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Borrelia burgdorferi Spirochetes That Harbor Only a Portion of the lp28-1 Plasmid Elicit Antibody Responses Detectable with the C_6 Test for Lyme Disease▽

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Detection of antibody to C_6, a peptide that reproduces the sequence of the sixth invariable region within the central domain of the VlsE protein of *Borrelia burgdorferi*, is used currently for the serologic diagnosis of Lyme disease in humans. *B. burgdorferi* isolates taken from infected humans can be categorized into specific genetic subtypes (designated RST1, -2, and -3) by restriction fragment length polymorphisms in the 16S to 23S rRNA spacer sequence. Many of these, usually categorized as RST2, retain only segments of the linear plasmid lp28-1, which encodes VlsE. The VlsE genetic region is retained, but altered expression of this molecule could affect diagnosis by the C_6 enzyme-linked immunosorbent assay (ELISA). Serum samples from patients infected with each of the three genotypes and from mice infected with three RST2 isolates were tested with the C_6 ELISA. Such isolates elicited marked C_6 responses in infected mice. The sensitivity of C_6 antibody detection in patients infected with RST2 spirochetes was statistically indistinguishable from detection of RST1 and RST3 infections. These findings demonstrate that diagnosis by C_6 ELISA remains effective for infection with all *B. burgdorferi* genotypes, including those with incomplete lp28-1 plasmids.

The clinical progression of Lyme disease, a tick-borne illness that is caused by the spirochete *Borrelia burgdorferi*, is divided into early localized, early disseminated, and late stages. During the early localized phase, the disease usually manifests by a characteristic skin lesion, erythema migrans (EM). Several days or weeks later, the spirochetes may spread hematogenously and patients may develop early disseminated disease with dermatologic, cardiac, neurologic, and/or rheumatologic involvement. During the early disseminated phase, some patients exhibit dermatologic signs that appear as multiple EM (24, 27, 29, 33). Late disease presents mainly with arthritis or neurologic manifestations (28).

Evidence has been put forward to suggest that a major determinant of the risk of *B. burgdorferi* dissemination in Lyme disease patients with EM is the genotype of the infecting spirochetal strain. Three genotypes have been identified on the basis of restriction fragment length polymorphisms in the rRNA spacer region and have been designated RST1, -2, and -3 (16). Patients infected with RST1 spirochetes had the highest proportion of blood culture positivity (43%), those infected with RST2 spirochetes were intermediate (20%), and those with RST3 had the lowest proportion (3%). In general, persons with an RST1 infection had more symptoms and symptoms of greater severity than those with either RST2 or RST3 infection (32). Mice that were inoculated with RST1 organisms were significantly more likely to yield cultivable spirochetes from different organs and showed a significantly higher prevalence of both carditis and arthritis than did animals inoculated with RST3 spirochetes (30); RST2 isolates were not assessed. When the plasmid contents of the different human infectious types were assessed, RST2 spirochetes emerged as almost uniformly lacking lp56, lp38, and fragments of lp28-1 (six of seven isolates assessed) (4). In contrast, spirochetes of RST1 either had no missing plasmids (six of eight isolates) or lacked a circular plasmid of the cp32 family (two of eight isolates). Spirochetes of RST3 were more heterogeneous, lacking assorted plasmids, but no more than two each. Only one of six isolates had a segment of lp28-1 missing (4).

VlsE is an antigenic variation protein encoded by lp28-1. This molecule undergoes variation by gene conversion within interspersed variable regions of a central cassette (34). VlsE could be abnormally expressed or not expressed at all by RST2 spirochetes given that many isolates harbor an incomplete lp28-1 plasmid. Detection of antibody to C_6, a peptide that reproduces the sequence of the sixth invariable region within the central domain of the VlsE protein of *B. burgdorferi*, is used currently for the serologic diagnosis of Lyme disease in humans (1, 5, 9, 12, 17, 18, 20–24) and in canines (3, 7, 8, 11, 14). As the C_6 test emerges as a possible substitute for the two-tiered serologic algorithm (1), the absence of portions of lp28-1 in RST2 isolates could represent a problem. Prior to the studies presented here, the possibility remained that the sensitivity of C_6 serology for infections with this spirochetal type could be diminished.

