Epitope-Specific Evolution of Human B Cell Responses to *Borrelia burgdorferi* VlsE Protein from Early to Late Stages of Lyme Disease

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Most immunogenic proteins of *Borrelia burgdorferi*, the causative agent of Lyme disease, are known or expected to contain multiple B cell epitopes. However, the kinetics of the development of human B cell responses toward the various epitopes of individual proteins during the course of Lyme disease has not been examined. Using the highly immunogenic VlsE as a model Ag, we investigated the evolution of humoral immune responses toward its immunodominant sequences in 90 patients with a range of early to late manifestations of Lyme disease. The results demonstrate the existence of asynchronous, independently developing, Ab responses against the two major immunogenic regions of the VlsE molecule in the human host. Despite their strong immunogenicity, the target epitopes were inaccessible to Abs on intact spirochetes, suggesting a lack of direct immunoprotective effect. These observations document the association of immune reactivity toward specific VlsE sequences with different phases of Lyme disease, demonstrating the potential use of detailed epitope mapping of Ags for staging of the infection, and offer insights regarding the pathogen's possible immune evasion mechanisms. *The Journal of Immunology*, **2016, 196: 1036–1043.**

Lyme disease is caused by spirochetes of the *Borrelia burgdorferi* species complex and is transmitted by the bite of infected ticks (1). Recently published estimates from the U.S. Centers for Disease Control and Prevention indicate that ∼300,000 cases are diagnosed annually in the United States (2). The infection is multisystemic and is usually described as occurring in three stages: early localized, early disseminated, and late disease (3). Early localized Lyme disease begins at the site of tick bite and is typically associated with a characteristic skin lesion, known as erythema migrans (EM) (3). Early disseminated disease occurs days or weeks after the tick bite when the bacteria have spread hematogenously and is associated with multiple skin EM lesions (4), as well as extracutaneous manifestations, including acute carditis and neurologic involvement (5). Late disease occurs months to years after the original exposure and can present as arthritis, late neuroborreliosis, and acrodermatitis chronica atrophicans (6, 7). The treatment guidelines from the Infectious Diseases Society of America recommend specific antibiotic regimens based on the stage or manifestation of the infection (3, 6), although there are currently no established biomarkers to stage the disease. Although antibiotic therapy resolves clinical symptoms in the majority of cases, ∼10% of patients with Lyme arthritis fail to respond to antibiotic therapy and continue to have persistent joint inflammation. Termed antibiotic-refractory Lyme arthritis, the condition often responds to immunomodulatory or anti-inflammatory agents (6). Distinct from antibiotic-refractory Lyme arthritis, some patients experience persistent symptoms of pain, fatigue, and/or difficulties with concentration and memory after standard antibiotic treatment and in the absence of evidence for ongoing infection (8–10). The condition, referred to as posttreatment Lyme disease syndrome, can be associated with considerable impairment in the health-related quality of life in some patients (11), but no diagnostic biomarkers or effective treatments are currently available.

Immunologic reaction to infection with *B. burgdorferi* includes a robust Ab response to a number of the organism's proteins and glycolipids. The generated Ab response to borrelial Ags is used extensively in serologic assays to aid the diagnosis of Lyme borreliosis (4). The two-tiered testing algorithm for Lyme disease, recommended since 1995, includes a screening ELISA and a subsequent supplemental Western blot analysis of serum Ab reactivity to a total antigenic extract of *B. burgdorferi* (12). More recently, a specific protein of *B. burgdorferi*, known as VlsE (variable major protein–like sequence expressed), has emerged as a useful solo Ag in serologic assays for Lyme disease (13–16). VlsE is a surface lipoprotein of *B. burgdorferi* that undergoes antigenic variation during infection. It consists of two invariable domains located at the N- and C-termini of the protein, as well as

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Abbreviations used in this article: EM, erythema migrans; IR, invariable region.

Table I. Demographic and clinical characteristics of study cohorts

Subject Group	No. of Subjects	Mean Age (years ± SD)	Gender Ratio (female:male)
Lyme disease	90	48.3 ± 15.4	34:56
Early localized (single EM)	18	46.5 ± 14.3	7:11
Early disseminated (multiple EM)	17	47.1 ± 16.4	8:9
Early neurologic	16	46.3 ± 9.6	5:11
Late neurologic	16	52.5 ± 16.9	6:10
Antibiotic-responsive arthritis	12	45.5 ± 15.5	4:8
Antibiotic-refractory arthritis	11	52.5 ± 20.9	4:7
Healthy	30	45.8 ± 16.2	13:17

six variable regions (VR1–VR6) and six invariable regions (IR1–IR6) within its central variable domain (Fig. 1A) (17). VlsE elicits a rapid and strong humoral response that can be detected throughout the course of the disease (18–20). The main immunodominant epitope of VlsE is located within the IR6 region (21, 22). C6, a peptide that reproduces the IR6 epitope, is now used in a commercial diagnostic test (23). Two other major epitopes of VlsE are located in the membrane-proximal N- and C-terminal regions of the protein's invariable domain (24). Ab responses to these membrane-proximal epitopes were found to be significantly higher in individuals with a history of Lyme disease and persistent symptoms than in those who did not have residual symptoms after antibiotic treatment for Lyme disease (24). It was hypothesized that Abs against the N- and C-terminal epitopes may be associated with later manifestations and more intractable forms of Lyme disease that would be more likely to lead to persistence of symptoms in some individuals (24).

In this study, we perform a comprehensive analysis of Ab responses toward all three major epitopes of VlsE in patients with a range of early to late manifestations of Lyme borreliosis and assess the immunoprotective potential of the generated Ab reactivities. Our findings have implications for gaining a more nuanced understanding of the evolution of the Ab response to VlsE in the context of *B. burgdorferi* persistence and its potential use as a source of information for staging the disease.

