Antibiotic-refractory Lyme arthritis is associated with HLA-DR molecules that bind a *Borrelia burgdorferi* peptide

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An association has previously been shown between antibiotic-refractory Lyme arthritis, the human histocompatibility leukocyte antigen (HLA)–DR4 molecule, and T cell recognition of an epitope of *Borrelia burgdorferi* outer-surface protein A ($OspA_{163–175}$). We studied the frequencies of HLA-DRB1-DQA1-DQB1 haplotypes in 121 patients with antibiotic-refractory or antibiotic-responsive Lyme arthritis and correlated these frequencies with in vitro binding of the $OspA_{163–175}$ peptide to 14 DRB molecules. Among the 121 patients, the frequencies of HLA-DRB1-DQA1-DQB1 haplotypes were similar to those in control subjects. However, when stratified by antibiotic response, the frequencies of DRB1 alleles in the 71 patients with antibiotic-refractory arthritis differed significantly from those in the 50 antibiotic-responsive patients (log likelihood test, $P = 0.006$; exact test, $P = 0.008$; effect size, $Wn = 0.38$). 7 of the 14 DRB molecules (DRB1*0401, 0101, 0404, 0405, DRB5*0101, DRB1*0402, and 0102) showed strong to weak binding of $OspA_{163–175}$, whereas the other seven showed negligible or no binding of the peptide. Altogether, 79% of the antibiotic-refractory patients had at least one of the seven known OspA peptide–binding DR molecules compared with 46% of the antibiotic-responsive patients (odds ratio = 4.4; $P < 0.001$). We conclude that binding of a single spirochetal peptide to certain DRB molecules is a marker for antibiotic-refractory Lyme arthritis and might play a role in the pathogenesis of the disease.

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Abbreviations used: CI, confidence interval; OR, odds ratio; OspA, outer-surface protein A; RA, rheumatoid arthritis.

Lyme arthritis, which is caused by the tick-borne spirochete *Borrelia burgdorferi* (1), can usually be treated successfully with 1 or 2 mo of oral antibiotic therapy, 2 or 4 wk of i.v. therapy, or both (2–4). However, in a small percentage of cases, proliferative synovitis of the knee continues for months or even several years after such courses of therapy, which is termed antibiotic-refractory Lyme arthritis. Although PCR results for *B. burgdorferi* DNA in joint fluid are usually positive before antibiotic therapy, they are frequently negative by the end of 2–3 mo of antibiotics (5), and they have been uniformly negative in synovial samples obtained at synovectomy months after treatment (6). Thus, Lyme arthritis may continue after the near or total eradication of spirochetes from the joint, perhaps sustained by retained spirochetal antigens or an infection-induced autoimmune response.

Most diseases with autoimmune features have HLA associations, particularly with class II MHC alleles. In our initial HLA study of 80 patients with Lyme arthritis (7), which was performed from 1977 to 1987, an association was shown between chronic Lyme arthritis, the HLA-DR4 serologic specificity, and the lack of response to antibiotic therapy; a secondary association was noted with DR2. Thereafter, we began to determine HLA alleles in patients with Lyme arthritis using contemporary molecular techniques. In a preliminary analysis (Steere, A.C., and L.A. Baxter-Lowe. 1998. Annual Meeting of the American College of Rheumatology), patients with antibiotic-refractory arthritis had an increased frequency of alleles associated with the severity of rheumatoid arthritis (RA), primarily the DRB1*0401, 0101, or 0404 alleles; those with

antibiotic-responsive arthritis more often had the 0801 or 1101 alleles. Antibiotic-refractory patients have not been noted to have increased frequencies of the HLA-B27 allele (7), as in reactive arthritis, or the DRB1*0801 or DRB1*11 alleles (Steere, A.C., and L.A. Baxter-Lowe. 1998. Annual Meeting of the American College of Rheumatology), as in pauciarticular juvenile RA (8).

Because the etiologic agent of Lyme arthritis is known, we have searched for a spirochetal antigen that may bind to certain HLA-DR molecules to trigger antibiotic-refractory arthritis. Cellular and humoral immune responses to *B. burgdorferi* outer-surface protein A (OspA) have been associated with this outcome (9–11). The spirochete expresses OspA primarily in the mid-gut of the tick and not in the early mammalian infection (12). However, in a mouse model, the organism up-regulates this protein in inflammatory foci (13), which seems to be the case in the majority of patients with Lyme arthritis. When archival serum samples were tested from patients seen in the late 1970s before the use of antibiotic therapy for this infection, 70% of the patients had OspA antibody responses near the beginning of prolonged episodes of arthritis (9), and the levels of OspA antibody correlated directly with the severity and duration of arthritis (10). In contrast, there was no correlation between arthritis duration and antibody levels to eight other spirochetal proteins. In a study of T cell responses to spirochetal antigens, OspA was preferentially recognized by T cell lines from patients with antibiotic-refractory Lyme arthritis but was only rarely recognized by T cell lines from antibiotic-responsive patients (11). In contrast, the responses to four other spirochetal proteins did not differ between these two groups.

As shown definitively in DRB1*0401-transgenic mice, the immunodominant epitope of OspA presented by the 0401 molecule was located at amino acids 163–175 ($OspA_{163–175}$; reference 14). When preliminary HLA data (Steere, A.C., and L.A. Baxter-Lowe. 1998. Annual Meeting of the American College of Rheumatology) were used to select five purified HLA-DR molecules for in vitro $OspA_{163–175}$ peptide binding studies, the DRB1*0401, 0101, and 0404 molecules, which were associated with antibiotic-refractory arthritis, bound the peptide, whereas the DRB1*0801 and 1101 molecules, which were more often found in antibiotic-responsive patients, did not (15). In addition, PBL from Lyme arthritis patients with the DRB1*0401, 0404, 0101, or 0102 alleles more often reacted with $OspA_{163–175}$ than did PBL from patients with other alleles, and patients in the former group were more likely to have an antibiotic-refractory course (16). Finally, as determined using tetramer reagents, DRB1*0401-positive patients often had increased precursor frequencies of $OspA_{163–175}$-reactive T cells in joint fluid (17). Thus, in patients with certain DRB1 alleles, $OspA_{163–175}$-reactive T cells were frequently concentrated in affected joints, and these individuals were more likely to have an antibiotic-refractory course.

