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CONTRIBUTION OF HLA ALLELES IN THE REGULATION OF ANTIBODY PRODUCTION IN LYME DISEASE

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1. ABSTRACT

A small subset of patients infected with *Borrelia burgdorferi (Bb)* does not produce *Bb* specific antibody. Our research provides additional evidence of a genetic predisposition for seronegativity in some individuals with Lyme disease. Because human leukocyte antigen (HLA) class II, a heterodimeric glycoprotein, plays an essential role in the regulation of antibody production, we investigated the difference in HLA genes between seropositive and seronegative patients with Lyme disease (LD). Our results show that HLA-DR7 was associated with anti-*Bb* antibody production. Nine out of the 22 seropositive LD patients (40.9%) had HLA-DRB1*0701, *0703, *0704 (HLA-DR7); only 1 out of the 18 seronegative LD patients (5.6%) had HLA-DR7 (odds ratio (OR)=11.8, P=0.0126). HLA-DRB1*01021and HLA-DRB1*0101, *0104, *0105 (HLA-DR1) contributed negatively to anti-*Bb* antibody production. Seven of 18 seronegative LD patients had HLA-DR1, only 1of 22 seropositive LD patients had HLA-DR1 (38.9% vs. 4.5%, OR=13.4, P=0.0138). These results suggest that the presence and or lack of production of specific antibody to *Bb* infection may be associated with particular HLA specificities of the Class II.

2. INTRODUCTION

Lyme disease is a multi-system illness caused by *Borrelia burgdorferi (Bb).* Diagnosis of the disease is based on clinical manifestations and serological tests (1). Soon after infection, a specific immunoglobulin (IgM) is produced. This response peaks between 3 to 6 weeks and is followed by the production of IgG specific antibody to *Bb* (1). It has been reported that some patients who exhibit signs and symptoms highly suggestive of Lyme disease do not produce antibodies to *Bb* infection (2,3,4,5,6).

The immune response is dependent on the ability of the immune system to discriminate "self" from "non-self" enabling the host to recognize and kill harmful microorganisms. Specialized leukocytes including macrophages, monocytes, Langerhan's and Kupffer's cells process and present antigen to T-cells thereby mediating an immune response (7). The HLA class II antigen is a transmembrane, polymorphic glycoprotein that assists in presenting antigenic peptides to $CD4^+$ helper T cells. The activated $CD4^+$ helper T cells stimulate B-cells to produce specific antibody. Polymorphism displayed by the HLA molecules influences the immune response and may predispose the individual to disease (8). Certain HLA antigens are associated with a higher risk of contracting a particular disease (9,10,11,12). For instance, of Caucasian ankylosing spondylitis patients, 88% have HLA-B27 while only 8% of American Caucasian controls have HLA-B27 (8). Seronegative spondyloarthropathies in association with HLA-B27 have also been documented (13). In addition, an increased frequency of HLA-DR4 in antibiotic treatment resistant chronic Lyme arthritis has been reported (14).

We investigated whether the presence or absence of certain HLA class II-linked genes correlated with antibody production in LD. To examine our hypothesis, we determined the immunogenetic profiles of seropositive LD patients, seronegative LD patients and normal controls.

3. MATERIALS AND METHODS

Patients were recruited from the Lyme Disease Center at Long Island Jewish Medical Center. Healthy donors were recruited from the hospital and physician's office staff. There were three groups of patients studied: [1] Seropositive LD patients with a history of exposure,

Table 1. Clinical profile, serology and OspA PCR product of *seropositive* Lyme disease patients

