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Ornstein, Katharina; Ostberg, Yngve; Bunikis, Jonas; Noppa, Laila; Berglund, Johan; Norrby, Ragnar; Bergström, Sven

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Differential Immune Response to the Variable Surface Loop Antigen of P66 of *Borrelia burgdorferi* Sensu Lato Species in Geographically Diverse Populations of Lyme Borreliosis Patients

Katharina Ornstein,1 Yngve Östberg,2 Jonas Bunikis,3 Laila Noppa,2 Johan Berglund,1 Ragnar Norrby,4 and Sven Bergström2*

Department of Medical Microbiology, Dermatology, and Infection, Lund University, Lund,1 Department of Molecular Biology, Umeå University, Umeå,2 and Swedish Institute for Infectious Disease Control, Stockholm,4 Sweden, and Departments of Microbiology, Molecular Genetics, and Medicine, University of California Irvine, Irvine, California3

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We have studied the immune response to a variable surface-exposed loop region of the P66 outer membrane protein from *Borrelia burgdorferi* sensu stricto and *B. afzelii* and *B. garinii*, respectively.

Lyme borreliosis is the most common tick-borne infection in Europe and North America (7, 15). In two recent reviews, genospecies distribution was shown to be associated with geographic origin (11, 19). *Borrelia burgdorferi* sensu stricto is the only species found in North American Lyme disease, while three species, *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii*, are associated with Lyme borreliosis in Europe (11, 19). In earlier studies, a correlation between the serological response among Lyme borreliosis patients living in different geographic regions to antigens from various *B. burgdorferi* sensu lato genospecies has been shown (2, 10, 13).

The P66 membrane protein has a surface-exposed loop region including a hypervariable immunogenic determinant that is polymorphic among *Borrelia* species (3, 4, 5). That this antigen is species specific was suggested by its reactivity in immunoblot assays with P66 protein derived from a *B. burgdorferi* sensu stricto strain from North American patients with Lyme disease (5). The aim of this investigation was to further characterize this antigenic site by determining the effect of sequence polymorphism on the ability of anti-P66 antibodies to detect P66 in Lyme borreliosis patients from Sweden and the United States.

The strains used were *B. burgdorferi* sensu stricto B31 (ATCC 35210) and *B. garinii* Ip90 (12) and Swedish strains *B. afzelii* LU81 and *B. garinii* LU59, LU116, LU118, LU170, LU185, LU190, and LU222 (16).

The sequence diversity of the P66 loop of Lyme borreliosis strains recovered in Sweden was studied by performing partial p66 gene sequence analysis by PCR and cycle sequencing. A primer pair targeting the P66 loop was chosen (5′-GAAATT TCAAGCtATGAGAC-3′ and 5′-CTCATATGTCTTGTTGGATCAATGTTAGATTAG-3′ for *B. garinii* Ip90, and 5′-GCGAATTCAAATGTCAGATTAGG-3′ for *B. burgdorferi* sensu stricto, 5′-GGACTGGGTTGGATCCACATCTATCG GTC-3′ and 5′-TCAATTGCAATCAAATGTTAAATTAATG-3′ for *B. afzelii*, and 5′-GGACTAGTGGATCCACATCTATCG TATCGGTC-3′ and 5′-TCATTGGCAATTCAATGTTAGATTAG-3′ for *B. garinii*). The endonuclease restriction site [1]) is underlined in each primer sequence.

Serum samples were collected from two geographically separated Lyme borreliosis populations, i.e., 100 patients from southern Sweden (50 with neuroborreliosis, 25 with arthritis, and 25 with acrodermatitis) and 38 patients from North America (23 with erythema migrans and 15 with disseminated Lyme disease [kindly provided by Martin Schäfer, Centers for Disease Control and Prevention, Fort Collins, Colo.]). Diagnoses were based on established case definitions (6). Serum samples from healthy Swedish blood donors (*n* = 100) collected in a tick-free area in Sweden were used to define the cutoff value for seropositivity in the EIAs.