In this study, we determined HLA-DRB1, DQA1, and DQB1 haplotype and allele frequencies in 121 consecutive patients with Lyme arthritis (all were Caucasian) who were seen in our clinic over a 16-yr period. They were all treated with antibiotics according to the guidelines now recommended by the Infectious Diseases Society of America (18). The haplotype and allele frequencies were first compared between all 121 patients with Lyme arthritis and European-American control subjects, and then between Lyme arthritis patients stratified by antibiotic response. Finally, in vitro binding of the $OspA_{163–175}$ peptide to 14 recombinant or purified DRB molecules was correlated with the clinical outcome. Our results showed a marked correlation between antibiotic-refractory Lyme arthritis and HLA-DR molecules that bound the *B. burgdorferi* $OspA_{163–175}$ epitope.

RESULTS

Haplotype frequencies in Lyme arthritis patients and control subjects

We first compared the frequencies of HLA-DRB1-DQA1-DQB1 haplotypes (the most detailed genetic analysis) in 121 Caucasian patients with Lyme arthritis (242 alleles) to the published frequencies of these haplotypes in 1,899 European-American bone marrow donors (3,798 alleles; reference 19). The laboratory that determined the HLA profiles in Lyme arthritis patients also participated in the typing of the control population. In these cases and control groups, 85 distinct haplotypes were identified, 30 of which were common enough for meaningful individual comparisons (Table I). When these haplotypes were compared between Lyme arthritis patients and the control population, a difference of borderline significance was found (log likelihood test, $P = 0.05$), but the effect size statistic was low ($Wn = 0.13$), suggesting that the two groups had only minimal overall differences in HLA types. Moreover, the differences were primarily in unusual alleles, which were each found in only one or a few patients. Furthermore, populations of bone marrow donors tend to be enriched for more common alleles because a match can be more readily found. Therefore, we concluded that the overall frequency of HLA-DRB1-DQA1-DQB1 haplotypes was similar in Lyme arthritis patients and the control population.

Haplotype and allele frequencies in Lyme arthritis patients by antibiotic response

When the 121 patients with Lyme arthritis were stratified by antibiotic response, 15 DRB1-DQA1-DQB1 haplotypes were common enough for individual comparisons (Table I). These haplotype frequencies differed significantly in patients with antibiotic-refractory or antibiotic-responsive arthritis (log likelihood test, $P = 0.009$; exact test, $P = 0.01$; effect size, $Wn = 0.35$). Moreover, when the DRB1 and DQA1-DQB1 alleles were analyzed separately, the frequencies of the DRB1 alleles showed a highly significant difference (log likelihood test, $P = 0.006$; exact test, $P = 0.008$) with a large effect size ($Wn = 0.38$; Table II). The alleles that differed most were the DRB1*0101 and 0401 alleles, which were more common in antibiotic-refractory patients, and the 0801,

Table I. DRB1-DQA1-DQB1 haplotype frequencies in all patients with Lyme arthritis and in European-American control subjects or Lyme arthritis patients stratified by antibiotic response

DRB1–DQA1–DQB1	Haplotype frequencies		G value	Haplotype frequencies		G value
	European-American subjects[a]	All Lyme arthritis patients[b]		Lyme arthritis patients		
				Antibiotic refractory[c]	Antibiotic responsive[d]	
	%	%		%	%	
0101-0101-0501	9.1	7.9	0.4	10.6	4	3.5
0102-0101-0501	1.4	1.7	0.1			
0103-0101-0501	0.5	2.1	6.7	0.7	4	3.1
0301-0501-0201	13.1	12.0	0.2	10.6	14	0.6
0401-03-0301	5.4	6.6	0.6	7.7	5	0.7
0401-03-0302	4.9	3.3	1.3	4.9	1	3.2
0402-03-0302	1.0	1.7	0.9			
0403-03-0302	0.4	0	1.9			
0404-03-0302	3.9	5.0	0.6	5.0	5	0.0
0405-03-0302	0.3	0.8	1.5			
0407-03-0301	0.9	1.2	0.3			
0701-0201-0202	11.1	9.5	0.5	11.3	7	1.2
0701-0201-0303	3.7	1.2	5.0			
0801-0401-0402	2.2	2.9	0.4	0.7	6	5.9
0901-03-0303	0.8	0.4	0.5			
1001-0104-0503	0.7	0.0	3.3			
1101-0501-0301	5.6	5.4	0	2.8	9	4.1
1103-0501-0301	0.3	0.4	0			
1104-0501-0301	2.7	3.3	0.3	0.7	7	7.4
1201-0501-0301	1.1	2.5	2.9	2.8	2	0.2
1301-0103-0603	5.6	3.7	1.6	3.5	4	0.0
1302-0102-0604	3.4	2.5	0.6	2.1	3	0.2
1302-0102-0609	0.7	1.2	0.7			
1303-0501-0301	0.7	2.5	5.6	2.1	3	0.2
1305-0501-0301	0.3	0.4	0.2			
1401-0104-0503	2.0	1.7	0.1			
1501-0102-0602	14.2	10.7	2.1	12.0	9	0.5
1501-0102-0603	2.1	0.8	2.2			
1502-0103-0601	0.7	0.8	0.1			
1601-0102-0502	1.0	2.1	2.2	2.1	2	0.0
Combined[e]	1.3	3.7		12.0	20.0	
Total	100.0	100.0	42.8	100.0	100.0	30.8
P value			0.05			0.009[f]
Degree of freedom			29			15
Wn effect size statistic			0.13			0.35

The 42 rare haplotypes that were found only in the control population are not shown here, but a complete listing of haplotypes in the control population has been published previously (19). Of the 13 rare haplotypes in patients with Lyme arthritis, 12 (0101-0102-0504, 0403-03-0304, 0408-03-0301, 0409-03-0301, 0416-03-0302, 1102-0501-0301, 1104-0101-0301, 1104-0103-0603, 1301-0103-0614, 1302-0102-0501, 1401-0104-0502, and 1501-0102-0502) were each found in only one patient, and one (0404-03-0402) was found in two patients. For comparison of the groups, 1.3% of the haplotypes in normal control subjects and 3.7% of those in all Lyme arthritis patients were excluded from analysis. For the analysis of patients by antibiotic response, 12% of those in the refractory group and 20% of those in the responsive group were excluded.