To ascertain whether the C_6 peptide was expressed in a serologically detectable manner by RST2 spirochetes, we inoculated mice with three different RST2 isolates and serially assessed their C_6 antibody responses in relation to that of an RST1 isolate. In addition, the serologic sensitivity of the C_6

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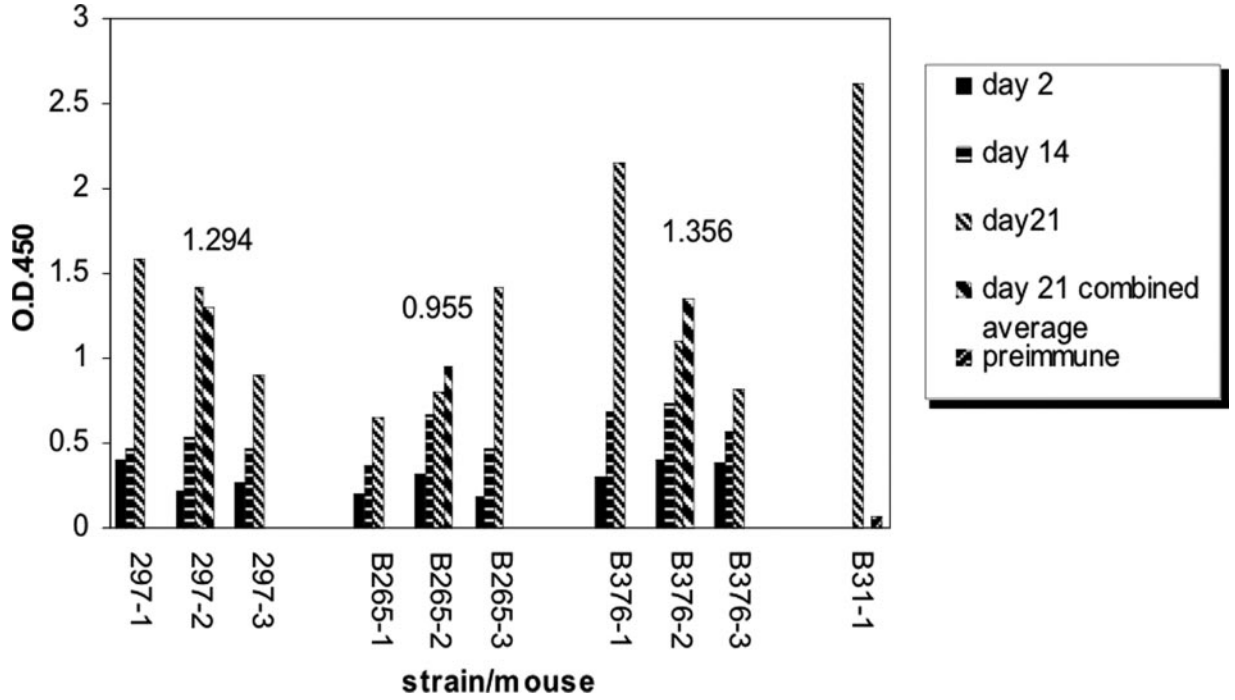

FIG. 1. Serum antibody reactivity to the C_6 peptide by mice infected with RST2 *B. burgdorferi* spirochetes. Optical density at 450 nm (O.D.450) for the combined value of triplicate samples is shown. Serum optical density values for each mouse and each time point are plotted individually, where the strain and mouse are shown in the *x* axis, e.g., B265-1 indicates strain B265 and mouse 1. The center bars in each set include the combined average values from three mice at the day 21 time point.

enzyme-linked immunosorbent assay (ELISA) was assessed in two groups of Lyme disease patients with EM infected with RST1 (or RST3) and RST2 spirochetes, respectively. Two *B. burgdorferi* RST2 clinical isolates, B265 and B376 (4), and the strain 297 (also RST2 [32]) were cultured in Barbour-Stoenner-Kelly–H (BSK-H) medium (Sigma, St. Louis, MO) and grown at 34°C to late log phase. Three C3H/HeN mice per isolate were infected with 10^4 cultured organisms via subcutaneous needle inoculation. Ear punch biopsy specimens were collected at 1 week postinfection and cultured in BSK-H. At 2, 14, and 21 days postinfection, blood was collected from each animal, with euthanasia and exsanguinations at day 21. The heart, bladder, spleen, axillary lymph nodes, tibiotarsal joints, and skin from the ears were collected, and ~1- to 2-mm^2 pieces of each tissue were placed in BSK-H medium for culture at 34°C. Cultures were examined 7 to 10 days later for the presence of spirochetes.

The antibody responses of infected mice to the C_6 region of VlsE were determined by peptide ELISA essentially as described previously (10). Mouse serum from each time point was assayed in triplicate at a dilution of 1:200. Goat anti-mouse immunoglobulin G (IgG) plus IgA plus IgM (heavy plus light chains) conjugated to horseradish peroxidase (Zymed Laboratories, San Francisco, CA) was used as the secondary antibody. Progression of the anti-C_6 response, beginning at day 2 postinfection, was measured for each mouse individually. As controls, preimmune and day 21 sera from a mouse infected with B31.5A19 (an RST1 clonal isolate possessing all plasmids) were included.

