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Dominant Epitopes of the C6 Diagnostic Peptide of *Borrelia burgdorferi* Are Largely Inaccessible to Antibody on the Parent VlsE Molecule▽

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Lyme borreliosis (LB) is a disease for which antibody-based detection assays are often required for diagnosis. The variable surface molecule VlsE and IR6, one of its invariable regions, are commonly targeted by the antibody response in infected individuals. A series of enzyme-linked immunosorbent assays was performed to comparatively examine the antibody responses of North American LB patients ($n = 37$) to VlsE and invariable segments of this molecule. Both immunoglobulin M (IgM) and IgG responses to full-length VlsE and to peptides reproducing invariable regions 2, 4, and 6, as well as the invariable domains at the amino and carboxyl termini of VlsE, were assessed. The proportions and specificities of reactivity to the invariable segments were tested by using cognate peptides as competitors for VlsE binding by patient serum antibodies. IR6 epitopes (by the C6 peptide) were found to dominate the response to invariable segments. IR6 (C6)-specific antibodies were detected in 78% of the serum specimens, whereas <40% of patients generated antibodies that bound the N- or C-terminal domain and <12% of patients responded to either IR2 or IR4. Interestingly, 15 of 37 patients generated IgG antibodies that reacted with C6 but not with VlsE. Conversely, IgM responses were frequent for VlsE but not for invariable segments. A representative number of the serum specimens ($n = 8$) that contained IgG antibodies reacting with both C6 and VlsE was assessed in competition experiments, using C6 as a competitor. Only half of these specimens contained IgG antibodies whose binding to VlsE could be inhibited >50% by competition with the added C6 peptide. The median percent inhibition was 45.5%. These findings indicate that IR6 epitopes are largely concealed from the VlsE molecular surface and that full-length VlsE-based diagnosis likely detects antibodies to conformational and/or variable region epitopes.

Infection with the spirochete *Borrelia burgdorferi* causes the multisystem disease known as Lyme borreliosis. The diagnosis of Lyme borreliosis is made by a combination of clinical observations and laboratory tests. In areas where Lyme disease is endemic, the presence of erythema migrans (EM), an expanding annular skin rash, is considered sufficient to diagnose early Lyme disease. When patients present with later manifestations of Lyme disease that are not specific, reliable laboratory tests are necessary to support the diagnosis (1). Ideally, infection would be confirmed by culture or PCR detection of *B. burgdorferi* in skin biopsy or blood specimens. In practice, these invasive or time-consuming methods are not sensitive enough for a negative result to rule out *B. burgdorferi* infection. Spirochete recovery from 2- to 4-mm skin biopsy samples of an EM lesion can be achieved, on average, for only 40 to 50% of untreated patients (1). Antibody detection is thus the most frequently used laboratory test to assist in the diagnosis of Lyme disease.

The variable surface protein VlsE is an immunogenic molecule of *B. burgdorferi* that engages in antigenic variation. Two invariable domains, one at the amino and the other at the carboxyl terminus, together encompass approximately one-half of the molecule's length. Antigenic variation occurs through gene conversion events that involve regions within the central domain (12). This domain contains six variable regions and six invariable regions (IRs), named IR1 to IR6. The six IRs remain unchanged during antigenic variation, and available sequence data indicate that they are conserved among *B. burgdorferi* sensu lato genospecies and strains (4, 14). The carboxyl- and amino-terminal domains of VlsE also remain invariant as infection proceeds (13).

In previous studies, the antibody responses to the IRs of VlsE in different host species were examined. Infected humans, monkeys, dogs, and mice either responded to IR6 and not to the other IRs or responded more vigorously to IR6 (7). Some individuals generated responses to peptides C2 and C4 (which comprise IR2 and IR4, respectively). In these studies, a limited selection of serum specimens from Lyme borreliosis patients was tested for immunoglobulin G (IgG) responses only. As with IR6, the C-terminal domain (Ct peptide) of VlsE was also immunodominant in these animal species (5), but this region's antigenicity was not as conserved as that of IR6 (5). Thus far, a systematic study of the relative contributions of the IRs and invariable domains of VlsE to the overall antigenicity of this protein has not been performed. In particular, the IgM response to invariable segments and the antigenicity of the N-terminal domain of VlsE have never been assessed.

Over the last 5 years, both the full-length VlsE molecule and the IR6 portion (the synthetic peptide C6) have emerged as diagnostic antigens in enzyme-linked immunosorbent assay (ELISA) tests that are comparatively sensitive and specific (2).