[a] $n = 3{,}798$.

[b] $n = 242$.

[c] $n = 142$.

[d] $n = 100$.

[e] When ≤5 individuals with Lyme arthritis or ≤10 individuals in the control population had a given haplotype, the values were excluded from analysis (empty cells).

[f] For log likelihood statistic verification, the 2×15 table was also calculated by an exact test; $P = 0.01$.

Table II. DRB1 allele frequencies in patients with antibiotic-refractory or antibiotic-responsive Lyme arthritis

DRB1[a]	Allele frequencies		G value
	Lyme arthritis patients		
	Antibiotic refractory[b]	Antibiotic responsive[c]	
	%	%	
0101	11.3	4	4.1
0102	*1.4*	*2.0*	
0103	0.7	4	3.1
0301	10.6	14	0.6
0401	12.7	6	2.8
0402	*2.8*	*0*	
0403	*0.7*	*0*	
0404	6.3	6	0.0
0405	*1.4*	*0*	
0407	*2.1*	*0*	
0408	*0*	*1*	
0409	*0.7*	*0*	
0416	*0*	*1*	
0701	12.0	9	0.5
0801	0.7	6	5.9
0901	0	1	
1101	2.8	9	4.1
1102	*0.7*	*0*	
1103	*0.7*	*0*	
1104	1.4	7	5.0
1201	2.8	2	0.2
1301	4.2	4	0.0
1302	2.1	7	3.4
1303	2.1	3	0.2
1401	1.4	3	0.7
1501	14.1	9	1.3
1502	*1.4*	*0*	
1601	2.1	2	0.0
Combined	*7.4*	*5*	
Total	100.0	100.0	31.9
P value			0.006[a]
Degree of freedom			15
Wn effect size statistic			0.38

When less than or equal to five individuals with Lyme arthritis had a given haplotype, the values were excluded from analysis (empty cells). These rare alleles, which consisted of 7.4% of the total alleles in the refractory group and 5.0% of those in the responsive group, were excluded from the statistical analysis. Rare DRB1 alleles and their combined sum are listed in italics.

[a]For G statistic verification, the 2 × 15 table was also calculated by an exact test; P = 0.008.

[b]$n = 142$.

[c]$n = 100$.

1101, 1104, and 1302 alleles, which were more frequent in antibiotic-responsive patients. A comparison of the overall frequencies of the DQA1-DQB1 alleles alone did not quite reach formal statistical significance (log likelihood test, P = 0.07; Wn = 0.27; unpublished data), and the alleles that differed most were linked with DRB1 alleles that had even greater differences. Thus, several DRB1 alleles accounted for most of the differences between antibiotic-refractory and antibiotic-responsive patients.

Comparison with past patients

The results in the current 121 patients (seen from 1987 to 2004) were compared with those in our initial HLA study of 80 patients with Lyme arthritis (seen from 1977 to 1987; reference 7). None of the 80 previous patients were included in the current study. In the previous analysis, the HLA profiles were determined by serologic typing methods. In addition, during the earlier period, we were still learning about the cause and treatment of infection. Because some patients were not treated with antibiotics and because others received therapy that would be considered inadequate today, the patients were stratified according to the duration of the longest single attack of arthritis. Analogous to the current definition of antibiotic-responsive arthritis, arthritis of ≤3-mo duration in past patients was defined as arthritis of brief duration, and, similar to the current definition of antibiotic-refractory arthritis, arthritis lasting 4 mo to 4 yr was defined as arthritis of moderate or prolonged duration.

When the DRB1 allele subtypes in the current patients were combined according to past HLA-DR serologic specificities, arthritis of moderate or prolonged duration in past patients and antibiotic-refractory arthritis in the current patients were both associated primarily with DRB1*04 alleles (Table III). In addition, each of the alleles that now make up the former DR2 specificity was identified slightly more often in past and current patients with more prolonged or antibiotic-refractory arthritis. Association of the DRB1*0101 molecule with antibiotic-refractory arthritis was only apparent in current patients. However, results in the current patients differed according to the subtype: 80% of those with the DRB1*0101 allele were in the antibiotic-refractory group; those with the DRB1*0102 allele were equally distributed between the two groups; and 80% of those with the DRB1*0103 allele were in the antibiotic-responsive group (P = 0.02). This may have obscured an association in past patients. In both past and current patients, alleles that make up the former DR5 specificity (except for the DRB1*1201 allele) and the DR3 specificity were more common in patients with arthritis of brief duration or antibiotic-responsive arthritis. Thus, although the typing methods and definitions for patient stratification differed in the two studies, similar HLA associations were found among the 201 study patients with Lyme arthritis tested over a 26-yr period.

OspA peptide binding to HLA-DR molecules

14 recombinant or purified HLA-DRB1, DRB4, or DRB5 molecules were available for in vitro peptide binding studies. Nearly 80% of the patients had one or two of these DRB molecules. Of the 14 molecules, seven showed strong to weak binding with the $OspA_{163–175}$ peptide, whereas the other seven showed negligible or no binding of the peptide (Figs. 1 and 2).

