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Evidence That the Variable Regions of the Central Domain of VlsE Are Antigenic during Infection with Lyme Disease Spirochetes

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It has been postulated that the *vls* system of the Lyme disease spirochetes contributes to immune evasion through antigenic variation. While it is clear that *vlsE* undergoes sequence change within its variable regions at a high frequency during the early stages of infection, a definitive role in immune evasion has not been demonstrated. In this report we assessed the murine and human humoral immune response to recombinant (r)-VlsE variants that originally arose during infection in mice. Immunoblot analyses of r-VlsE variants were conducted by using serum samples collected from mice infected with *Borrelia burgdorferi* clones that carried different *vlsE* variants. All of the r-VlsE variants were recognized by infection sera regardless of the identity of the infecting clone or isolate. In addition, all variants were immunoreactive with a panel of human Lyme disease patient serum samples. It is evident from these analyses that the infection-induced VlsE variants share common epitopes that reside within conserved segments of these proteins. However, preabsorption experiments revealed that the variable regions of the central domain of VlsE, which undergo rapid mutation during infection, also influence the antigenic properties of the protein. A subset of the antibodies elicited against *vlsE* variants that differ in the sequences of their variable regions were found to be variant specific. Hence, in spite of a robust antibody response to conserved segments of VlsE, infection-induced sequence changes within the variable regions alter the antigenicity of VlsE. These results provide the first direct evidence of antigenic variation in the VlsE protein.

The chronic nature of Lyme disease and the genetic and antigenic diversity of the Lyme disease spirochetes suggest that antigenic variation may play an important role in immune evasion. Two distinct gene families have been demonstrated to undergo sequence change or mutation during infection (17, 21). Infection-induced antigenic variants of OspE protein family members have been demonstrated to arise specifically during infection (17). The molecular mechanisms involved in generating OspE variation include the development of point mutations that alter the amino acid sequence and recombination among members of the *ospE* gene family, resulting in the formation of hybrid genes. VlsE has also been postulated to be involved in antigenic variation (21). The *vls* system is comprised of a single expression locus and a series of nonfunctional, partial *vls* cassettes that exhibit tightly defined regions of conservation and variability. Unidirectional gene conversion events between the silent cassettes and the *vlsE* gene have been demonstrated to generate new *vlsE* variants (19). The development of post-gene conversion point mutations also plays a role in generating *vlsE* variation (18).

In this study we assess the specificity of the humoral immune response in infected mice and humans to infection-induced variants of VlsE. Consistent with previous reports, the data indicate that immunodominant epitopes of VlsE reside within its conserved segments (7, 8, 12, 13). However, we also demonstrate that the variable regions of the central domain of VlsE influence the overall antigenicity of the protein and are presumably antigenic during infection. Sequence changes in the variable regions also lead to the generation of a subset of antibodies that are variant specific. This represents the first direct demonstration of the involvement of the variable regions of VlsE in antigenic variation.

MATERIALS AND METHODS

Bacterial strains and cultivation. Clones of *Borrelia burgdorferi* B31MI were obtained by cultivation of spirochetes from an ear punch biopsy from C3H-HeJ mice that had been infected for 3 months. As soon as growth was evident, the cultures were subsurface plated. The methods for these procedures have been previously described (17).

Cloning and expression of recombinant VlsE protein variants. In a previous analysis, we PCR amplified, cloned, and sequenced several variant *vlsE* genes that were recovered from a series of *B. burgdorferi* clones recovered from mice infected with *B. burgdorferi* B31MI or *B burgdorferi* B31MIpc53 (18). These amplicons were originally cloned into the pT7Blue-2 vector, but attempts to generate recombinant (r)-protein by IPTG (isopropyl-β-D-thiogalactopyranoside) induction were unsuccessful, as expression of the proteins proved toxic to *Escherichia coli*. To generate recombinant protein, the *vlsE* genes carried by these *E. coli* clones were PCR amplified with the following primers: LIC70(+) (GAC ACG ACA AGA TGG CTG ATA AGG ACG ACC CAA CA) and LIC1080(−) (GAG GAG AAG CCC GGT ATT CAA GGC AGG AGG TGT TTC). These primers target conserved regions of *vlsE* and were designed with tail sequences (underlined) that complement the single-stranded overhangs of the pET-32 LIC vector (Novagen). Treatment of the amplicons to regenerate the single-stranded overhangs prior to annealing into the pET-32 LIC vector was performed as previously described (17). The annealed products were then trans-

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TABLE 1. Properties of infection-induced VlsE variants

Postinfection clone designation	Original clone used to infect mouse	VlsE variant designation	No. of amino acid differences relative to:	
			Original infecting clone	B31MIpc
B31MIpc	B31MI	$VlsE_{pc}$	ND[a]	NA[b]
B31MIpc17	B31MIpc	$VlsE_{pc\text{-}c17}$	14	14
B31MIpc53	B31MIpc	$VlsE_{pc\text{-}c53}$	25	25
B31MIc53e13	B31MIpc53	$VlsE_{c53\text{-}e13}$	28	21
B31MIc53e72	B31MIpc53	$VlsE_{c53\text{-}e72}$	29	23

[a] ND, not determined.
[b] NA, not applicable.

formed into *E. coli* BL21(DE3)pLysS cells by standard methods and plated on Luria-Bertani plates supplemented with ampicillin (50 μg ml^{-1}) and chloramphenicol (34 μg ml^{-1}). The resulting colonies were inoculated into Terrific Broth with antibiotics (ampicillin, 50 μg ml^{-1}; chloramphenicol, 34 μg ml^{-1}), and the expression of the recombinant proteins was induced by using the protocol for induction of toxic proteins provided by the vector manufacturer (Novagen). The general properties or features of the VlsE variants used in this study are summarized in Table 1.

