An Immunodominant Conserved Region Within the Variable Domain of VlsE, the Variable Surface Antigen of *Borrelia burgdorferi*[1]

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Antigenic variation is an effective strategy evolved by pathogenic microbes to avoid immune destruction. Variable Ags such as the variable major protein of *Borrelia hermsii*, the variant surface glycoprotein of African trypanosomes, and the pilin of *Neisseria gonorrhoeae* include an immunodominant variable domain and one or more invariable domains that are not antigenic. Short, nonantigenic, invariable regions also may be present within the variable domain. VlsE (variable major protein-like sequence, expressed), the variable surface Ag of *Borrelia burgdorferi*, the Lyme disease spirochete, also contains both variable and invariable domains. In addition, interspersed within the VlsE variable domain there are six invariable regions (IR_{1-6}) that together amount to half of this portion's primary structure. We show here that these IRs are conserved among strains and genospecies of the *B. burgdorferi* sensu lato complex. Surprisingly, unlike the invariable regions of variable major protein, variant surface glycoprotein, and pilin, which are not antigenic in natural infections, the most conserved of the IRs, IR_6, is immunodominant in Lyme disease patients and in monkeys infected with *B. burgdorferi*. IR_6 is exposed on the surface of VlsE, as assessed by immunoprecipitation experiments, but is inaccessible to Ab on the spirochete's outer membrane, as demonstrated by immunofluorescence and in vitro killing assays. VlsE thus significantly departs from the antigenic variation paradigm, whereby immunodominance is only manifest in variable portions. We submit that IR_6 may act as a decoy epitope(s) and contribute to divert the Ab response from other, perhaps protective regions of VlsE. *The Journal of Immunology,* **1999, 163: 5566–5573.**

To maintain chronic infection, parasitic microbes have evolved strategies to avoid or suppress the host immune response. One of the most effective evasive strategies is antigenic variation. Several pathogenic organisms, including species of protozoa and bacteria, undergo antigenic variation. The African trypanosome *Trypanosoma brucei* evades the immune response by successively expressing more than 100 antigenically distinct variant surface glycoprotein molecules (VSG)[3] (1, 2). The VSG appears to be the only antigenic structure exposed on the surface of the organism (3). The bacterium *Neisseria gonorrhoeae* avoids immune attack by activating silent variable pilin genes (4). Pilin is a major component of the pilus, a predominant surface structure of *N. gonorrhoeae* that allows adhesion of the bacterium to various host cells (5). Relapsing spirochetemia and fever occur when the spirochete *Borrelia hermsii* alternates expression of at least 25 different antigenic forms of a surface-exposed and abundant lipoprotein called variable major protein (Vmp) (6, 7).

Borrelia burgdorferi, the spirochete that causes Lyme disease, expresses a surface lipoprotein, VlsE (Vmp-like sequence, expressed), that also undergoes antigenic variation (8). In the B31 strain of *B. burgdorferi*, this lipoprotein has a predicted molecular mass of 34 kDa and is expressed from a site (*vlsE*) that is located near the right telomere of the linear plasmid lp28-1 (8). The sequences of the 5′- and 3′-end segments of the *vlsE*-coding region, each segment encompassing less than one-third of this molecule's length, are preserved during the course of *vlsE* variation (9). The central portion, namely the cassette segment, which encodes the whole variable domain, recombines unidirectionally with 15 similar cassette segments that are located upstream (8, 9) from the *vlsE* locus. The unidirectional recombination events that result in VlsE antigenic variation only affect six regions of the VlsE variable domain (8). These six variable regions are ~16, 22, 6, 18, 13, and 6 aa in length, respectively (9). The remainder of the VlsE variable domain, which is composed of invariable regions of about 9, 16, 7, 25, 10, and 26 aa, remains essentially unchanged during the process of antigenic variation (9).

The VSG, pilin, and Vmp each can be divided also into invariable and variable domains (10–12). As with VlsE, small invariable regions are scattered within the latter domains. Although the variable domains are highly immunogenic and serve as the major target of the host immune response (13), the small invariable regions within them as well as the larger invariable domains are not antigenic in natural infections and tend to be conserved among different isolates of the organism (10, 11). Conserved portions are probably responsible for the maintenance of a functional molecular conformation (10, 11). Abs directed to the variable domains are able to effectively kill the organisms that express the corresponding Ags. However, the organisms escape killing by successively expressing different variable domains. Pathogens thus use these variable Ags to divert the host immune response from other Ags that may be protective (13).

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[3] Abbreviations used in this paper: VSG, variant surface glycoprotein; Vmp, variable major protein; VlsE, Vmp-like sequence, expressed; PI, postinfection; CDC, Centers for Disease Control and Prevention; Ospa, outer surface protein A.

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Nothing is known about the functional or antigenic properties of the invariable regions within the variable domain of the VlsE. As we analyzed the Ab response to a recombinant protein (P7-1), encoded by a cassette segment of the *vls* locus of the IP90 strain of *Borrelia garinii*, we made the following observation. In rhesus monkeys that had been infected with spirochetes of either the JD1 or the B31 strain of *B. burgdorferi* sensu stricto, Ab to P7-1 appeared within the first 3 wk postinfection (PI) and persisted thereafter, at least until wk 10 PI, the longest time point examined in this initial experiment. How could an Ag fragment so promiscuously and rapidly altered as the variable domain of VlsE (8, 14) elicit such a long-lasting Ab response to a cassette segment from another genospecies? A straightforward answer to this question was that one or more of the invariable regions within the variable domain are antigenic themselves and sufficiently conserved among *Borrelia* strains and genospecies to retain antigenic cross-reactivity.

To investigate this possibility, we analyzed the antigenicity of the invariable regions of the IP90 cassette segment using standard computer algorithms and determined the percent identity of their amino acid sequences with the corresponding invariable regions of published VlsE cassette segments of *B. burgdorferi* sensu stricto strains B31 (8) and 297 (15). One of the invariable regions (IR_s), namely IR_6, showed both the highest levels of antigenicity by the Hopp-Woods algorithm (16) and amino acid sequence identity among *Borrelia* genospecies and strains. We synthesized a peptide (C_6) that encompassed the amino acid sequence of IR_6 and assessed its antigenicity by ELISA in *B. burgdorferi*-infected mice, monkeys, and humans. We also performed antigenic competition experiments, using C_6 as competitor, to determine what proportion of the mouse, monkey, and human Ab response to P7-1 was directed to IR_6. Exposure of IR_6 on the surface of the VlsE molecule was determined by immunoprecipitation of native VlsE of IP90 with a rabbit antiserum raised against the C_6 peptide. Exposure of IR_6 on the spirochetal surface was assessed by direct immunofluorescence with FITC-labeled anti-C_6 Ab and by Ab-dependent, in vitro killing experiments using anti-C_6 Ab and IP90 spirochetes. Here we report the results of these studies.