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TABLE 1. Sequences of VlsE-derived peptides used as antigens and competitors in ELISAs

VlsE region	Peptide name	Peptide sequence (length)
IR2	C2	GIAKGIKEIV EAA (13-mer)
IR4	C4	GDSEAASKAA GAVSAVSGEQ ILSAIV (26-mer)
IR6	C6	MKKDDQIAAA IALRGMAKDG KFAVK (25-mer)
C terminus	Ct	KAEGAIKGAA ESAVRKVLGA ITGLIGDAVS SGLRKVGDSV KAASKETPPA LNK (53-mer)
N terminus		
Positions 19 to 57	N1	CKSQVADKDD PTNKFYQSVI QLGNGFLDVF TSFGGLVAE (39-mer)
Positions 48 to 86	N2	FTSFGGLVAE AFGFKSDPKK SDVKTYFTTV AAKLEKTKT (39-mer)
Positions 76 to 114	N3	VAAKLEKTKT DLNSLPKEKS DISSTTGKPD STGSVGTAV (39-mer)

We hypothesized that the antigenicity of VlsE was largely concentrated on that of IR6, to the exclusion of other IRs and invariable domains of the molecule. This result could occur either because other invariant segments are simply not antigenic or because their epitopes are not accessible for antibody binding on the VlsE molecule. To test these possibilities, we set out to evaluate the antigenicity of VlsE in comparison to that of invariant segments known to be immunogenic in some individuals (7) and exposed on the VlsE surface, per available X-ray crystallography (3) and immunoprecipitation (6) data. We chose to test IR2, IR4, and IR6, represented by peptides C2, C4, and C6, respectively. Both the carboxyl and amino termini of VlsE, represented by peptide Ct and the triad of overlapping peptides N1 to N3, were also included in this study. To evaluate relative antigenicity among VlsE segments a reference Lyme disease human serum panel available from the Centers for Disease Control and Prevention (CDC) was used. Where possible, peptide antigen competition for binding of serum antibodies to full-length VlsE was assessed.

MATERIALS AND METHODS

Human serum. A panel of 42 serum samples, collected from 37 Lyme borreliosis patients and 5 healthy blood donors, was obtained from the CDC. All Lyme disease patients met the CDC surveillance case definition for national reporting of Lyme disease (11). Each patient presented either with EM or with at least one late manifestation (musculoskeletal, nervous, or cardiac) and laboratory confirmation of infection. Patients were categorized into the "early localized" disease group by the presence of a single EM, culture of *B. burgdorferi* from a skin biopsy specimen, and a time interval between onset and diagnosis of <2 months. Patients with "disseminated" disease, most commonly Lyme arthritis, were diagnosed clinically with serologic support for the diagnosis by two-tiered serology. The diagnosis of neuroborreliosis was supported in two instances by culture of *B. burgdorferi* from cerebrospinal fluid (see Table 2). Two-tiered serologic testing consisted of an initial ELISA (Lyme Screen II [LYT]; bioMérieux, Durham, NC) followed by immunoblotting (Lyme Disease Marblot, MarDx; Trinity Biotech, Carlsbad, CA), when appropriate. All patients were treated with antibiotics prior to blood donation for the samples used in this study.

Peptide ELISAs. All peptide-based ELISAs were performed in the same manner as that described previously (7). Peptides used for the following experiments (sequences are shown in Table 1; all were derived from VlsE of *B. burgdorferi* strain B31) consisted of free peptides and N-terminal biotin-conjugated peptides (Genemed Synthesis, South San Francisco, CA). Briefly, 96-well plates were coated with streptavidin and incubated overnight. Biotinylated peptides were added to plates in a blocking buffer of 5% nonfat dry milk in phosphate-buffered saline (PBS). Human serum was diluted 1:200 (with blocking buffer) and assayed for binding to the substrate-fixed peptides. Both IgG and IgM were detected, separately, with the horseradish peroxidase-conjugated secondary antibodies goat anti-human IgG (heavy plus light chains), at 0.1 μg/ml, and goat anti-human IgM (μ-chain specific), at 0.1 μg/ml for C2, C4, and C6 peptides and 0.067 μg/ml for Ct and Nt peptides (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The bound antibody was detected with the TMB microwell peroxidase substrate system (Kirkegaard & Perry Laboratories). Following the addition of a stop solution (1 M H_3PO_4), the optical density at 450 nm (OD_{450}) was determined for the contents of each well. Results presented are the averages for triplicate wells.