Generation and preabsorption of infection sera. Most of the infection sera (murine) employed in these analyses were generated as part of a previous study (16). The anti-*B. burgdorferi* 297 and anti-*B. burgdorferi* N40 antisera were generated as part of this study by subcutaneous inoculation of 10^3 spirochetes. Infection of the mice was confirmed by the positive cultivation of spirochetes from ear punch biopsies at 1 month postinoculation. Serum samples were collected from these mice at 2-week intervals out to 3 months. All murine infection sera were preabsorbed to remove nonspecific antibodies as follows. The infection sera were diluted 1:1,000 (in phosphate-buffered saline with 5% nonfat dried milk and 0.2% Tween 20), 1 unit of optical density at 600 nm of *E. coli* (lysed by boiling) was added, and the samples were incubated overnight at 4°C with shaking. Cellular debris and bound antibodies were removed by low-speed centrifugation. The resulting pellet was discarded, and the supernatant was subjected to a second round of preabsorption (room temperature, 4 h) followed by centrifugation. In some cases, the infection sera were preabsorbed with *E. coli* expressing various r-VlsE variants. In all cases the murine infection sera were used at a dilution of 1:1,000.

Human serum samples. Serum samples were collected from patients after informed consent was obtained. All patients met the Centers for Disease Control and Prevention (CDC) case definition for the diagnosis of Lyme disease, had an acute syndrome consistent with Lyme disease as diagnosed by an experienced physician, and had positive immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) and Western blot tests for anti-*Borrelia burgdorferi* antibodies interpreted according to the CDC/Association of State and Territorial Public Health Laboratory Directors criteria (3). All patients were infected in the Northeastern United States.

Immunoblot analyses. Cell lysates of *E. coli* that had been induced with IPTG to express the r-VlsE variants were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12.5% gels. The proteins were then transferred onto Immobilon-P membranes by electroblotting as previously described. Immunoblot analyses using the murine infection sera, the human infection sera, or the S-Tag protein were conducted as previously described (16).

RESULTS

Description of VlsE variants. To further investigate the possible contribution of VlsE in immune evasion, we analyzed the specificity of the antibody response to VlsE variants in naturally infected humans and in experimentally infected mice. The VlsE variants employed in these analyses were obtained from a set of *B. burgdorferi* B31MI-derived clones recovered from infected mice and had been previously characterized by PCR amplification and DNA sequencing (18). The clones were originally obtained by the cultivation and subsequent subsurface plating of spirochetes derived from ear punch biopsies from C3H-HeJ mice infected with *B. burgdorferi* B31MIpc or B31MIpc53 (17). Figure 1 presents a schematic of the experimental flow chart. The *B. burgdorferi* clones and salient features of their *vlsE* genes-proteins are summarized in Table 1, and the amino acid sequences of variable regions 1 through 5 are aligned in Fig. 2. Note that the sequence of variable region 6 was not included because this segment of VlsE had not been sequenced in our earlier analyses of this protein (18). Zhang and colleagues have demonstrated that segmental gene conversion events play an important role in generating sequence variation within the variable regions (19, 21), and consistent with this, most of the sequence changes in the VlsE variants analyzed in this report appear to have arisen by this mechanism and to be localized to the variable regions (18). Point mutations outside the variable regions also resulted in some sequence changes, but overall the invariable segments of VlsE remain highly conserved (18). The nomenclature employed to differentiate the VlsE variants is as follows. Using the VlsE variant $VlsE_{pc\text{-}c53}$ as an example, the first part of the subscript indicates the identity of the infecting clone, while the designation following the hyphen indicates the designation assigned to the clone recovered from the infected mouse.

Expression of recombinant VlsE proteins in *E. coli*. Through the use of a vector that allows for tight regulation of expression of inserted genes, we were able to circumvent initial problems with toxicity and obtain efficient expression of forms of VlsE lacking only their signal peptide. Expression of the VlsE variants was confirmed through immunoblot analysis using S-Tag protein (Fig. 3). All r-VlsE variants were expressed at comparable levels in *E. coli*. It was also noted that the recombinant proteins are somewhat labile, as a significant amount of degradation product was observed with all expressed variants.

Analysis of the humoral immune response to VlsE during experimental infection of mice with *B. burgdorferi*. The recombinant proteins were screened with serum samples collected at different time points from mice infected with *B. burgdorferi* B31MIpc. Serum samples, preabsorbed with *E. coli*, were used to screen identical immunoblots of cell lysates of *E. coli* that had been induced to express the VlsE variants. Immunoreactivity with the prebleed sample was not observed. All of the VlsE variants were strongly immunoreactive with B31MIpc infection sera (week 4) (Fig. 4), including those variants ($VlsE_{c53\text{-}e13}$, $VlsE_{c53\text{-}e72}$, and $VlsE_{c53}$) that developed in mice infected with *B. burgdorferi* B31MIpc53. The mouse from which the B31MIpc infection serum was collected had never been exposed to $VlsE_{c53\text{-}e13}$ or $VlsE_{c53\text{-}e72}$, indicating that the

