BRIEF REPORT

A Case-Control Study to Examine HLA Haplotype Associations in Patients with Posttreatment Chronic Lyme Disease

Mark S. Klempner,[1] Gary H. Wormser,[5] Karen Wade,[3] Richard P. Trevino,[1] Jianming Tang,[4] Richard A. Kaslow,[4] and Christopher Schmid[2]

[1]Boston University School of Medicine and Boston Medical Center, and [2]Tufts–New England Medical Center, Boston, Massachsetts; [3]RhoFED Inc., Chapel Hill, North Carolina; [4]University of Alabama, Birmingham; [5]New York Medical College, Valhalla, New York

(See the editorial commentary by Radolf, on pages 948–9.)

In a comparison of 95 patients with systemic symptoms that persisted after antibiotic treatment for acute Lyme disease (posttreatment chronic Lyme disease) and 104 control subjects without such symptoms after antibiotic treatment, we sought associations between human leukocyte antigen class II (*DRB1* and *DQB1*) markers and posttreatment chronic Lyme disease. No strong association between posttreatment chronic Lyme disease and any class II allele or genotype was found.

Lyme disease is caused by infection with the tickborne bacterium *Borrelia burgdorferi.* Antibiotic treatment is highly effective for the acute symptoms of Lyme disease and is also effective for late septic manifestations [1]. After standard treatment with antibiotics, some patients have persistent symptoms. There appear to be at least 2 distinct syndromes in patients with persistent symptoms after antibiotic treatment. One syndrome has localized symptoms that are similar to pretreatment symptoms. Patients with this syndrome often have recurrent episodes of arthritis/synovitis. Results of synovial fluid cultures and polymerase chain reaction (PCR) for *B. burgdorferi* are negative [2]. Patients generally feel well aside from their arthritis symptoms. Specific HLA haplogroups (i.e., HLA-DR4 and HLA-DR2) have been associated with the failure to respond to antibiotics in this group of patients, and their arthritis may be due to molecular mimicry between a dominant epitope of outer surface protein A (OspA) of *B. burgdorferi* and lymphocyte function–associated antigen–1 (LFA-1) [3]. A much more common syndrome of persistent symptoms is a systemic illness that is characterized by profound fatigue, myalgias, polyarthralgias without arthritis, paresthesias, and mood and memory disturbances. This syndrome has been variously referred to as "chronic Lyme disease," "post–Lyme disease syndrome," and "posttreatment chronic Lyme disease" (PTCLD). The cause of the persistent systemic symptoms in these patients is unknown. However, we have reported elsewhere that the impact that PTCLD has on health-related quality of life was highly significant and that treatment with placebo or 90 days of additional antibiotics did not differentially affect patients' health-related quality of life [4]. We also did not find evidence of persistent infection with *B. burgdorferi* or exposure to other tickborne infectious agents that could explain the persistent systemic symptoms.

A case-control study was conducted to determine whether HLA class II polymorphisms are associated with PTCLD, as is suggested by the established relationship between specific HLA alleles and relapsing Lyme arthritis. Such evidence would support an autoimmune mechanism in PTCLD.

Participants, materials, and methods. All study participants were 18 years of age or older, provided informed consent, and had a history of treatment with a recommended antibiotic regimen appropriate for the clinical features of Lyme disease at the time of presentation. Case patients with PTCLD were identified from a study of 2 previous cohorts of seronegative and seropositive subjects, as defined by the Centers for Disease Control and Prevention criteria for the interpretation of serological results of Lyme disease testing [4]. All case patients had been treated with a recommended regimen of antibiotics and had persistent systemic symptoms that were attributed to PTCLD at the time of enrollment into a trial. Persistent symptoms were defined as those that first appeared within 6 months of the initial infection with *B. burgdorferi* and persisted for at least 6 months after the initial infection. The symptoms attributed to PTCLD included 1 or more of the following: widespread musculoskeletal pain and fatigue that interfered with usual function; symptoms of memory impairment that interfered with usual function; and radicular pain, paresthesias, and/or dysesthesias that interfered with usual function and were attributed to Lyme disease.

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Reprints or correspondence: Dr. Mark S. Klempner, Boston University School of Medicine, Collamore 738, 715 Albany St., Boston, MA 02118 (klempner@bu.edu).

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Table 1. Baseline and demographic characteristics of the study population.