Materials and Methods

Patients and controls

Serum samples were obtained with written informed consent under Institutional Review Board–approved protocols at the National Institute of Allergy and Infectious Diseases (National Institutes of Health) and New York Medical College. This study was approved by the Institutional Review Board of Columbia University Medical Center. Serum samples were from 90 individuals with a range of early to late manifestations of Lyme disease (Table I) and were collected when the clinical manifestations listed were present. All patients met the U.S. Centers for Disease Control and Prevention case definition for Lyme disease (25). Patients with EM had culture evidence of *B. burgdorferi* infection. Early neurologic Lyme disease was defined as the presence of compatible objective clinical findings (e.g., cranial nerve palsy, lymphocytic meningitis, and/or radiculoneuritis) in conjunction with current or recent EM and/or serologic evidence of the infection. Late neurologic Lyme disease was defined based on the presence of a compatible objective clinical finding (e.g., encephalopathy, polyneuropathy, or encephalomyelitis) in association with serologic evidence of borrelial infection. Lyme arthritis was defined as the presence of clinically compatible joint swelling in conjunction with serologic evidence of the infection. Antibiotic-responsive Lyme arthritis was defined as the resolution of symptoms within 3 mo after the start of treatment with up to 4 wk of i.v. antibiotics or up to 8 wk of oral antibiotics. Antibiotic-refractory Lyme arthritis was defined as persistent joint swelling for >3 mo after the initiation of treatment with at least 4 wk of i.v. antibiotics or at least 8 wk of oral antibiotics. Serum samples from 30 healthy subjects without clinical or serologic evidence of Lyme disease were also included in the study. Serum specimens were kept at −80°C to maintain stability.

Preparation of peptides representing the VlsE epitopes

For immunoassays, biotin-labeled peptides representing the sequences of the three major epitopes of the VlsE protein of *B. burgdorferi* B31 (21, 24) were synthesized by using Fmoc chemistry (Sigma-Aldrich). These sequences were 1) $VlsE_{274}$ (aa 274–298, representing the IR6 epitope: MKKDDQIAAAIALRGMAKDGKFAVK), 2) $VlsE_{21}$ (aa 21–44, representing the N-terminal epitope: SQVADKDDPTNKFYQSVIQLGNGF), and 3) $VlsE_{336}$ (aa 336–349, representing the C-terminal epitope: LRKVGDSVKAASKE). Peptide notation was based on the amino acid number of the first residue of each peptide in the protein sequence for *B. burgdorferi* B31 VlsE protein (NCBI AAC45733). For animal immunizations, a cysteine was added to the N terminus of a nonbiotinylated version of each peptide to use the thiol group of this amino acid for conjugation to keyhole limpet hemocyanin (ProSci).

National Center for Biotechnology Information's three-dimensional structure database coordinates, based on the published crystal structure of VlsE (26), were used to visualize the spatial location of the three epitopes. Images were rendered with the visual molecular dynamics molecular graphics program (27).

Preparation of recombinant VlsE proteins

The procedure for preparation of the recombinant full-length VlsE protein has been described previously (17). Preparation of a recombinant sequence

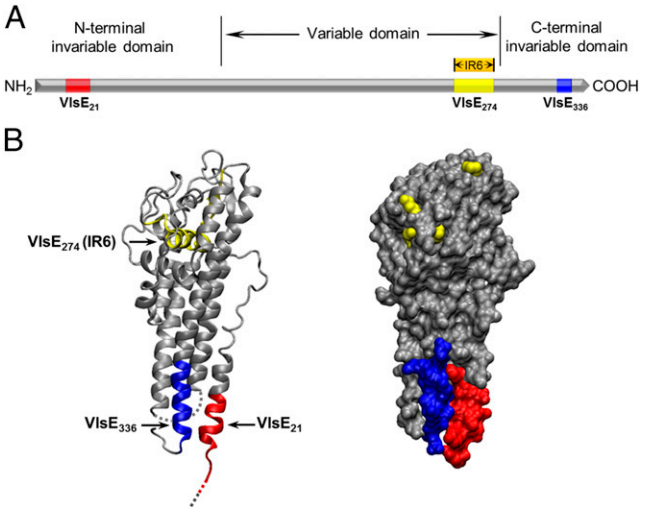

FIGURE 1. (**A**) Diagrammatic illustration of the VlsE sequence showing the location of the peptides representing the protein's three dominant epitopes, which were examined for immunoreactivity in this study. These included $VlsE_{21}$ (aa 21–44; N-terminal epitope), $VlsE_{274}$ (aa 274–298; IR6 epitope), and $VlsE_{336}$ (aa 336–349; C-terminal epitope) of the VlsE protein from *B. burgdorferi* B31. (**B**) Spatial position of the three sequences in the crystal structure of the VlsE protein. A ribbon diagram (*left*) and an orthographic molecular surface representation (*right*) of VlsE monomer are depicted using the visual molecular dynamics molecular graphics program, based on National Center for Biotechnology Information's three-dimensional structure database coordinates. Parts of the protein that were missing from the three-dimensional structure database coordinates are represented as dashed lines in the ribbon diagram. The *bottom* of the figure represents the membrane proximal region.

