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Dominant Recognition of a *Borrelia burgdorferi* Outer Surface Protein A Peptide by T Helper Cells in Patients with Treatment-Resistant Lyme Arthritis

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In an earlier study, we found that T-cell lines (TCL) from five patients with treatment-resistant Lyme arthritis preferentially recognized *Borrelia burgdorferi* outer surface protein A (OspA), but TCL from four patients with treatment-responsive arthritis only rarely recognized this protein. Dominant T-cell recognition of an arthritogenic OspA epitope is one way in which the immune response against OspA might be involved in the pathogenesis of treatment-resistant Lyme arthritis. In an effort to test this hypothesis, we mapped the epitopes of 31 OspA-specific TCL and five T-cell clones derived from the synovial fluid or peripheral blood samples of three patients with treatment-resistant Lyme arthritis. Although each patient's TCL recognized a broad array of OspA peptides with different individual patterns, two regions of OspA were dominantly recognized. Each patient's TCL dominantly recognized a C-terminal epitope of OspA, ranging from amino acids (aa) 214 to 233 in one patient to 244 to 263 in another, and the TCL of all three patients dominantly recognized an epitope between aa 84 and 113. These dominant regions were confirmed by clonal analysis in one patient. Thus, the region of OspA between aa 84 and 113 was the dominant T-cell epitope shared by these three patients with treatment-resistant Lyme arthritis. If the T-cell response to OspA is involved in the pathogenesis of treatment-resistant Lyme arthritis, an epitope contained within aa 84 to 113 is a potentially arthritogenic epitope.

Lyme borreliosis is a chronic multisystem disease caused by the tick-borne spirochetes *Borrelia burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*. These spirochetes affect primarily the skin, nervous system, heart, or joints (1, 2, 14). Within months after disease onset, approximately 60% of untreated North American patients develop intermittent attacks of monoarticular or oligoarticular arthritis, primarily in large joints. Lyme arthritis coexists with strong and specific humoral and cellular immune responses to *B. burgdorferi* (4). While patients with Lyme arthritis usually respond to antibiotic therapy, about 10% of patients have persistent arthritis for months or even years after antibiotic therapy (32). Thus, patients may be divided into two groups, those with treatment-responsive Lyme arthritis, defined as arthritis resolving within 3 months after the initiation of appropriate antibiotic treatment, and those with treatment-resistant arthritis that lasts for more than 3 months after the initiation of treatment (20, 32). Two hypotheses that could explain treatment-resistant Lyme arthritis are spirochete-mediated pathology due to persistent infection and infection-induced immunopathology (reviewed in reference 17). It is important to emphasize that the two hypotheses of persistent infection and infection-induced immunopathology are not mutually exclusive. In untreated patients with chronic Lyme arthritis, spirochete-mediated pathology and infection-induced immunopathology surely occur together. The latter might be mediated by *Borrelia*-induced cytokine imbalance or autoimmunity.

Arguments for persistent *B. burgdorferi* infection include the following: (i) the antibody response to *B. burgdorferi* in untreated patients, including the immunoglobulin M (IgM) response, expands for months or even years after the onset of infection; (ii) *B. burgdorferi* DNA can be detected in the synovial fluid (SF) of most patients prior to antibiotic treatment, and *B. burgdorferi* has been detected occasionally in the synovium or ligaments of patients with Lyme arthritis; (iii) late manifestations of Lyme disease, including chronic neuroborreliosis and acrodermatitis chronica atrophicans, can usually be cured with antibiotic treatment; (iv) local production of antibodies in the cerebrospinal fluid in the absence of a detectable antibody response in the serum indicates that *B. burgdorferi* persists at privileged sites; and (v) disease severity has been linked to spirochete burdens in murine models of Lyme arthritis.