The rP66 peptides were used as antigens in the EIAs. Preparation of the microtiter plates and the EIA protocol were performed as previously described (13). A protein concentration of 5 μg/ml was used. Serum samples from patients were diluted 1:200. Each plate contained a positive control. Samples were run in duplicate. An index was calculated for each sample by subtracting the background activity (estimated by using an antigen-free well) from the mean optical density value and thereafter divided by the positive control. The cutoff values were calculated as the ≥95th percentile of the blood donor index value. The seroreactivity in the Lyme borreliosis groups was compared by using the Mann-Whitney rank sum test. The proportions of seropositive samples were compared by using McNemar's chi-square test (paired proportions) and the contingency tables chi-square test (independent proportions). A *P* value of <0.05 was considered statistically significant. The
were included in the statistical comparison. Therefore, only patients with disseminated disease reacted more strongly to the P66 loop region of the Swedish Lyme borreliosis strains identified as a late immune response, since the American patient group with disseminated disease showed no significant lower in the B31 P66 EIA, with 1 positive sample (1.0%), compared to the positivity rate in the B31 P66 EIA.

Comparisons of the seropositivity rates obtained between the North American group, with six positive samples (40%), than in the Swedish group, which contained only one positive sample (1%) (P < 0.001). The seropositivity rate of the Swedish group in the EIAAs based on B. afzelii and B. garinii was higher, although not significantly so, than that of the North American group. The cross-reactivity of the Swedish Lyme borreliosis group in the B. afzelii and B. garinii EIAAs (46%) was extensive, and occasional cross-reactivity was also observed in the North American group.

Within the Swedish group, the seropositivity rate was significant lower in the B31 P66 EIA, with 1 positive sample (1.0%), than in the LU81 P66 EIA, with 11 positive samples (11.0%); the Ip90 P66 EIA, with 19 positive samples (19.0%); and the LU59 P66 EIA, with 10 positive samples (10.0%) (P < 0.001, 0.004, and 0.004, respectively). In the North American group, the seropositivity rate was significant higher in the B31 P66 EIA than in the LU59 P66 EIA but not in the LU81 and Ip90 P66 EIAAs. Six North American samples were positive in the B31 P66 EIA (40%), one sample was also positive in the LU81 and Ip90 P66 EIAAs (6.7%), and no sample was positive in the LU59 P66 EIA (0%) (P = 0.131, 0.131, and 0.041, respectively).

Our sequence analysis of the p66 gene from Swedish B. afzelii and B. garinii human isolates confirms the variable species-specific sequence epitope-containing region of the P66 loop. The species-specific nature of the immune response to B. burgdorferi sensu stricto P66 was supported by the preferential immunoglobulin G antibody response among the North American patients in the EIA that used the Borrelia species causing Lyme disease in North America. In contrast, the two species known to be present in Sweden, B. afzelii and B. garinii, reacted more often in the Swedish patient group. The two latter genospecies have been isolated from skin and cerebrospinal fluid from Swedish patients (16). B. burgdorferi sensu stricto has been identified in cerebrospinal fluid by PCR in Sweden (17).

The cross-reactivity observed among the Swedish samples in the assays based on B. afzelii and B. garinii could be caused by multiple Borrelia infections or peptide similarities in the epitope. Our finding indicates that the P66 loop antigen of B. afzelii and B. garinii, but not B. burgdorferi sensu stricto, might be sufficiently cross-reactive for detection by antibodies to P66 from patients with Lyme borreliosis. P66 has been identified as one of five proteins with discriminative potential in immunoblots for late disease testing in Switzerland (18) but has not been recognized as an important diagnostic immunoblot marker in Europe (9). The general utility of P66 as a diagnostic tool in Europe needs to be further evaluated.

The location of immunogenic epitopes in the P66 protein has been discussed in two different studies (3, 14). Both studies propose that the C-terminal portion, including the loop region, is immunoglobulin G immunoreactive. Ntchobo et al. have proposed multiple antibody epitopes throughout B. burgdorferi P66 protein (14). Retention of the native conformation of P66 (Oms66) was found to be essential for eliciting bactericidal P66 antibodies by Exner et al. (8). Further analysis of the immune response to P66-specific peptides could provide additional information needed to localize the species-specific immunoreactive epitopes of the P66 membrane protein.

In summary, our findings confirm the species specificity of the P66 loop protein and that the immune response to the B. burgdorferi sensu stricto P66 loop is species specific. In addition, the findings suggest that the loop antigen of P66 of B. garinii and B. afzelii is somewhat cross-reactive. This study supports the notion that B. afzelii and B. garinii are the major causative agents of Lyme borreliosis in Sweden.

**Nucleotide sequence accession numbers.** The sequences determined in this study were deposited in the GenBank database and assigned accession numbers AY090472 (LU59), AY090473 (LU81), AY090474 (LU116), AY090475 (LU118), AY090476 (LU170), AY090477 (LU185), AY090478 (LU190), and AY090479 (LU222).
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