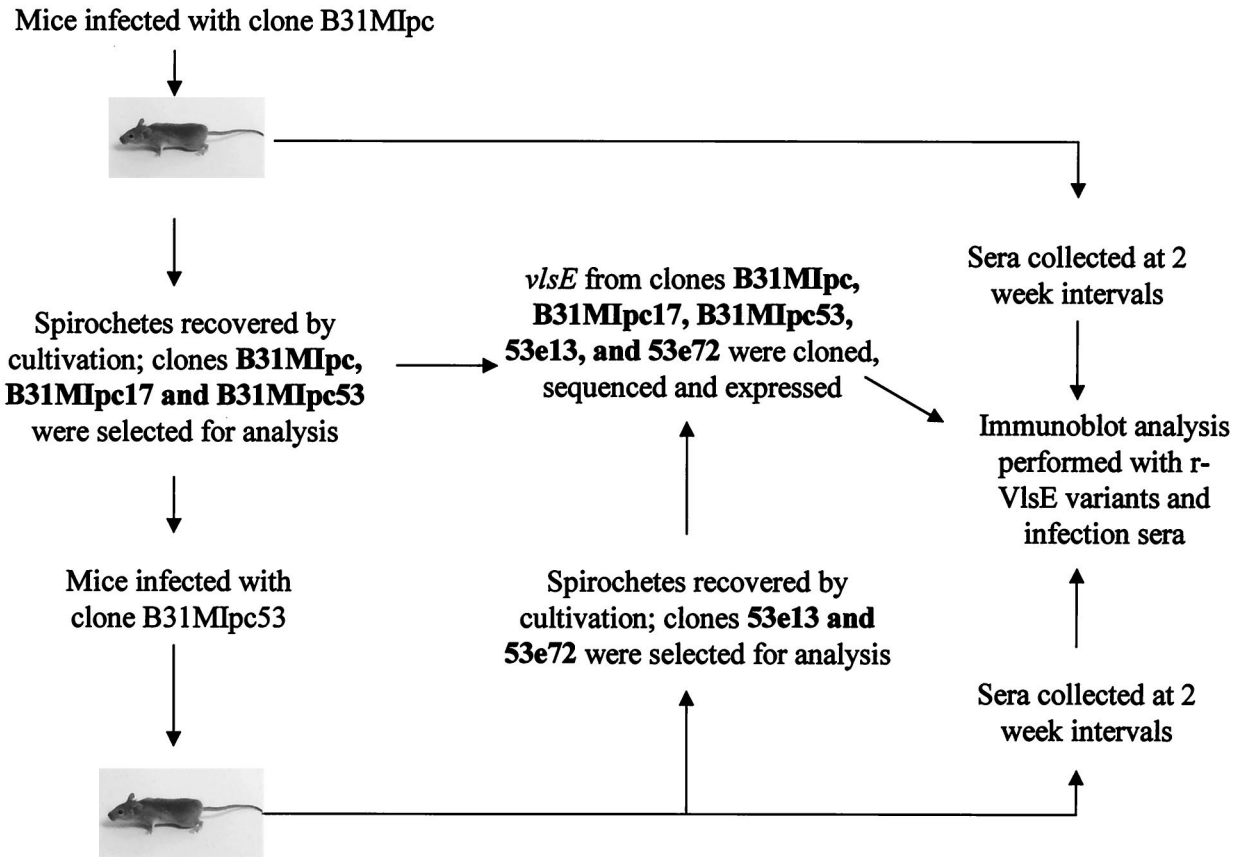

FIG. 1. Experimental flow chart. The schematic depicts the approach employed in our analysis of the immune response to VlsE. All bacterial isolates and clones used in these analyses were *B. burgdorferi*.

antibodies that recognize these variants target conserved, common epitopes. A similar result was obtained with the B31MIpc53 antiserum. This infection serum also carried antibodies that recognized all variants including those to which the B31MIpc53-infected mouse had never been exposed (i.e., $VlsE_{pc}$ and $VlsE_{pc\text{-}c17}$). Hence, in spite of the numerous infection-induced sequence changes in the variable regions of these proteins (Table 1), all were immunoreactive with infection sera regardless of the identity of the infecting clone. This observation indicates that VlsE variants share common epitopes that presumably reside within the conserved domains of these proteins. Based on the strong IgG response observed with the 4-week infection, it can also be concluded that VlsE is expressed during early infection.

	VR-1	VR-2	VR-3
$VlsE_{pc}$	ANAGAAKAADKDSVK	GGSEKLK-VAAAKGENNK	VGDA-AG
$VlsE_{pc\text{-}c17}$	.DDN.....E...	.....A....T..-.E	....-..
$VlsE_{pc\text{-}c53}$	--DN.....T	.....-....T.....	A..LLCW
$VlsE_{c53\text{-}e13}$	--DND.....A..T	R.....-.....-G.E	A..DN..
$VlsE_{c53\text{-}e72}$	--DND..V...E..T	R.....-.....-G.E	A..DN..

	VR-4	VR-5
$VlsE_{pc}$	KAAG--AAEQDGKKPAEAK	DA-DGADF-KDE
$VlsE_{pc\text{-}c17}$	....--....E.....	..E...E.-...
$VlsE_{pc\text{-}c53}$	....EA.GD.E....ED..	.KDGD.E.G-.G
$VlsE_{c53\text{-}e13}$	T...--.....E..GD..	N.D.....N.EG
$VlsE_{c53\text{-}e72}$	T...--.....E..GD..	N.D.....N.EG

FIG. 2. Sequence alignments of the variable regions 1 through 5 of infection-induced variants of *B. burgdorferi* VlsE. The individual variable regions (VR) are indicated. The clone or isolate of origin is indicated by the subscript.

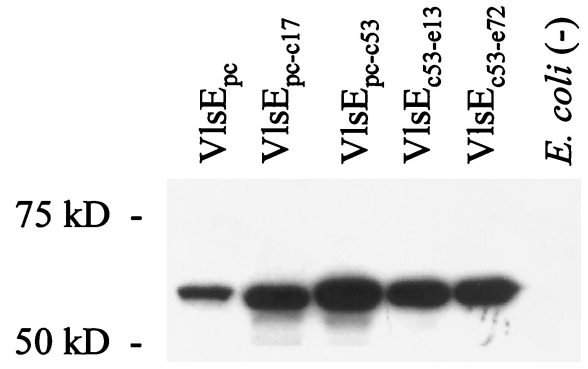

FIG. 3. Demonstration of the expression of r-VlsE variants in *E. coli*. Infection-induced variants of the *vlsE* gene were cloned and expressed in *E. coli* as described in the text. Cell lysates of the IPTG-induced cultures were fractionated by SDS-PAGE, immunoblotted, and screened with S-Tag protein.

Analysis of the antibody response to VlsE variants in mice infected with heterologous isolates and species. To further assess the degree to which VlsE variants share common epitopes, lysates of *E. coli* expressing the VlsE variants described above were immunoblotted and screened with infection sera from mice infected with the heterologous isolates, *B. burgdorferi* 297 and N40 (16). All VlsE variants reacted strongly with the anti-*B. burgdorferi* 297 and N40 antiserum (Fig. 5). Sera from mice infected with the relapsing fever spirochetes *Borrelia turicatae* OZ1, *Borrelia parkeri* (Fig. 5), and *Borrelia hermsii* (data not shown) also reacted with the VlsE proteins. These analyses demonstrate that divergent *Borrelia* isolates and species express proteins that are antigenically related to VlsE. This finding is of importance in evaluating the potential utility of the anti-VlsE antibody response in Lyme disease diagnostics.