representing the membrane-proximal region of VlsE was as follows. A coding fragment of human complement factor C3b was obtained by PCR amplification of human cDNA (NCBI BQ717819) using primers 5′-ATA TCA TGA AAA AAC TAG TGC TGT CCA GTG AGA-3′ and 5′-TAT CTC GAG ATA ATC CAT GGC TCG GAT CTT CCA CTG GCC CAT GTT GAC-3′. The amplicon was digested with PagI and XhoI and ligated with pET24d-N (28). The resulting expression vector was designated pET24d-N-C3b. The expression plasmid for His-C3b-VlsE(Δ1–21, Δ45–335) was assembled in two steps. Initially, PCR was conducted using primers 5′-ATA GGT CTC ATC TCC AGA GAA AGA GAA GGC TGA GGG GGC-3′ and 5′-GCA GGT CTC CTC TCA AAT CCG TTA CCT AAT TGT ATG AC-3′ and a plasmid containing the full-length *vlsE1* (17). The product was digested with Eco31I and ligated to obtain the mutated expression construct. In a second step, the coding sequence of the deletion

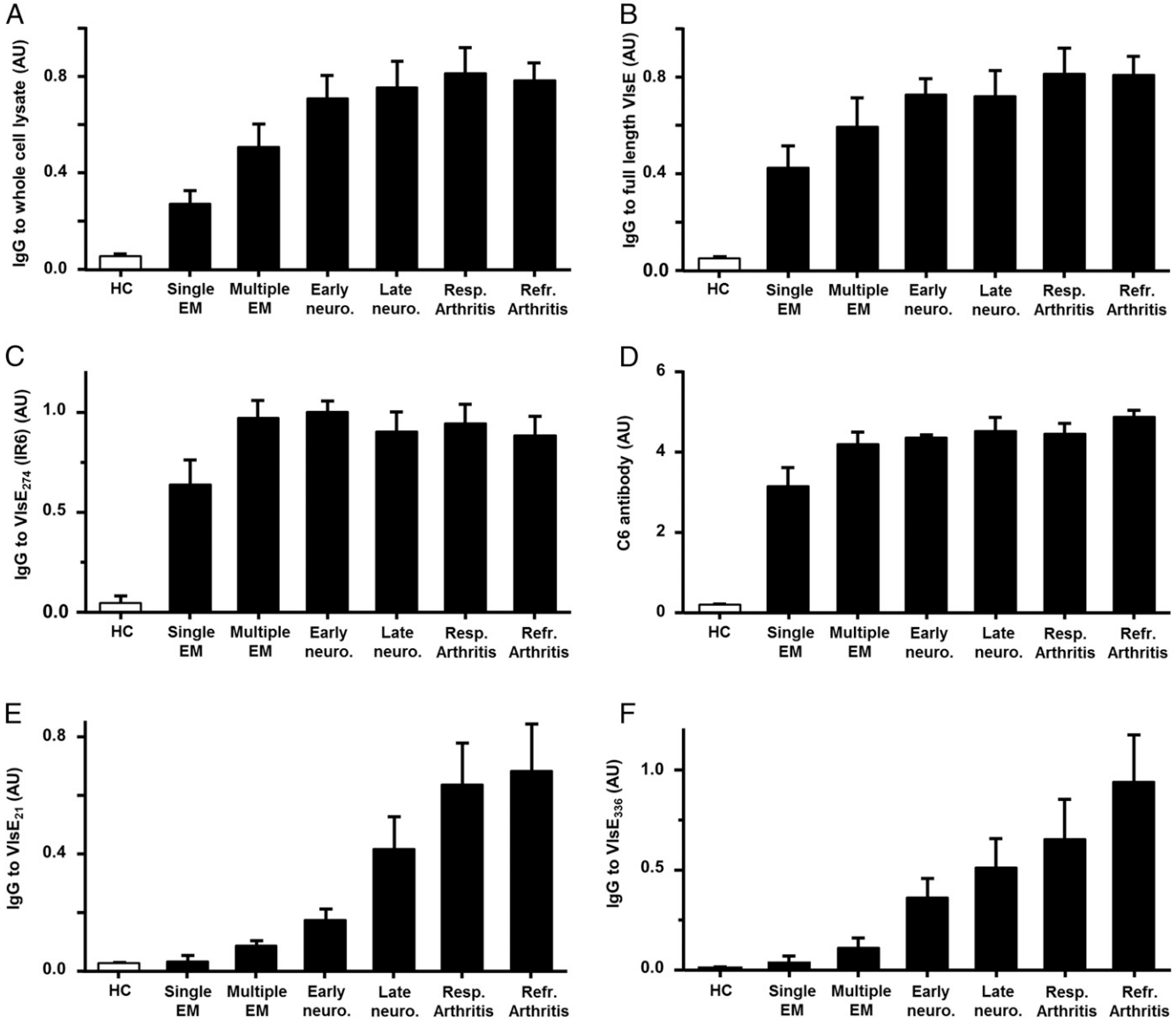

FIGURE 2. Ab response to *B. burgdorferi* (whole-cell lysate), full-length VlsE, and individual VlsE epitopes. Mean levels of IgG reactivity to *B. burgdorferi* (whole-cell lysate) (**A**), IgG to full-length recombinant VlsE protein (**B**), IgG to $VlsE_{274}$ (**C**), C6 Ab (**D**), IgG to $VlsE_{21}$ (**E**), and IgG to $VlsE_{336}$ (**F**), as measured by ELISA, in healthy controls (HC) ($n = 30$) and in patients with single EM ($n = 18$), multiple EM ($n = 17$), early neurologic ($n = 16$), late neurologic ($n = 16$), antibiotic-responsive arthritis ($n = 12$), and antibiotic-refractory arthritis ($n = 11$) manifestations of Lyme disease. Error bars represent the SEM. Levels of statistical significance for differences in Ab reactivity are as follows. (A) Healthy versus multiple EM, early neurologic, late neurologic, responsive arthritis, and refractory arthritis: $p < 0.001$, $p < 0.0001$, $p < 0.0001$, $p < 0.0001$, and $p < 0.0001$, respectively; single EM versus early neurologic, late neurologic, responsive arthritis, and refractory arthritis: $p < 0.01$, $p < 0.01$, $p < 0.0001$, and $p < 0.0001$, respectively. (B) Healthy versus single EM, multiple EM, early neurologic, late neurologic, responsive arthritis, and refractory arthritis: $p < 0.01$, $p < 0.001$, $p < 0.0001$, $p < 0.0001$, $p < 0.0001$, and $p < 0.0001$, respectively; single EM versus early neurologic, late neurologic, responsive arthritis, and refractory arthritis: $p < 0.01$ for each comparison. (C and D) Healthy versus individual Lyme disease subgroups: $p < 0.0001$ for each; single EM versus other Lyme disease subgroups: $p < 0.05$ for each. (E) Healthy versus late neurologic, responsive arthritis, and refractory arthritis: $p < 0.01$, $p < 0.0001$, and $p < 0.0001$, respectively; single EM versus late neurologic, responsive arthritis, and refractory arthritis: $p < 0.01$, $p < 0.0001$, and $p < 0.0001$; multiple EM versus late neurologic, responsive arthritis, and refractory arthritis: $p < 0.01$, $p < 0.0001$, and $p < 0.0001$, respectively; early neurologic versus late neurologic, responsive arthritis, and refractory arthritis: $p < 0.05$, $p < 0.001$, and $p < 0.0001$, respectively. (F) Healthy versus late