Antigenic Conservation of an Immunodominant Invariable Region of the VlsE Lipoprotein among European Pathogenic Genospecies of *Borrelia burgdorferi* SL

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Lyme disease is caused by genetically divergent spirochetes, including 3 pathogenic genospecies: *Borrelia burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*. Serodiagnosis is complicated by this genetic diversity. A synthetic peptide (C_6), based on the 26-mer invariable region (IR_6) of the variable surface antigen of *B. burgdorferi* (VlsE), was used as ELISA antigen, to test serum samples collected from mice experimentally infected with the 3 genospecies and from European patients with Lyme disease. Regardless of the infecting strains, mice produced a strong antibody response to C_6, which indicates that IR_6 is antigenically conserved among the pathogenic genospecies. Twenty of 23 patients with culture-confirmed erythema migrans had a detectable antibody response to C_6. A sensitivity of 95.2% was achieved, with serum samples collected from patients with well-defined acrodermatitis chronica atrophicans. Fourteen of 20 patients with symptoms of late Lyme disease also had a positive anti-IR_6 ELISA. Thus, it is possible that C_6 may be used to serodiagnose Lyme disease universally.

Lyme borreliosis is the most prevalent tick-borne disease in Europe, the United States, and parts of Asia [1–4]. It is a complex multisystem disorder with early manifestations, such as erythema migrans (EM), acute meningitis or meningopolyneuritis and acute arthritis, and late signs and symptoms that include chronic arthritis, chronic neurologic abnormalities, and acrodermatitis chronica atrophicans (ACA) [5–7].

Lyme disease is caused by a group of genetically diverse spirochetes collectively termed *Borrelia burgdorferi* sensu lato. This complex includes several genospecies, of which 3 are known to be pathogenic to humans. They are *B. burgdorferi* sensu stricto, *B. garinii,* and *B. afzelii* [8, 9]. Thus far, all the North American pathogenic strains that have been identified are *B. burgdorferi* sensu stricto [9]. In contrast, in Europe [8, 9] and China [4], all 3 genospecies are found, with *B. garinii* and *B. afzelii* being the most prevalent isolates. Clinical and pathological manifestations of Lyme disease vary geographically, according to which is the predominant infectious geno-

species. Borrelial lymphocytoma, ACA, and encephalomyelitis are found mainly in Europe, whereas disseminated early infection, secondary EM, and arthritis are more common in the United States [10–13].

Although it is recommended that Lyme disease diagnosis be based on the patient's clinical history (including symptoms and exposure to the tick vector), physical findings, and laboratory data other than serology [14], serologic tests are used widely as confirmatory. ELISA and immunoblotting are the techniques most commonly used for Lyme disease serology, both in Europe and in the United States. In the United States, the Centers for Disease Control and Prevention (CDC) have recommended a 2-tiered diagnostic approach. This entails an initial ELISA of relatively high sensitivity but low specificity followed, when positive, by an immunoblot [15]. Several advantages are associated with this approach, such as enhanced specificity and the opportunity to estimate duration of infection. The need to include the immunoblotting technique, however, will increase the cost of Lyme disease diagnosis and probably will further enhance inter- and intralaboratory discrepancies, because the test is itself more difficult to perform than a standard ELISA, and because its outcome may depend on subjective interpretation of the banding pattern.

There is evidence indicating that antigens prepared from different *B. burgdorferi* strains and genospecies influence reactivity with human antibody, both in ELISA and in immunoblot assays. A single serum sample or a serum pool from patients with

Lyme disease shows remarkably different banding patterns on immunoblots that are prepared with antigens from different strains of *B. burgdorferi* sensu lato [16–19]. ELISA antigens prepared from different spirochete strains also significantly influence antibody titers of human serum [20, 21]. Because of these reasons and because the infecting strain or genospecies is usually unknown [17], serodiagnosis of Lyme disease is even more problematic in Europe than in the United States [17–20]. This difficulty is compounded by the fact that subclinical infections are common in Europe; thus, serodiagnosis is often used in lieu of clinical diagnosis [22, 23]. A simple, sensitive, and specific test that is applicable, regardless of the infecting strain, is urgently needed in Europe.