Analysis of the antibody response to VlsE variants in human Lyme disease patients. The specificity of the IgG response to VlsE in human Lyme disease patients was also assessed. A panel of previously characterized human Lyme disease serum samples were used to screen immunoblot strips of the r-VlsE variants (15). All serum samples were obtained from patients in the United States (15). To remove nonspecific antibody, the serum samples were preabsorbed with *E. coli*. Whole-cell lysates of *B. burgdorferi* B31MIpc served as a positive control in the immunoblot analyses to reconfirm exposure of these patients to the Lyme disease spirochetes. Sera collected from healthy adults (samples provided by the CDC) served as the negative controls. All of the preabsorbed Lyme disease patient sera were immunoreactive with the B31MIpc cell lysate to various degrees, consistent with previous exposure to *B. burgdorferi* and a diagnosis of Lyme disease. The serum specimens from healthy adults were negative in all cases. The immunoblot data are summarized in Table 2. Of the Lyme disease-positive serum samples, eight did not react with the r-VlsE proteins. It is unclear if this is due to the expression of an antigenically

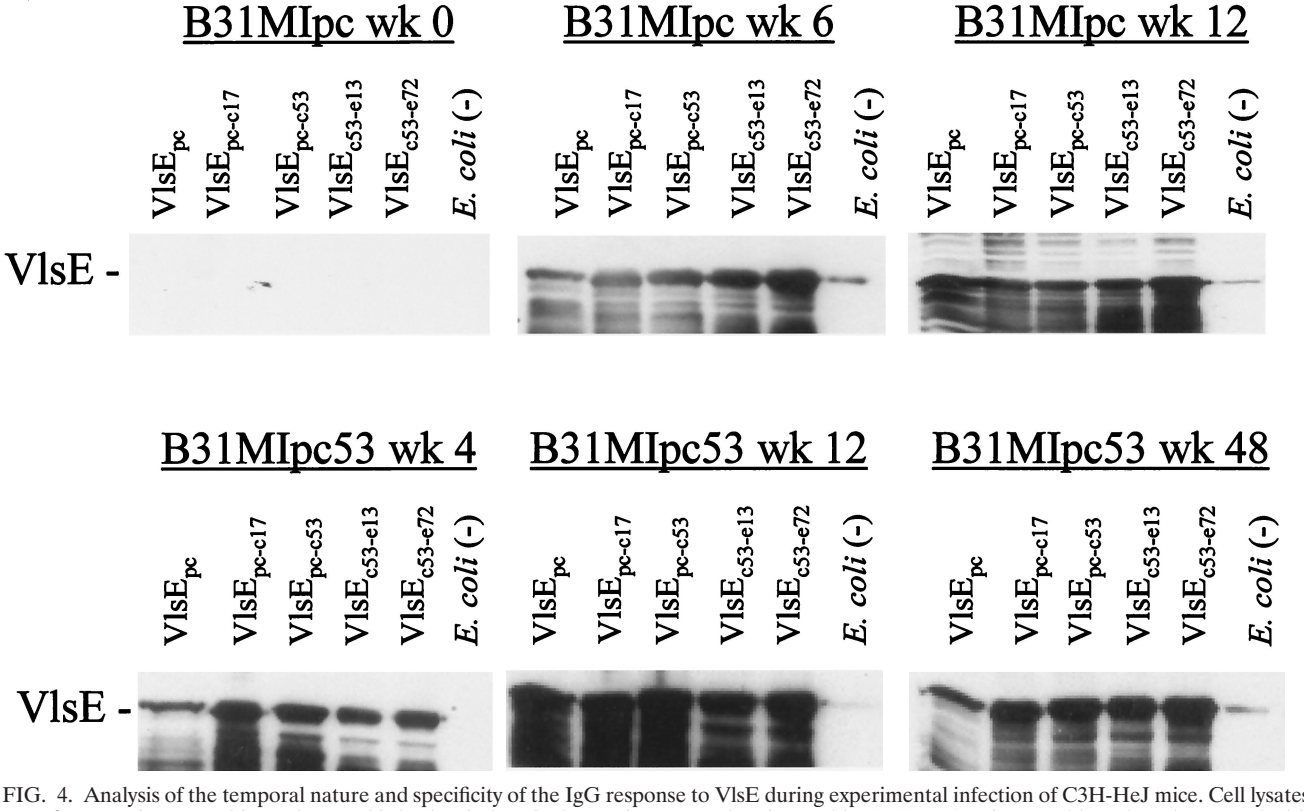

FIG. 4. Analysis of the temporal nature and specificity of the IgG response to VlsE during experimental infection of C3H-HeJ mice. Cell lysates of *E. coli* expressing recombinant forms of infection-induced VlsE variants were fractionated by SDS-PAGE, immunoblotted, and screened with infection serum samples collected at different time points from mice infected with either B31MIpc or B31MIpc53. The different VlsE variants are indicated above each lane. *E. coli* (-), lysates of uninduced cultures of *E. coli.*