Materials and Methods

Animals, animal infections, and spirochetal strains

Rhesus monkeys (2–4 years old, *Macaca mulatta*) were infected by the bite of *Ixodes scapularis* nymphal ticks that were themselves infected with *B. burgdorferi* sensu stricto strain JD1 or B31. Mice (6–8 wk old, C3H/HeN, The Jackson Laboratory, Bar Harbor, ME) were infected with *B. burgdorferi* sensu stricto strain Sh-2-82 (low passage, a gift from Denee Thomas, University of Texas Health Science Center, San Antonio, TX) by s.c. needle inoculation with 1×10^8 spirochetes administered in 1 ml of BSK-H medium (Sigma, St. Louis, MO) or by the bite of B31-infected *Ixodes scapularis* nymphal ticks. *B. garinii* strain IP90 (low passage) and *B. burgdorferi* sensu stricto strain HB19 (high passage, lacking the lp28-1 plasmid encoding VlsE) were originally obtained from the Centers for Disease Control and Prevention (CDC; Fort Collins, CO). When required, spirochetes were cultivated in BSK-H medium as described previously (17).

Cloning, sequencing, and expression of the 7-1 cassette segment of IP90

A library of randomly sheared total DNA from *B. garinii* IP90 was constructed in the λZAP II bacteriophage vector (Stratagene, La Jolla, CA) following a procedure described previously (18). The library was screened with a pool of plasma collected from rhesus monkeys within the first 10 wk after tick inoculation with *B. burgdorferi* JD1. On immunoblots of whole cell extracts of *B. garinii* IP90 this plasma pool reacted strongly only with three components, namely flagellin, an unidentified 60-kDa protein, and an Ag that was the IP90 homologue of the 34-kDa VlsE of B31 (8). After several rounds of screening, 11 clones were rescued into the pBluescript phagemid (Stratagene), and the recombinant plasmids were purified and used to transform cells of the SURE strain of *E. coli* (Stratagene). Several transformants were selected from each original clone, the presence of the insert was confirmed, and one such transformant from each clone was grown, induced for expression, lysed, and analyzed by immunoblot analysis with the original plasma pool. One of the 11 cloned fragments (named 7-1) hybridized to all others by dot-blot hybridization. This fragment was selected for overexpression and purification on the basis of the strong reactivity of the expressed protein with the plasma Abs. Sequencing of the 7-1 insert was performed using standard procedures (19) after generating nested *Bal*31 deletions and subcloning the partially deleted fragments. Ab from the original plasma pool that was affinity purified using the recombinant protein (P7-1) expressed by clone 7-1 as immunoabsorbant reacted with the putative IP90 VlsE on immunoblot of *B. garinii* lysates. The 7-1 insert was subcloned into the pQE expression system for overexpression and purification of the polypeptide (Qiagen, Chatsworth, CA). The protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA) with BSA as a standard.

P7-1 ELISA

This protocol was initially used to monitor monkey Ab responses to P7-1. Ninety-six-well ELISA plates (Corning, Corning, NY) were coated with 100 μl/well of a solution of P7-1 at 0.1 μg/ml in coating buffer (0.1 M carbonate buffer, pH 9.2) at 4°C overnight. Plates were blocked with 200 μl/well of 5% FCS in PBS/T (10 mM sodium phosphate, 150 mM NaCl, and 0.1% Tween 20, pH 7.4) for 1 h at room temperature. After washing three times with PBS/T, 50 μl/well of serum samples from *B. burgdorferi*-infected monkeys diluted 1/100 in 5% FCS in PBS/T were incubated for 1 h at 37°C. Plates were washed and incubated with 50 μl of 1) a mixture of biotinylated goat anti-human IgG (γ-chain-specific) and IgM (μ-chain-specific) Abs at the dilution recommended by the manufacturer (Vector Laboratories, Burlingame, CA) for 1 h at 37°C, 2) avidin-HRP complex also at the dilution recommended by the manufacturer (Vector) for 30 min at 37°C, and 3) a solution containing 2 g/l ortho-phenylenediamine and 0.03% hydrogen peroxide (both from Sigma) in 0.1 M citrate-sodium-phosphate buffer, pH 5.0, for 10 min at room temperature. The reaction was stopped with 50 μl of 4 N H_2SO_4. The OD at 490 nm was determined. Goat anti-human IgG and IgM cross-react with the corresponding monkey Igs.

Identification of conserved sequences and prediction of antigenicity of invariable regions of the VlsE variable domain

The deduced amino acid sequence of P7-1 was compared with sequences available in the GenBank database (National Center for Biotechnology Information, Rockville, MD) using the BlastP algorithm (20). The antigenicity of the entire P7-1 polypeptide was analyzed using the Hopp-Woods scale (16), and identities of invariable regions of P7-1 with homologous cassette segments from strains B31 and 297 were calculated after aligning the cassette segments using the pam250 algorithm and MacVector 5.0 computer software (Eastman Kodak, New Haven, CT).

Peptide synthesis and conjugation to biotin

A 25-mer peptide (C_6 = MKKDDQIAAAMVLRGMAKDGQFALK) and a 19-mer (C_2 = DAASVNGIAKGIKGIVDAA) were prepared using the fluorenylmethoxycarbonyl synthesis protocol (21). N-terminal biotinylation was performed by the maleimide carboxylate method. The maleimide reagent was obtained from Molecular Probes (Eugene, OR), and the protocol suggested by the manufacturer was followed.

Human serum samples

A panel of 41 human serum samples was provided by the CDC. All samples were collected from Lyme disease patients who had signs and symptoms that satisfied the CDC clinical case definition (22). Four serum samples from chronic Lyme disease patients were obtained from the National Institutes of Health. Ninety-seven serum samples obtained from hospitalized patients in an area not endemic for Lyme disease were used as negative controls.