The variable surface antigen of *B. burgdorferi*, VlsE, contains a 26-mer invariable region (IR_6) [24]. The IR_6 sequence remains unchanged during antigenic variation [25] and, on the basis of the data available thus far, is conserved among 3 strains of 2 genospecies of *B. burgdorferi* sensu lato [24]. IR_6 is highly immunogenic. Experimental infections of monkeys and mice elicit in all the infected animals an early, strong, and persistent antibody response to a peptide (C_6) that reproduces the IR_6 sequence [24, 26]. This same peptide, when used in a diagnostic ELISA, detects antibody in serum samples from US patients with early Lyme disease with a sensitivity of 85% and a sensitivity ≤100% in patients with chronic disease, with a specificity of 99% [26]. In the current study, serum samples from mice experimentally infected with European strains of *B. burgdorferi* sensu lato were used to evaluate antigenic conservation of IR_6. In addition, human serum samples from European patients with either early or late Lyme disease were tested, to assess sensitivity of the C_6 peptide ELISA in a European setting.

Materials and Methods

Spirochete strains, ticks, mice, and mouse infection protocol. Female BALB/c mice (6–8 weeks old) were infected by subcutaneous needle inoculation with 10^5 spirochetes of *B. garinii* strains Pbi, Pohm, or *B. burgdorferi* sensu stricto strain B31 or by tick bite. Before needle inoculation, spirochetes were cultured in BSK-II medium, which was supplemented with 10% rabbit serum (Sigma Chemical, St. Louis). Nymphal *Ixodes ricinus* were field collected in Malonne, Belgium, and Neuchâtel, Switzerland, by flagging vegetation. Infection rates were 33% (7/21) for ticks from Neuchâtel, as assessed by immunofluorescence and *Borrelia* isolation, and 20% (9/45) for ticks from Malonne, as assessed by polymerase chain reaction (PCR). Eleven (Neuchâtel origin) or 15 (Malonne origin) ticks were applied to hollow plastic caps placed on the back of each mouse. *B. burgdorferi* infection by tick bite was confirmed by ear punch–biopsy culture and/or xenodiagnosis. Immunofluorescence, PCR, spirochete isolation, ear punch–biopsy culture, and xenodiagnosis were performed as described elsewhere [27, 28]. Infecting strains were classified into genospecies on the basis of a standard protocol described elsewhere [8]. Blood samples were drawn at 4 weeks after inoculation via needle or tick bite.

Spirochete isolation from patients with EM and genospecies classification. Skin punch biopsies were taken from the erythematous border of the EM lesions after local anesthesia and were cultured at 34°C in BSK-II medium supplemented with 10% rabbit serum. Cultured fluid was monitored weekly for the presence of spirochetes under a darkfield microscope for 1 month. Grown spirochetes were classified into genospecies on the basis of procedure and standards described elsewhere [8].

Human Lyme disease serum sources. Two sources of human serum were used in this study, one from Austria and the other from Italy. Two serum panels were obtained from each source, one from patients with early Lyme disease and the other from patients with late disease manifestations. The early serum panel from Austria consisted of 29 specimens collected from 11 patients with EM. Two or 3 specimens were serially collected from each patient. Skin biopsies from all but 1 of the Austrian patients were cultured. All the 10 cultured biopsies were positive for *B. burgdorferi*. The late serum panel from this source consisted of 21 specimens from 21 IgG-seropositive patients with clinically and histologically diagnosed ACA. The Italian panel of early serum specimens consisted of 20 samples collected from 13 patients with EM with culture-confirmed *B. burgdorferi* infection. As many as 2 samples per patient were serially collected. Chronic serum specimens from Italy were from 20 patients with clinically diagnosed signs and symptoms that were consistent with late neuroborreliosis, arthritis, or ACA and contained antibody to *B. burgdorferi*, as detected by ELISA [29]. Early specimens were collected serially, starting at disease onset and continuing thereafter for 3 months, during and after antibiotic treatment.

Control serum specimens. Control serum specimens were collected randomly from hospital patients in 2 areas of the world where Lyme disease is not endemic, Louisiana ($n = 20$) and Sicily, Italy ($n = 21$).

ELISA diagnosis. A commercial ELISA kit (DAKOPATTS A/S, Copenhagen, Denmark) was used to test the serum specimens from Austrian patients with early and late Lyme disease, according to the manufacturer's instructions. The antigen was native flagellar protein purified from cultured *B. afzelii* strain DK-1.