VlsE ELISAs. For VlsE ELISAs, recombinant VlsE, cloned from *B. burgdorferi* strain B31, expressed, and purified as described previously (2), was bound to 96-well plates overnight in 0.1 M Na_2HPO_4, pH 9.0, at 50 ng/well for IgG and 100 ng/well for IgM. Following a wash in PBS, blocking buffer (5% nonfat dry milk in PBS) was added and allowed to block each plate for 2 h. Patient serum was then added at a 1:200 dilution for IgG detection and a 1:100 dilution for IgM detection in 5% milk-PBS and incubated for 1 h at room temperature in a rotary shaker set at 150 rpm. After three washes in PBS, the goat anti-human secondary antibodies were added at 0.1 μg/ml. The detection procedure was the same as that for peptide ELISAs.

Negative control samples from healthy blood donors (NA1 to NA5) were assayed in each plate. All serum samples were run in triplicate. The cutoff OD value was calculated for each plate as the mean OD of the control serum specimens plus 3 standard deviations of that mean. Where possible, sera from previously tested patients (A21 and 2A) were included as positive controls.

Peptide competition for VlsE binding. The ability of individual peptides (C2, C4, C6, Ct, N1, N2, and N3) to compete for antibody binding to VlsE was evaluated in all patient samples that were positive for both anti-VlsE antibodies and antibodies against the peptide in question (IgG only). Recombinant VlsE was bound to 96-well plates overnight, and the plates were blocked for nonspecific binding of antibody as described above. Serum samples were diluted 1:200 in PBS and incubated for 1 h with peptides at the following final concentrations: 0, 10, 100, and 1,000 ng/well in a total volume of 100 μl. These mixtures were added directly to the blocked plates and incubated for 1 h. Following four PBS washes, the secondary antibodies were added and detected as in the VlsE ELISA. Equivalent concentrations of a mixture of unrelated peptides (simian immunodeficiency virus mac239 envelope peptides, each of 20 amino acids in length) were used as negative controls. The percent inhibition for each patient sample tested was calculated as follows: % inhibition = $(OD_0 - OD_{1000})/(OD_0 - OD_{c/avg})$, where OD_0 is the patient serum OD_{450} value with no peptide added, OD_{1000} is the patient serum OD_{450} value with 1,000 ng peptide/well, and $OD_{c/avg}$ is the combined average of OD_{450} values of serum specimens from each of the five healthy donors.

RESULTS

C6 epitopes dominate IgG responses to VlsE IRs and invariable domains. Lyme disease patient serum antibody reactivity to VlsE invariable segment epitopes was evaluated by ELISAs that utilize, as antigen, peptides that reproduce those invariant portions (Table 1), including Ct, a 52-mer peptide that encompasses the C-terminal domain; three overlapping 39-mers, N1, N2, and N3, that span the sequence of the N terminus; and C2, C4, and C6, which reproduce the sequences of the corresponding IRs.

A majority of patients (78%) tested IgG positive for C6 reactivity (Tables 2 and 3); a preponderance of anti-C6 reactivity was seen in samples from patients with disseminated infections compared with samples from patients with early localized disease. Markedly fewer patients (10.8% for C2 and 8.1% for C4) generated IgG antibodies against epitopes within the other IRs of VlsE. A smaller proportion of patients elicited

TABLE 2. ELISA values for Lyme disease patient serum samples tested for IgG reactivity to VlsE and VlsE-derived peptides