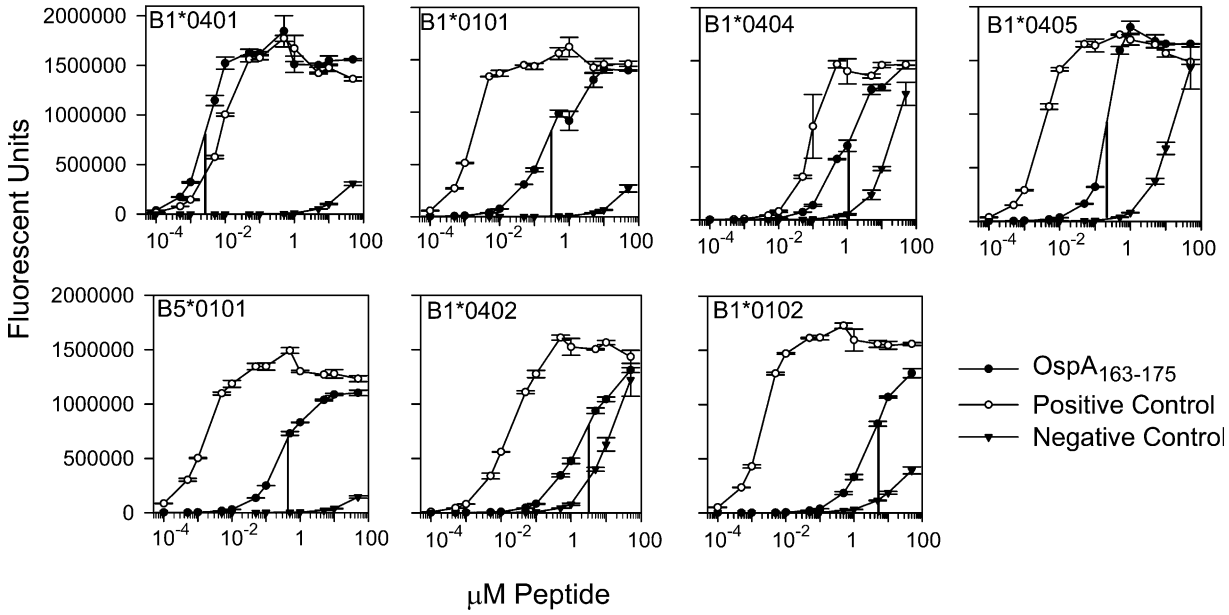

Figure 1. Relative binding avidity of the $OspA_{163-175}$ peptide to seven HLA-DRB molecules that demonstrated strong to weak binding of the peptide. The half max binding concentration of the *B. burgdorferi* OspA peptide for each MHC molecule is shown with error bars (SD). The sequence of the OspA peptide was KGYVLEGTLTAEK. The positive control peptide for the B1*0401, 0402, 0404, and 0405 molecules was glutamic acid decarboxylase $65_{555-567}$ (NFIRMVISNPAAT). For the B1*0101 and 0102 molecules, it was artificial peptide-0102 (PKYVKLNALKLAT), and for the B5*0101 molecule, it was influenza $hemagglutinin_{307-319}$ (PKYVKQNTLKLAT). The negative control peptide for the B1*0101 and 0102 and B5*0101 molecules was $OspA_{165A}$ (KGAVLEGTLTAEK). For the B1*0401, 0404, and 0405 molecules, it was retinal S-antigen peptide-14 (HVIFKKISRDKS), and for the B1*0402 molecule, it was Herpes simplex viral protein 16_{34-44} (PLYATGRLSQA). Data are not depicted for six HLA-DRB molecules that had negligible binding (DRB1*1101; half max = 48 μM) or no detectable binding (half max of ≥50 μM; DRB1*0301, 0701, 1104, 1501, and DRB4*0101) of the OspA peptide. With each of these DRB molecules, the positive control peptide showed strong binding, and the negative control peptide showed no detectable binding.

The DRB1*0401 molecule bound the peptide strongly (half maximal MHC–peptide-binding concentration [half max] = 0.003 μM), the DRB1*0101, 0404, and 0405 molecules bound it moderately well (half max = 0.2–0.3 μM), and the DRB1*0402 or 0102 molecules bound it weakly (half max = 4–6 μM). In addition, separate testing of the genetically

Table III. Comparison of HLA-DR specificities and alleles according to disease course in past and current patients with Lyme arthritis[a]

HLA-DR specificity	Past patients with Lyme arthritis		OR (95% CI)	HLA-DRB1 alleles	Current patients with Lyme arthritis		OR (95% CI)
	Moderate or prolonged[b]	Brief[c]			Antibiotic refractory[d]	Antibiotic responsive[e]	
	%	%			%	%	
DR1	21	18	1.17 (0.35, 3.89)	0101, 0102, 0103	28	18	1.79 (0.75, 4.27)
DR2	41	18	3.18 (0.99, 10.04)	1501, 1502, 1601	32	20	1.92 (0.83, 4.43)
DR3	21	23	0.89 (0.28, 2.77)	0301	20	32	0.51 (0.23, 1.19)
DR4	40	9	6.75 (1.54, ND)[f]	401/02/03/04/05/06/07/08/09/16	46	26	2.47 (1.13, 5.37)
DR5	17	32	0.45 (0.15, 1.34)	1101/02/03/04, 1201	17	36	0.36 (0.16, 0.84)
DR7	14	18	0.72 (0.20, 2.52)	0701	23	18	1.33 (0.54, 3.24)

[a]The results in past patients were published previously (7). The 80 patients were stratified according to the longest single attack of arthritis. Analogous to the current definition of antibiotic-responsive arthritis, arthritis of ≤3-mo duration in past patients was defined as arthritis of brief duration, and analogous to the current definition of antibiotic-refractory arthritis, arthritis lasting 4 mo to 4 yr in past patients was defined as arthritis of moderate or prolonged duration.
[b]$n = 58$.
[c]$n = 22$.
[d]$n = 71$.
[e]$n = 50$.
[f]The number of patients with the DR4 specificity in the brief group was too small to calculate a reliable upper CI.

linked DRB1*1501/DRB5*0101 molecules showed that the DRB5 molecule but not the DRB1 molecule bound the peptide moderately well (half max = 0.4 μM).