Arguments for infection-induced immunopathology include the following: (i) while *Borrelia* DNA can be amplified reliably from pretreatment samples of SF, most patients with treatment-resistant arthritis yield consistently negative PCR tests of SF after antibiotic treatment (3, 26); (ii) the HLA-DR4 specificity and the level of IgG reactivity against outer surface protein A (OspA) were found to be risk factors for treatment-resistant Lyme arthritis, and the presence of both factors doubled the risk of this outcome (32); (iii) *B. burgdorferi* OspA was preferentially recognized by T-cell lines (TCL) from five patients with treatment-resistant Lyme arthritis but not by TCL from four patients with treatment-responsive arthritis (20); and (iv) hamsters vaccinated with Formalin-inactivated *B.*

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TABLE 1. Clinical characteristics of three patients with treatment-resistant Lyme arthritis

Patient	Age (yr)	Sex[a]	Arthritis duration (mo)		IgG titer against *B. burgdorferi*	PCR for *B. burgdorferi* DNA	HLA allele(s)		
			Before therapy	After therapy			DRB1	DQB1	DPB1
1	52	M	8	8	1:800	Negative	0701, 1201	0201, 0301	0402, 0602
2	36	F	11	15	1:3,200	Negative	0301, 0701	0201	0201, 0402
3	30	M	6	15	1:3,200	ND[b]	0301, 1501	0201, 0602	0401, 0402

[a] M, male; F, female.
[b] ND, not done.

burgdorferi and then challenged with a homologous *B. burgdorferi* isolate prior to developing protective antibody titers or challenged with other isolates even after high levels of borreliacidal antibodies were detectable developed T-cell-mediated destructive arthritis (21, 22).

Earlier data strongly indicate that a T-cell response to OspA does not necessarily protect from Lyme arthritis (4). On the contrary, our finding that OspA is recognized primarily by TCL from patients with treatment-resistant Lyme arthritis is compatible with the hypothesis that the T-cell response to OspA is involved in the pathogenesis of chronic synovial inflammation in these patients (20). Recognition of an arthritogenic OspA epitope is one way in which the T-cell response against OspA might lead to autoimmunity and consequently an antibiotic-resistant arthritis. In this study we examined the T-cell recognition of OspA epitopes by TCL and T-cell clones from three patients with treatment-resistant Lyme arthritis.

(This work is part of the Ph.D. thesis of B. Lengl-Janßen.)

MATERIALS AND METHODS

Patients. The three patients met established clinical and serologic criteria for the diagnosis of Lyme arthritis. Their clinical characteristics have been described earlier in detail (20), and a summary is given in Table 1.

Antigens. The N40 strain of *B. burgdorferi* was propagated in BSK medium (Sigma, St. Louis, Mo.) with 6% rabbit serum (Sigma), and lysates were made as previously described (20). OspA was produced as a maltose-binding protein (MBP) fusion protein, as previously described (20). A set of 20-mer peptides overlapping by 10 amino acids each (e.g., 24 to 43, 34 to 53, etc.) derived from the published sequence of *B. burgdorferi* ZS7 (GenBank accession number X16467) was synthesized by conventional Merrifield solid-phase chemistry with t-Boc (tert-butoxy-carbonyl) or Fmoc (9-fluorenylmethoxycarbonyl) amino acids. Peptides were cleaved from the synthesis substrate, desalted by gel filtration, lyophilized, purified by reverse-phase high-pressure liquid chromatography, subjected to amino acid analysis, resuspended in phosphate-buffered saline, sonicated, and sterilized by filtration (0.22-μm-pore-size filter).