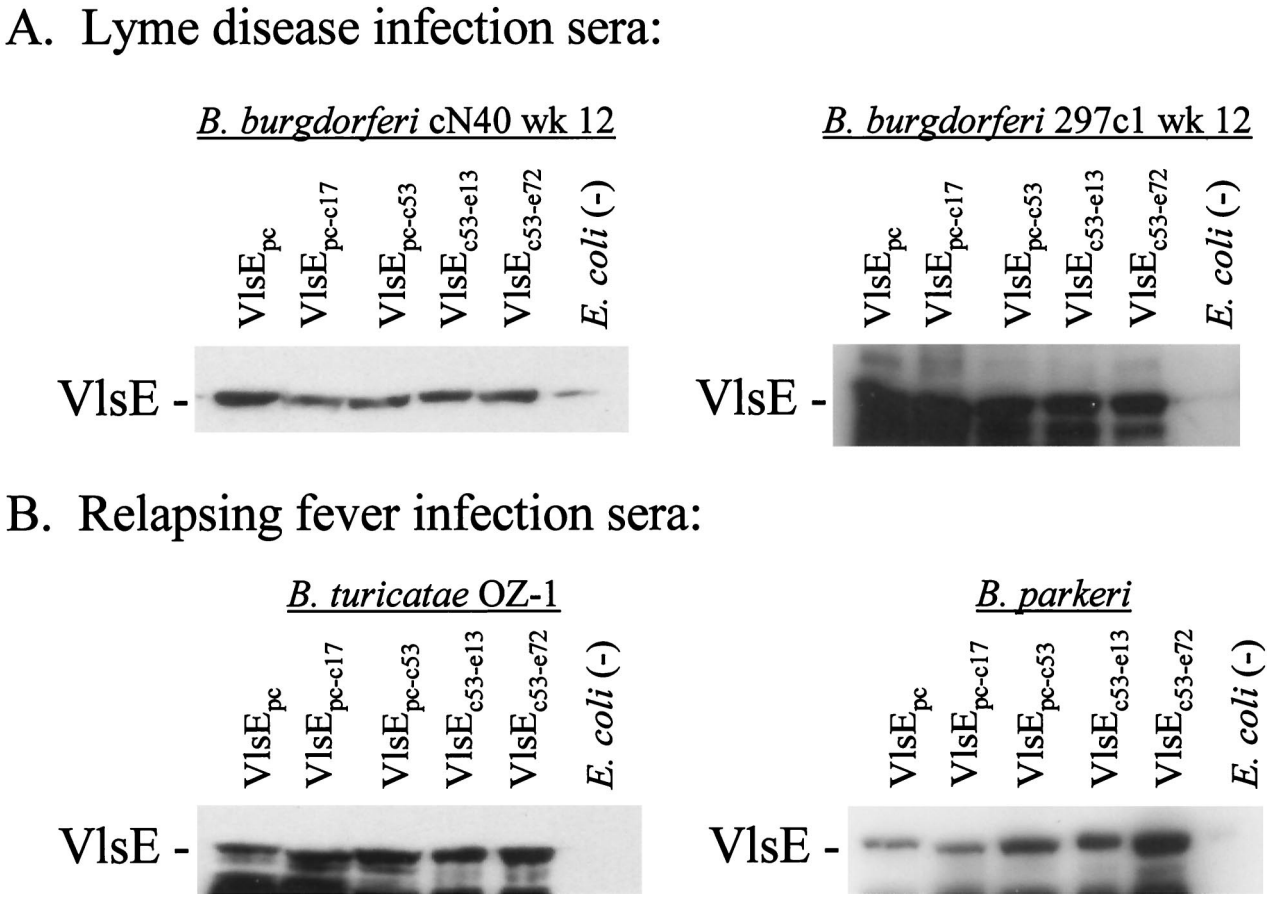

FIG. 5. Detection of anti-VlsE antibodies in mice infected with different *Borrelia burgdorferi* isolates and *Borrelia* species. Immunoblot analyses of the r-VlsE variants were performed as described in the text. The blots in panel A were screened with sera from mice infected with different *Borrelia burgdorferi* isolates and those in panel B were screened with sera from mice infected with relapsing fever spirochete species as indicated. *E. coli* (-), lysates of uninduced cultures of *E. coli.*

unique variant of VlsE by the infecting isolate in these patients or if it reflects lack of expression of VlsE. We cannot distinguish between these possibilities. The 14 serum samples that reacted with VlsE$_{pc}$ also reacted with VlsE$_{pc-c53}$ and VlsE$_{c53-e72}$. Of these, 10 also reacted with VlsE$_{pc-c17}$. It can be concluded that in a majority of Lyme disease cases the infecting strains elicit the production of anti-VlsE IgG antibodies and that at least one component of this humoral response is directed at conserved and shared epitopes.

Preabsorption of infection sera with VlsE variants: impact of sequence variation in the variable regions of VlsE on the antigenicity of the protein. To further define the epitopes of VlsE, immunoblots of the recombinant proteins were screened with an infection serum that had been preabsorbed with *E. coli* expressing individual VlsE variants, the rationale being that since most of VlsE is conserved in sequence, antibodies targeting the conserved domains could be absorbed out, leaving behind antibodies that target VlsE variant-specific epitopes. Since the only significant sequence differences in these variants lie within variable regions 1 through 6, any variant-specific antibodies would therefore target the variable regions or epitopes whose conformations are influenced by the sequences of the variable regions. Preabsorption of the B31MIpc infection sera with *E. coli* expressing VlsE$_{c53-e13}$ removed all antibodies that recognize VlsE$_{c53-e13}$, demonstrating that the preabsorption procedures were completely effective (Fig. 6). Not surprisingly, the reactivity of VlsE$_{c53-e72}$, which differs from VlsE$_{c53-e13}$ at only a few amino acid positions, was also completely abolished. Preabsorption decreased signal intensity but did not remove all antibodies that target VlsE$_{pc}$, VlsE$_{pc-c17}$, and VlsE$_{pc-c53}$. The decreased signal intensity is presumably due to the removal of antibodies that target the common epitopes among VlsE variants. Antibodies that were not absorbed out likely target the variable regions of VlsE$_{pc}$, VlsE$_{pc-c17}$, and VlsE$_{pc-c53}$ which differ significantly in sequence from the variable domain sequences of VlsE$_{c53-e72}$ and VlsE$_{c53-e13}$. Preabsorption of the B31MIpc infection sera with VlsE$_{c53-e72}$ yielded similar results (data not shown) in that the immunoreactivities of VlsE$_{c53-e72}$ and VlsE$_{c53-e13}$ were abolished while the immunoreactivities of VlsE$_{pc}$, VlsE$_{pc-c53}$, and VlsE$_{pc-c17}$ remained. These observations indicate that in the B31MIpc-infected mouse VlsE$_{pc}$, VlsE$_{pc-c53}$, and VlsE$_{pc-c17}$ were expressed and antigenic and elicited variant-specific antibodies. The B31MIpc infection serum was also preabsorbed with VlsE$_{pc-c53}$. This effectively removed antibodies that recognize VlsE$_{c53-e13}$, VlsE$_{c53-e72}$, and VlsE$_{pc-c53}$, but antibodies that rec-