In contrast, the DRB1*1101 molecule showed negligible binding of the peptide (half max = 48 μM), and the DRB1*0301 and 1104 molecules and separate testing of the genetically linked DRB1*0701/DRB4*0101 molecules showed no detectable binding of the peptide (half max of ≥50 μM; Fig. 2). Although the DRB1*0801 molecule was not available for study here, no binding of the OspA peptide to this molecule was demonstrated previously when the molecule was obtained from a homozygous B-lymphoblastoid cell line (15). Thus, 40% of the DRB alleles in patients with Lyme arthritis showed strong to weak binding of the OspA peptide, 39% showed negligible or no binding of the peptide, and, for 21% of the alleles, DR molecules were not yet available for testing.

Correlation of OspA peptide binding and HLA-DR frequencies

In general, the DRB molecules that bound the $OspA_{163–175}$ peptide were more common in antibiotic-refractory patients, whereas those that did not bind it were more frequent in antibiotic-responsive patients (Fig. 2 and Table IV). The exceptions were the weak OspA peptide–binding DRB1*0102 molecule, which was found in two patients each with antibiotic-refractory or -responsive arthritis, and the genetically linked, non-OspA peptide–binding DRB1*0701/DRB4*0101 molecules, which were found slightly more often in the antibiotic-refractory group. Altogether, 79% of the patients with antibiotic-refractory arthritis had at least one of the seven known OspA peptide–binding HLA-DR molecules compared with 46% of those with antibiotic-responsive arthritis (odds ratio [OR] = 4.4; $P < 0.001$; Table IV). Moreover, among the subgroup of 31 patients in whom the binding potential of both alleles was known, the patients with two known OspA peptide–binding alleles were 11.3 times more likely to have an antibiotic-refractory course than those with two non-OspA peptide–binding alleles ($P = 0.008$).

Frequencies of RA alleles in Lyme arthritis patients

The severity of RA is associated with DRB1 alleles that have a shared sequence in the third hypervariable region of the DRB1 chain (20–22). In Caucasian populations, this sequence is found most often in the DRB1*0401, 0404, 0405, 0408, 0101, and 0102 alleles. Except for the 0408 molecule, which was not available for testing, these DRB1 molecules bound the $OspA_{163–175}$ peptide. In addition, 43 of the 71 patients (61%) with antibiotic-refractory Lyme arthritis had one or two of these RA alleles compared with 16 of the 50 patients (32%) with antibiotic-responsive arthritis (OR = 3.3; $P = 0.004$). In a previous analysis (14), PBL from patients with RA did not respond to $OspA_{163–175}$, and, therefore, we think that OspA immunity is not a feature of RA. Nevertheless,

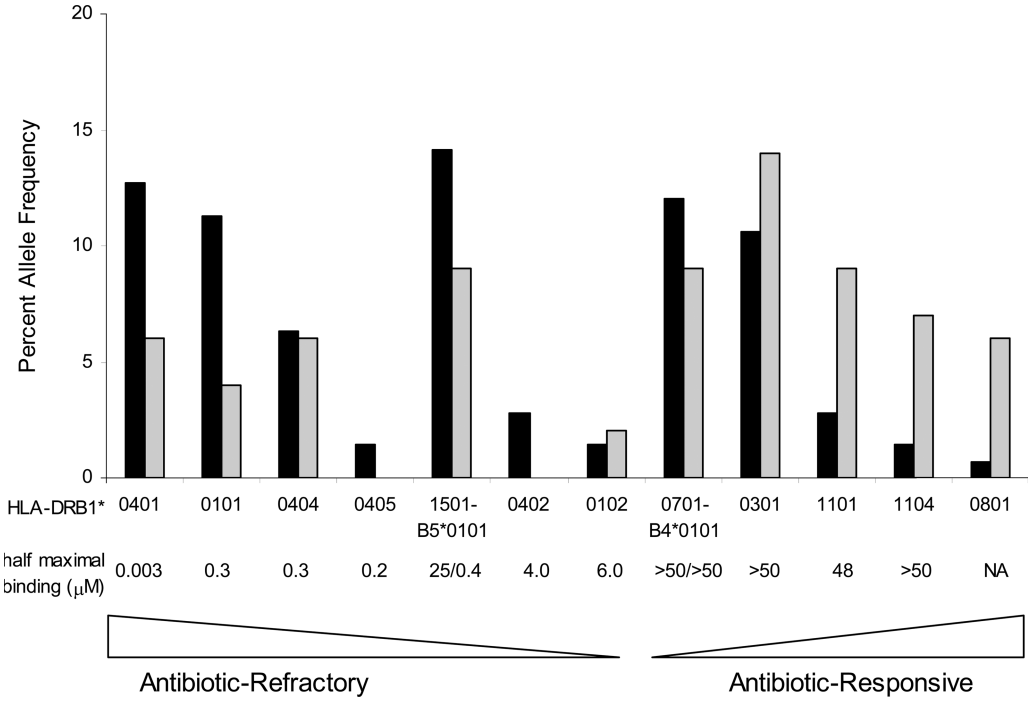

Figure 2. Correlation of the relative binding avidity of the $OspA_{163–175}$ peptide for 14 HLA-DR molecules with percent allele frequencies of these HLA-DR molecules in patients with antibiotic-refractory or antibiotic-responsive Lyme arthritis. Although the DRB1*0801 molecule was not available (NA) for study here, no binding of the OspA peptide was demonstrated in a previous analysis in which this molecule was obtained from a homozygous B-lymphoblastoid cell line (15).