Cell culture and assay conditions. *B. burgdorferi*-specific TCL and Epstein-Barr virus-transformed B-cell lines were derived and maintained as previously described (20). T-cell clones were generated from TCL by limiting dilution. T cells were plated on 96-well plates (Costar Corp., Cambridge, Mass.) at a theoretical density of 0.3 cells per well together with 5×10^5 irradiated autologous Epstein-Barr virus-transformed B cells and *B. burgdorferi* sonicated lysate (10 μg/ml) in a final volume of 200 μl in complete medium containing RPMI 1640 (GIBCO BRL, Gaithersburg, Md.) with 5% human AB serum (Sigma), 10 mM glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer. Four days later, human recombinant interleukin-2 (IL-2; final concentration, 5 U/ml; Cetus, Emeryville, Calif.) and IL-4 (final concentration, 5 U/ml; Schering-Ploegh, Bloomfield, N.J.) were added to plates. Clones were expanded in the same medium for 18 to 21 days. Subsequent additions of medium, IL-2, and IL-4 were necessary during this period. After this growth period, viable T cells were retrieved through Ficoll-Hypaque separation. Clones were maintained and tested exactly as described above for TCL. Antigen recognition was tested by a standard proliferation assay, as described previously (20). Briefly, 2.5×10^4 T cells and the same number of irradiated autologous Epstein-Barr virus-transformed B cells were plated in triplicate on 96-well plates (Costar) with complete medium alone or in the presence of antigen. MBP–β-galactosidase-α and the MBP-OspA fusion protein were used at 0.03 to 3 μM, and peptides were used at 1 to 3 μM. After a 72-hour incubation, [^{3}H]thymidine (1 μCi per well) in 50 μl of complete medium was added to each well. Cells were harvested 18 h later with an automated cell harvester, and incorporated thymidine was detected in a liquid scintillation counter (Top Count; Packard, Meridien, Conn.). The results reported are the mean values of triplicate cultures and were calculated in counts per minute with the background subtracted (Δcpm) or as stimulation indices (SI), test/control (in counts per minute). A result was considered positive if the SI was at least 2 and the Δcpm was more than 1,000.

Sequence alignments. Sequence alignments were done by using the fasta and tblastn programs of the Genetics Computer Group package (8).

RESULTS

Clinical characteristics. The clinical characteristics of the three patients with treatment-resistant arthritis have been described in a previous study (20). Briefly, each patient had inflammatory arthritis affecting a knee. All three had elevated IgG antibody responses to *B. burgdorferi*, as determined by enzyme-linked immunosorbent assay (Table 1), and they had reactivities with 12 or more spirochetal proteins, including OspA, as determined by Western blotting (immunoblotting). All three patients had arthritis for 8 to 15 months after treatment with oral and intravenous antibiotics, and their T cells were obtained months after antibiotic therapy. At that time, *B. burgdorferi* was not detected in the SF samples of the two patients tested. Each patient had a different HLA type.

OspA peptide recognition by TCL. In a previous study, 30 *B. burgdorferi*-specific TCL were derived from peripheral blood (PB) samples and 50 *B. burgdorferi*-specific TCL were derived from SF samples of these three patients (Table 2). The majority of these TCL recognized OspA. Seventeen randomly selected TCL, including nine of those reported here, were analyzed by immunofluorescent staining and flow cytometry. They were all $CD3^+$ $CD4^+$ $TCR\alpha/\beta^+$ $CD8^-$ $TCR\gamma/\delta^-$ (20).

OspA epitope mapping was done with 31 of the OspA-specific TCL from SF or PB samples of these patients. For patient 1, 5 to 19 different OspA peptides (mean, 11) were recognized by 18 OspA-specific TCL (Fig. 1A). Peptides 214-233 (residues 214 to 233) and 84-103 induced the strongest T-cell proliferation, and three regions of the protein, amino

TABLE 2. Numbers of *B. burgdorferi*-specific and OspA-specific TCL tested from three patients with treatment-resistant Lyme arthritis

Patient	No. of *B. burgdorferi*-specific TCL		No. of OspA-specific TCL		No. of OspA-specific TCL tested with peptides	
	PB	SF	PB	SF	PB	SF
1	8	23	4	21	0	18
3	5	12	5	12	3	7
2	17	15	5	8	1	2
Total	30	50	14	41	4	27

