetic peptides have proven to be superior to assays currently used for LB serodiagnosis (1, 13, 24).

In the present study, we used the pJuFo phage surface display method (6) to identify *Borrelia* antigens with affinity for immunoglobulin G (IgG) from *Borrelia*-infected patients. This technology has previously been applied successfully to identify allergens from cDNA libraries of *Aspergillus fumigatus* (5), *Cladosporium herbarum* (29), peanuts (12), and mites (8) by employing IgE antibodies from patients. Genomic phage surface display libraries of three different *Borrelia* strains and one mixed library consisting of all three strains were constructed by helper phage superinfection according to a published protocol (2). The strains (*Borrelia burgdorferi* N40, *Borrelia afzelii* VS461, and *Borrelia garinii* PSTH), which were kindly provided by T. Kamradt (Berlin, Germany), were grown in BSK-H medium (Sigma-Aldrich, Deisendorf, Germany) as described previously (7), and the genomic DNAs were purified (QiaAmp tissue kit; QIAGEN, Hilden, Germany). The DNAs were partially digested with MboI, ligated into the BglII-restricted pJuFo vector (6), and electrotransformed into *Escherichia coli* XL1-Blue. Recombinant phage were enriched by five cycles of affinity selection with a pool of sera from six LB patients (Table 1). Microtiter plates were coated with anti-human IgG monoclonal antibodies (Zymed Laboratories Inc., San Francisco, Calif.), blocked with 3% nonfat dry milk, and incubated with LB patient sera (20 μl/well in 80 μl Tris-buffered saline) overnight at 4°C. After washing of the plates, 1.8×10^{11} to 1.6×10^{12} CFU of each library was separately added and incubated for 2 h at 37°C. After 10 (cycles one to three) or 20 (cycles four and five) consecutive washing steps, adherent phage were eluted by a pH shift (100 mM glycine-HCl, pH 2.2), and *E. coli* was reinfected for further cycles of affinity enrichment. Phage enrichment was monitored by titration of ampicillin-resistant CFU.

After five cycles of selection, the phagemid DNAs from 124 randomly picked *E. coli* clones were analyzed by restriction with PstI and sequencing. Fifty clones contained *Borrelia* sequences with the incorrect orientation in the pJuFo vector, and three clones could not be correlated with any published *Borrelia* sequence. Among the remaining clones with *Borrelia*-specific sequences, 10, 14, 4, and 43 were derived from the *B. burgdorferi*, *B. garinii*, *B. afzelii*, and mixed libraries, respectively. These clones carried inserts of 16 different sizes, which could be matched with nine genomic sequences of *B. burgdorferi* B31, namely, BB0182, BB0272, BB0335, BB0371, BB0713, BB0786, BBA26, BBK32, and BBL38. One sequence encoded the fibronectin binding protein BBK32, a well-established immunogenic protein (9) with high potential as a target for serodiagnosis of human LB (11, 13). Another sequence identified from the *B. burgdorferi* and mixed libraries encoded the ribosomal protein L25. This protein is part of the 50S ribosomal subunit that binds to a specific portion of the 5S rRNA called loop E (28). Although L25 has conserved regions involved in

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TABLE 1. Clinical data for six panning pool sera from LB patients

Patient no.[a]	Age (yr)	Tick bite recall	Presence of erythema migrans	ELISA titer	IgG Western blot reactivity (bands [kDa])	LB symptom(s)
1	61	+	+	1:320	39, 41, 58, 66, 75, 100	Lyme arthritis (knee), myocarditis
2	35	−	−	1:80	30, 41, 58, 100	Lyme arthritis (knee, elbow), arthralgia
3	58	−	−	1:640	17, 30, 35, 41, 58, 75, 100	Lyme arthritis
4	35	−	−	1:40	58	Lyme arthritis, ACA
5	59	+	−	1:160	30, 58, 75, 100	Lyme arthritis (knee, fingers), neuropathy
6	28	−	−	1:80	25, 30, 39, 41, 58, 66, 75, 100	Lyme arthritis (knee), palpitation

[a] Patients were selected by an experienced general practitioner.

RNA binding (28), the amino acid residues interacting with the 5S rRNA show no conservation among different eubacterial species (17). Among *Borrelia* species, the deduced amino acid sequence of L25 from *B. burgdorferi* N40 is 98% and 90% identical with those from *B. burgdorferi* B31 and *B. garinii* PBi, respectively.