Table IV. $OspA_{163–175}$ peptide binding of HLA-DRB1 genotypes in patients with antibiotic-refractory or antibiotic-responsive Lyme arthritis

DRB genotypes	Number of positive patients		OR (95% CI)	P value
	Antibiotic refractory	Antibiotic responsive		
	%	%		
Two known OspA-binding alleles versus two known nonbinding alleles	$n = 18$	$n = 13$		
(1) Both alleles bind	15 (83)	4 (31)	11.3 (2.14, 58.8)	0.008
(2) Neither allele binds	3 (17)	9 (69)		
One known OspA-binding allele versus one known nonbinding or unknown binding allele	$n = 53$	$n = 37$		
(3) One binding/one non- or unknown binding allele[a]	41 (77)	19 (51)	3.2 (1.3, 8.0)	0.013
(4) One nonbinding/one unknown binding allele	12 (23)	18 (49)		
All patients	$n = 71$	$n = 50$		
1 and 3: one or two known binding alleles	56 (79)	23 (46)	4.4 (1.99, 9.66)	<0.001
2 and 4: no known binding alleles	15 (21)	27 (54)		

[a]Among the antibiotic-refractory patients, 22 had one known OspA-binding allele and one known non-OspA–binding allele, and 19 had one known OspA-binding allele and one allele that was not yet possible to test for binding. Among the antibiotic-responsive patients, 16 had a known OspA-binding allele and one known nonbinding allele, and three had one known OspA-binding allele and one allele that was not possible to test for binding.

primarily DRB1 molecules associated with RA bound $OspA_{163–175}$ and were also associated with antibiotic-refractory Lyme arthritis.

DISCUSSION

In this study, we first compared the frequencies of DRB1-DQA1-DQB1 haplotypes in patients with Lyme arthritis with those in a Caucasian control population, which did not show substantial differences. However, when the patients were stratified by antibiotic response, the two patient groups differed. Moreover, this study is unique in examining the in vitro binding of a single spirochetal epitope, $OspA_{163–175}$, to recombinant forms of most DRB molecules found in this patient population, all of whom had *B. burgdorferi* infection of the joints. When the DRB frequencies and binding results were correlated, patients with stronger OspA peptide–binding DRB molecules were three or four times more likely to have antibiotic-refractory arthritis, and those with weaker binding molecules were one or two times more likely to have this outcome. When the seven known $OspA_{163–175}$-binding DRB alleles were combined, a marked correlation was found with antibiotic-refractory arthritis.

Although this series of 121 patients with Lyme arthritis is the largest tested to date, the number of patients was not large enough to show differences between refractory and responsive patients in the frequencies of individual alleles. Moreover, only small numbers of patients had more unusual alleles, some of which bound the $OspA_{163–175}$ peptide, such as the DRB1*0402, 0405, and 0102 molecules. Despite this problem, groups were analyzed by the log likelihood test because it tests the overall difference between two distributions and reveals individual contributions to any difference but avoids the problem of multiple comparisons when the frequencies of alleles or haplotypes are examined individually. However, because the number of samples in the tests was relatively small, resulting in sparse tables, the log likelihood statistic [G value] was verified by an exact test. Both methods showed similar results.

We were able to test $OspA_{163–175}$ peptide binding of the four most common DR4 subtypes (0401, 0404, 0405, and 0402), each of which showed binding of the OspA peptide and would explain the primary association of antibiotic-refractory arthritis with DR4 alleles. Among 80 previous patients, arthritis of moderate or prolonged duration was associated primarily with the HLA-DR4 serologic specificity (7). In the current patients, the DRB1*1501 allele (formerly DR2) was slightly more common among antibiotic-refractory patients, and, in the previous study, a secondary association was noted with the DR2 specificity. The OspA peptide binding studies would explain this weak association. This peptide does not bind the DRB1*1501 molecule but does bind the nearly equally expressed DRB5*0101 molecule to which it is linked (23, 24). Therefore, additional DRB loci in some HLA class II haplotypes may play a critical role in enlarging the peptide-binding repertoire.

Although the DR1 serologic specificity was not increased in frequency in the previous study (7), differential binding of the OspA peptide to the three DRB1*01 subtypes may explain this result. Among the current patients, the DRB1*0101 molecule, which was strongly associated with antibiotic-refractory arthritis, bound the OspA peptide well. In comparison, the DRB1*0102 molecule, which was distributed equally between antibiotic-refractory and antibiotic-responsive patients, bound the OspA peptide weakly. This molecule differs from the DRB1*0101 molecule in only two amino acids (25), which are predicted to make the P1-binding pocket smaller (26). This difference would be likely to make binding of the OspA peptide's large aromatic tyrosine in this

position less favorable. Compared with the DRB1*0101 molecule, the DRB1*0103 molecule, which was identified almost exclusively in antibiotic-responsive patients, has three amino acid substitutions in the P4 pocket (25), which would be likely to alter the peptide-binding properties of that molecule. Thus, small differences in the three quite similar alleles of the DR1 specificity, leading to differential binding of the OspA peptide, would provide an explanation for differences in disease outcomes among these patients.

The antibiotic-refractory group also had a slightly increased frequency of the genetically linked DRB1*0701/DRB4*0101 molecules, neither of which bound the OspA peptide. In the previous study (7), no association was seen between the duration of arthritis and the DR7 specificity. Because all of the current DRB1-0701–positive, antibiotic-refractory patients had the linked DQB1*0202 molecule, it is possible that this molecule or a linked DP molecule may bind the OspA peptide weakly, but we are not yet able to test this hypothesis. Alternately, the slightly increased frequency of these molecules in the current patients may have occurred by chance.

Might another spirochetal epitope show a similar or even better correlation between DRB frequencies and peptide binding? The *B. burgdorferi* genome contains sequences for 1,639 known or predicted proteins (27, 28), and a given protein may have as many as seven predicted T cell epitopes. In a recent computer search, the closest match with $OspA_{163–175}$ was an epitope of *B. burgdorferi* glycerol kinase, which contained the OspA epitope's first five core amino acids (YVLEG). However, the glycerol kinase epitope did not stimulate patients' T cells (unpublished data). We also identified predicted T cell epitopes of 10 known immunogenic proteins of *B. burgdorferi* and selected eight epitopes for binding studies. The $OspA_{163–175}$ peptide and one epitope of fibronectin-binding protein ($BBK32_{392–404}$) had similar binding patterns, and $BBK32_{392–404}$ even showed weak binding of the DRB1*0701 and DRB4*0101 molecules. However, patients' T cells did not respond to the BBK32 epitope (unpublished data). Finally, Lyme borreliosis worldwide is caused by three related pathogenic *Borrelia* species: *B. burgdorferi*, *B. afzelii*, and *B. garinii* (29). In a recent study, the sequences of the core $OspA_{165–173}$ epitope differed among the three species, and lymphocytes from patients with antibiotic-refractory Lyme arthritis proliferated only in response to the *B. burgdorferi* peptide (30). Thus, we have not yet found a borrelial epitope other than $OspA_{163–175}$ that shows a correlation with the HLA data and is preferentially recognized by T cells in antibiotic-refractory patients (16, 17).