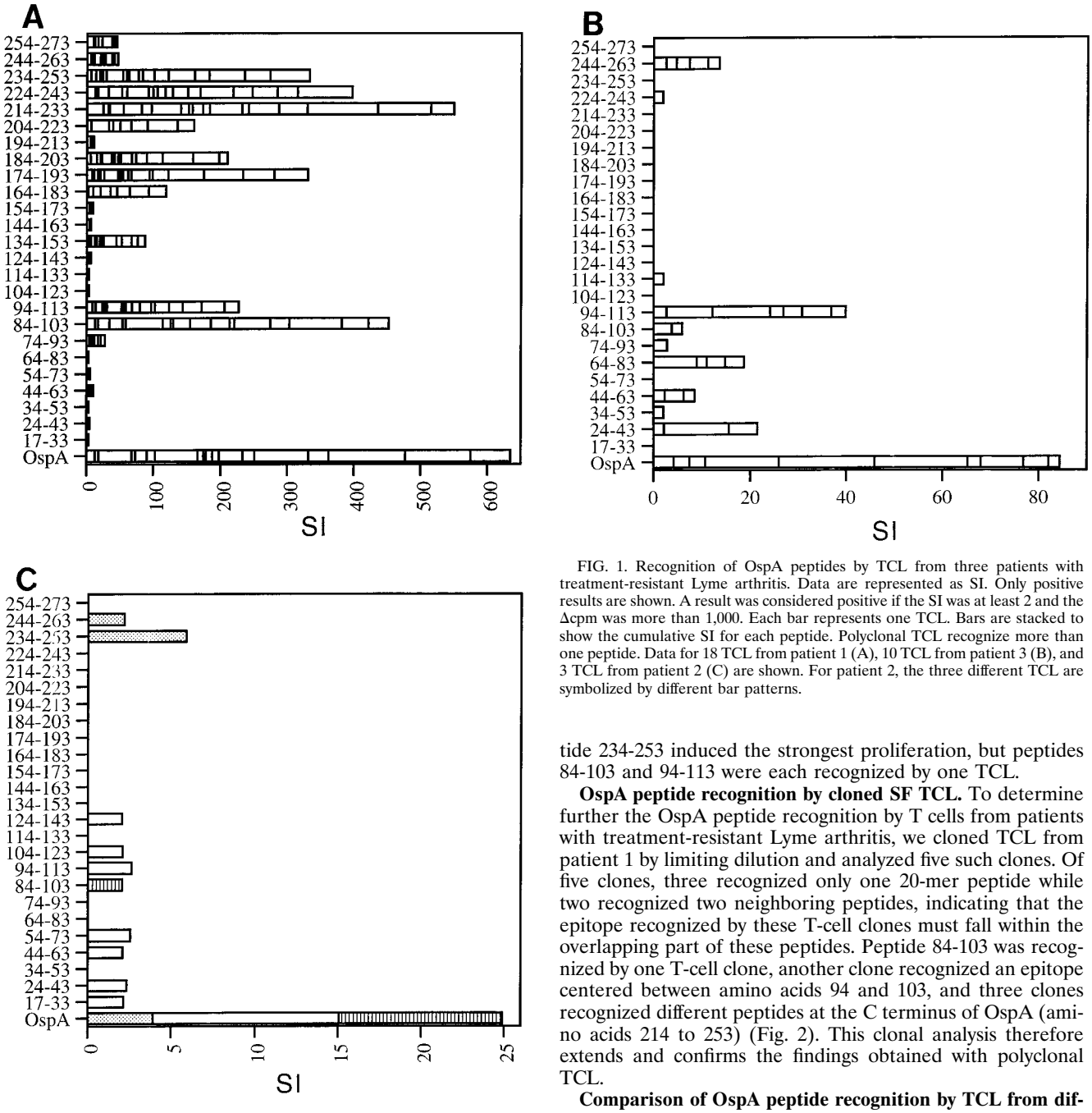

FIG. 1. Recognition of OspA peptides by TCL from three patients with treatment-resistant Lyme arthritis. Data are represented as SI. Only positive results are shown. A result was considered positive if the SI was at least 2 and the Δcpm was more than 1,000. Each bar represents one TCL. Bars are stacked to show the cumulative SI for each peptide. Polyclonal TCL recognize more than one peptide. Data for 18 TCL from patient 1 (A), 10 TCL from patient 3 (B), and 3 TCL from patient 2 (C) are shown. For patient 2, the three different TCL are symbolized by different bar patterns.