Immunoblot diagnosis. A commercial immunoblot assay kit (MRL Diagnostics, Cypress, CA) was employed to analyze the Austrian serum panel obtained from patients with early Lyme disease, according to the manufacturer's recommendations. The antigen consisted of whole-cell sonicates of cultured *B. garinii* strain 20047. A test was considered to be positive when ≥1 of the 23- or 39-kDa IgM bands or 4 of the 21-, 23-, 37-, 39-, 41-, 45-, or 93-kDa IgG bands were visible on the immunoblots.

To analyze serum specimens from the early Lyme disease Italian patients with increased sensitivity, 3 local strains—*B. garinii* BITS, *B. afzelii* BL3, and *B. burgdorferi* sensu stricto Alcaide—were individually used as a source of immunoblot antigens. The anti–*B. burgdorferi* antibodies were probed with goat anti–human IgG or IgM as secondary antibodies. Diagnosis was made on the basis of criteria described elsewhere [18, 29].

Peptide and biotinylated peptide preparation. A 26-mer peptide (C_6 [CMKKDDQIAAAMVLRGMAKDGQFALK]), which reproduced the IR_6 sequence of VlsE cloned from *B. garinii* strain IP90, was synthesized and conjugated to biotin, as described elsewhere [24].

C_6-peptide–based ELISA. The C_6-peptide–based ELISA was performed as described elsewhere [24]. Ninety-six-well ELISA plates were coated with 100 μL per well of 4 μg/mL streptavidin (Pierce
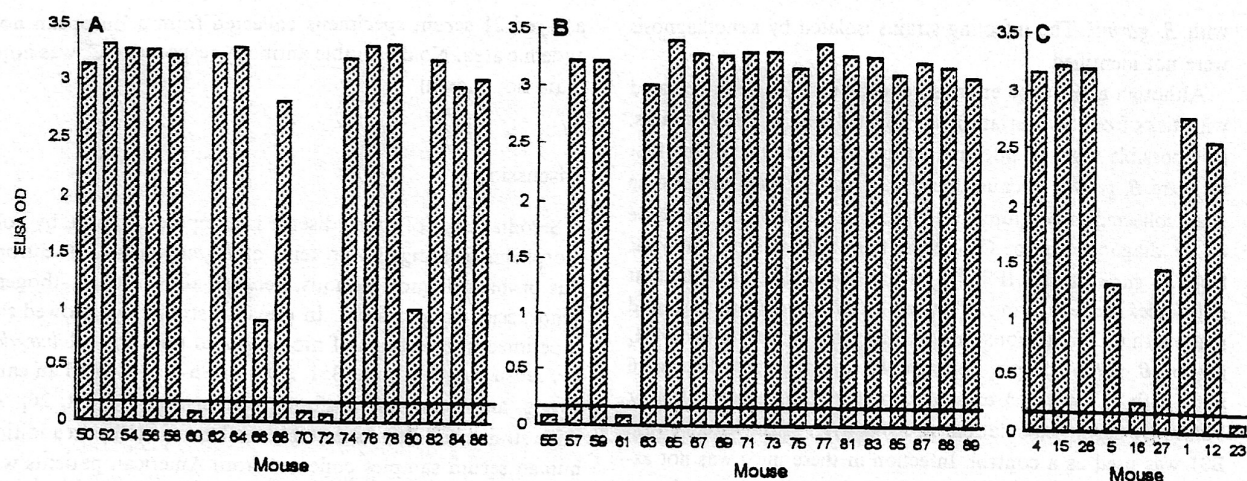
Figure 1. Antigenic conservation of a 26–amino acid invariable region (IR_6) of the variable surface antigen of *Borrelia burgdorferi* (VlsE) among pathogenic *B. burgdorferi* genospecies. Mice were infected with European *B. burgdorferi* sensu lato strains by tick bite *(A and B)* or by needle inoculation *(C)*. Ticks were field collected in Belgium *(A)* and Switzerland *(B)*. Blood samples were drawn at 4 weeks after inoculation. Antibody response to IR_6 was assessed with the C_6 ELISA. Cutoff value (0.171) was based on the mean ELISA optical density (OD) plus 3 SD of serum samples collected from 35 uninfected mice. Sixteen of the 19 animals in *A* were infected, as confirmed by ear punch–biopsy culture, but the infecting strains were not classified. Mice 60, 70, and 72 were not infected. All but 2 of the mice in *B* (mice 55 and 61) were infected, as determined by ear punch–biopsy culture and/or xenodiagnosis. Infecting strains were classified into genospecies. Mice 67 and 75 were positive by xenodiagnosis only. Infecting strains were not identified. Mouse 59 was infected with a *B. garinii* strain. Remaining mice were infected with *B. afzelii* strains. In *C*, animals were infected with *B. garinii* strains PBi (mice 4, 15, and 26) or Pohm (mice 5, 16, and 27) or *B. burgdorferi* sensu stricto strain B31 (mice 1, 12, and 23) by needle inoculation.