Four basic hypotheses have been proposed to explain the association of $OspA_{163–175}$ peptide–binding DRB alleles with antibiotic-refractory Lyme arthritis: (a) persistent infection, (b) retained spirochetal antigens, (c) infection-induced autoimmunity resulting from molecular mimicry between a spirochetal and host epitope, or (d) from bystander activation of autoreactive T cells (31). According to the persistent infection hypothesis, an HLA-linked immune response to *B. burgdorferi* in antibiotic-refractory patients may be ineffective in eradicating spirochetes from a protected niche in the joint even though these patients have higher levels of IFN-γ, TNF-α, and other proinflammatory cytokines in infected joints than antibiotic-responsive patients (Shin, J.J., L.J. Glickstein, G. McHugh, and A.C. Steere. 13th Annual Conference of the International Cytokine Society. 2005. Abstr. P3-60; Fawcett, P.T., C.D. Rose, V.L. Maduskuie, J.J. Sanderson, P.A. Stanek, T. Stetson, A. Brescia, and L.B. Fawcett. 2005. Annual Meeting of the American College of Rheumatology. Abstr. 172). In that case, a preferential T cell response to $OspA_{163–175}$, a protein expressed only in inflamed joints (13), may simply be a marker for more prolonged infection in refractory than responsive patients. However, if small numbers of spirochetes remain in the synovial tissue of refractory patients, they are below the limits of detection by PCR because PCR results have been uniformly negative in their synovectomy samples (6). Alternately, MHC molecules in synovial tissue may preferentially retain OspA antigens after spirochetal killing, and T cell recognition of these antigens, along with high levels of proinflammatory cytokines (Shin, J.J., L.J. Glickstein, G. McHugh, and A.C. Steere. 13th Annual Conference of the International Cytokine Society. 2005. Abstr. P3-60; Fawcett, P.T., C.D. Rose, V.L. Maduskuie, J.J. Sanderson, P.A. Stanek, T. Stetson, A. Brescia, and L.B. Fawcett. 2005. Annual Meeting of the American College of Rheumatology. Abstr. 172), may continue to induce synovial inflammation for months after spirochetal killing.

According to the molecular mimicry hypothesis of autoimmunity, molecular mimicry between the $OspA_{163–175}$ epitope and a similar sequence of a self protein might serve as a bridge to activate an autoimmune T cell or linked B cell response within the proinflammatory milieu of inflamed joints (31). We originally proposed human $LFA\text{-}1\alpha_{L332–340}$, which has partial sequence homology with $OspA_{163–175}$, as a candidate autoantigen in antibiotic-refractory Lyme arthritis (14). However, we later showed that the LFA-1 peptide was only a weak, partial agonist for $OspA_{163–175}$-reactive T cells (32), and the LFA-1 peptide does not bind the refractory arthritis-associated DRB1*0101 molecule (15). Thus, we now think that this peptide is unlikely to be a relevant autoantigen. Although it has been a formidable challenge to identify relevant infectious and self molecular mimics in any disease, the association of antibiotic-refractory Lyme arthritis with a single spirochetal epitope could still be explained by such a mechanism, which may depend on structural similarity between $OspA_{163–175}$ and a self epitope rather than simple sequence similarity (33). Alternately, T cell recognition of the $OspA_{163–175}$ epitope in genetically susceptible individuals may lead to especially high levels or inadequate regulation of proinflammatory cytokines, which might cause T cell activation to a structurally unrelated or shielded self epitope. However, recognition of the OspA epitope alone is clearly insufficient to induce disease because among nearly 10,000 individuals vaccinated with OspA in the deltoid muscle, autoimmune arthritis was not observed (34, 35). Recognition of the epitope

within the proinflammatory milieu of infected joints would presumably be necessary.

Regardless, we do not think that a single mechanism explains persistent arthritis after 2–3 mo of antibiotic therapy in all patients. Although most of the antibiotic-refractory patients who lacked one of the seven known OspA peptide–binding DR molecules had molecules that are not yet available in recombinant form for in vitro studies, three patients had two alleles that are known not to bind this peptide. Moreover, after the clinical classification was made, it became clear that at least one patient classified in the refractory group still had persistent infection. The patient, who had the DRB1*0301 and 1302 alleles and lacked OspA reactivity, had the resolution of arthritis 6 mo after the initiation of a 2-mo course of oral antibiotic therapy. Later, she had two recurrences of arthritis, and the first was accompanied by a positive PCR result for *B. burgdorferi* DNA in joint fluid. Nevertheless, for this study, we retained the initial classification of each patient, which was made before HLA typing. We would emphasize that the inclusion of such patients would bias against showing an association between antibiotic-refractory arthritis and $OspA_{163–175}$-binding DRB alleles, yet an association was still demonstrated.

It is of great interest that primarily DRB1 molecules associated with RA bound $OspA_{163–175}$ and were also associated with antibiotic-refractory Lyme arthritis. However, several non-RA–associated DRB1 molecules also bound the OspA peptide, including the DRB1*0402 and DRB5*0101 molecules, and RA patients' PBLs did not respond to the OspA peptide (14). Although OspA immunity is not a feature of RA, the similar HLA associations in both diseases raise the possibility that specific HLA molecules or other HLA-linked immune responses, such as TNF-α levels, are important in the pathogenesis of both diseases.