TABLE 2. Summary of data[a] from VlsE immunoblot analyses using human Lyme patient sera

Patient serum no.	B31 lysate	VlsE variant			
		$VlsE_{pc}$	$VlsE_{pc\text{-}c53}$	$VlsE_{pc\text{-}c17}$	$VlsE_{c53\text{-}e72}$
1	++	+++	+	++	++
2	+	−	−	−	−
3	+++	+++	+++	+++	+++
4	+++	−	−	−	−
5	+++	−	−	−	−
6	+	+/−	−	−	−
7	++	+/−	−	−	−
8	+	−	−	−	−
9	+++	+++	++	+++	+++
10	+++	+++	++	++	++
11	++	+++	++	++	++
12	++	+++	++	++	++
13	+++	+++	++	++	++
14	+++	+++	++	++	++
15	+++	+++	++	++	++
16	++	−	−	−	−
17	++	−	−	−	−
18	++	++	++	+++	++
19	++	++	++	−	++
20	++	+	+	−	+
21	++	+++	+++	−	+++
22	++	+++	+++	−	+++

[a] Reactivity: none (−), slight (+/−), moderate (+), strong (++), very strong (+++).

ognize $VlsE_{pc}$ and $VlsE_{pc\text{-}c17}$ remained. These data can be explained as follows. Complete removal of anti-$VlsE_{pc\text{-}c53}$ antibodies is expected, since this variant was used to preabsorb. The complete removal of antibodies to $VlsE_{c53\text{-}e13}$ and $VlsE_{c53\text{-}e72}$ is also expected, since the B31MIpc-infected mouse had never been exposed to these variants. Hence, antibodies recognizing these variants must be directed at conserved epitopes. All antibodies to $VlsE_{pc}$ and $VlsE_{pc\text{-}c17}$ could not be absorbed because these variants elicited the production of variant-specific antibodies that target their unique variable regions in the B31MIpc-infected mouse. Preabsorption of the B31MIpc53 sera with $VlsE_{c53\text{-}e72}$ also effectively removed antibodies against $VlsE_{c53\text{-}e72}$ and $VlsE_{c53\text{-}e13}$ but not against $VlsE_{pc\text{-}c53}$, which is the variant carried by the B31MIpc53 infecting clone. Antibodies to other variants that arose during infection were also not absorbed. These data provide further evidence that VlsE variants that arise during infection possess epitopes that are antigenically distinct from those of their predecessors. Collectively, we interpret the data as demonstrating that the variable regions of VlsE are antigenic and elicit an antibody response during infection. It follows that extensive sequence changes within these regions could result in the generation of VlsE variants that possess antigenically modified variable region epitopes and that this process could be important in Lyme disease pathogenesis. However, an alternative hypothesis that cannot be rejected is that the variable regions are not in and of themselves antigenic and that instead sequence changes within the variable regions lead to structural changes in the proteins that alter the antigenic properties of distal epitopes. However, since the immunoblots were of recombinant proteins fractionated under denaturing conditions, an approach that would presumably disrupt most structurally defined epitopes, we consider this possibility unlikely.

DISCUSSION

In view of the variation that arises in *vlsE* during infection, it has been postulated that infection-induced sequence changes alter the antigenic properties of VlsE and that as a consequence, VlsE contributes to immune evasion through antigenic variation (18–21). In other well-defined antigenic variation systems, immunodominant epitopes reside within the variable regions of the antigenic variation protein and infection-induced sequence changes result in the new variants being antigenically distinct from their parental predecessors. However, the data presented here and in earlier studies indicate that the immunodominant epitopes of VlsE reside within its conserved regions (7–9, 11, 13). To date, the antigenicity of the variable

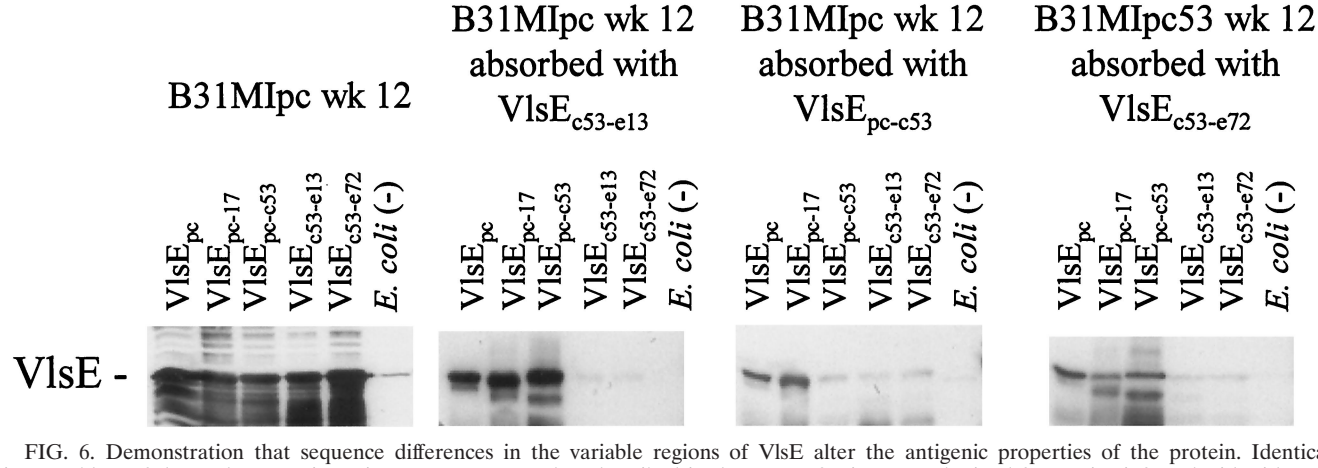

FIG. 6. Demonstration that sequence differences in the variable regions of VlsE alter the antigenic properties of the protein. Identical immunoblots of the r-VlsE protein variants were generated as described in the text. Infection sera obtained from mice infected with either *B. burgdorferi* B31MIpc or B31MIpc53 (as labeled above each immunoblot) were employed in these analyses. To remove antibodies targeting shared epitopes, the infection sera, with the exception of that in the left-hand panel, were preabsorbed with *E. coli* lysates that had been induced to express specific r-VlsE variants (as indicated). All infection serum samples were collected at 12 weeks after inoculation of the mice. Uninduced *E. coli* were included on each blot as a control.

regions and the impact of sequence changes in these regions on the antigenicity of VlsE overall have not been demonstrated.