In order to investigate the diagnostic value of L25, the protein was expressed as a six-histidine-tagged recombinant protein in *E. coli* M15 (QIAGEN). As a control, the antigenic protein OspC was expressed in *E. coli* BL21 (Stratagene, La Jolla, Calif.). The full-length coding sequence of L25 was amplified from the genomic DNA of *B. burgdorferi* N40 by PCR with primers including a BamHI and a KpnI site (forward primer, CGGGATCCGGACGTCGACAAGTGGTAAG; reverse primer, GGGGTACCAAATCACTTTATAATAACAA CTTCC) and then ligated into the pQE30 expression plasmid (QIAGEN). The sequence of OspC was amplified with primers including an EcoRI and an XhoI site (CCGGAATTCATGA AAAAGAATACATTAAGTGC and CCGCTCGAGCTTAT AATATTGATCTTAATTAAGG) and then ligated into the pTYB12 plasmid (New England Biolabs, Ipswich, Conn.). Protein expression of L25 and OspC was induced for 20 h at 16°C. Recombinant L25 (rL25) was purified by Ni^{2+}-chelate affinity chromatography in the presence of 8 M urea, and rOspC was purified according to the manufacturer's instructions (NEB) with an elution buffer containing 8 M urea. For serological analysis, 80 serum samples from patients with late-stage LB, i.e., Lyme arthritis, neuroborreliosis, or acrodermatis chronica atrophicans (ACA), were collected in the southwest of Germany by an experienced physician. The diagnosis of LB was based on characteristic clinical findings, a history of exposure, and an antibody response, which was further confirmed by IgG ELISA based on a C_6 peptide antigen of the VlsE protein (16). A peptide with the sequence CMKKDDQIAAAMVLRGMA KDGQFALK was synthesized in the Department of Analytical Chemistry (M. Przybyslki, University of Konstanz, Germany). Control serum samples from 75 healthy donors without any signs of LB and no serum reactivity against the C_6 peptide were collected in the same area of Germany.

For the determination of anti-rL25 and anti-rOspC antibodies, specific ELISAs were established and optimized. In brief, the wells of a microtiter plate (Nunc) were coated with 0.5 μg recombinant protein overnight at 4°C. After being washed with PBST (10 mM sodium phosphate, 140 mM NaCl, 0.1% Tween 20), the wells were blocked with 5% nonfat dry milk for 2 h. Human serum samples were diluted 1:200 in blocking solution, and 100 μl was added to the wells and incubated for 3 h. After four washing steps, the wells were incubated with 100 μl horseradish peroxidase-conjugated rabbit anti-human IgG antibody (Dako, Denmark) diluted 1:5,000 in blocking solution for 45 min. The substrate (3,3′,5,5′-tetramethylbenzidine; Sigma-Aldrich) was added after eight washing steps. The reaction was stopped with 50 μl 1 M H_2SO_4, and the optical density (OD) was measured at 450 nm. To determine the specific reactivities of rL25 and rOspC, each OD value for serum samples added to wells coated with the negative control (chromatography elution fractions of lysates from *E. coli* M15 or BL21 transformed with pQE30 or pTYP12) was subtracted from the OD values for serum samples added to wells coated with the respective protein of interest. The cutoff value for each protein evaluated by ELISA was defined as the mean OD plus 3 standard deviations for all control sera.

As shown in Fig. 1, rL25 specifically bound IgG antibodies in serum samples from LB patients. For the LB patients, 18 of 80 serum samples were positive when rL25 was used as the antigen and 54 of 80 were positive for rOspC, resulting in sensitivities of 23% and 68%, respectively. Of the 75 control serum samples, only one reacted with rL25 and another reacted with rOspC, corresponding to a specificity of 99% for both antigens. Even though a substantial number of patient sera showed reactivity only with the more sensitive rOspC antigen, 7 of 80 samples reacted exclusively with rL25, which might be of potential value for the serodiagnosis of late-stage LB. The observed sensitivity of 68% for rOspC in IgG serology was higher than those reported for other European seroepidemiological studies (10, 20) but was in agreement with results from North American studies (18, 19), where an OspC protein with a sequence 99% identical to ours was used. To our knowledge,

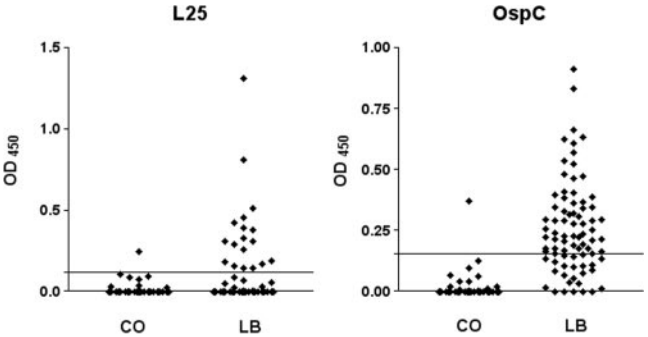

FIG. 1. IgG ELISA OD values for reactivities of rL25 (BB0786) and rOspC from *B. burgdorferi* N40 with serum samples from 80 patients with different phases of LB and 75 control (CO) serum samples from healthy blood donors. The cutoff value (mean for control serum samples plus 3 standard deviations) is indicated by a horizontal line.

the diagnostic value of L25 has not been recognized so far. It can be assumed that our patients acquired LB in Europe. For the southwest region of Germany, we have recently shown that the predominant genospecies in ticks are *B. burgdorferi* (11%), *B. garinii* (18%), and *B. afzelii* (53%), while mixed infections (18%) also occur (22). Although the available sequence data indicate that L25 is rather conserved among species, we cannot exclude the possibility that the use of *B. garinii* or *B. afzelii* L25 would lead to increased sensitivity. However, the low sensitivity of L25 limits its ability as a stand-alone diagnostic antigen, while its high specificity qualifies the protein as a useful antigen for LB serodiagnosis when combined with other antigens.

Nucleotide sequence accession number. The nucleotide sequence of L25 from *B. burgdorferi* N40 has been submitted to GenBank database with the accession number DQ400710.

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