Chemical, Rockford, IL) in coating buffer and were incubated at 4°C overnight. The remaining steps were conducted in a rotatory shaker at room temperature. After 2 3-min washes with 200 μL per well of PBS/T at 200 rpm, 200 μL of 5 μg/mL biotinylated peptide dissolved in blocking solution (PBS/T supplemented with 5% nonfat dry milk) was applied to each well. The plate was shaken at 150 rpm for 2 h. After 3 washes with PBS/T, as described above, 50 μL of mouse or human serum, diluted 1:200 with blocking solution, was added to each well. The plate was incubated at 150 rpm for 1 h and then washed 3 times with PBS/T. Each well then received 100 μL of 0.5 μg/mL goat anti–mouse IgG (heavy and light-chain specific; Sigma) or 0.1 μg/mL of anti–human IgG (heavy- and light-chain specific; Pierce Chemical), each conjugated to horseradish peroxidase and dissolved in blocking solution. The plate was incubated for 1 h while shaking. After 4 washes with PBS/T, each for 3–6 min, the antigen-antibody reaction was probed using the TMB Microwell Peroxidase Substrate System (Kirkegaard and Perry Laboratories, Gaithersburg, MD), and color was allowed to develop for 10 min. The enzyme reaction was stopped by the addition of 100 μL of 1 M H_3PO_4. Optical density was measured at 450 nm.

Results

Antigenic conservation of IR_6 among 3 pathogenic B. burgdorferi *genospecies.* Although the IR_6 amino acid sequence is conserved among strains and genospecies of *B. burgdorferi* sensu lato, as shown with sequence data from 3 strains of 2 genospecies, *B. burgdorferi* sensu stricto and *B. garinii* [24], a single amino acid substitution may destroy an epitope or diminish antibody-

binding affinity. Moreover, no VlsE sequence data are available from *B. afzelii* strains. To address these issues in relation to the diagnostic potential of the C_6 ELISA, we conducted extensive experimental infection studies with different *B. burgdorferi* strains. Previously, 10 rhesus monkeys and 13 mice were experimentally infected by tick or needle inoculation with *B. burgdorferi* sensu stricto strains B31, JD1, or Sh-2-82; all the animals produced an early and persistent antibody response to IR_6 [24, 26].

In the current study, the anti–C_6 antibody response elicited by European spirochetal strains was investigated in experimentally infected mice. Two sets of mice were infected by tick bite. The ticks were field collected in 2 European countries where both *B. garinii* and *B. afzelii* are endemic. The first set (*n* = 19) was inoculated with ticks collected in Belgium, and the second set (*n* = 18) was inoculated with ticks from Switzerland. Sixteen mice from the first set were infected, as demonstrated by ear punch–biopsy culture, and 16 from the second set were also infected, as assessed by ear punch–biopsy culture and/or xenodiagnosis. All the mice that proved to be infected by culture and/or xenodiagnosis produced a strong antibody response to C_6, whereas mice negative by the former criteria were C_6-antibody negative (figure 1*A* and 1*B*). Fourteen mice from the second set were culture positive, whereas mice 67 and 75 of this set were positive by xenodiagnosis. Speciation was performed only with the 14 isolates cultivated from the second set. Thirteen of these mice were infected with *B. afzelii* strains. Mouse 59 was infected

with *B. garinii.* The infecting strains isolated by xenodiagnosis were not identified.