C_6 peptide ELISA

Ninety-six-well ELISA plates were coated with 100 μl/well of 4 μg/ml streptavidin (Pierce, Rockford, IL) in coating buffer and incubated at 4°C overnight. The remaining steps were conducted in a rotatory shaker at room temperature. After two 3-min washes with 200 μl/well of PBS/T at 200 rpm, 200 μl of 5 μg/ml biotinylated peptide dissolved in blocking solution (PBS/T supplemented with 5% nonfat dry milk (Carnation, Nestle Food, Glendale, CA)) was applied to each well. The plate was shaken at 150 rpm for 2 h. After three washes with PBS/T as described above, 50 μl of serum (mouse, monkey, or human) diluted 1/200 with blocking solution was

added to each well. The plate was incubated at 150 rpm for 1 h and then washed three times with PBS/T. Each well then received 100 μl of 0.2 μg/ml goat anti-monkey IgG (γ-chain specific; Kirkegaard & Perry Laboratories, Gaithersburg, MD), 0.5 μg/ml anti-mouse IgG (heavy and light chain specific; Sigma), or 0.1 μg/ml anti-human IgG (heavy and light chain specific; Pierce), each conjugated to HRP and dissolved in blocking solution. The plate was incubated for 1 h while shaking. After four washes with PBS/T, each for 3–6 min, the Ag-Ab reaction was probed using the TMB Microwell Peroxidase Substrate System (Kirkegaard & Perry), and color was allowed to develop for 10 min. The enzyme reaction was stopped by addition of 100 μl of 1 M H_3PO_4. OD was measured at 450 nm.

Competitive ELISA

Ninety-six-well ELISA plates were coated with 100 μl/well of 0.3 μg/ml of purified recombinant P7-1 dissolved in coating buffer at 4°C overnight. After blocking with blocking solution, 25 μl of this solution with 0, 1, 4, 16, 64, 256, or 1024 ng of the C_6 or C_2 peptides was added to each well. A 25-μl volume of a 1/100 dilution of the appropriate serum in blocking solution also was added to each well. The remaining steps were performed as related in the section describing the C_6 peptide ELISA.

Preparation of rabbit anti-C_6 peptide antiserum

The C_6 peptide was covalently linked to keyhole limpet hemocyanin by the *N*-succinimidyl maleimide carboxylate method after adding a cysteine residue to the peptide's N-terminus. The maleimide reagent was obtained from Molecular Probes (Eugene, OR), and the protocol suggested by the manufacturer was followed. Six-month-old New Zealand White rabbits were given three injections at biweekly intervals of 200 μg of conjugated Ag emulsified with CFA (first injection) or IFA (remaining injections). Ten days after the last injection the Ab titer was determined by the peptide ELISA and immunoblot analysis using IP90 spirochete whole cell lysates as Ag.

Immunoprecipitation and immunoblot

Immunoprecipitation was conducted at 4°C. About 1.5×10^{10} IP90 spirochetes harvested at stationary growth phase were extracted in 4.5 ml of solubilization buffer (50 mM Tris-HCl, 1% Triton X-100, and 1 mM EDTA, pH 7.6) for 30 min. The mixture was centrifuged at 13,000 × *g* for 30 min, and the supernatant was collected. Each 1.5 ml of this supernatant was mixed with 30 μl of preimmune or immune rabbit serum and incubated for 30 min. Fifty microliters of drained ImmunoPure Immobilized Protein G (Pierce) pre-equilibrated in solubilization buffer was then added and allowed to incubate for an additional 30 min. After washing the gel twice with excess volumes of this buffer by centrifugation at 3000 × *g* for 20 min, 150 μl of nonreducing SDS-PAGE sample buffer (125 mM Tris-HCl, 3% SDS, and 20% glycerol, pH 6.8) was added. The suspension was incubated at room temperature for 30 min and then centrifuged at 16,000 × *g* for 30 min. Ten microliters of supernatant was loaded onto each of 10 lanes of a SDS-12% polyacrylamide minigel. Separated proteins were electrotransferred to nitrocellulose in Towbin transfer buffer. After incubating in blocking solution for 2 h, the blot was incubated for 1 h in rabbit anti-C_6 serum diluted 1/2,000 with blocking solution. After three washes with PBS/T, the blot was incubated in blocking solution with 0.5 μg/ml goat anti-rabbit IgG conjugated to HRP (Pierce) for 1 h. The Ag-Ab reaction was probed in PBS/T supplemented with 0.05% 4-chloro-naphthol (Sigma), 0.015% hydrogen peroxide, and 17% methanol.

Direct immunofluorescence

Rabbit IgG was purified from preimmune or immune serum using conventional ammonium sulfate precipitation. The purity and concentration of IgG preparations were assessed using SDS-PAGE and the Bio-Rad protein assay kit, respectively. Purified IgG was conjugated to FITC according to the manufacturer's instruction (Pierce). For labeling, spirochetes were either unfixed or acetone fixed. For the fluorescent labeling of unfixed spirochetes, $\sim 10^8$ spirochetes that were harvested from 1.0 ml of IP90 or HB19 culture grown to stationary phase by centrifugation at 4,000 × *g* for 20 min were gently resuspended in 100 μl of PBS supplemented with 2 μg of rabbit anti-C_6 IgG-FITC conjugate, and incubated for 1 h. After two washes with excess volumes of PBS by centrifugation at 16,000 × *g* for 5 min, spirochetes were resuspended in 100 μl of PBS, applied to microscope slides, and counted under both darkfield and fluorescent microscopes. The ratio of fluorescent to total spirochetes was thus calculated. The same procedure was performed on acetone-fixed spirochetes. For this purpose, organisms harvested from 1.0 ml of culture fluid were suspended in 1.5 ml of acetone, incubated for 20 min, and then centrifuged at 16,000 × *g* for 5 min. After one wash with PBS, the fixed spirochetes were stained with fluorescent anti-C_6 Ab as described for unfixed spirochetes. Goat anti-*B. burgdorferi* Ab FITC conjugate (Kirkegaard & Perry Laboratories) was used as a positive control.