acids 84 to 113, 164 to 203, and 214 to 253, were dominantly recognized. The other peptides were recognized less frequently, but each peptide was recognized by at least one polyclonal TCL.

For patient 3, one to nine different peptides (mean, three) were recognized by 10 OspA-specific TCL (Fig. 1B). Peptides 94-113 and 244-263 were most frequently recognized, and peptide 94-113 induced the strongest T-cell proliferation. Two others, peptides 24-43 and 64-83, also induced strong proliferation in this patient's TCL.

For patient 2, one to seven different peptides (mean, three) were recognized by three OspA-specific TCL (Fig. 1C). Peptide 234-253 induced the strongest proliferation, but peptides 84-103 and 94-113 were each recognized by one TCL.

OspA peptide recognition by cloned SF TCL. To determine further the OspA peptide recognition by T cells from patients with treatment-resistant Lyme arthritis, we cloned TCL from patient 1 by limiting dilution and analyzed five such clones. Of five clones, three recognized only one 20-mer peptide while two recognized two neighboring peptides, indicating that the epitope recognized by these T-cell clones must fall within the overlapping part of these peptides. Peptide 84-103 was recognized by one T-cell clone, another clone recognized an epitope centered between amino acids 94 and 103, and three clones recognized different peptides at the C terminus of OspA (amino acids 214 to 253) (Fig. 2). This clonal analysis therefore extends and confirms the findings obtained with polyclonal TCL.

Comparison of OspA peptide recognition by TCL from different patients. Our data show that each OspA peptide was immunogenic since each peptide was recognized by at least one TCL. Furthermore, each patient and each TCL had individual peptide recognition patterns. For example, TCL from patient 3 reacted strongly with peptides representing the N-terminal third of OspA, whereas only TCL from patient 1 recognized the region from amino acid 164 to 203 of OspA. Nevertheless, two common motifs became apparent. First, each patient mounted a strong T-cell response to epitopes in the C-terminal region of OspA, albeit to different epitopes (234 to 263 for patient 2, 244 to 263 for patient 3, and 214 to 253 for patient 1). Second, each patient preferentially recognized peptides 84-103 and/or 94-113. Clonal analysis of the response in patient 1 showed that the dominant epitope is likely to be centered between residues 94 and 103.

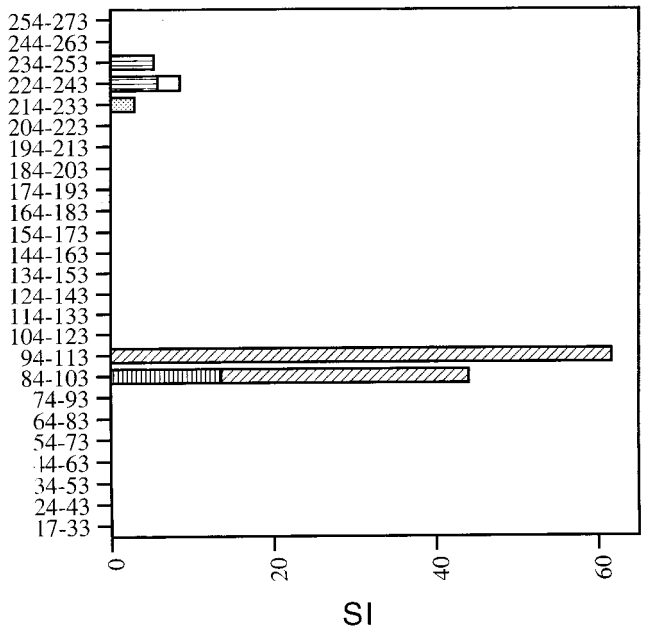

FIG. 2. Recognition of OspA peptides by five T-cell clones from patient 1. Data are presented as SI. Only positive results are shown. A result was considered positive if the SI was at least 2 and the Δcpm was more than 1,000. Each T-cell clone is symbolized by a different bar pattern.