Although almost the entire second group of mice (inoculated with ticks from Switzerland) was infected with *B. afzelii* strains, it is possible that the infecting strains in some mice of the first set were *B. garinii,* because the ticks that were used in this case were collected in Belgium. In light of the fact that the sequence of the diagnostic probe C_6 used in this study was part of VlsE from *B. garinii* strain IP90 [24], it is reasonable to assume that antibodies elicited during infections with *B. garinii* strains should react with C_6. To demonstrate the antigenic conservation of IR_6 among *B. garinii* strains, however, we experimentally infected 6 mice with *B. garinii* strains other than IP90—namely, Pbi or Pohm, by needle inoculation. *B. burgdorferi* sensu stricto strain B31 was used as a control. Infection in these mice was not assessed by culture or xenodiagnosis. Antibody to C_6 was found in mice, regardless of the inoculated strains (figure 1*C*). These results further confirmed the antigenic conservation of IR_6.

Sensitivity of the C_6 ELISA in patients with early Lyme disease. Twenty of 23 patients with culture-confirmed EM (87%) had a detectable anti–IR_6 antibody response (figure 2). One patient (A3) with positive EM was C_6 ELISA negative (figure 2). No culture was performed with this patient. Results obtained with commercially available immunoblot and ELISA kits show that the C_6 ELISA detected more positive results than did any of these tests, used either alone or combined (figure 2, *Austrian panel*). The C_6 ELISA also performed, in terms of diagnostic sensitivity, better than any of the 3 antigen preparations that were used to diagnose the Italian serum panel (figure 2, *Italian panel*). Bacterial isolation and classification revealed that the patients were infected with either *B. garinii* or *B. afzelii* strains (figure 2). This result provided additional evidence that IR_6 is antigenically conserved across the genospecies barriers of the *B. burgdorferi* sensu lato complex.

Sensitivity of the C_6 ELISA for serodiagnosing late Lyme disease. Twenty (95%) of 21 Austrian patients with histologically confirmed ACA and positive IgG serology with commercially available tests showed a strong antibody response to IR_6 (figure 3). A sensitivity of 70% (14/20) was obtained when a panel of serum samples from Italian patients with signs and/or symptoms consistent with late Lyme disease was tested. These 14 positive results included 3 of 4 patients with late arthritis, 8 of 12 with late neurologic manifestations, and 3 of 4 with ACA, which indicate that the C_6 ELISA was able to detect different manifestations that might be caused by various *B. burgdorferi* strains. It should be pointed out, however, that clinical diagnosis of late Lyme disease can be difficult because of the absence of well-defined clinical criteria, especially in the case of neurologic manifestations.

Specificity of the C_6 ELISA. We previously assessed diagnostic specificity of the C_6 ELISA with an array of 77 serum samples from patients with other spirochetal infections or autoimmune or neurologic diseases [26]. To extend this survey, we assayed 21 serum specimens collected from a European nonendemic area. No detectable antibody response to C_6 was noted (data not shown).

Discussion

Serodiagnosis of Lyme disease is hampered, in part, by polymorphism of antigenic proteins of *B. burgdorferi.* In Europe, this problem is more serious, because all 3 of the pathogenic genospecies are prevalent. In previous studies, we showed that experimental infections of monkeys and mice with *B. burgdorferi* sensu stricto strains B31, JD1, or Sh-2-82 elicited an early, strong, and persistent antibody response to IR_6 [24, 26]. All these strains had been isolated in the United States. In addition, human serum samples collected from American patients with Lyme disease yielded sensitivities of 85% (117/138) and 100% (59/59) for early and late Lyme disease, respectively, when assayed with the C_6 ELISA [26]. In the present study, European strains were investigated. Mice responded strongly to IR_6, regardless of whether they were infected with *B. garinii* or *B. afzelii* strains by tick or needle inoculation. In humans, sensitivities of 83% (20/24) and 95% (20/21) were obtained from European patients with clinically well defined, early (EM, culture confirmed) and late (histologically confirmed) Lyme disease (ACA), respectively (figures 2 and 3). Several independent groups have noted that European patients show restricted antibody responses to *B. burgdorferi* antigens, which, in part, contributes to the relatively lower sensitivity in European serodiagnosis [17–20]. This difference was not apparent when the C_6 ELISA was used, because similar diagnostic sensitivities were obtained with North American [24, 26] and European serum samples (figures 2 and 3). The exception was the panel of serum samples collected from Italian patients with chronic symptoms, in which the C_6 ELISA yielded a sensitivity of only 70% (figure 3). Clinical diagnosis of late Lyme disease, however, is compounded by the absence of pathognomonic signs and the virtual impossibility of confirming diagnosis by culture. Our current and previous studies [24, 26] thus demonstrate that the VlsE immunodominant IR_6 is antigenically conserved among the 3 *B. burgdorferi* genospecies and thus potentially can serve as a universal probe for the serodiagnosis of Lyme disease.