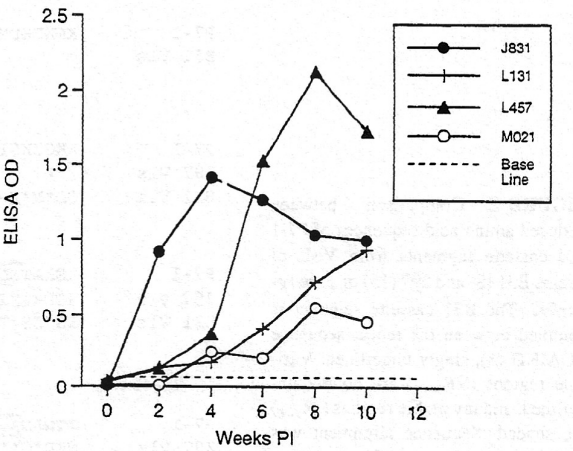

FIGURE 1. Ab response to P7-1 in monkeys. Serum samples from two rhesus macaques that were infected with the JD1 strain of *B. burgdorferi* (J831 and L131) and two that were infected with B31 strain spirochetes (L457 and M021) were serially collected. The Ab level was measured by ELISA using P7-1 as Ag. The baseline represents the mean ELISA OD of serum samples collected from all of the animals before infection + 3 SD.

In vitro Ab killing assay

As a source of complement, serum samples were collected from normal rhesus macaques, pooled, and stored in small aliquots at −70°C until used. Serum chosen for this purpose did not contain cross-reactive anti-*B. burgdorferi* Abs as determined by immunoblot analysis using whole cell lysates of *B. burgdorferi* as Ag. To perform the killing assay, spirochetes were cultured in BSK-H medium until they reached the mid-logarithmic phase ($\sim 2 \times 10^7$ cells/ml). A total of $\sim 5 \times 10^5$ spirochetes in 25 μl of BSK-H medium was added to each well of a 96-well plate (Corning). A volume of 50 μl of heat-inactivated (56°C, 30 min) serum sample appropriately diluted in the same medium was already dispensed in each well. The plate was incubated at 34°C for 30 min before the addition of 25 μl of complement preparation (normal monkey serum). After 24 h of incubation at 34°C in a humidified atmosphere of 3% CO_2, 5% O_2, and the balance of N_2, 5 μl of each sample was removed, and dead (nonmotile) and live (motile) spirochetes were counted under a darkfield microscope. Monkey anti-outer surface protein A (OspA) antiserum was used as a positive control (23).

Results

The invariable regions of the VlsE variable domain are conserved among strains and genospecies of B. burgdorferi

As we assessed the Ab response to the recombinant P7-1 polypeptide in monkeys that had been tick inoculated with either B31 or JD1 *B. burgdorferi* spirochetes, we noticed that the response was detectable within the first 3 wk PI, and that it persisted at least until wk 10 PI, the longest time point measured in this initial experiment (Fig. 1). We hypothesized that one or more of the invariable regions within the P7-1 variable domain were antigenic themselves and sufficiently conserved among *Borrelia* strains and genospecies to exhibit antigenic cross-reactivity. The deduced amino acid sequence of the *B. garinii* P7-1 recombinant polypeptide is shown in Fig. 2. It is depicted aligned with sequences from VlsE cassette segments of *B. burgdorferi* strains B31 (8) and 297 (15). In B31, the cassette segment is comprised between the repeats EGAIKG (Fig. 2) (8). The six variable regions of the B31 VlsE cassette segment (8) are doubly underlined. The invariable regions IR_{1-6} of the IP90 VlsE variable domain (Fig. 2) are clearly conserved across both genospecies and strains of *B. burgdorferi* sensu lato. With the exception of IR_1, which is 78% conserved in strain B31

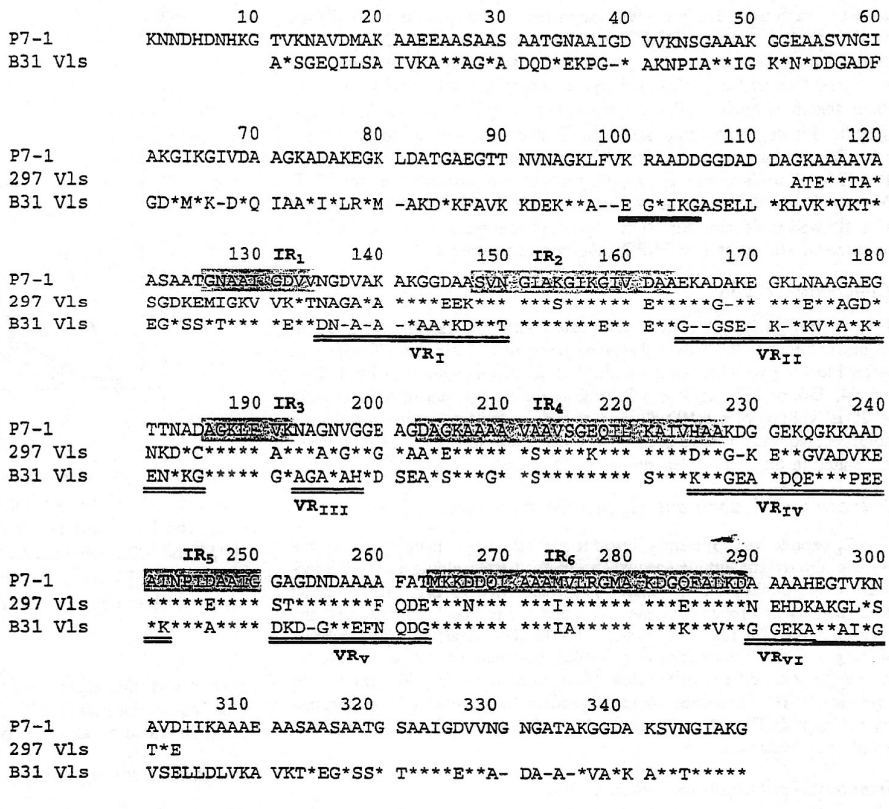

FIGURE 2. Comparison between deduced amino acid sequences of P7-1 and cassette segments from VlsE of strains B31 (8) and 297 (15) of *B. burgdorferi*. The B31 cassette segment is confined between the repeat sequence EGAIKG (8), singly underlined. Variable regions (VR_{I-VI}) are doubly underlined, and invariable regions (IR_{1-6}) are shaded. Sequence alignment was obtained with the pam250 algorithm. Identical amino acids are indicated with asterisks, and sequence gaps are shown with dashes; nonidentical residues are shown as letters following the single letter code.

and only 11% conserved in 297, all other invariable regions are between 80–90% conserved with respect to the P7-1 VlsE cassette segment of IP90 (Table I).