	Case patients (n = 95)	Control subjects (n = 104)	All (n = 199)	P
Sex				
Male	55 (58)	59 (57)	114 (57)	…
Female	40 (42)	45 (43)	85 (43)	.8868
Age group				
20–59 years	64 (67)	69 (66)	133 (67)	…
⩾60 years	31 (33)	35 (34)	66 (33)	>.999
Ethnic heritage				
American Indian	2 (2)	0	2 (1)	…
Asian/Pacific Islander	0	1 (1)	1 (1)	…
Black	1 (1)	2 (2)	3 (2)	…
Hispanic	1 (1)	3 (3)	4 (2)	…
White	89 (94)	96 (92)	185 (93)	…
Other/unknown	2 (2)	2 (2)	4 (2)	.5240

NOTE. Data are no. (%) of participants, unless otherwise indicated.

Control subjects consisted of a convenience sample of patients who had been treated for early Lyme disease associated with erythema migrans during the 12 months before entry into the study. None of the control subjects had PTCLD or relapsing Lyme arthritis. The diagnosis of acute Lyme disease in all control subjects was confirmed by recovery of *B. burgdorferi* in cultures of a skin and/or blood sample.

Ninety-four case patients had previously provided samples and had given permission for their stored samples to be used for additional testing for Lyme disease research. The samples were masked so that HLA class II typing could not be linked to a specific case patient. Site institutional review boards granted waivers of informed consent for the masked use of these samples.

One case patient and all control subjects underwent phlebotomy, during which 10-mL tubes of whole blood that was anticoagulated with EDTA or citrate were collected. Collected blood samples were divided into 0.5-mL aliquots, and tubes were affixed with study labels identifying only the sample type (whole blood), date of collection, study name, and subject study number.

High-molecular-weight genomic DNA was extracted from blood samples using the QIAamp Blood Kit and protocols recommended by the manufacturer (Qiagen). The 4-digit alleles at the *HLA-DQB1* locus were resolved by PCR with sequence-specific primers (Pel-Freez Clinical Systems). Sequencing-based typing of *HLA-DRB1* was facilitated by SequiTyper software (version 2.0) and the DRβ Plus kit, both designed for use with the ALFexpress automated sequencer (Amersham Pharmacia Biotech) [5]. After resolution of individual alleles at each locus, *DRB1-DQB1* haplotypes were assigned manually in accordance with known linkage disequilibria observed in major US populations [6, 7].

The objective of the study was to determine whether particular HLA class II alleles or haplotypes are associated with PTCLD. Adherence to Hardy-Weinberg equilibrium at each individual locus was evaluated for all case patients and control subjects separately and combined. Exact tests for Hardy-Weinberg equilibrium were performed using the permutation method [8]. Linkage disequilibrium was also tested for each pair of alleles [9, 10]. An omnibus likelihood ratio test was applied to the aggregated case and control populations, to detect an overall distortion of the allelic frequencies.

Primary analyses of individual alleles were conducted at the $\alpha = 0.05$ level, with no corrections for multiple corrections; P values for secondary analyses were considered to be descriptive. To test for an association between a genetic marker and PTCLD, allelic frequencies were compared across case patients and control subjects using Fisher's exact, multiallelic trend, and allele case-control tests. Logistic regression techniques were also used to compare allelic frequencies across case patients and control subjects, and potential confounding variables were controlled for. The outcome variable for each model was disease status (PTCLD or no PTCLD in case patients vs. control subjects). Alleles with $P < .25$ (Fisher's exact test) in the univariate analysis were included in the final model. If the model did not converge, alleles were entered 2 at a time and then 3 at a time, until the largest model that would converge was found. Haplotype frequencies were compared across case patients and control subjects in a similar manner as allelic frequencies.

Results. Data from 95 case patients and 104 control subjects were analyzed. Because these genetic analyses required anonymization of patients, the only demographic and baseline characteristics considered were sex, age group, and ethnic heritage. As is shown in table 1, there were no significant demographic differences between case patients and control subjects.

Neither *DQB1* nor *DRB1* genotypes in the study population departed from Hardy-Weinberg equilibrium: in case patients, for *DQB1,* $P = .4353$, and for *DRB1,* $P = .3044$; in control subjects,

for *DQB1,* $P = .6921$, and for *DRB1,* $P = .7550$; and in the combined cohort, for *DQB1,* $P = .1852$, and for *DRB1,* $P = .1911$. As was expected, a number of markers at the *DQB1* and *DRB1* loci were in tight linkage disequilibrium ($P < .0001$).