Antigenic conservation is different from molecular (or sequence) conservation, because a single amino acid substitution may destroy antigenic reactivity of an epitope but does not significantly alter sequence conservation. On the other hand, a peptide with even extensive amino acid substitutions may still retain antigenic reactivity of a particular epitope. Although limited molecular data from 3 strains of 2 genospecies of *B. burgdorferi* sensu lato indicate that the IR_6 sequence is conserved [24, 25], it is unknown whether VlsE is universally expressed by all pathogenic *B. burgdorferi* strains during a mammalian infection. To address these issues, 37 mice were infected by tick bite with European isolates. Field-caught ticks may
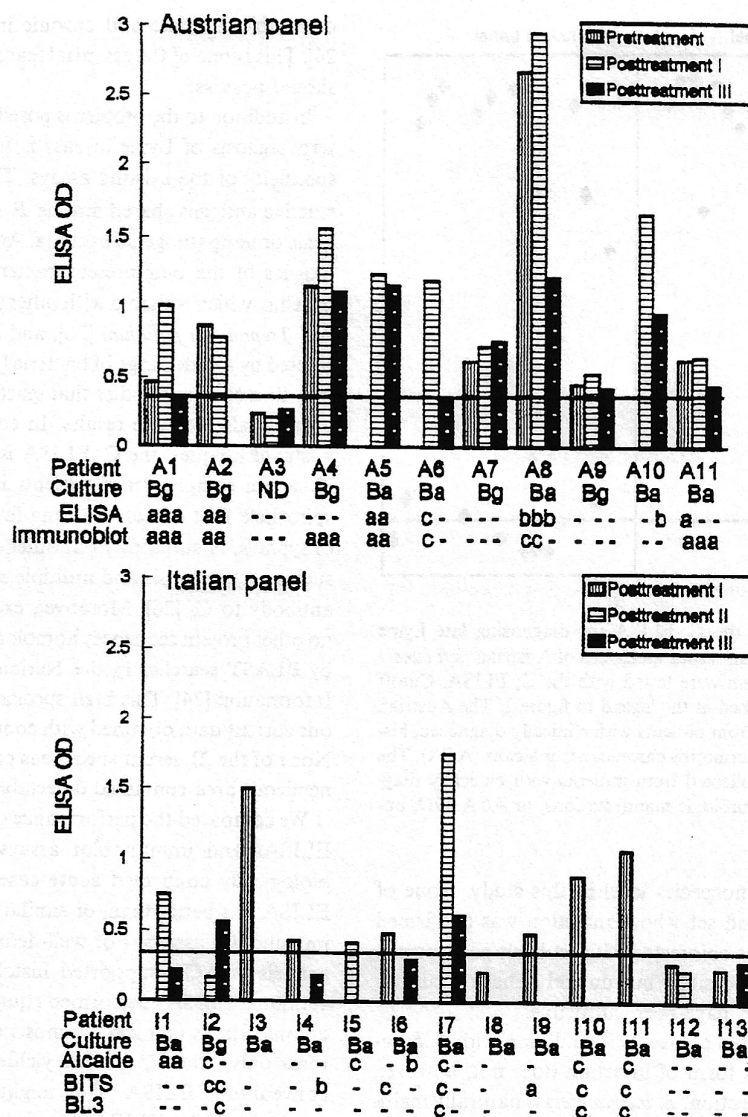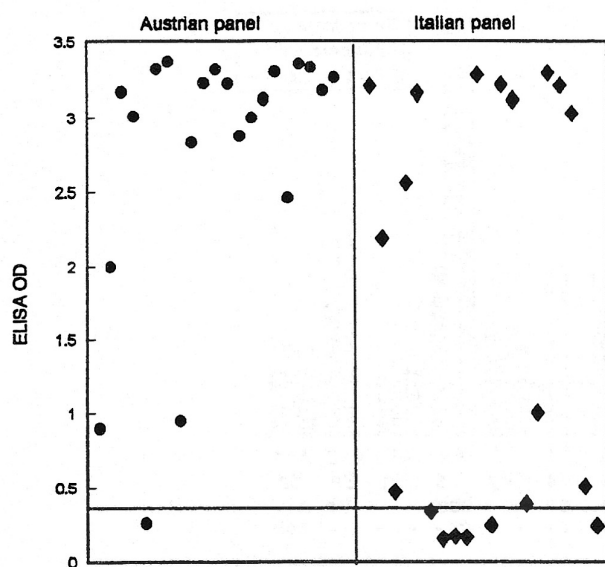
Figure 2. Sensitivity of the C_6 ELISA for diagnosing early Lyme disease. Two panels of human serum specimens of Austrian *(top panel)* or Italian *(bottom panel)* origin were tested with the C_6 ELISA. Cutoff value (0.380) was based on the mean ELISA optical density (OD) plus 3 SD of 20 human serum samples collected from hospitalized patients in an area nonendemic for Lyme disease in the United States. Except for serum A3 from a patient with erythema migrans (EM), for whom spirochetal culture was not performed, all the serum samples were collected from patients with culture-confirmed EM. Blood samples were collected from each patient at 1–3 time points after disease onset. For the Austrian panel, samples were collected before treatment (pretreatment), and at 1 (posttreatment I) and 3 months (posttreatment III) after antibiotic treatment. For the Italian panel, blood was collected at 1 (posttreatment I), 2 (posttreatment II), and 3 (posttreatment III) months after treatment. All the patients received antibiotic treatment. In some cases, not all 3 serial samples were available for this study. Spirochete isolation and classification, ELISA, and immunoblotting were performed, as described in Materials and Methods. For the Italian panel, 3 immunoblot antigens were prepared from *Borrelia garinii* strain BITS, *B. afzelii* strain BL3, and *B. burgdorferi* sensu stricto strain Alcaide, respectively. Ba, *B. afzelii;* Bg, *B. garinii;* ND, not performed; −, negative ELISA or immunoblot diagnosis. Letters "a," "b," and "c" indicate positive IgM, IgG, or both IgM and IgG serodiagnosis, respectively, by the method indicated.