The antigenicity of the invariable regions of P7-1 was assessed with the Hopp-Woods hydrophilicity algorithm (16). Reputedly, this method has been more successful than similar ones for identifying protein antigenic determinants (24). Overall, the invariable regions of the IP90 variable domain showed negative hydrophilicity values. Sequences with positive values were composed of fewer than six amino acids and thus were not likely to configure antigenic sites per se. In contrast, IR_6 contained six or more contiguous amino acids with relatively high positive hydrophilicity values (1.7). IR_6 was also slightly more conserved than other invariable regions, with a mean identity of 87% when using the IP90 sequence as the reference strain (Table I).

Experimental assessment of the antigenicity of IR_6.

To evaluate experimentally the antigenicity of IR_6, we synthesized a peptide (C_6) whose primary structure encompassed that of IR_6. Serum samples from blood obtained from 10 rhesus macaques that were infected either with JD1 (six animals) or B31 spirochetes were used to examine the antigenicity of IR_6 in monkeys. Anti-C_6 ELISA Ab levels shown in Fig. 3*A* were present in serum samples at 4–6 wk PI and remained as high or higher for up to 3 yr PI (data not shown).

Mice also responded vigorously to this region. Serum samples collected 4–6 wk PI from 10 mice infected with either *B. burgdorferi* strain Sh-2-82 (six animals) or B31 showed high levels of anti-C_6 Abs (Fig. 3*B*). This result further confirmed the antigenicity of IR_6 and reaffirmed this region's antigenic conservation.

In humans, the antigenicity of IR_6 was examined with the aid of a panel of 41 serum samples provided by the CDC. Ab directed to C_6 was found in 35 of the 41 samples (Table II). The result of this experiment also underscores both the antigenicity and the antigenic conservation of IR_6, as the serum specimens in the panel stemmed from several Lyme disease endemic areas in the United States and must therefore contain Abs to multiple strains of *B. burgdorferi*.

Mouse and monkey sera obtained both before and after *B. burgdorferi* infection were also reacted with an ELISA plate coated with streptavidin alone, with no C_6 peptide added. No difference between the reactivity of the samples collected before and after infection was detected (not shown), thus precluding that the anti-C_6 reactivity observed was nonspecific.

IR_6 may be the only immunodominant invariable region in both monkeys and humans

In addition to IR_6, the VlsE variable domain contains five other invariable regions (Fig. 2). To determine whether any of these regions is antigenic, a competitive ELISA was performed, using P7-1 as Ag attached to an ELISA plate, in the presence of increasing concentrations of the C_6 or C_2 peptides. The sequence of C_2, a 19 mer, was based on that of IR_2, plus three N-terminal amino acids (DAASVNGIAKGIKGIVDAA). P7-1 includes invariable

Table I. *Identity between invariable regions from VlsE cassette segments of* B. burgdorferi *strains B31 and 297 and* B. garinii *IP90 (P7-1)*

Invariable Region	Percent Identity (%)		
	B31 vs IP90	297 vs IP90	Mean
IR_1	78	11	45
IR_2	81	88	85
IR_3	86	86	86
IR_4	80	80	80
IR_5	80	90	85
IR_6	85	88	87

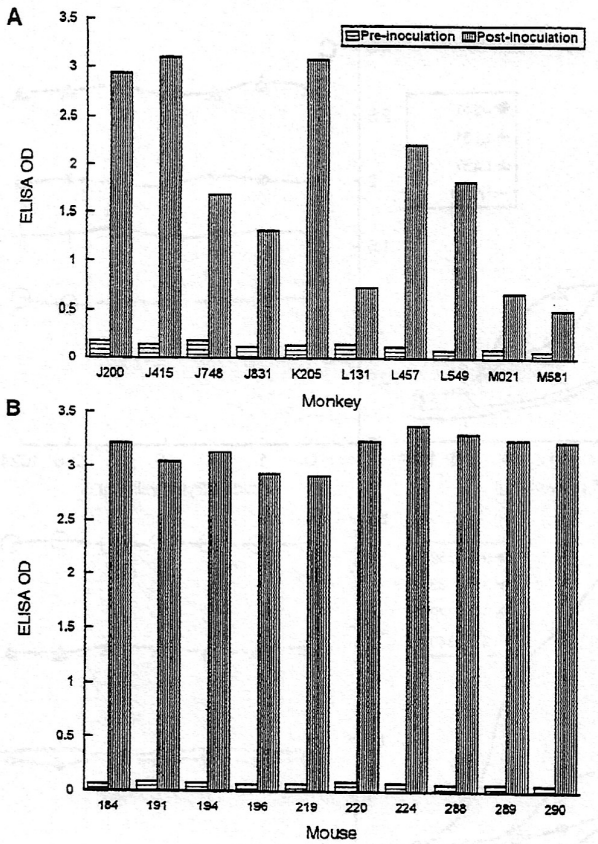

FIGURE 3. Antigenicity of IR_6 in monkeys (*A*) and mice (*B*). Serum samples were collected from monkeys or mice at 0 (pre) and 4–6 wk PI (post). Animals were infected with the JD1 strain of *B. burgdorferi* (monkeys J200, J415, J748, J831, K205, and L131), the B31 strain (monkeys L457, L549, M021, and M581; mice 184, 191, 194, and 196), or the Sh-2-82 strain (mice 219, 220, 224, 288, 289, and 290). Ab levels were assessed by the C_6 ELISA.

regions IR_{1-6} (Fig. 2). Ten serum samples from infected monkeys, four human serum samples randomly selected from the CDC panel, and an additional four serum samples from chronic Lyme disease patients provided by the National Institutes of Health were tested. Representative results for four human and four monkey serum samples are presented in Fig. 4. Addition of C_6 almost completely inhibited the binding of monkey and human serum Abs to P7-1 (Fig. 4, *A* and *B*). In contrast, addition of C_2 in the same range of concentrations caused no detectable inhibition (Fig. 4, *C* and *D*). Thus, inhibition of Ab binding to P7-1 by the C_6 peptide is specific and, more importantly, in both monkeys and humans IR_6 appears to be an immunodominant invariable region within the VlsE variable domain.