The distribution of *DQB1* alleles (table 2) did not differ between case patients and control subjects by the omnibus likelihood ratio test ($P = .1273$). The multiallelic trend test and the allele case-control test were equivalent, because no departure from Hardy-Weinberg equilibrium was detected and no additive effect of the alleles was found ($P = .2533$ and $P = .1935$, respectively). When they were examined individually, alleles *0201, *0301, *0302, *0303, *0306, *0602, *0604, and *0609 were present in a higher proportion of case patients than control subjects, but these differences were quite modest ($P = .224$ to $P > .999$). In the 199 participants analyzed, 73 *DQB1* genotypes (diplotypes) were observed. The most prevalent genotype was *0301/*0501, which occurred in 9% of the population and was equally distributed between case patients and control subjects. No other genotype occurred in >4% of the population.

For *DRB1,* the distribution of alleles (table 2) did not differ between case patients and control subjects by the omnibus likelihood ratio test ($P = .1706$), the multiallelic trend test ($P = .3372$), and the allele case-control test ($P = .2481$). DRB1*0401 showed the greatest relative difference (18% of case patients and 7% of control subjects) ($P = .017$), and DRB1*0403 showed a trend toward an association with a reduced risk of developing PTCLD ($P = .067$). In the absence of a significant deviation in the overall distribution of *DRB1* alleles or a hypothesis that there is an association between PTCLD and any single allele, and because of the large number of alleles examined, the finding that DRB1*0401 differed between case patients and control subjects at $P = .017$ is of unknown significance.

We examined the presence of *DRB3* (DRB1*03, *11, *12, *13, and *14), *DRB4* (DRB1*04, *07, and *09), and *DRB5* (DRB1*15 and *16) genes along with the remaining *DRB1* alleles (DRB1*01, *08, and *10). The frequencies of *DRB3, DRB4,* and *DRB5* genes did not differ between case patients and control subjects by the omnibus likelihood ratio test. There was no significant trend toward differences between case patients and control subjects who had 0, 1, or 2 copies of *DRB3, DRB4,* or *DRB5* ($P = .73$, $P = .73$, and $P = .61$, respectively; Cochran-Armitage trend test). When participants were classified as having the presence or absence of each gene, there was also no significant trend toward differences between case patients and control subjects (χ^2 test) (data not shown).

The distribution of haplotype frequencies did not differ between case patients and control subjects ($P > .999$, multiallelic trend test and genotypic case-control test; $P = .81$, allele case-control test). Because the global tests showed no evidence of an association between any haplotype and disease status, in-

Table 2. *DQB1* and *DRB1* allelic frequencies.

Allele	All (n = 199)	Test for heterogeneity: Case patients (n = 95)	Control subjects (n = 104)	P[a]
DQB1				
*0201	43 (22)	21 (22)	22 (21)	>.999
*0202	32 (16)	11 (12)	21 (20)	.123
*0301	64 (32)	35 (37)	29 (28)	.224
*0302	31 (16)	15 (16)	16 (15)	>.999
*0303	22 (11)	12 (13)	10 (10)	.508
*0305	2 (1)	0	2 (2)	.499
*0306	1 (<1)	1 (1)	0	.477
*0401	2 (1)	1 (1)	1 (1)	>.999
*0402	8 (4)	3 (3)	5 (5)	.723
*0501	54 (27)	22 (23)	32 (31)	.265
*0502	5 (3)	0	5 (5)	.061
*0503	9 (5)	3 (3)	6 (6)	.502
*0504	3 (2)	0	3 (3)	.248
*0601	3 (2)	2 (2)	1 (1)	.607
*0602	45 (23)	25 (26)	20 (19)	.241
*0603	26 (13)	12 (13)	14 (13)	>.999
*0604	19 (10)	11 (12)	8 (8)	.470
*0609	7 (4)	4 (4)	3 (3)	.711
DRB1				
*0101	31 (16)	12 (13)	19 (18)	.330
*0102	10 (5)	4 (4)	6 (6)	.750
*0103	10 (5)	4 (4)	6 (6)	.750
*0301	42 (21)	22 (23)	20 (19)	.602
*0313	1 (<1)	0	1 (1)	>.999
*0401	24 (12)	17 (18)	7 (7)	.017
*0402	6 (3)	4 (4)	2 (2)	.428
*0403	8 (4)	1 (1)	7 (7)	.067
*0404	8 (4)	5 (5)	3 (3)	.483
*0405	6 (3)	2 (2)	4 (4)	.685
*0407	1 (<1)	1 (1)	0	.477
*0413	1 (<1)	1 (1)	0	.477
*0701	48 (24)	22 (23)	26 (25)	.868
*0801	8 (4)	3 (3)	5 (5)	.723
*0802	1 (<1)	1 (1)	0	.477
*0806	1 (<1)	1 (1)	0	.477
*0901	5 (3)	1 (1)	4 (4)	.371
*1001	4 (2)	1 (1)	3 (3)	.623
*1101	21 (11)	10 (11)	11 (11)	>.999
*1102	1 (<1)	1 (1)	0	.477
*1103	7 (4)	4 (4)	3 (3)	.711
*1104	9 (5)	4 (4)	5 (5)	>.999
*1108	1 (<1)	0	1 (1)	>.999
*1201	7 (4)	2 (2)	5 (5)	.448
*1301	25 (13)	13 (14)	12 (12)	.674
*1302	28 (14)	16 (17)	12 (12)	.313
*1303	1 (<1)	1 (1)	0	.477
*1305	4 (2)	0	4 (4)	.123
*1401	8 (4)	3 (3)	5 (5)	.723
*1407	1 (<1)	0	1 (1)	>.999
*1501	42 (21)	23 (24)	19 (18)	.385
*1502	5 (3)	3 (3)	2 (2)	.671
*1503	1 (<1)	0	1 (1)	>.999
*1601	4 (2)	0	4 (4)	.123