In conclusion, we found a marked association between antibiotic-refractory Lyme arthritis and DRB molecules that bound the $OspA_{163–175}$ epitope of *B. burgdorferi*. This clinical correlation suggests that binding of a single spirochetal peptide, $OspA_{163–175}$, to certain DRB molecules, primarily those associated with RA, is a marker for antibiotic-refractory Lyme arthritis and might play a role in the pathogenesis of the disease.

MATERIALS AND METHODS

Study patients. From August 1987 through May 2004, we evaluated 121 consecutive patients with Lyme arthritis (ages 12–79) who were treated with antibiotics according to the guidelines now recommended by the Infectious Diseases Society of America (18). The study protocol, which included HLA typing, was approved by the Human Investigations Committees at Tufts-New England Medical Center (1987–2002), Massachusetts General Hospital (2002–2004), and the University of California, San Francisco (1999–2004); all patients gave written informed consent. All patients met the criteria of the Centers for Disease Control for the diagnosis of Lyme arthritis (36, 37).

50 patients (41%) had antibiotic-responsive arthritis, which was defined, as in previous studies (15, 16), as the resolution of arthritis within 3 mo after the start of no more than 4 wk of i.v. antibiotics or 8 wk of oral antibiotics. In this group, the median duration of antibiotic therapy was 4 wk (range of 4–8 wk), and the median duration from the start of antibiotics to the resolution of arthritis was also 4 wk (range of 2–11 wk). The remaining 71 patients (59%) had antibiotic-refractory arthritis, which was defined as persistent joint swelling for ≥3 mo after the start of ≥4 wk of i.v. antibiotics, ≥8 wk of oral antibiotics, or both. Their median duration of antibiotic therapy was 13 wk (range of 4–29 wk), and the median duration from antibiotic initiation to arthritis resolution was 11 mo (range of 4–48 mo).

Of the 55 patients who were referred before or during their first course of antibiotics, 50 (91%) had antibiotic-responsive arthritis, and five had an antibiotic-refractory course, a distribution similar to that found in the community (3). The remaining 66 study patients were first referred to us later in their course because of a lack of response to antibiotics. Thus, this distribution of refractory and responsive cases is reflective of our role as a referral center.

HLA typing procedures. HLA-DRB1, DQA1, and DQB1 alleles in the 121 patients were determined by high resolution molecular HLA typing methods using sequence-specific oligonucleotide probes, automated sequencing, or sequence-specific priming (Dynal) as previously described (38). In 1992, HLA typing was determined in patients seen in the previous 5 yr; thereafter, typing was determined on a yearly basis. Although typing methods were modified during the study as new typing techniques became available, the typing methods used throughout the study were capable of resolving the more common HLA alleles.

Production of recombinant HLA-DR molecules and peptides. Recombinant, soluble HLA-DR molecules were generated from Schneider S-2 cells that expressed the extracellular domains of DRα and DRβ chains linked to the leucine zipper dimerization domains of the transcription factors c-Fos and c-Jun, respectively (39). The transfected cells were expanded to a density of $4–6 \times 10^6$ cells/ml, and 1 mM $CuSO_4$ was added to induce production of the soluble class II molecules. Recombinant MHC molecules were purified by affinity chromatography using the HLA-DR–specific monoclonal antibody L243 (40). The peptides were synthesized and biotinylated as previously described (40). Two aminohexanomic acid spacers were placed between the biotin and peptide to inhibit steric hindrance.

MHC–peptide-binding assay. One 96-well plate (Costar) was coated with 100 μl (12.5 μg/ml) of the anti-DR monoclonal antibody L243 diluted in 12.5 mM borate buffer and incubated overnight at 4°C as previously described (15). In a second plate (Costar), 1 μl (50 μM) of each biotinylated peptide diluted in DMSO (Sigma-Aldrich) was placed in duplicate wells; 200 μl of purified MHC molecules (0.004 μg/ml) diluted in citrate phosphate buffer, pH 5.4, with 0.75% n-octyl-β-D-glucopyranoside (Sigma-Aldrich) and 1 mM PefaBloc (Roche) were then added to each well and incubated overnight at 37°C in a humidified chamber. The following day, the antibody plate was washed five times with 0.05% Tween-20 in PBS, pH 7.4, and blocked with 5% FCS in PBS for 3 h at room temperature. After washing, 50 μl of 50 mM Tris, pH 8.0, with 0.75% n-octyl-β-D-glucopyranoside was added to each well, and MHC–peptide complexes were transferred to the antibody plate and incubated overnight at 4°C. The following day, 0.1 mg/ml europium-labeled streptavidin diluted in assay buffer was added to each well and incubated for 30 min at room temperature followed by enhancement buffer (each from PerkinElmer) for 10–15 min. Fluorescence was then measured with a multilabel counter (Victor^2 1420; PerkinElmer). The half max binding concentration of $OspA_{163–175}$ was defined as the concentration of OspA peptide required for the binding of half of the MHC molecules compared with the positive control peptide.

Statistical analysis. The frequency of haplotypes and alleles in all patients with Lyme arthritis and control subjects and in Lyme arthritis patients stratified by antibiotic response were first compared by means of a 2 by k test for homogeneity. The overall deviations between the two frequency distributions were calculated with the G value, and the sum of the individual G values was used to evaluate the overall difference between the two distributions (41). Because the number of samples in the tests was relatively small, resulting

in sparse tables, the G statistic was verified by an exact test on the 2 by k distribution of the observed counts using the R language library Fisher test. The effect size statistic, Wn, a chi square–related statistic that ranges from 0 (no overlap) to 1 (complete identity), was also calculated as a measure of the absolute differences between the comparison groups (42, 43). For the comparisons of DR alleles with specificities and of $OspA_{163–175}$-binding or nonbinding DRB molecules in antibiotic-responsive or -refractory patients, ORs (cross product ratios) and 95% confidence intervals (CIs) were calculated. P values for the comparisons of patient frequencies were calculated by two-tailed exact tests.

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