Homologies of dominantly recognized OspA peptides with other proteins. Clinical observations suggest that arthritis is a more prominent manifestation of *B. burgdorferi* infection in North America than it is in Europe. In North America, all patient isolates to date have been *B. burgdorferi* sensu stricto strains, whereas all three species of this spirochete cause infections in Europe. Therefore, one may predict that there are differences in these critical epitopes among *B. burgdorferi* strains. Among *B. burgdorferi* sensu stricto strains, the OspA amino acid sequence is >99% conserved. When the ZS7 OspA sequence was compared with 13 other *B. burgdorferi* sensu stricto OspA sequences, the sequence from residues 84 to 113 was completely identical in 12 strains. Only one strain, California isolate CA8 (GenBank accession number L23144), differed (at amino residue 90) from ZS7. This was a conservative change from I → V. In contrast, four complete OspA sequences from *B. afzelii* strains had an average of 62 (range, 61 to 63) amino acid substitutions compared with ZS7 OspA. The prototypical *B. afzelii* strain, Pko (GenBank accession number X62161), OspA had 77.3% sequence identity with ZS7 OspA (Fig. 3). Peptides 84-103 (70%) and 94-113 (75%) showed the same degree of sequence identity. All peptides representing amino acids 214 to 273 showed 70 to 80% homology with ZS7 OspA. The OspA sequences of 10 *B. garinii* strains had an average of 55.4 (range, 51 to 58) amino acid substitutions compared with ZS7 OspA. As shown in Fig. 3 for characteristic strain ZQ1 (GenBank accession number X66065), the overall sequence identity was 80.3%, with the identities for amino acids 84 to 103 (70%) and 94 to 113 (80%) not being significantly different from that average. Similarly, the C terminus lies within this range (75% sequence homology with ZS7 OspA for amino acids 214 to 273). Thus, while amino acid residues 84 to 113 are almost 100% conserved among the *B. burgdorferi* sensu stricto strains found in North America and Europe, this part of the OspA molecule is different in the *B. garinii* and *B. afzelii* strains found in Europe.

DISCUSSION

We investigated a panel of 31 TCL and five T-cell clones from three different patients with treatment-resistant Lyme arthritis. We found that two regions of the molecule, repre-