Patient #	Age	Sex	Duration months	Signs & Symptoms	Serology			pre-OspA		post-OspA		Improvement
					G	M	E	Bl	CSF	Bl	CSF	
1	46	M	4	Tick bite, knee pain, knee effusion	+	+	+	–	NT	NT	NT	yes
2	58	F	4	EM rash, fatigue, encephalopathy	+	+	+	–	+	NT	–	yes
3	50	M	6	Bell's palsy	+	+	+	–	NT	NT	NT	yes
4	65	M	84	Bell's palsy, arthritis, myalgias	+	+	+	–	NT	NT	NT	yes
5	82	M	2	Arthralgias, fatigue, cognitive, ataxia	+	+	+	–	NT	NT	NT	yes
6	57	M	10	Flu-like, arthralgias, fatigue, depression, myalgias	+	+	+	–	–	NT	–	yes
7	35	M	4	Tick bite, EM rash, arthralgias, fatigue, headache, stiff neck	+	+	–	+	+	–	–	yes
8	80	M	1	Tick bite, Bell's palsy, joint swelling, cognitive	+	+	+	–	NT	NT	NT	yes
9	47	F	84	Tick bite, EM rash, arthralgias, fatigue, depression, headaches, cognitive, muscle pain	+	+	+	–	NT	NT	NT	yes
10	51	M	1	EM rash	+	+	+	–	NT	NT	NT	Yes
11	66	M	3	Headaches, stiff neck, cognitive	+	–	+	–	NT	NT	NT	yes
12	48	M	2	Tick bite, EM rash, flu-like, arthralgias, knee effusion, cognitive	+	+	+	–	NT	NT	NT	yes
13	58	F	1	Disseminated EM, flu-like, headaches	+	+	+	+	–	–	NT	yes
14	57	F	84	Arrythmia, encephlopathy	+	+	+	–	+	NT	–	yes
15	49	F	48	Tick bite, flu-like, arthralgias, fatigue, depression, cognitive, stiff neck, dizziness	+	–	+	–	–	NT	NT	yes
16	37	M	?	Arthralgias, fatigue, headaches, dizziness	+	NT	+	–	NT	NT	NT	yes
17	83	F	?	Arthralgias, muscle pain	+	–	+	–	NT	NT	NT	yes
18	44	M	18	Bell's palsy, flu-like, knee effusion, polysynovitis, fatigue, depression, stiff neck, muscle pain	+	–	+	–	–	NT	NT	yes
19	57	F	?	EM rash, Bell's palsy, arthralgias, joint swelling, arthritis, neuropathy, muscle pain	+	–	+	–	NT	NT	NT	yes
20	50	F	3	EM rash, Bell's palsy, flu-like, arthralgias, fatigue	+	+	+	–	–	NT	NT	yes
21	57	F	2	Bell's palsy, depression, arthralgias, fatigue, myalgias, headaches, memory problem, irritability	+	+	+	–	–	NT	NT	yes
22	63	M	2	Bell's palsy, arthralgias, fatigue, myalgias, headaches, dizziness, cognitive	+	+	+	–	–	NT	NT	yes

G–IgG; M–IgM; E–ELISA; Bl–blood (monocyte or lymphocyte); Pre–before antibiotic treatment; post–after antibiotic treatment ; EM–erythema migrans; NT–not tested. ? – Duration unknown

clinical signs and symptoms and confirmatory serologies (positive ELISA and Western blot, Table 1); [2] Seronegative LD patients with signs and symptoms and exposure to Bb without confirmatory serologies (Table 2); and [3] normal controls from healthy donors without LD exposure. After obtaining informed consent from subjects, patients and healthy donors had blood drawn for ELISA, Western Blot and outer surface protein A (OspA) PCR determinations. Additionally, 21 patients with neurological symptoms had lumbar punctures with cerebrospinal fluid obtained for OspA PCR assay, antiborrelial antibody titers and CSF protein determination and cell counts.

3.1. Serological Tests

Tests of *Bb*-specific immunoglobulin G (IgG) and IgM antibodies were performed using *Bb* IgG/IgM Enzyme-Linked Immunosorbent Assay (ELISA, Wampole Laboratories, Cranbury, NJ) and *Bb* IgG/IgM Marblot Strip Test System (Western blot kit, MarDx Diagnostic Inc., Carlsbad, CA) according to the manufacturer's instructions. A positive threshold for the Lyme ELISA assay was determined by adding the mean optical density (OD) of normal sera and three standard deviations. Western blots were defined as positive when they showed any 5 bands of 18, 23, 28, 30, 39, 41, 45, 58, 66, or 93 kilo Dalton (kDa) for IgG and any 2 bands of 23, 39, or 41 kDa for IgM.

3.2. Cell Isolation

Macrophages and lymphocytes were isolated and tested for *Bb*. In brief, 25 ml of whole blood from LD patients and healthy donors were collected in heparinized tubes. Peripheral blood mononuclear cells (PBMC) were isolated by centrifuging whole blood over Lymphoprep (GIBCO Life Technologies, Gaithersburg, MD); the mononuclear cell layers were cultured in 25 cm^2 flasks for 48 to 72 hours (2) (RPMI 1640 medium with 10% fetal bovine serum, 5% CO_2 incubation at 37°C). Non-adherent cells (lymphocytes) were separated and washed. Nested PCR was performed on both adherent and non-adherent cells.