Patient	Diagnosis[a]	ELISA result (value) for diagnosis[b]	Immunoblot result[c]		IgG ELISA result (value)[b,d]							
			IgM	IgG	Ct	N1	N2	N3	C2	C4	C6	VlsE
NA6	LA	P (6.90)	N	P	**P (1.602)**	N (0.146)	**P (0.754)**	N (0.107)	N (0.119)	N (0.097)	**P (1.353)**	**P (0.275)**
NA7	Clinical	P (1.93)	N	P	N (0.157)	N (0.119)	N (0.144)	N (0.102)	N (0.069)	N (0.105)	**P (0.224)**	N (0.081)
NA8	Clinical	P (4.41)	P	P	**P (2.057)**	**P (0.803)**	N (0.097)	N (0.160)	N (0.103)	N (0.106)	**P (1.031)**	**P (0.319)**
NA9	EM, LA	P (5.75)	N	P	N (0.091)	N (0.127)	N (0.0890)	N (0.087)	N (0.152)	N (0.144)	**P (0.293)**	N (0.090)
NA10	LA	P (7.48)	N	P	**P (1.026)**	N (0.207)	**P (2.105)**	N (0.168)	N (0.241)	N (0.154)	**P (2.263)**	**P (1.450)**
NA11	Clinical	P (3.51)	N	P	**P (1.007)**	N (0.321)	**P (0.3)**	N (0.099)	N (0.135)	N (0.214)	**P (0.770)**	**P (0.440)**
NA12	EM, clinical	P (4.33)	P	P	**P (1.800)**	N (0.079)	N (0.076)	N (0.117)	N (0.096)	N (0.093)	**P (0.899)**	**P (0.639)**
NA13	LA	P (8.45)	P	P	**P (2.429)**	**P (0.433)**	**P (1.268)**	**P (1.495)**	**P (0.270)**	**P (0.271)**	**P (1.428)**	**P (1.605)**
NA14	LA	P (5.49)	N	P	N (0.174)	N (0.305)	**P (0.208)**	N (0.175)	N (0.239)	**P (0.258)**	**P (0.454)**	**P (0.192)**
NA15	EM, clinical	P (5.98)	P	P	**P (2.522)**	N (0.331)	**P (0.191)**	**P (2.086)**	N (0.15)	N (0.13)	**P (2.101)**	**P (2.136)**
NA16	LA	P (8.18)	N	P	**P (0.872)**	N (0.123)	**P (0.690)**	N (0.061)	N (0.105)	N (0.113)	**P (0.591)**	**P (0.207)**
NA17	LA	P (3.77)	N	P	N (0.077)	N (0.152)	N (0.123)	N (0.082)	N (0.123)	N (0.111)	**P (1.025)**	**P (0.220)**
NA20	CSF C+	P (5.82)	P	P	**P (2.241)**	**P (0.602)**	**P (0.802)**	**P (0.275)**	N (0.118)	N (0.116)	**P (1.458)**	**P (0.853)**
NA22	CSF C+	P (1.24)	N	P	N (0.133)	N (0.191)	N (0.094)	N (0.066)	N (0.106)	N (0.135)	**P (0.390)**	N (0.090)
NA18	EM, C+	P (2.58)	N	P	N (0.062)	N (0.233)	N (0.071)	N (0.064)	N (0.103)	N (0.088)	**P (0.684)**	N (0.123)
NA19	EM, C+	P (1.89)	P	P	N (0.092)	N (0.213)	N (0.110)	N (0.087)	**P (0.316)**	N (0.139)	**P (0.609)**	N (0.112)
NA21	EM, C+	P (3.87)	P	P	**P (1.661)**	E (0.420)	N (0.153)	**P (0.225)**	N (0.185)	N (0.171)	**P (1.464)**	**P (0.195)**
NA23	EM, C+	P (4.77)	P	P	N (0.180)	**P (0.486)**	N (0.101)	N (0.083)	N (0.147)	N (0.176)	**P (2.543)**	**P (1.045)**
NA24	EM, C+	P (3.91)	P	N	N (0.306)	N (0.256)	N (0.132)	N (0.127)	N (0.190)	N (0.158)	**P (1.993)**	**P (0.160)**
NA25	EM, C+	P (3.49)	P	N	N (0.154)	N (0.152)	N (0.103)	N (0.082)	N (0.149)	N (0.126)	**P (1.309)**	N (0.106)
NA26	EM, C+	P (2.80)	P	N	**P (0.935)**	**P (1.073)**	**P (0.507)**	**P (0.483)**	**P (0.485)**	**P (0.435)**	**P (1.025)**	N (0.150)
NA27	EM, C+	P (1.45)	P	N	N (0.121)	**P (0.460)**	N (0.120)	N (0.096)	**P (0.302)**	N (0.123)	**P (0.415)**	N (0.099)
NA28	EM, C+	P (2.27)	P	N	**P (0.665)**	N (0.281)	N (0.135)	N (0.109)	N (0.178)	N (0.168)	**P (1.054)**	N (0.137)
NA29	EM, C+	N (0.53)	P	N	N (0.109)	N (0.131)	N (0.094)	N (0.073)	N (0.117)	N (0.109)	**P (0.676)**	N (0.084)
NA30	EM, C+	P (1.75)	N	N	N (0.129)	N (0.186)	N (0.107)	N (0.075)	N (0.113)	N (0.104)	N (0.202)	N (0.106)
NA31	EM, C+	E (0.99)	P	N	N (0.102)	N (0.106)	N (0.067)	N (0.065)	N (0.092)	N (0.093)	N (0.159)	N (0.065)
NA32	EM, C+	E (0.95)	P	N	**P (0.766)**	N (0.289)	N (0.140)	N (0.125)	N (0.143)	N (0.142)	**P (0.425)**	N (0.117)
NA33	EM, C+	P (1.30)	N	N	N (0.226)	**P (0.547)**	N (0.150)	N (0.144)	N (0.202)	N (0.187)	N (0.238)	N (0.126)
NA34	EM, C+	P (1.58)	P	N	N (0.195)	N (0.133)	N (0.074)	N (0.082)	N (0.113)	N (0.143)	**P (1.153)**	N (0.110)
NA35	EM, C+	P (1.59)	P	N	N (0.102)	N (0.091)	N (0.065)	N (0.088)	N (0.1)	N (0.089)	**P (0.432)**	N (0.089)
NA36	Rash, C+	P (1.29)	N	N	N (0.183)	N (0.127)	N (0.088)	N (0.121)	N (0.131)	N (0.126)	N (0.180)	N (0.137)
NA37	EM, C+	N (0.18)	N	N	N (0.114)	N (0.233)	N (0.072)	N (0.075)	N (0.124)	N (0.135)	N (0.159)	N (0.073)
NA38	EM, C+	N (0.59)	N	N	N (0.407)	N (0.357)	N (0.153)	N (0.130)	N (0.173)	N (0.167)	**P (0.637)**	N (0.119)
NA39	EM, C+	N (0.05)	N	N	N (0.157)	N (0.098)	N (0.077)	N (0.081)	N (0.115)	N (0.116)	N (0.173)	N (0.087)
NA40	EM, C+	N (0.57)	N	N	N (0.212)	N (0.275)	N (0.077)	N (0.075)	N (0.102)	N (0.130)	**P (0.356)**	N (0.081)
NA41	EM, C+	N (0.58)	N	N	N (0.167)	N (0.186)	N (0.111)	N (0.129)	N (0.194)	N (0.183)	N (0.318)	N (0.133)
NA42	EM, C+	E (0.93)	N	N	N (0.096)	N (0.101)	N (0.109)	N (0.079)	N (0.116)	N (0.202)	N (0.270)	N (0.115)