The goals of this study were to further assess the specificity and targets of the humoral immune response to VlsE during infection. The antigenic test substrates for these analyses were a series of infection-induced VlsE variants that developed in mice infected with B31MIpc or one of its clonal derivatives. These variants had been previously characterized (18) and are described in Table 1 and Fig. 2. Anti-*B. burgdorferi* antisera, collected from mice infected with clonal populations carrying *vlsE* genes of known sequence, were screened for anti-VlsE antibodies. The antisera employed were collected at 2-week intervals over the course of infection. A strong anti-VlsE IgG response was evident by week 4 of infection, indicating that VlsE is expressed during the early stages of infection. Interestingly, it has been demonstrated that VlsE is expressed in culture and in unfed ticks but only weakly in ticks 48 and 96 h after they have fed to repletion (5). This suggests that VlsE is downregulated during tick feeding and then upregulated upon establishment of infection in the mammalian host. Some of the VlsE antigenic test substrates used in this analysis differ significantly in the sequences of their variable regions. In spite of this, all variants were recognized by antibodies that developed in mice infected with different clones or isolates of *B. burgdorferi.* Antibodies that were immunoreactive with all VlsE variants were also detected in sera from mice infected with the relapsing fever spirochetes *B. hermsii*, *B. parkeri*, and *B. turicatae*. Serum samples from human Lyme disease patients also possessed anti-VlsE antibodies that were immunoreactive with all or most of the VlsE antigenic test substrates. These observations indicate that there are conserved epitopes among VlsE variants that are antigenic during infection in humans. These findings are consistent with studies by others who have demonstrated that most Lyme disease patients develop an anti-VlsE antibody response (6, 14). Lawrenz et al. employed r-VlsE in an ELISA format and demonstrated diagnostic sensitivities of 63% for culture-confirmed erythema migrans cases and 92% for late-stage infections (6). Using a peptide-based ELISA, Liang and Philipp demonstrated that invariable region 6 is antigenic in humans, monkeys, and mice infected with the Lyme disease spirochetes (12). This peptide-based ELISA was found to have diagnostic sensitivities of 100% in late-stage Lyme disease (14) and 74% in acute- or early-stage Lyme disease. These observations would seemingly argue against a role for VlsE in antigenic variation since conserved regions appear to be immunodominant.

While it is now clear that invariable region 6 of VlsE is immunodominant, until this report evidence for the impact of sequence changes in the variable regions on the antigenicity of the protein had not been provided. Through preabsorption of infection sera with VlsE variants, we were able to selectively and completely remove antibodies that target conserved segments of VlsE. These preabsorbed infection sera no longer carried antibodies that recognized the protein that was used to preabsorb but retained antibodies that recognized variants encoded by heterologous isolates or clones. We interpret the data as indicating that the remaining antibodies target variant-specific epitopes that reside within or are defined by the variable regions of VlsE. In either case it can be concluded that sequence changes within the variable regions alter the antigenic properties of VlsE.

While the data presented above suggest that it is possible that VlsE sequence variation could contribute to immune evasion, such a role is confounded by the immunodominant immune response to the invariable segments of the protein (8, 12). The robust antibody response to invariable segments of a protein presumably involved in antigenic variation is paradoxical and without precedent. For example, the antibody response to antigenic variation proteins in other organisms such as *Trypanosoma brucei*, *Anaplasma marginale*, and *B. hermsii* is directed solely at the immunodominant variable domains (1, 2, 4). If VlsE is involved in antigenic variation, then the questions are the following: what is the biological impact or significance of the antibody response to the conserved segments of the protein and what conditions lead to the development of this response? In a recent study it was demonstrated that invariable region 6 of VlsE does not serve as a target for a protective immune response and that antibodies to this region are not able to bind to intact unfixed spirochetes and are not bactericidal (10). The authors suggested that this could be due to inaccessibility of this region during natural infection. While this may be the case, it is difficult to rationalize the mechanism by which a strong humoral immune response to this domain of VlsE develops during infection. It is possible that the antibody response to invariable region 6 develops as a result of phagocytosis of cells followed by the presentation of epitopes that are otherwise not exposed during infection. However, in view of the strength of the observed IgG antibody response to VlsE during early infection, at a time when spirochete numbers are low, this possibility seems unlikely. In addition, a similar phenomenon has not been observed for other Lyme disease spirochete outer surface proteins. For example, an analysis of the antibody response to the naturally exposed epitopes of OspE and OspF has demonstrated that only certain domains are recognized during infection. An antibody response to naturally unexposed domains of these outer surface proteins was not found to develop in mice even after long-term infection (M. S. Metts, J. V. McDowell, K. McLeod-Saalih, and R. T. Marconi, unpublished data). Collectively the data indicate that the phenomenon alluded to above is unlikely to account for the observed early antibody response to the invariable regions. Here we raise an alternative hypothesis that warrants testing. It is our hypothesis that VlsE or VlsE fragments that originate from possible processing of the protein are secreted into the host environment. These polypeptides may then elicit the development of an antibody response to the invariable regions that would otherwise be hidden in the native, membrane-anchored protein. It is tempting to speculate that this could function as an immune decoy and represent an additional mechanism of immune evasion in the Lyme disease spirochetes.

While it is clear that sequence changes in the variable regions of VlsE alter the antigenic characteristics of this protein, the overall contribution of this process to immune evasion remains unclear. It had been suggested that as many as 10^{30} different *vls* variants could arise as a result of VlsE gene conversion (20). However, we demonstrated that 62% of the amino acid positions in the variable regions of these proteins are stable during infection and do not undergo sequence change (18). These observations indicate that the hypothetical

capacity for VlsE variation, while certainly significant, may be smaller than previously thought. We also demonstrated that the net number of sequence changes within the variable domain stabilizes after initial infection as a result of the occurrence of sequence reversions in the variable regions (18). This is perhaps a feature that would not be expected if the variable regions were key contributors to antigenic variation.