neurologic, responsive arthritis, and refractory arthritis: $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively; single EM versus late neurologic, responsive arthritis, and refractory arthritis: $p < 0.05$, $p < 0.01$, and $p < 0.001$; multiple EM versus late neurologic, responsive arthritis, and refractory arthritis: $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively; early neurologic versus responsive arthritis and refractory arthritis: $p < 0.05$ and $p < 0.01$, respectively. Differences for other group comparisons were not statistically significant.

Table II. Rates of positive Ab reactivity to whole-cell *B. burgdorferi* Ags, full-length VlsE protein, and immunodominant VlsE epitopes in patients representing early to late manifestations of Lyme borreliosis

Lyme Disease Subgroup	Whole Cell (IgG)[a]	Full-Length VlsE Protein (IgG)[a]	C6 (IgG/IgM)[a]	$VlsE_{274}$ (IR6 epitope) (IgG)[a]	$VlsE_{21}$ (N-Terminal Epitope) (IgG)[a]	$VlsE_{336}$ (C-Terminal Epitope) (IgG)[a]	$VlsE_{mp}$ (IgG)[a]
Single EM (n = 18)	7 (38.8)	12 (66.7)	13 (72.2)	15 (83.3)	1 (5.6)	1 (5.6)	1 (5.6)
Multiple EM (n = 17)	10 (58.8)	13 (76.5)	16 (94.1)	16 (94.1)	2 (11.8)	2 (11.8)	2 (11.8)
Early neurologic (n = 16)	14 (87.5)	15 (93.8)	16 (100)	16 (100)	6 (37.5)	7 (43.7)	6 (37.5)
Late neurologic (n = 16)	16 (100)	16 (100)	16 (100)	16 (100)	9 (56.3)	9 (56.3)	9 (56.3)
Responsive arthritis (n = 12)	12 (100)	12 (100)	12 (100)	12 (100)	10 (83.3)	9 (75.0)	9 (75.0)
Refractory arthritis (n = 11)	11 (100)	11 (100)	11 (100)	11 (100)	10 (90.9)	9 (81.8)	9 (81.8)
Healthy control (n = 30)	0	0	0	0	0	0	0

[a]Rates of positivity are shown as number (%).

mutant was amplified with primers 5′-ATA CCA TGG GAT CCA GCC AAG TTG CTG ATA AGG AC-3′ and 5′-ATA CTC GAG TTA CTT ATT CAA GGC AGG AGG TGT TTC-3′. The resulting amplicon was digested with NcoI and XhoI following ligation with pET24d-N-C3b. The constructs were confirmed by DNA sequencing. His-C3b-VlsE(Δ1–21, Δ45–335) was expressed and purified under denaturing conditions as described for another protein (28). Identity and purity of the final protein preparation, referred to thereafter as $VlsE_{mp}$, were assessed by mass spectrometry-assisted peptide mass mapping (29) and SDS-PAGE.

Analysis of serum Ab reactivities

This study focused on the IgG Ab response because our initial data indicated minimal IgM response in patients against VlsE, which is consistent with the findings of prior studies (22).

IgG Ab reactivity against the whole cell extract of borrelial Ags was measured by ELISA, as described previously (30).

IgG Abs against the specific IR6, N-terminal, and C-terminal immunoreactive sequences were measured separately by ELISA, using the biotinylated peptides and following the previously described protocol (24). Optimal serum dilution factor (1:300) was determined to yield absorbance results on the linear portion of the standard curve (derived from serial dilutions of a serum sample with highly elevated Ab reactivity). In addition, the commercial C6 ELISA kit (Immunetics) was used to measure total Ab reactivity to the IR6 invariable region of VlsE, according to the manufacturer's instructions (31).