[a] C+, culture positive; CSF, cerebrospinal fluid; LA, Lyme arthritis. Clinical diagnoses were made as described in Materials and Methods.
[b] P, positive; N, negative; E, equivocal (indeterminate). Bold values (positive) indicate samples with OD values greater than the mean plus 3 standard deviations of the negative control values.
[c] Lyme disease Marblot (MarDx).
[d] The values for control patients NA1 to NA5 were used to calculate the cutoff value (mean + 3 standard deviations). These were run in tandem on the ELISA plates with patient samples NA6 to NA31 or NA32 to NA42. The means + standard deviations (cutoff values) for these patients were as follows: for patients NA6 to NA31, 0.128 + 0.046 (0.265), 0.1842 + 0.079 (0.421), 0.096 + 0.024 (0.168), 0.116 + 0.025 (0.190), 0.150 + 0.039 (0.267), 0.126 + 0.032 (0.222), 0.115 + 0.036 (0.223), and 0.079 + 0.016 (0.127) for Ct, N1, N2, N3, C2, C4, C6, and VlsE, respectively; and for patients NA32 to NA42, 0.194 + 0.101 (0.497), 0.180 + 0.082 (0.425), 0.097 + 0.026 (0.173), 0.111 + 0.019 (0.168), 0.146 + 0.049 (0.292), 0.136 + 0.042 (0.260), 0.150 + 0.065 (0.344), and 0.085 + 0.025 (0.158) for Ct, N1, N2, N3, C2, C4, C6, and VlsE, respectively.

TABLE 3. Patient serum IgG reactivities to VlsE-derived peptides

Peptide	% of serum samples with IgG reactivity		
	Early localized disease[a]	Disseminated disease[b]	Total
Ct	17	64	35
N1	17	21	19
N2	4	57	24
N3	9	21	14
C2	13	7	11
C4	4	14	8
C6	65	100	78
VlsE	13	79	38

[a] Patients NA18, NA19, NA21, and NA23 to NA42.
[b] Patients NA6 to NA17, NA20, and NA22.

responses to other segments of VlsE. Serum specimens exhibited reactivity to Ct (35%) and N2 (27%), but the responses to the two other N-terminal peptides were less frequent (19% and 13.5% for N1 and N3, respectively). Early-localized-phase patient serum IgG was most frequently detected with C6, but 17% of these patients also generated antibodies to the N1 and Ct portions. Only one patient was C6 negative and N1 positive, so the impact of combining these peptides on diagnostic sensitivity would probably be negligible. In summary, among the selected invariable segments, C6 was found to detect responses in a majority of patients. A much smaller fraction of the patient population produced antibodies that were detectable by the C-terminal (35%) and N-terminal (N1 plus N2 plus N3 = 37.8%) peptides. Responses to C2 and C4 were negligible, with only a few patients producing antibodies detected by these peptides.