In conclusion, the data presented here demonstrate that the overall antibody response to VlsE is robust and develops early and that sequence changes in the variable regions alter the antigenicity of the VlsE protein. In future studies it will be important to determine if the antibody response to the variable regions is bactericidal and to decipher the biological significance of the robust antibody response to the in vivo-masked epitopes that reside within the invariable regions of VlsE.

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REFERENCES

1. **Barbet, A. F., and T. C. McGuire.** 1978. Crossreacting determinants in variant specific surface antigens of African trypanosomes. Proc. Natl. Acad. Sci. USA **75:**1989–1993.
2. **Borst, P., and G. A. M. Cross.** 1982. Molecular basis for trypanosome antigenic variation. Cell **29:**291–303.
3. **Centers for Disease Control and Prevention.** 1995. Recommendations for test performance and interpretation from the 2nd National Conference on Serologic Diagnosis of Lyme Disease. Morb. Mortal. Wkly. Rep. **44:**590–591.
4. **Hagblom, P., E. Segal, E. Billyard, and M. So.** 1985. Intragenic recombination leads to pilus antigenic variation in *Neisseria gonorrhoeae*. Proc. Natl. Acad. Sci. USA **315:**156–158.
5. **Indest, K. J., J. K. Howell, M. B. Jacobs, D. School-Meeker, S. J. Norris, and M. T. Philipp.** 2001. Analysis of *Borrelia burgdorferi vlsE* gene expression and recombination in the tick vector. Infect. Immun. **69:**7083–7090.
6. **Lawrenz, M. B., J. M. Hardham, R. T. Owens, J. Nowakowski, A. C. Steere, G. P. Wormser, and S. J. Norris.** 1999. Human antibody response to VlsE antigenic variation protein of *Borrelia burgdorferi*. J. Clin. Microbiol. **37:** 3997–4004.
7. **Liang, F. T., E. Aberer, M. Cinco, L. Gern, C. M. Hu, Y. N. Lobet, M. Ruscio, P. E. Voet, Jr., V. E. Weynants, and M. T. Philipp.** 2000. Antigenic conservation of an immunodominant invariable region of the VlsE lipoprotein among European pathogenic genospecies of *Borrelia burgdorferi* SL. J. Infect. Dis. **182:**1455–1462.
8. **Liang, F. T., A. L. Alvarez, Y. Gu, J. M. Nowling, R. Ramamoorthy, and M. T. Philipp.** 1999. An immunodominant conserved region within the variable domain of VlsE, the variable surface antigen of *Borrelia burgdorferi*. J. Immunol. **163:**5566–5573.
9. **Liang, F. T., L. C. Bowers, and M. T. Philipp.** 2001. C-terminal invariable domain of VlsE is immunodominant but its antigenicity is scarcely conserved among strains of Lyme disease spirochetes. Infect. Immun. **69:**3224–3231.
10. **Liang, F. T., M. B. Jacobs, and M. T. Philipp.** 2001. C-terminal invariable domain of VlsE may not serve as target for protective immune response against *Borrelia burgdorferi*. Infect. Immun. **69:**1337–1343.
11. **Liang, F. T., R. H. Jacobson, R. K. Straubinger, A. Grooters, and M. T. Philipp.** 2000. Characterization of a *Borrelia burgdorferi VlsE* invariable region useful in canine Lyme disease serodiagnosis by enzyme-linked immunosorbent assay. J. Clin. Microbiol. **38:**4160–4166.
12. **Liang, F. T., and M. T. Philipp.** 1999. Analysis of antibody response to invariable regions of VlsE, the variable surface antigen of *Borrelia burgdorferi*. Infect. Immun. **67:**6702–6706.
13. **Liang, F. T., and M. T. Philipp.** 2000. Epitope mapping of the immunodominant invariable region of *Borrelia burgdorferi* VlsE in three host species. J. Bacteriol. **68:**2349–2352.
14. **Liang, F. T., A. C. Steere, A. R. Marques, B. J. Johnson, J. N. Miller, and M. T. Philipp.** 1999. Sensitive and specific serodiagnosis of Lyme disease by enzyme-linked immunosorbent assay with a peptide based on an immunodominant conserved region of *Borrelia burgdorferi* VlsE. J. Clin. Microbiol. **37:**3990–3996.
15. **Lin, B., R. Noring, A. C. Steere, M. S. Klempner, and L. T. Hu.** 2000. Soluble CD14 levels in the serum, synovial fluid, and cerebrospinal fluid of patients with various stages of Lyme disease. J. Infect. Dis. **181:**1185–1188.
16. **McDowell, J. V., S.-Y. Sung, G. Price, and R. T. Marconi.** 2001. Demonstration of the genetic stability and temporal expression of select members of the Lyme disease spirochete OspF protein family during infection in mice. Infect. Immun. **69:**4831–4838.
17. **Sung, S. Y., J. McDowell, J. A. Carlyon, and R. T. Marconi.** 2000. Mutation and recombination in the upstream homology box flanked *ospE* related genes of the Lyme disease spirochetes results in the development of new antigenic variants during infection. Infect. Immun. **68:**1319–1327.
18. **Sung, S. Y., J. V. McDowell, and R. T. Marconi.** 2001. Evidence for the contribution of point mutations to *vlsE* variation and for apparent constraints on the net accumulation of sequence changes in *vlsE* during infection with the Lyme disease spirochetes. J. Bacteriol. **168:**5855–5861.
19. **Zhang, J. R., and S. J. Norris.** 1998. Genetic variation of the *Borrelia burgdorferi* gene *vlsE* involves cassette-specific, segmental gene conversion. Infect. Immun. **66:**3698–3704.
20. **Zhang, J. R., and S. J. Norris.** 1998. Kinetics and in vivo induction of genetic variation of *vlsE* in *Borrelia burgdorferi*. Infect. Immun. **66:**3689–3697.
21. **Zhang, J.-R., J. M. Hardham, A. G. Barbour, and A. G. Norris.** 1997. Antigenic variation in Lyme disease borreliae by promiscuous recombination of vmp-like sequence cassettes. Cell **89:**275–285.

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