Measurement of serum IgG Ab reactivity to each of the recombinant VlsE proteins was also done by ELISA. Round-bottom polystyrene plates (Nunc) were coated with 50 μl/well of a 0.01 mg/ml solution of full-length VlsE or $VlsE_{mp}$ in 0.1 M carbonate buffer (pH 9.6) or were left uncoated to serve as control wells. After incubation at 37°C for 1 h, all wells were washed and blocked by incubation with 1% BSA in PBS containing 0.05% Tween 20 for 1.5 h at room temperature. The remainder of the protocol was the same as for measuring Abs to the VlsE peptides.

Determination of IgG subclass distribution

Contribution of each IgG subclass to the detected Ab reactivity against $VlsE_{274}$ and $VlsE_{mp}$ was determined in positive sera according to the protocols described above for measurement of total IgG to each sequence, except that different secondary Abs were used. The subclass-specific monoclonal secondary Abs were HRP-conjugated anti-IgG1 (clone HP6069), anti-IgG2 (clone HP6014), anti-IgG3 (clone HP6047), and anti-IgG4 (clone HP6025) (Life Technologies), each used at a final concentration of 0.5 μg/ml.

Preparation of Abs against VlsE epitopes

New Zealand White rabbits were immunized, using the described keyhole limpet hemocyanin–conjugated peptides (two per each peptide) (ProSci). Immunizations were carried out with injection of 200 μg of peptide conjugate in CFA on day 0, and 100 μg of the conjugate in IFA on days 14, 28, and 42. Collection of serum for immunoblotting and immunoflouorescence analyses was done on day 0 (preimmunization) and day 55 (postimmunization). Abs from pooled sera for each peptide immunization were purified by affinity chromatography, using peptide-coupled affinity columns, as described previously (32). Reactivity of the generated rabbit sera and affinity-purified Abs to each peptide was confirmed at serial dilutions by ELISA, using the same procedure as described above, except HRP-conjugated donkey anti-rabbit IgG (GE Healthcare Life Sciences) was used as the secondary Ab.

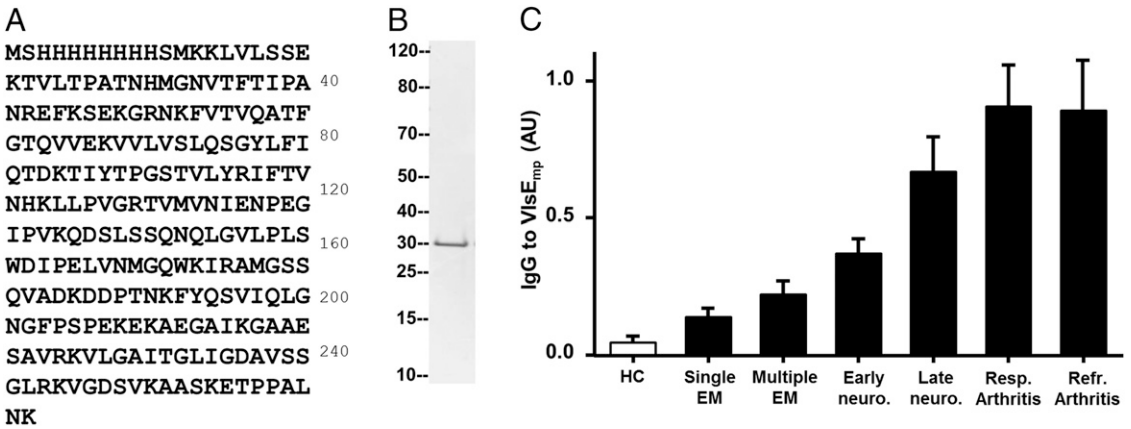

FIGURE 3. Ab response to $VlsE_{mp}$. (**A** and **B**) A single contiguous molecule containing only the membrane-proximal region of VlsE and its associated $VlsE_{21}$ and $VlsE_{336}$ epitopes was cloned and expressed as a C3b fusion protein: the expected—and mass spectrometry confirmed—sequence (A) and the gel electrophoresis (denaturing) profile (B) of the expressed protein that was used for subsequent Ab assays. (**C**) Mean IgG Ab reactivity to $VlsE_{mp}$, as measured by ELISA, in healthy controls (HC) (n = 30) and in patients with single EM (n = 18), multiple EM (n = 17), early neurologic (n = 16), late neurologic (n = 16), responsive arthritis (n = 12), and refractory arthritis (n = 11) manifestations of Lyme disease. Error bars represent the SEM. Levels of statistical significance for differences in Ab reactivity to $VlsE_{mp}$ are as follows. Healthy versus early neurologic, late neurologic, responsive arthritis, and refractory arthritis: $p < 0.05$, $p < 0.0001$, $p < 0.0001$, and $p < 0.0001$, respectively; single EM versus late neurologic, responsive arthritis, and refractory arthritis: $p < 0.001$, $p < 0.0001$, and $p < 0.0001$, respectively; multiple EM versus late neurologic, responsive arthritis, and refractory arthritis: $p < 0.01$, $p < 0.0001$, and $p < 0.0001$, respectively; early neurologic versus late neurologic, responsive arthritis, and refractory arthritis: $p < 0.05$, $p < 0.01$, and $p < 0.01$, respectively. Differences for other group comparisons were not statistically significant.

Immunoblotting

Binding of the generated Abs to the full-length recombinant VlsE protein was assessed by immunoblotting as described previously (24). In addition to the purified Abs against VlsE274, VlsE21, and VlsE336 (1:100–1:1000), a commercially available rabbit polyclonal Ab to full-length recombinant VlsE protein (Rockland) was used as a control. The secondary Ab was an AP-conjugated goat anti-rabbit IgG (Millipore).