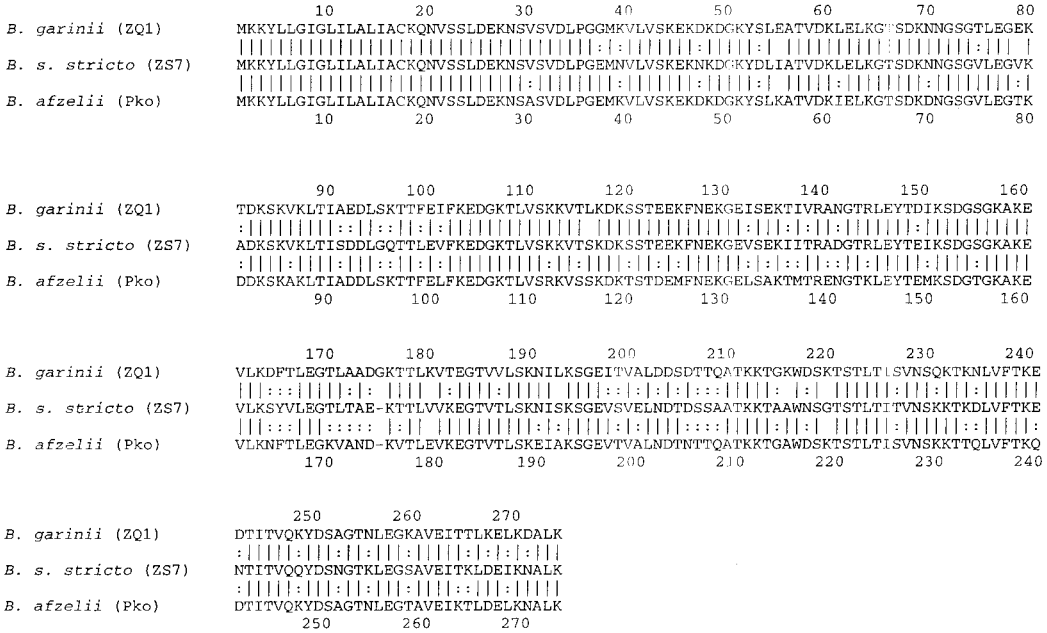

FIG. 3. Alignment of the OspA sequences of the following three *B. burgdorferi* sensu lato strains: ZS7 (GenBank accession number X16467; *B. burgdorferi* sensu stricto [*B. s. stricto*]), *B. garinii* ZQ1 (GenBank accession number X66065), and *B. afzelii* Pko (GenBank accession number X62161). Vertical bars indicate identical amino acids; colons indicate conservative amino acid changes.

sented by amino acids 84 to 113 and the C-terminal third of OspA, were dominantly recognized. Other investigators have mapped the OspA epitopes recognized by monoclonal antibodies (23, 27, 28), by sera from mice infected with *B. burgdorferi* or immunized with OspA (28), and by sera from patients with Lyme disease (15, 27–29). The C-terminal third of the molecule was found to be dominantly recognized by these different antibodies and antisera. In addition, sera from patients with Lyme disease were also found to recognize the N-terminal (15) and middle (15, 27, 28) thirds of OspA. Protection of rodents against experimental Lyme disease has been correlated with the development of antibodies recognizing conformationally dependent epitopes between amino acids 133 and 273 (28) and between amino acids 143 and 273 (13, 33), respectively.

One group has mapped OspA epitopes recognized by six T-cell clones from three patients with Lyme arthritis. Three clones recognized epitopes in the C-terminal third of the molecule (29), and three recognized epitopes in the N-terminal region. Of the latter, two recognized peptide 61-78 and one recognized peptide 73-90 (19). However, this analysis did not allow for comparison of treatment-resistant and treatment-responsive patients.

Thus, while the recognition of C-terminal OspA epitopes is common in patients with Lyme arthritis, antibodies and T-cell responses against this region developed during the course of infection do not protect against Lyme arthritis. Active and passive immunizations with OspA protect rodents from experimental infection with homologous strains of *B. burgdorferi* (6, 31). The first report of clinical trials using recombinant OspA as a vaccine against Lyme disease was encouraging (18). However, the arthritogenicity of OspA and OspB in rats (9), vaccine-induced T-cell-mediated immunopathology in hamsters (22), and our data on differential recognition of OspA in patients with treatment-resistant or treatment-responsive Lyme arthritis (20) together raise the specter of immunopathology.