When the competitive ELISA was used to analyze infected mouse serum, the maximum inhibition resulting from the addition of C_6 was ~40%, depending on the animal. Unlike with monkeys and humans, however, addition of C_2 competitively inhibited binding of mouse Abs to P7-1 (up to 40%; data not shown). This indicates that other invariable regions may be antigenic in mice.

Exposure of IR_6 on the surface of VlsE

We examined the exposure of IR_6 on the surface of the VlsE protein by immunoprecipitation with the rabbit anti-C_6 antiserum. VlsE from IP90 spirochetes was extracted in solubilization buffer and precipitated with protein G-agarose in the presence of either rabbit anti-C_6 antiserum or serum obtained from the same rabbit before immunization. The presence of VlsE in the immunoprecipitates was then assessed on immunoblots reacted with the anti-C_6 antiserum. The VlsE of IP90 was immunoprecipitable with the anti-C_6 antiserum, but not with normal preimmune serum (Fig. 5). This result indicates that IR_6 is exposed on the VlsE surface.

Table II. *Antigenicity of IR_6 in human patients whose signs and symptoms satisfied the CDC Lyme disease case definition*[a]

Serum ID No.	C_6ELISAOD	SerumIDNo.	C_6ELISAOD
90-2111	P 3.055	91-1352	P 1.763
90-2436	P 2.541	91-1353	P 0.550
90-2622	P 3.099	91-1354	P 1.140
90-2631	N 0.276	91-1841	P 0.607
90-2668	P 1.625	91-1458	P 1.056
91-0521	P 1.423	91-1842	P 1.423
91-0531	P 3.330	91-1843	P 1.733
91-0532	P 1.942	91-1844	P 1.879
91-0533	P 2.178	91-1845	N 0.222
91-0544	P 3.091	91-1846	N 0.338
91-0794	P 0.600	91-1847	P 1.676
91-0865	P 3.274	92-0057	P 1.735
91-0900	P 2.386	92-1682	N 0.404
91-0943	P 2.959	92-1941	N 0.432
91-1104	P 1.642	92-1982	P 0.832
91-1222	P 2.274	93-0206	P 0.633
91-1347	P 0.960	93-0208	P 0.600
91-1348	P 3.233	93-1414	P 1.025
91-1349	N 0.448	93-1426	P 0.832
91-1350	P 1.246	94-0357	P 0.739
91-1351	P 0.843		

[a] N, negative result; P, positive result. The cutoff OD value (0.500) was defined as the mean + 3 SDs of 97 serum samples obtained from hospitalized patients in an area not endemic for Lyme disease. C_6 ELISA was performed as described in *Materials and Methods.*

IR_6 is not accessible to Ab on the outer membrane of the spirochete

Exposure of IR_6 on the spirochetal surface was assessed by immunofluorescence. While FITC-conjugated anti-C_6 Ab extensively labeled all the acetone-fixed IP90 spirochetes (Fig. 6*B*), it labeled few (<5%) of the unfixed spirochetes (Fig. 6*A*). The unfixed spirochetes that were labeled exhibited a discontinuous fluorescent pattern, whereas fixed spirochetes fluoresced uniformly (Fig. 6*B*). In contrast, neither unfixed nor fixed spirochetes of the *B. burgdorferi* sensu stricto strain HB19 high passage, which do not express VlsE, fluoresced with the FITC-conjugated anti-C_6 Ab (Fig. 6, *C* and *D*). Control anti-*B. burgdorferi* Ab-FITC conjugate labeled both fixed and unfixed spirochetes (data not shown). These results indicate that IR_6 is not accessible to Ab despite the fact that VlsE is itself surface exposed (8). This is consistent with the result of the in vitro Ab killing assay; anti-C_6 antiserum had no significant killing activity compared with preimmune serum, although monkey anti-OspA antiserum killed all spirochetes in the same experiment (data not shown). In vitro killing experiments were performed at least twice with the same results.

Discussion

We have demonstrated that the amino acid sequences of the six invariable regions described previously in the cassette segments of VlsE (8) are conserved, at least in two *Borrelia* genospecies, *B. garinii* and *B. burgdorferi* sensu stricto, and we have illustrated with data from the literature that such conservation is also retained among strains of the latter genospecies. This is evident from the

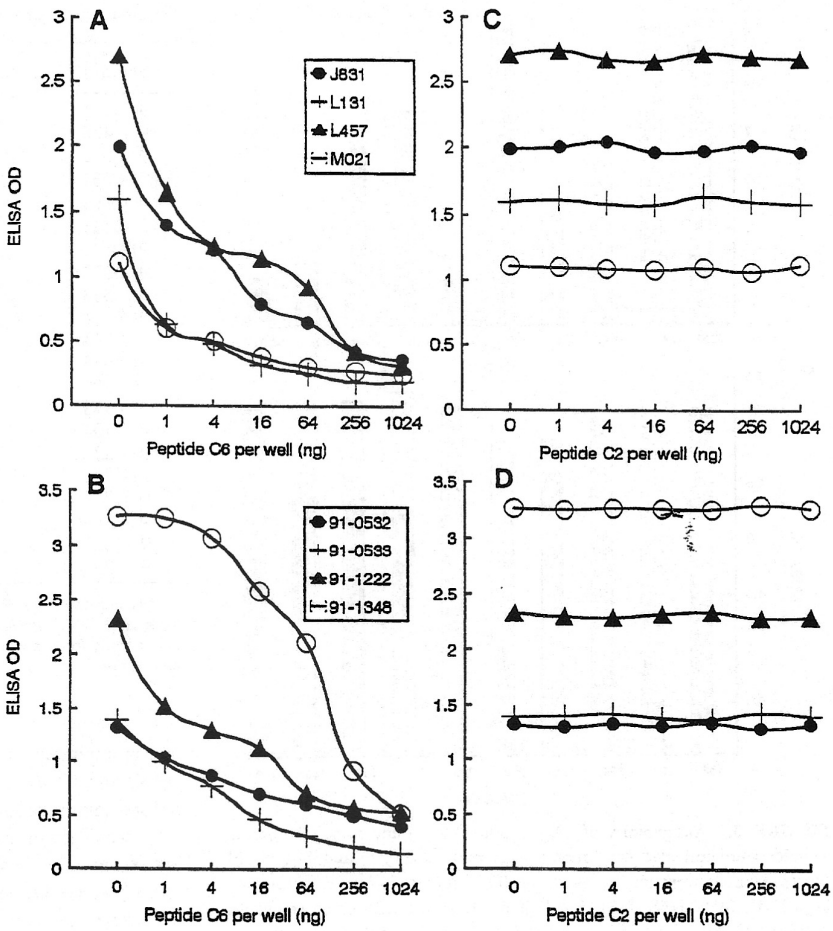

FIGURE 4. Immunodominance of IR_6 in monkeys and humans. Serum samples were obtained 4–6 wk PI from monkeys (*A* and *C*) inoculated with spirochetes of the JD1 strain of *B. burgdorferi* (J831 and L131) or the B31 strain (L457 and M021), and from humans (*B* and *D*) with an acute *B. burgdorferi* infection (91-1222 and 91-1348) or a chronic infection (91-0532 and 91-0533). Ab levels to P7-1 were assessed in the presence of increasing concentrations of the C_6 peptide (*A* and *B*) or the C_2 peptide (*C* and *D*) by the competitive ELISA procedure.