TABLE 4. Patient serum IgM reactivities to VlsE-derived peptides

Peptide	% of serum samples with IgM reactivity		
	Early localized disease	Disseminated disease	Total
Ct	9	7	8
N1	9	0	5
N2	4	0	3
N3	0	0	0
C2	0	0	0
C4	0	0	0
C6	9	0	5
VlsE	48	14	35

TABLE 5. Patient serum samples that tested positive for either IgG or IgM antibodies to VlsE and VlsE peptides

Peptide	% of serum samples with IgM or IgG reactivity		
	Early localized disease	Disseminated disease	Total
Ct	17	64	35
N1	22	21	22
N2	9	57	27
N3	9	21	14
C2	13	7	11
C4	4	14	8
C6	65	100	78
VlsE	48	86	62

Serum IgM responses to conserved segments of VlsE are uncommon. Very few patients generated IgM antibodies against epitopes in the tested portions of VlsE (Table 4). Even among those patients with early localized Lyme disease, not many serum samples exhibited positive reactivity to the invariable VlsE segments. The highest proportion of IgM-positive samples was for those antibodies directed against Ct, but even that percentage was low (8.11%). At the time of serum collection, no patient carried IgM antibodies at levels above the cutoff values derived from healthy blood donors which recognized the C2 or C4 IR or the N3 portion of the N-terminal domain. Only 1 or 2 of 37 patients had significant amounts of IgM antibodies against epitopes within the N2 and C6 or N1 peptide, respectively.

Serum responses to full-length VlsE include IgG and IgM isotypes. VlsE reactivity, in contrast, involved IgM responses in 35% of patients. Many of these were in the early localized phase. IgG responses to VlsE were present in only 38% of samples and were more frequent in disseminated-phase patients. Thus, unlike the antibody responses to invariant regions and domains, the IgG and IgM responses together had a combined effect on *Borrelia* exposure detection such that 62% of patients were either IgG or IgM positive for antibody to VlsE (Table 5). The sensitivity of detection for VlsE IgG and IgM combined, however, was significantly lower than that determined for IgG antibodies against C6 (78%) in this patient population (McNemar chi-square value of 6.32; df = 1; P = 0.012).

C6 and full-length VlsE reactivities lack concordance. The proportion of serum specimens with IgG antibodies that reacted with C6 (78%) was much higher than the fraction that reacted with full-length recombinant VlsE (38%). As many as 15 patients of the total of 37 possessed serum IgG antibodies that reacted with C6 and not with VlsE (Tables 2 and 3). Several of these patients (seven in total) exhibited no reactivity to full-length VlsE with either antibody isotype but were positive for anti-C6 IgG antibodies. Therefore, C6 must have epitopes that are frequently recognized by human patients but are not available for binding on VlsE, at least in the molecular structure adopted by VlsE in the ELISA. We explored the accessibility of C6 epitopes further with competition experiments.

C6 competition for VlsE reactivity varies from patient to patient. A group of eight serum specimens that reacted with both C6 and VlsE via IgG over a range of VlsE ELISA values was chosen. Increasing concentrations of C6 peptide were added to each of the serum specimens prior to performing the ELISAs, as described in Materials and Methods. Only half of these specimens contained IgG antibodies whose binding to VlsE could be inhibited more than 50% by competition with added C6 peptide. The median percent inhibition was 45.5%, with a range of 10.1% to 95.4% (Fig. 1). This disparity in antipeptide and anti-VlsE antibody reactivities in a considerable portion of patients was not unique to C6. In fact, no other IR/invariable domain peptide was able to compete for VlsE binding at a level above 20% inhibition in greater than half of the patients tested. Therefore, we show data only for competition with the C6 peptide. In five of nine patient samples, some level of inhibition of VlsE binding with the Ct peptide was observed, with a median inhibition of 22%. Only two of eight serum specimens were inhibited for binding to VlsE by the C2 peptide (13% for NA10 and 39% for NA20). Interestingly, only one patient (NA13) of those responsive to both VlsE and N-terminal peptides produced antibodies that were ~30% inhibited for VlsE binding; this applied to N1 and N3 but not N2. Thus, while several patients generated antibodies against these invariable portions, those antibodies comprised only a minor proportion of total VlsE reactivity.