Cell culture and immunofluorescence

B. burgdorferi strain B31 cells (clone A3), used in a previous study (33) and maintained at −80°C in medium containing glycerol, were brought to room temperature and grown in modified Barbour–Stoenner–Kelly culture medium containing 6% rabbit serum (Sigma-Aldrich) at 25°C and pH 7.8 for optimal VlsE expression (34). Culture tubes used were 8 ml polystyrene round-bottom tubes with screw caps (BD Falcon). Cells were harvested during the exponential phase (~50×10^6 spirochetes/ml) by centrifugation at 9600 × *g* for 10 min and seeded onto Superfrost Plus microscope slides (Fisher Scientific) at 1.5–2 × 10^5 spirochetes/slide. Spirochetes were fixed by immersion of slides in acetone at 4°C for 10 min. For unfixed staining, this step was omitted. Slides were blocked for 1 h with PBS containing 0.2% BSA. This was followed by incubation for 1 h with affinity-purified rabbit polyclonal Abs specific for VlsE21, VlsE274, or VlsE336 as well as the corresponding preimmunization rabbit sera (1:20–1:100). Rabbit Abs to a whole-cell protein extract of *B. burgdorferi* B31 (Virostat) and to full-length recombinant VlsE protein (Rockland) were used as controls. After washing in PBS, slides were incubated in Alexa Fluor 488–labeled chicken anti-rabbit IgG (Molecular Probes) at 1:500 for 1 h in the dark. Slides were then washed, air-dried, and mounted with Vectashield HardSet mounting reagent (Vector Laboratories). Slides were viewed and images captured with an EVOS FL Cell Imaging System (Life Technologies).

Data analysis

Group differences were analyzed by one-way ANOVA, with post hoc testing and correction for multiple comparisons. Positivity cutoffs for the ELISA data were assigned as 3 SDs above the mean for the healthy control group. All *p* values were two-sided, and $p < 0.05$ was considered to be statistically significant. Statistical analyses were performed with Prism 6 (GraphPad) and Minitab 17 (Minitab) software.

Results

Patients and controls

The demographic and clinical characteristics of patients and healthy controls in this study are shown in Table I.

B cell response to VlsE is characterized by asynchronous targeting of distinct epitopes during early to late stages of Lyme disease

The diagrammatic representation of the VlsE sequence and the three-dimensional crystal structure of VlsE, showing the specific epitopes, are depicted in Fig. 1. Mean levels of Ab reactivity to each Ag for the patient and control cohorts are shown in Fig 2. The *p* values for the various comparisons in each analysis are provided in the Fig. 2 legend. Table II shows the frequency of positive Ab reactivity to each Ag in the patient and control groups.

The anti-*B. burgdorferi* (whole cell) IgG levels in the multiple EM, early neurologic, late neurologic, antibiotic-responsive arthritis, and antibiotic-refractory arthritis patient subgroups were significantly higher than for the healthy control group, and the differences between these five patient subgroups did not reach statistical significance (Fig. 2A). Similarly, the anti-VlsE (full length) IgG levels in the multiple EM, early neurologic, late neurologic, responsive arthritis, and refractory arthritis patient subgroups were significantly higher than for the healthy control group, whereas the differences between these patient subgroups were not statistically significant (Fig. 2B).

The mean Ab response to the IR6 region (as detected by the $VlsE_{274}$ ELISA and the commercial C6 assay) for every Lyme disease subgroup (single EM, multiple EM, early neurologic, late neurologic, responsive arthritis, and refractory arthritis) was significantly higher than for the healthy control group (Fig. 2C, 2D). With the exception of the single EM group, there was not a significant difference in mean Ab reactivity or rate of positivity between the patient subgroups representing the various manifestations of Lyme disease (Fig. 2C, 2D, Table II). The data demonstrate the consistent presence of Abs against the IR6 sequence throughout the course of Lyme disease, except for the very early stage of infection. It should be noted that, although the C6 assay is designed to detect both IgG and IgM Abs, previous work has shown the contribution of IgM isotype to total Ab reactivity against the IR6 epitope in Lyme disease patients to be minor (22). Our data are confirmatory because we found no substantive difference between the results of the commercial C6 test and the in-house $VlsE_{274}$ IgG assay.

In stark contrast to Ab response against the IR6 epitope, Ab reactivity to the membrane-proximal N- and C-terminal epitopes of VlsE ($VlsE_{21}$ and $VlsE_{336}$) increased sharply from early to late Lyme disease (Fig. 2E, 2F, Table II). Ab reactivity to the N-terminal $VlsE_{21}$ epitope was mostly absent in patients with early localized and early disseminated Lyme disease (5.6% rate of positivity in single EM and 11.8% in multiple EM) but became prevalent in patients with late neurologic manifestations (56.3%), responsive arthritis (83.3%), and refractory arthritis (90.9%), whereas none of the healthy controls were positive (Table II).