[a] Fisher's exact test to test for a difference between case patients and control subjects.

dividual haplotypes were not examined. Additionally, because the distribution of allelic frequencies for both markers showed little evidence of an association with disease status, and because the haplotype frequencies were very low, no logistic regression models were fit.

Discussion. In this study, we sought to identify HLA class II alleles or haplotypes that might be associated with PTCLD. Case patients and control subjects were similar in age, sex, and ethnic heritage and appeared to differ only in whether, after antibiotic treatment for acute Lyme disease, they had persistent systemic symptoms. Our analyses did not reveal any unequivocally strong relationship between PTCLD and either *DQB1* or *DRB1*.

Previous analyses have suggested that there is an increased frequency of DRB1*0401, DRB1*0101, and related alleles in subjects with antibiotic treatment–resistant Lyme arthritis [11]. On the strength of this association and the evidence of T cell reactivity to the predicted immunodominant epitope of OspA presented by the DRB1*0401 molecule in 15 of 16 patients with antibiotic treatment–resistant Lyme arthritis, an autoimmune basis for relapsing Lyme arthritis has been hypothesized [3, 12]. In the present study, the higher frequency of DRB1*0401 in case patients was nominally significant ($P = .05$). In a previous study, we did not find evidence of arthritis or other inflammatory manifestations in patients with PTCLD [4], and allele DRB1*0401 has been associated with only relapsing Lyme arthritis. Thus, a meaningful association between disease status and this allele is difficult to accept without reservation.

In the absence of biological or epidemiological evidence of the involvement of any single genetic marker (e.g., on the basis of a selective response to a class II allele–specific peptide), the usual problem of multiple comparisons will arise in the interpretation of the results. Because most of these analyses were directed at generating hypotheses about the effect that genetic factors have on the risk of developing PTCLD, Bonferroni or another *P* value correction procedure is not appropriate for these studies. The *P* values generated from these hypothesis-generating analyses were interpreted as simple statistics describing the relative weight of evidence of an association between a genetic marker and the risk of developing PTCLD. Any effect of HLA that is observed for the first time in this study should be interpreted cautiously, and the multiple comparisons should be taken into account. In this case, a strong association in any direction (typically, an association evidenced by $P \leqslant .01$) should be taken as a guide to conducting further research at the biological level. In general, the analyses at the 2-digit resolution (the serotype equivalent) provided the same results as the analyses at the 4-digit resolution (performed by molecular typing). Results from the logistic regression models should be interpreted cautiously. Because frequencies were generally low for all alleles, fitting the logistic regression models was computationally difficult.

The cause of PTCLD remains elusive. To date, there has been little evidence that its symptoms are caused by persistent viable *B. burgdorferi,* as judged on the basis of negative results of culture and PCR of serum and cerebrospinal fluid (CSF); coinfection with another agent transmitted by *Ixodes scapularis* at the time of initial infection with *B. burgdorferi* (e.g., *Babesia microti* or *Anaplasma phagocytophila*), as judged on the basis of the absence of serological evidence of such an infection; damage to a specific neural locus; or an active inflammatory process, as judged on the basis of the lack of clinical signs and symptoms of inflammation or laboratory evidence of increased peripheral white blood cell count, CSF pleocytosis, or increased erythrocyte sedimentation rate [4, 13]. To the extent that many autoimmune processes are characterized by associations with specific HLA class II alleles, the data presented here do not clearly support an autoimmune mechanism in PTCLD in these patients.

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