The level of inhibition did not appear to directly correlate with relative OD values of peptide IgG versus VlsE IgG. For example, the highest level of inhibition with C6 (95.4%) was obtained with serum from patient NA10, which showed a high OD value for C6 IgG (2.263) and a high OD value for VlsE IgG (1.450) (Table 2). Patient NA13 serum also exhibited high OD values for both C6 and VlsE IgG (1.428 and 1.605, respectively), but this translated into a C6 inhibition of only 38.9%.

DISCUSSION

Over the past few years, the C6 test has come to the fore of Lyme disease diagnosis, either as a single test or in combination with other assays, such as immunoblotting (2, 8–10). To date, the remaining IRs and invariable domains have not been explored completely for their general antigenicity. The relative contributions of these portions and C6 to the overall VlsE response have likewise not been evaluated. The results obtained in this study reflect a snapshot of the antibodies that were present in an individual at the time that serum was collected. Furthermore, we tested for reactivity to an antigenic variation protein molecule, VlsE, by quantifying antibodies bound to a single recombinant variant of that molecule. Thus,

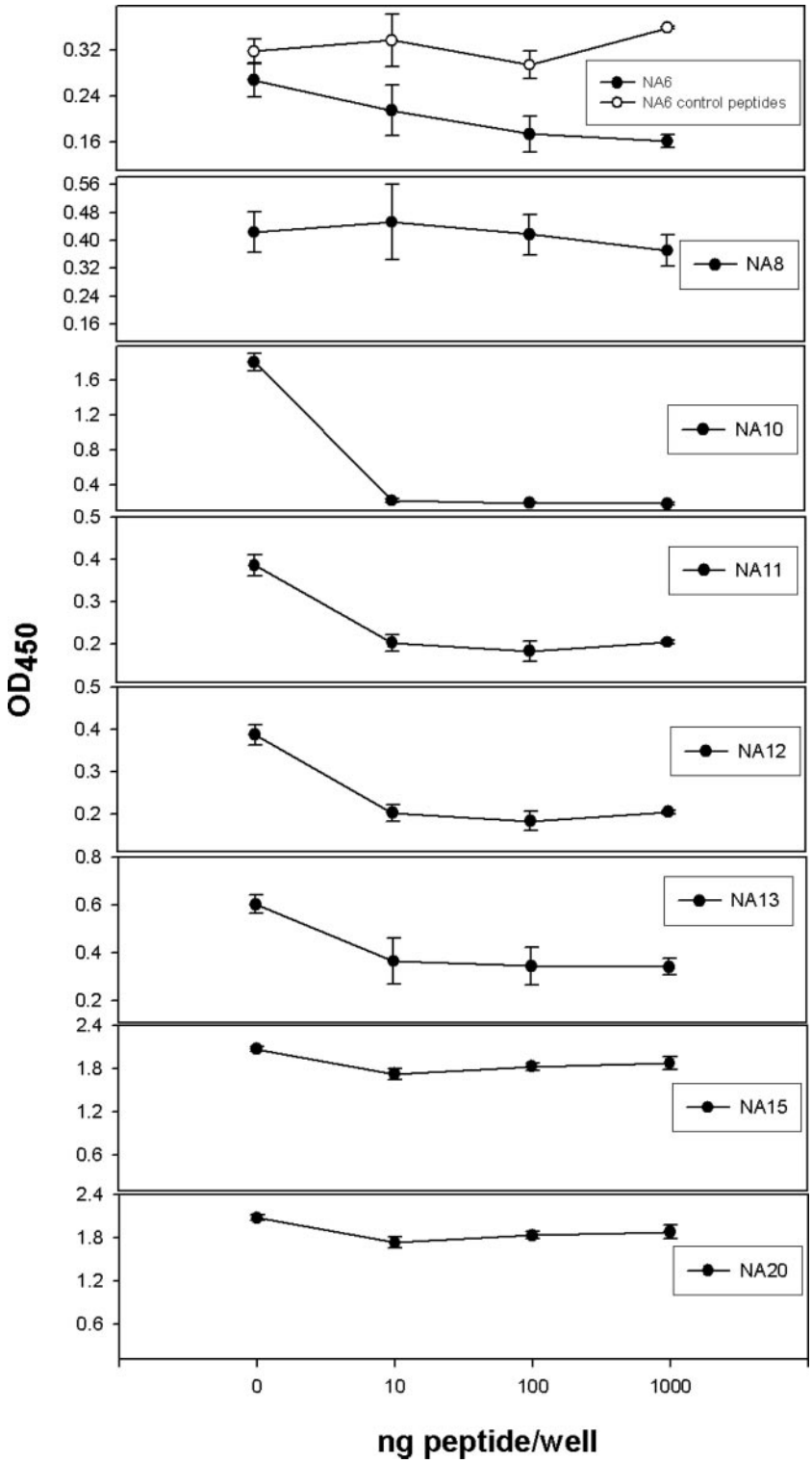

FIG. 1. Competition for VlsE binding with the C6 peptide. Individual serum samples were incubated with increasing concentrations of peptide for 1 h prior to their addition to VlsE antigen-coated 96-well plates. Shown are OD_{450} values obtained for each sample. Error bars represent the standard deviations of the means of triplicate determinations. The levels of inhibition with 1,000 ng C6 peptide/well added were as follows: NA6, 69%; NA8, 17%; NA10, 95%; NA11, 66%; NA12, 53%; NA13, 39%; NA15, 10%; and NA20, 15%.

the responses to variable regions not present in that variant would not have been detected. Despite these limitations, we have obtained relevant findings with respect to the invariable segments and VlsE antibody responses in humans.

In this report, we examined a panel of serum specimens from Lyme borreliosis patients for antibody responses to all of the invariable sections of VlsE either predicted or proven to be largely exposed on the surface of the VlsE molecule. With

respect to the IRs, C6 was found to elicit responses in a large fraction (78%) of patients, but responses to C2 and C4 were few. Of the eight patients who tested negative for C6 reactivity, seven did not show reactivity to any other portion of VlsE or the whole molecule itself. Each of these patients had culture-confirmed diagnosis, but serum collection may have been too early for the detection of specific antibodies to *B. burgdorferi* VlsE epitopes. Invariable domains at the VlsE termini were targeted by antibodies in a minority of the patient population, with 35% generating antibodies to the C-terminal domain and 37.8% generating antibodies to the N-terminal domain.