high level of identity between the deduced amino acid sequences of the invariable regions within the variable domain of IP90 (*B. garinii*), which we cloned, sequenced, and identified ourselves, and those of the B31 and 297 cassette segments (8, 14) (Fig. 2 and Table I).

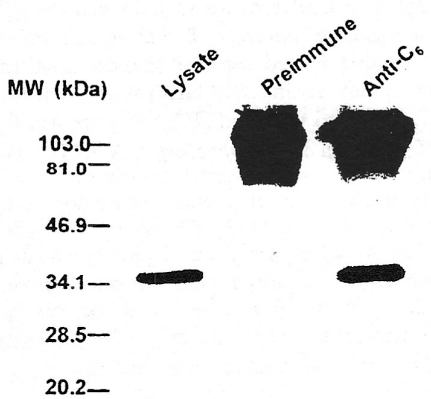

FIGURE 5. Exposure of IR_6 on the VlsE surface. VlsE from *B. garinii* strain IP90 spirochetes was extracted in solubilization buffer and immunoprecipitated with protein G-agarose in the presence of rabbit anti-C_6 antiserum or preimmune serum. Solubilized immunoprecipitates and a whole cell lysate of IP90 spirochetes were electrophoresed on a SDS-12% polyacrylamide minigel and blotted onto nitrocellulose. VlsE was visualized with the rabbit anti-C_6 antiserum. Besides the VlsE band (*bottom*), single rabbit IgG bands (*top*) are visible, because nonreducing SDS-PAGE sample buffer was used.

The sequence conservation of the six invariable regions across strain and genospecies barriers indicates that these regions are important in whichever role VlsE may play in the physiology of *B. burgdorferi*. One would therefore expect that such sequences are not antigenic in hosts with a chronic *B. burgdorferi* infection or would be otherwise inaccessible to Ab, either because they are conformationally buried within the VlsE molecule or are unavailable on the spirochetal surface. The Hopp-Woods algorithm indicated that, with the exception of IR_6, all the other invariable regions were either not antigenic or had lower antigenicity.

The predicted antigenicity of IR_6 was confirmed in humans, monkeys, and mice (Fig. 3 and Table II). Sera from all these hosts reacted with the C_6 peptide early and persistently in the course of infection, thus indicating that IR_6 contains one or more epitopes that may be broadly antigenic regardless of host species. The antigenicity of IR_6 was not only manifest independently of host species, but also regardless of whether the animals had been infected with the JD1, B31, or Sh-2-82 strains of *B. burgdorferi* sensu stricto. In addition, 35 of 41 human serum samples collected in the Northeast and Midwest of the U.S. from patients with acute or chronic Lyme disease also reacted with the C_6 peptide. The five serum samples that had no detectable anti-C_6 Ab were obtained from patients who were in the early stages of infection. Hence, the negative results may reflect the presence of very low serum Ab titers rather than the absence of cross-reactivity. The C_6 peptide

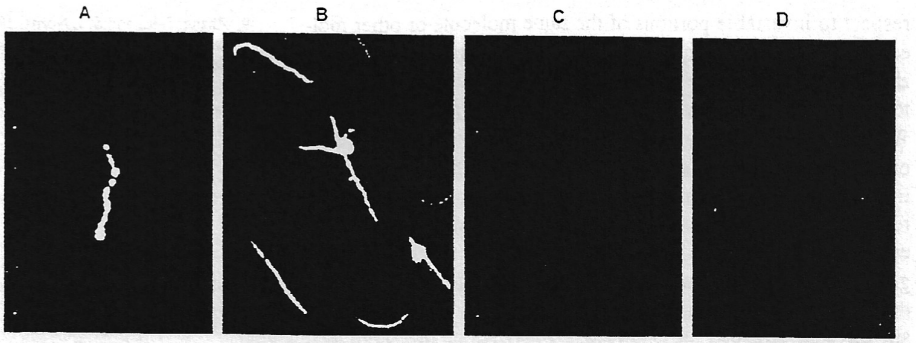

FIGURE 6. Lack of exposure of IR_6 on the spirochetal surface. *B. garinii* strain IP90 spirochetes (*A* and *B*) or *B. burgdorferi* sensu stricto strain HB19 high passage, lacking VlsE expression (*C* and *D*) were either left unfixed (*A* and *C*) or were fixed with acetone (*B* and *D*) and labeled with rabbit anti-C_6 Ab conjugated to FITC.

may thus serve as a global diagnostic probe. We are currently investigating this possibility.

No Ab reactivity was observed when the C_6 peptide was not included in the assay, i.e., when plates were coated with streptavidin. Hence, the anti-C_6 reactivity is specific and cannot be construed as originating from polyclonal B cell stimulation during *B. burgdorferi* infection (25, 26).

The absence of or relatively low antigenicity of IR_{1-5} was underscored by the results of the competition experiments (Fig. 4). The fact that the binding of essentially all the human and monkey Ab that reacted with P7-1 could be inhibited simply by adding an excess of the C_6 peptide indicates that in these hosts IR_6 is possibly the only immunodominant invariable region within the VlsE variable domain. In mice the results were different, as we were unable to fully inhibit the P7-1 reactivity of mouse anti-Sh-2-82 or anti-B31 Abs with an added excess of the C_6 (or C_6 and C_2) peptide(s). This result entails that some of the other IRs may be antigenic in mice. Indeed, when synthetic peptides based on the five remaining IRs listed in Fig. 2 were used as ELISA Ags, Ab responses to IR_2 and IR_4 were evident in infected mice, but not in monkeys or humans (34). The immunodominance of IR_6 was further underscored by the long term persistence of anti-C_6 Abs in infected monkeys and in patients with chronic Lyme disease (not shown).