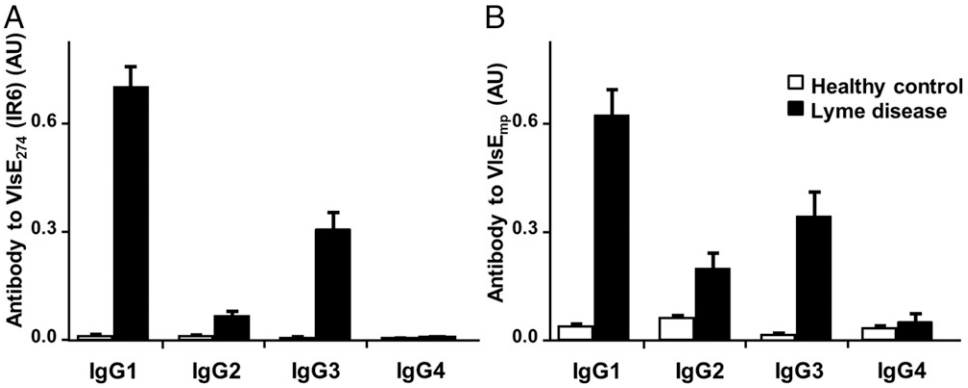

FIGURE 4. IgG subclass Ab responses to the IR6 and the membrane-proximal epitopes, as represented by $VlsE_{274}$ and $VlsE_{mp}$. Mean levels of IgG1, IgG2, IgG3, and IgG4 Ab reactivity to $VlsE_{274}$ (**A**) and $VlsE_{mp}$ (**B**) in healthy controls ($n = 30$) and in Lyme disease patients found to be positive for total IgG reactivity to each peptide ($n = 86$ for $VlsE_{274}$, $n = 36$ for $VlsE_{mp}$). In comparison with the healthy control group, IgG1, IgG2, and IgG3 reactivities to each peptide were significantly increased in the Lyme disease patient group ($p < 0.0001$, $p < 0.01$, and $p < 0.0001$, respectively for both peptides). Error bars represent the SEM.

Levels of serum Ab reactivity to $VlsE_{21}$ were significantly higher in the individual late neurologic, responsive arthritis, and refractory arthritis groups when compared with each of the single EM, multiple EM, and early neurologic Lyme disease groups or to healthy controls. Compared with healthy controls, there was not a statistically significant increase in Ab reactivity to $VlsE_{21}$ in the single EM, multiple EM, or early neurologic Lyme disease groups. The profile of serum Ab reactivity to $VlsE_{336}$ was similar to $VlsE_{21}$.

To reproduce a single contiguous molecule that contains only the membrane-proximal region of VlsE and its associated epitopes, we generated a recombinant protein, referred to as $VlsE_{mp}$, in which the aa 45–335 were omitted. We speculated that the recombinant generation of this region may have a greater chance of maintaining the conformation found in native VlsE than chemical synthesis. The expected and mass spectrometry–confirmed sequence of the expressed fusion protein, as well as its SDS-PAGE pattern, are shown in Fig. 3A and 3B. Ab reactivity to this molecule largely resembled and confirmed what was seen with individual N- and C-terminal epitopes of VlsE ($VlsE_{21}$ and $VlsE_{336}$) (Fig. 3C, Table II). Levels of serum Ab reactivity to $VlsE_{mp}$ were significantly higher in the individual late neurologic, responsive arthritis, and refractory arthritis cohorts when compared with each of the single EM, multiple EM, and early neurologic Lyme disease groups or to healthy controls. There was not a statistically significant difference when comparing the single and multiple EM groups to the healthy control group.

Ab response to the membrane-proximal region is primarily of IgG1 and IgG3 subclasses

In comparison with the healthy control group, Lyme disease patients exhibited higher levels of IgG1, IgG2, and IgG3 reactivity to both the $VlsE_{mp}$ and VlsE274 (IR6) sequences at the same levels of statistical significance ($p < 0.0001$, $p < 0.01$, and $p < 0.0001$, respectively) (Fig. 4). Differences in IgG4 reactivity were not statistically significant. As such, the IgG subclass contributions did not appear to be substantially different between the Ab response to the IR6 epitope and that against the membrane-proximal region.

Abs to the membrane-proximal region cannot access their respective epitopes on the VlsE protein of intact B. burgdorferi

When assessed by immunoblotting, Abs against all three epitopes (VlsE21, VlsE274, and VlsE336), as well as Abs against the full-length VlsE protein, bound to the recombinant VlsE on membrane, which is believed to be partially denatured (Fig. 5A). When tested by immunofluorescence following acetone fixation (which can result in cell permeabilization and some protein denaturation), rabbit Abs against each of the three epitopes (VlsE21, VlsE274, and VlsE336), as well as Abs to the full-length VlsE protein, were found to bind some *B. burgdorferi* spirochetes, although the staining was not as strong or frequent as that with anti-*B. burgdorferi* (whole-cell proteins) Abs (Fig. 5B). In contrast, unfixed *B. burgdorferi* could not be stained with Abs against VlsE21, VlsE274, or VlsE336 but maintained staining with Abs to full-length VlsE protein and to *B. burgdorferi* (whole-cell proteins) (Fig. 5B). The data indicate that Abs to the N- and C-terminal membrane-proximal regions, similarly to those against IR6, cannot access their respective epitopes on the native VlsE protein of intact spirochetes.

Discussion

The results of our study demonstrate the development of asynchronous B cell responses toward two specific regions of the VlsE protein during early to late stages of Lyme disease. In contrast to the Ab response against the IR6 region, which is generated early on and remains elevated following the dissemination of Lyme disease, the responses toward the membrane-proximal epitopes in the N- and C-terminal invariable domains, particularly the $VlsE_{21}$ epitope, were found to be largely absent in the early stage and to increase sharply only when infection progresses to later stages. The Ab responses to both the IR6 and the membrane-proximal regions were primarily composed of IgG1 and IgG3, IgG subclasses that can be strongly effective at opsonization to enhance phagocytosis, as well as activation of the complement cascade.