Culture of ear biopsy specimens at 1 week postinfection and organ tissues at 21 days postinfection revealed dissemination of RST2 isolates within infected mice (data not shown). Spirochetes were cultured from the 7-day-postinfection ear biopsy specimens of all mice (three of three and three of three, respectively) infected with B265 and B376 but no mice (zero of three) infected with 297. At 21 days, the ear skin, heart, lymph nodes, and tibiotarsal joints of all mice (nine of nine) bore spirochetes. Several splenic (five of nine) cultures and one bladder culture did not contain spirochetes.

Mouse serum antibodies from days 2, 14, and 21 postinfection were tested by the C_6 ELISA. As shown in Fig. 1, all mice generated an anti-C_6 response that was weaker at day 14 but markedly elevated by day 21. Some variation existed between individual mice, but no difference in the responses generated by individual RST2 isolates was evident. The C_6 antibody level of the B31.5A19-infected mouse was higher, but this may simply reflect the ability of RST1 spirochetes to rapidly proliferate and disseminate in mice (30).

The patient population consisted of 79 patients who presented to the Lyme Disease Practice of the Westchester Medical Center between June 1991 and July 2000 with either a single EM (early localized disease, $n = 61$) or multiple EM (early disseminated disease, $n = 18$). This is a subgroup of a patient population ($n = 120$) that was C_6 tested in a previous study (24). RST typing was available for 79 of these 120 patients; the former were thus included in the present study solely on this basis. A skin biopsy specimen or blood specimen from each patient was shown to contain cultivable *B. burgdorferi* spirochetes. The genotype of each cultured isolate was determined by PCR amplification of a portion of the 16S to

TABLE 1. Results of C_6 antibody tests on Lyme disease patients infected with different subtypes of *B. burgdorferi*

Patient category	Total no. of patients	No. (%) of C_6-positive patients	No. of C_6-negative patients
All patients	79	69 (87)	10
Single EM	61	52 (85)	9
Multiple EM	18	17 (94)	1
RST1 infected	24	21 (88)	3
RST2 infected	35	31 (89)	4
RST3 infected	20	17 (85)	3
RST1 and RST3 infected	44	38 (86)	6

23S rRNA spacer followed by restriction fragment length polymorphism analysis as previously described (15, 16).

For human samples, C_6 antibody titers were determined exactly as described previously (24). The serum from each of these patients was tested for reactivity to the C_6 peptide by ELISA. All of the combined results are presented in Table 1. Of 79 patients, 69 tested positive for C_6 antibodies on acute- or convalescent-phase serum samples, yielding an overall response in 87% of patients. This fraction is essentially the same as that obtained in the above-mentioned study that included the 120 patients from which 79 were RST typed (88%) (24). Thus, the selection of these 79 patients on the basis of availability of their RST typing did not bias the outcome with regard to overall sensitivity of C_6 antibody detection. Among these patients, 88% of RST1-infected individuals, 89% of RST2-infected patients, and 85% of RST3-infected persons were positive for C_6 antibodies (Table 1). The combined proportion of patients infected with types 1 and 3 (86.3%) that were C_6 positive was lower than the proportion of RST2-infected patients that were C_6 positive (88.5%, or approximately 89%). However, this difference was not statistically significant: a Z test for statistical analysis yielded, for Z of 0.2925, a two-tailed *P* value of 0.7707 and a one-tailed *P* value of 0.3962.

The lp28-1 plasmid has previously been shown to be required for persistent infection in mice (6). Thus, clinical isolates taken from Lyme disease patients probably must possess at least some part of lp28-1 to survive. The presence of VlsE in that portion of lp28-1 that is retained is evident from PCR and Southern blot analyses of isolates (4). In this report, the demonstration of C_6 antibody responses by mice infected with isolates known to harbor incomplete lp28-1 plasmids proves not only that VlsE is present in these isolates but also that it is antigenic in a manner equivalent to that of VlsE expressed by *B. burgdorferi* spirochetes that have complete lp28-1 plasmids.

The diagnosis of Lyme borreliosis is complicated by the genetic and subsequent antigenic variability that exists among *B. burgdorferi* species. The presence of multiple strains in an area of Lyme disease endemicity could also complicate diagnosis (2, 13, 19, 25, 26, 31). An antigen that contains epitopes which remain conserved and expressed across the *B. burgdorferi* sensu lato population is of extraordinary importance to diagnostic sensitivity and accuracy. The sixth invariable region of VlsE, from which the C_6 ELISA was developed, is an immunogenic segment with those properties. In this series of experiments, we sought to assess the ability of the C_6 test to detect infection with organisms of different genotypes with variable plasmid contents. The results revealed that the C_6 test remains a viable option for serological diagnosis of Lyme disease regardless of RST genotype.

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