The Hopp-Woods algorithm also predicted, as expected, that some variable regions of the cassette segment are strongly antigenic (data not shown). Thus, at least theoretically, the VlsE variable regions could satisfy the antigenic variation paradigm that variable domains are immunodominant. In the case of VlsE, however, variable region immunodominance of individual VlsE molecules may be difficult to achieve, for the recombination process that underlies the mechanism of VlsE variation occurs very rapidly (14). Despite strong antigenicity, no variable region might at any time be represented on a large enough number of bacterial cells or otherwise be expressed long enough to become immunodominant. Indeed, within the first 4 days PI, as many as 11 predicted amino acid changes per VlsE variant can occur during a *B. burgdorferi* B31 infection in C3H/HeN mice (14), and unlike with *T. brucei*, *N. gonorrhoeae*, and *B. hermsii*, VlsE serotypes of *B. burgdorferi* have never been found. In fact, it has been estimated that amino acid substitutions within the variable domain of VlsE could result in as many as 10^{30} different combinations (9), a number much larger than the diversity of the Ab repertoire of vertebrate host species (10^9–10^{11}). Even though some of the combinations may share antigenic specificities, it seems unrealistic that VlsE serotypes may emerge during infection.

Our finding that IR_6 is exposed on the VlsE surface (Fig. 5) but not on the spirochetal surface (Fig. 6) is in agreement with the antigenicity and immunodominance of this region. Features of protein domains, such as surface accessibility, hydrophilicity, flexibility, and proximity to a site recognized by Th cells, are all important in positively determining domain antigenicity (27). Except for the possibility that solubilization with Triton X-100 may have partially denatured VlsE and thus artificially exposed IR_6, the result of the immune precipitation experiment indicates that IR_6 is accessible on the surface of native VlsE. It is also hydrophilic, as assessed by the Hopps-Wood algorithm. The molecular surface exposure of IR_6 was indicated also by the fact that a small proportion of unfixed spirochetes fluoresced when incubated with FITC-labeled anti-C_6 Ab (Fig. 6*A*).

Most of the unfixed spirochetes failed to label with the FITC-conjugated anti-C_6 Ab (Fig. 6*A*). This indicates that IR_6 is not exposed on the spirochetal surface. The small proportion of unfixed spirochetes that bound anti-C_6 Ab probably had some degree of membrane damage. Although our procedure for labeling of unfixed spirochetes entailed very gentle manipulations, it is possible that portions of the VlsE molecule not normally exposed on the spirochete surface were nonetheless uncovered in a fraction of the spirochetes. The fragility of the outer membrane of *B. burgdorferi* has been noted by other investigators (28). Loss of the lp28-1 plasmid, which encodes VlsE, during in vitro cultivation (8) also could explain a negative labeling result. However, the fact that nearly 100% of acetone-fixed spirochetes from the same culture were labeled allows us to rule out this possibility (Fig. 6*B*). Acetone fixation probably exposed regions of the VlsE not accessible to Ab on an intact spirochete. As expected, the HB19 spirochetes not expressing VlsE did not bind anti-C_6 Ab regardless of whether the organisms were fixed or unfixed (Fig. 6, *C* and *D*). Hence, the anti-C_6 reactivity is singularly specific.

The result of the in vitro Ab killing experiment was entirely consistent with our interpretation of the immunofluorescent observations, as absence of killing is most likely due to failure of the anti-C_6 Ab to bind to the spirochetal surface. Complement-dependent killing of *B. burgdorferi* is facilitated by anti-surface Ab binding and does not depend on the complement-activating properties of the Ab. *B. burgdorferi* spirochetes are able to activate complement through an Ab-independent mechanism (29). Taken together, our immunofluorescence and in vitro killing results indicate that IR_6 is inaccessible to Ab on the spirochetal surface. Because VlsE is surface exposed and is a lipoprotein (8), it is probably anchored to the spirochetal membrane only by its N-terminal lipid moiety and is not otherwise embedded in the membrane. It follows that VlsE is probably densely packed on the spirochetal surface, interacting with either neighboring VlsEs or other vicinal surface-exposed molecules.

In the variable Ags that have been thoroughly investigated to date, such as the VSG, Vmp, or pilin, the function attributed to variable portions, because of their immunodominance, has been to act both as target of the immune response and as decoy with

respect to invariable portions of the same molecule or other molecules. On the other hand, the role ascribed to these Ags' invariable portions, which are characteristically nonantigenic, has been to contribute to preserving a functional conformation (10, 11). What, then, is the role of the antigenicity and immunodominance of IR_6? It has been hypothesized that chronic host exposure to immunodominant Ags or epitopes diverts the immune system from responding to less antigenic but functionally important Ags or epitopes, thus serving as a protective strategy for persistent pathogens (30). Recently, it was demonstrated that when the immunodominant V3 loop epitope of gp120 of HIV-1 is masked through site-directed targeting of *N*-linked glycosylation, the dominant, type-specific, neutralizing Ab response is shifted away from V3 to epitopes in the first variable domain (V1) of gp120 (31). Ab responses to conserved domains of gp120 also were observed (31).

We submit that IR_6 may act as a decoy epitope and contribute to subvert the Ab response to *B. burgdorferi*. As mentioned before, it is conceivable that the promiscuity of VlsE antigenic variation is such that variable regions of VlsE do not, or do not always, become immunodominant. The variation may serve to inhibit the formation of high avidity Abs to the variable VlsE regions, but not to divert the response away from invariable VlsE regions and invariable domains or other less antigenic, but functionally neutralizing, *B. burgdorferi* Ag. It should be considered that only about half the length of the mature VlsE protein is variable, compared with more than two-thirds of proteins such as VSG, pilin, and Vmp. Moreover, more than half the variable domain of VlsE is encompassed by invariable regions, including one immunodominant IR_6 identified in this study. Conserved regions of VlsE other than IR_6 thus may be exposed, per force, on the spirochete surface. IR_6 would serve as the decoy epitope for such domains by suppressing protective immune responses through mechanisms such as clonal restriction and/or idiotypic dysregulation, as has been invoked for HIV-1's V3 (32, 33).

Acknowledgments

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