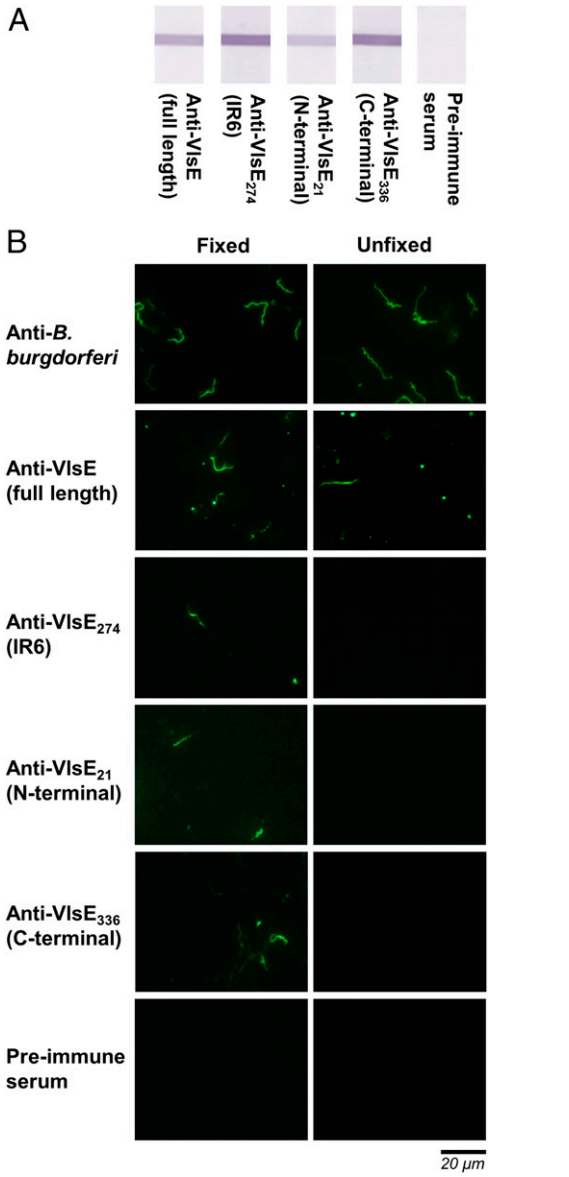

FIGURE 5. Accessibility of major VlsE immunogenic regions to Abs. (**A**) Assessment of binding of Abs to partially denatured VlsE protein by immunoblotting. IgG Abs to VlsE274, VlsE21, and VlsE336 bound to the localized VlsE on membrane, as did the control Ab to full-length recombinant VlsE. IgG from preimmunization sera showed no binding (representative image shown). (**B**) Immunofluorescence assessment of binding of IgG Abs to fixed or unfixed *B. burgdorferi* B31 cells. Staining of *B. burgdorferi* spirochetes with IgG Abs against VlsE21, VlsE274, and VlsE336 was only observed if the cells were fixed. The anti-*B. burgdorferi* (whole-cell lysate) and anti-VlsE (full-length) IgG Abs stained both fixed and unfixed cells. IgG from preimmunization sera did not stain cells (representative images shown).

assuming they can bind their target epitopes. However, the described epitopes were found to be inaccessible to Abs on intact spirochetes.

There is evidence that VlsE is required for optimal infectivity and persistence of the spirochete in the mammalian host (35–37). The earliest Ab response to the protein is directed at its IR6 epitope and is maintained at elevated levels throughout the course of infection. It has been suggested that the potent immunogenicity of the IR6 region may be part of *B. burgdorferi*'s immune evasion mechanism in the mammalian host (21). Although patients with Lyme disease develop a vigorous Ab response to IR6, these Abs cannot bind the intact spirochete, as shown in this and earlier studies, and are therefore unlikely to exert a protective effect (21, 26). This is probably partly because the IR6 region is mostly buried in the membrane-distal region of the protein, with little surface exposure (26).

The Ab responses to the membrane proximal domain of VlsE, which develop during later manifestations of *B. burgdorferi* infection, may be a part of the process of epitope expansion, aimed at exerting greater protective immunity. This epitope expansion may be accentuated in part by the inflammatory environment within host tissue and mediated by IFN-γ, which has been shown to be associated with late Lyme disease and to contribute to VlsE recombination (38). Greater reactivity to the membrane-proximal epitopes may also be driven via enhanced Ag processing during later stages of Lyme disease. Although the identified $VlsE_{21}$ and $VlsE_{336}$ epitopes are surface exposed, they are located in the membrane-proximal part of the monomeric form of VlsE (26). This may explain why, despite the prominent IgG response to the membrane proximal epitopes in late Lyme disease, the generated Abs cannot bind their respective targets on the intact organism. It is possible that this is another protective mechanism of the spirochete for persistence in the human host.

The sequences of the membrane-proximal N-terminal $VlsE_{21}$ and the C-terminal $VlsE_{336}$ epitopes in this study were from the B31 strain of *B. burgdorferi*. B31 belongs to the rRNA integral spacer type 1 group of *B. burgdorferi*, which has been shown to be associated with greater inflammation and more severe Lyme disease (39). Some of the other strains of *B. burgdorferi* contain poorly conserved sequences for these regions, which would be expected to lead to differences in immunoreactivity. As such, the observed Ab responses to the specific membrane-proximal epitope sequences derived from *B. burgdorferi* B31 may also reflect the increased likelihood of infection with rRNA integral spacer type 1 genotype organisms that are associated with later manifestations and more severe forms of the disease.

In summary, the observations from this study document the complexity of immune response to VlsE in the human host, delineating the evolution of epitope-specific B cell responses through the various stages of infection and yielding novel insights regarding the potential role of the protein in the context of spirochetal persistence. The results also demonstrate the possible use of detailed epitope mapping of Ab responses in Lyme disease for information regarding disease stage. Further work is needed to determine whether the measurement of Ab reactivity against the membrane-proximal epitopes of VlsE and other borrelial antigenic determinants of interest can become useful for devising algorithms to stage Lyme disease or to identify patients at greater risk for developing persistent posttreatment symptoms.

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