harbor multiple spirochetal strains. Therefore, the infecting strains could represent a broad spectrum of European *B. burgdorferi* sensu lato strains. Only the mice whose infection was confirmed by ear punch–biopsy culture and/or xenodiagnosis

responded strongly to IR$_6$, which indicates that VlsE is generally expressed by, and that IR$_6$ is antigenically conserved among, European strains. Some of the mice might have been coinfected with multiple strains, because the infecting spirochetes were

Figure 3. Sensitivity of the C_6 ELISA for diagnosing late Lyme disease. Two panels of human serum specimens of Austrian *(left panel)* or Italian *(right panel)* origin were tested with the C_6 ELISA. Cutoff value was defined as described in the legend to figure 2. The Austrian serum panel was collected from patients with clinically diagnosed, histologically confirmed acrodermatitis chronica atrophicans (ACA). The Italian serum panel was collected from patients with clinically diagnosed late arthritis, late neurologic manifestations, or ACA. OD, optical density.

classified at only the genospecies level in this study. None of the 14 mice in the second set whose infection was confirmed by culture appeared to be coinfected with multiple genospecies, although it is formally possible, but unlikely, that certain infectious isolates may not have been cultivable.

Mice are readily infected by needle inoculation with *B. burgdorferi* spirochetes. This form of infection does not, however, fully mimic a natural infection. *B. burgdorferi* is naturally maintained in enzootic cycles that involve *Ixodes* ticks and vertebrate hosts. The spirochete alters its pattern of protein expression during these cycles. One typical example is the switch of outer surface protein (Osp) A and OspC expression [30–32]. The organism makes or residually expresses OspA, but not OspC, in the midgut of unfed ticks. The feeding process induces OspC expression and either turns off or attenuates OspA expression. This may be one of the reasons why anti–OspA antibody is rarely detectable in early infection by tick bite [33, 34]. In contrast, OspA is abundantly expressed in cultured spirochetes, and needle inoculation induces a strong early anti-OspA response [33, 34]. Therefore, infection by tick bite was evaluated first. Although VlsE expression in the tick, mammalian hosts, and in vitro culture has not been studied extensively, the data from our previous and current studies strongly indicate that this lipoprotein is consistently expressed during an infection in humans, monkeys, or mice, because anti–IR$_6$ antibody is readily

detectable in acute and chronic infections in these hosts [24, 26]. This is one of the essential features that a diagnostic antigen should possess.