Could the T-cell response against OspA be involved in the pathogenesis of treatment-resistant Lyme arthritis? Gondolf et al. found OspA and OspB to be arthritogenic in rats (9). We found differential recognition of OspA by TCL from patients with treatment-resistant or treatment-responsive Lyme arthritis. While OspA was preferentially recognized by TCL from patients with treatment-resistant arthritis, it was only rarely recognized by TCL from patients with treatment-responsive arthritis (20). Consequently, we asked if there was an arthritogenic epitope on OspA which might lead to autoreactivity, perhaps via molecular mimicry (7). We found that the OspA epitope(s) represented by amino acid residues 84 to 113 was dominantly recognized by the TCL from three different patients with treatment-resistant Lyme arthritis. Searches of the GenBank, EMBL, and SwissProt databases did not reveal significant sequence homology between OspA or amino acids 84 to 113 of OspA and any human protein. Obviously, these databases include only a minority of possible self antigens. Furthermore, a recent study has shown that simple sequence alignment does not suffice to identify molecular mimics (34). In that study, T-cell clones from multiple sclerosis patients, specific for an immunodominant myelin basic protein epitope, were used to identify the structural requirements for major histocompatibility complex binding and T-cell receptor recognition of the peptide and databases were then searched for potentially cross-reactive peptides. Of the seven bacterial and viral peptides found to activate MBP-specific T-cell clones, only one would have been found by sequence alignment. Using OspA-specific T-cell clones from patients with treatment-resistant Lyme arthritis to determine the structural requirements for binding of an immunodominant OspA epitope to HLA and recognition of the peptide-HLA complex by the T-cell receptor should allow us to test the possibility of molecular mimicry further.

Even if, as we hypothesize, T-cell recognition of an arthritogenic epitope on OspA plays a role in the pathogenesis of treatment-resistant Lyme arthritis, other factors must be involved (20). Cytokine production is an obvious possibility. Predominant gamma interferon production upon stimulation with *B. burgdorferi* has been found in arthritis-susceptible mice, and predominant IL-4 production has been found in arthritis-resistant mice (24). Furthermore, *B. burgdorferi* can induce Th1 phenotype differentiation in naive T-cell receptor transgenic T cells (16). Clearly, not only T-cell cytokines are important; the balance of IL-1β and IL-1β-RA is disturbed in the SF of patients with treatment-resistant Lyme arthritis but not in the SF of those with treatment-responsive Lyme arthritis (25). There have been similar findings for other chronic inflammatory diseases, such as rheumatoid arthritis (5). Furthermore, *B. burgdorferi* is mitogenic for B cells (reviewed in reference 4) and costimulatory for incompletely activated T cells (30) and thus might provide a potential means to overcome self-tolerance.

All North American *B. burgdorferi* isolates to date have been classified as *B. burgdorferi* sensu stricto, whereas all three species of *B. burgdorferi* sensu lato occur in Europe. It has been suggested that the species differences partly account for some of the differences in the clinical manifestations of Lyme borreliosis on these two continents. For example, acrodermatitis chronica atrophicans is rarely seen in North America but is commonly found in Europe in association with *B. afzelii* infection. In contrast, arthritis seems to be a more prominent feature of this illness in North America than it is in Europe (10–12).

Could different OspA sequences, associated with different *Borrelia* species, evoke different immune responses? On the basis of the deduced amino acid sequences of 39 *B. burgdorferi* sensu lato strains in the GenBank and EMBL databases, we found that the OspA sequences of different *B. burgdorferi* sensu stricto strains had >99% sequence identity. In contrast, they had only 77.3 and 80.3% sequence identities with *B. afzelii* and *B. garinii* OspA sequences, respectively. Importantly, the amount of sequence variation within amino acids 84 to 113 was similar to that in the whole molecule, indicating no selective pressure to either maintain or alter this sequence. Whether *B. afzelii* or *B. garinii* causes treatment-resistant Lyme arthritis, as defined for *B. burgdorferi* sensu stricto, is currently the subject of investigation.

In summary, our data show that an epitope contained within amino acid residues 84 to 113 of OspA was dominantly recognized by TCL and T-cell clones from patients with treatment-resistant Lyme arthritis. This epitope is highly conserved among *B. burgdorferi* sensu stricto strains and is conserved to a lesser degree among other *B. burgdorferi* sensu lato strains. If the T-cell response to OspA is involved in the pathogenesis of treatment-resistant Lyme arthritis, an epitope contained within residues 84 to 113 is a potentially arthritogenic epitope.

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