Responses to variable regions are likely more represented in the samples with detectable IgM against VlsE. Depending on the time of serum collection, the most recently encountered antigens will elicit IgM and may thus be more prevalent in early-localized-phase patients. We did observe VlsE-specific IgM more frequently in these patients but must point out that VlsE variant antigens resulting from recombination can arise throughout persistent infection, thus possibly resulting in continuous generation of anti-VlsE IgM antibodies. Among invariable segments of VlsE, only Ct elicited appreciable, yet meager (8.1% of patients), IgM responses. Presumably, because these are invariant regions and IgG titers already exist, the initial levels of IgM may have already declined due to isotype switching.

The IR2, IR4, and IR6 fragments are suitably exposed on the surface of the VlsE lipoprotein such that antibodies that bind those epitopes can immunoprecipitate VlsE (4, 6). X-ray crystallography of VlsE indicates that the IR6 region exhibits limited surface exposure. Specifically, only 13.7% of this region's theoretical surface area is predicted to be solvent exposed, whereas 35.8% of the IR4 surface area is exposed (3). We thus expected that a significant proportion of antibodies from patients that bound the C4 peptide would be able to compete for VlsE binding in the competition assay. We did not find this to be the case for C2 or C4, and competition with the C6 peptide reduced VlsE reactivity, with a median inhibition among patients of 45.5%. The inhibition with C6 peptide varied substantially from patient to patient. These results suggest that (i) only a moderate proportion of the antibodies that recognize invariable segment peptide epitopes bind to the full-length plate-bound VlsE molecule and/or (ii) these epitopes are so subdominant compared to VlsE that their cognate antibodies' competition for binding is masked. While both explanations may apply to the C2 and C4 segments, the immunodominance of C6 implies the former.

Patient serum antibody reactivities to invariable segment peptides, specifically C6, were compared to full-length VlsE molecule reactivities. Of the 29 patients who were positive for IgG antibodies to the IR6 component of VlsE, 15 (52%) were negative for full-length VlsE reactivity. We can thus conclude that humans generate antibodies to epitopes of C6 that are not exposed on the molecular surface of VlsE, at least as VlsE exists in the ELISA.

The outcome of the competition experiments further supports the concept that during *B. burgdorferi* infection in humans, VlsE antigen processing leads to an anti-C6 response that, in many patients, involves epitopes that are not accessible to binding on the VlsE molecule. To conclude, in testing all plausibly exposed invariable portions of VlsE for host antibody responses, we found reactivity to C6 to be the most common by far. This was followed by that to the much larger C- and N-terminal domains, which reacted with fewer than half of the specimens. Our most interesting finding is the lack of concordance between C6 and VlsE (IgG and IgM) reactivities. The diagnostic sensitivities of the two assays with this patient population were significantly different, at 78% versus 62%, respectively, and the epitopes involved are most frequently not the same.

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