In addition to the problems posed by antigenic polymorphism, serodiagnosis of Lyme disease is further hampered by the low specificity of the existing assays. This may be caused by cross-reactive antigens shared among *B. burgdorferi* and other pathogenic or nonpathogenic bacteria. Among these antigens are homologues of the omnipresent bacterial heat-shock proteins [35], flagellin, which is shared with other spirochetes, such as *B. hermsii* and *Treponema pallidum* [36], and a 60-kDa antigen that is expressed by a wide range of bacteria [37]. Other bacterial infections may thus elicit antibodies that react with *B. burgdorferi* antigens, causing false-positive results. In contrast to conventional diagnostic techniques, the C_6 ELISA is highly specific. None of the 77 serum samples from patients infected with *B. hermsii*, the spirochete that causes relapsing fever, or *T. pallidum,* the agent of syphilis, or suffering from other chronic infections or diseases, such as tuberculosis and multiple sclerosis, contained detectable antibody to C_6 [26]. Moreover, except for *B. burgdorferi* VlsE, no other protein sequences homologous to IR$_6$ could be identified by BLAST searches in the National Center for Biotechnology Information [24]. This high specificity was further confirmed by our current data obtained with control European serum samples. None of the 21 serum specimens collected from a European nonendemic area contained detectable antibody to IR$_6$.

We compared the performance of the C_6 ELISA with existing ELISAs and immunoblot assays. For clinically and microbiologically confirmed acute cases (EM plus culture), the C_6 ELISA was better than, or similar to, conventional ELISA and immunoblot assays. For well-defined chronic cases (clinical diagnosis of ACA supported histologically), both C_6 and conventional ELISAs performed equally well. For less well-defined chronic illness (clinical diagnosis of nonpathognomonic symptoms only), the C_6 ELISA yielded a lower sensitivity than a conventional ELISA. This might have resulted from the low specificity of this ELISA. More chronic cases need to be studied by us, to sort out the possible cause of this difference. Studies conducted by several independent groups, however, indicate that inter- or even intralaboratory variation in Lyme disease diagnosis, using commercially available serological methods, is frequently observed [38–40] and that the performances of such tests differ dramatically from one another [41]. This makes any comparisons of serologic data difficult to interpret.

Recently, the initiation of Lyme disease immunoprophylaxis in humans through the use of the OspA vaccine has made obsolete those diagnostic assays based on whole-cell antigens, because these preparations include OspA. Predictably, the C_6 ELISA does not detect anti–OspA antibodies [26]. Hence, approval and use of the OspA vaccine in Europe will not affect the performance of our test.

Finally, perhaps the most compelling advantage of the C_6 ELISA is its simplicity in terms of antigen preparation and

assay utilization. A 26-mer peptide is easy and inexpensive to synthesize. In terms of diagnostic utilization, ≤100 bands may be counted on *B. burgdorferi* whole-cell immunoblots that are reacted with Lyme disease patient serum samples, making it often difficult to identify bands that are diagnostic markers [42]. Antigenic polymorphism is part of the problem. For example, the Bdr antigen family is generally expressed by spirochetes of the genus *Borrelia,* including the *B. burgdorferi* sensu lato species, *B. hermsii, B. turicatae,* and *B. parkeri* [43]. The Bdr family contains a large number of members that share antigenic determinants and range in molecular weight from 10.7 to 38.5 kDa on immunoblots. A single *B. burgdorferi* sensu stricto isolate (strain B31) carries 17 distinct *bdr* alleles. Because of antigenic cross-reactivity, antiserum to a single Bdr member reveals multiple bands on an immunoblot [43]. This problem is even more complicated in Europe, where multiple criteria for immunoblot diagnosis have to be adopted [18, 19]. In contrast, the C_6 ELISA depends on a single antigen, and the test's format may be adapted to yield a strictly quantitative readout or a more rapid type with a semiquantitative readout. The antigenic conservation of IR_6 that we demonstrate here and the results of our initial assessment of sensitivity and specificity augur well for the successful use of C_6-based diagnostic tests in Europe.

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