3.3. PCR

Ten µ1 of TE buffer was added to each of the pellets from 1/5 the macrophage fraction and 1/10 the lymphocyte fraction, then boiled for 30 minutes. CSF was centrifuged at 16,000 x g for 30 min, 10 µl TE buffer was added to the pellets then boiled for 30 min. $MgCl_2$ was added to saturate EDTA in the TE buffer. The nested PCR was modified from a previously published procedure (2,15). For OspA PCR, the first reaction mixture of 50 µl contained 10 µl cell lysate, l x PCR buffer, 1.5 mM MgCl2, 200 µM dNTP, 0.25 µM of each external primer

Table 2. Clinical profile, serology and OspA PCR product of *seronegative* Lyme disease patients

Patient #	Age	Sex	Duration months	Signs & Symptoms	Serology			pre-OspA		post-OspA		Improvement
					G	M	E	Bl	CSF	Bl	CSF	
1	55	F	12	Cognitive, fatigue, radiculopathy	–	–	–	+	–	–	–	yes
2	29	F	12	Arthralgias, fatigue	–	–	-	+	–	–	NT	yes
3	39	F	16	Flu-like, arthralgias & swelling, headaches, fatigue, depression, cognitive, paresthesias	–	–	–	+	–	–	NT	yes
4	43	F	2	Tick bite, fatigue, arthralgias, headaches	–	–	–	+	NT	–	NT	yes
5	61	F	6	Knee pain, effusion, headaches	–	–	–	+	–	–	NT	yes
6	45	M	36	Headaches, fatigue, knee effusion, cognitive, paresthesias	–	–	–	+	NT	–	NT	yes
7	58	F	72	Tick bite, EM rash, arthralgias	–	–	–	+	NT			Did not finish treatment
8	55	M	120	Tick bite, EM rash, arthralgias, depression, myalgias, cognitive	–	–	–	+	–	–	NT	yes
9	44	M	132	Tick bite, arthralgias, fatigue, headaches, cognitive	–	–	–	+	NT	–	NT	yes
10	45	F	24	Tick bite, knee pain, headaches, depression, cognitive	–	–	–	+	NT	–	NT	yes
11	35	F	5	EM rash, arthralgias, knee & elbow pain	–	–	–	+	NT			Did not finish treatment
12	71	F	12	Knee pain & effusion, arthralgias, shoulder pain, fatigue	–	–	–	+	NT	–	NT	yes
13	33	M	96	Flu-like, arthralgias, fatigue, myalgias, headaches, fatigue, palpitation	–	–	–	+	–	–	NT	yes
14	62	F	120	Paresthesias, radiculitis	–	–	–	+	+	NT	–	yes
15	63	F	12	Bell's palsy, flu-like, arthralgias, neuropathy, fatigue	–	–	–	–	+	–	NT	yes
16	37	F	60	Multiple tick bites, arthralgias, ankle swelling, cognitive	–	–	–	–	+	–	–	yes
17	41	F	12	Flu-like, arthralgias, knee pain, cognitive	–	–	–	–	+	–	–	yes
18	24	F	1	Seizure, cognitive, bilat knee effusions	–	–	–	–	+	NT	NT	yes

G–IgG; M–IgM; E–ELISA; Bl–blood (monocytes or lymphocytes); pre–before antibiotic treatment; post–after antibiotic treatment; EM–erythema migrans; NT–not tested.

and 1.5 U of Taq DNA polymerase. The amplification condition was denatured 1.5 min at 94ºC, annealing 2 min at 45ºC, and elongation was 2 min at 72ºC, for 30 cycles. Ten µl of product from the first reaction was added to a new PCR mixture containing l x PCR buffer, 1.5 mM MgCl2, 200 µM dNTP, 0.25 µM of each internal primer and 1.5 U of Taq DNA polymerase. Amplification conditions were changed to 35 cycles and annealing temperature to 55°C. PCR amplification was performed in a Perkin-Elmer DNA thermal cycler. The sequences of OspA PCR primers used and expected size of PCR products were external primers, 5'-GGGAATAGGTCTAATATTAGCC-3' (forward) and 5'-CACTAATTGTTAAAGTGGAAGTGCC-3' (reverse), 622 bp; and internal primers, 5'-GCAAAATGTTAGCAGCCTTGACG-3' (forward) and5'-CTGTGTATTCAAGTCTGGTTCC-3' (reverse), 392 bp. PCR products were analyzed on a 1.5% agarose gel. Ten fg of Bb B31 DNA served as positive control and water was used as a negative control. The PCR hoods were UV sterilized for at least 30 minutes prior to use. Each step in the nested PCR was done in a different room using a different hood. Both negative and positive controls were used in each assay. The positive control was never added in the same room in which the PCR was performed.

3.4. HLA Typing

DNA was extracted from PBMCs using the Invitrogen Easy-DNA kit (Invitrogen, San Diego, CA). All specimens were genotyped for HLA-DRB by polymerase chain reaction-sequence specific oligonucleotide (PCR-SSO) (Dynal A. S, Oslo, Norway). The Dynal RELI SSOHLA-DRB strip was banded with 36 probes (35 specific and 1 control). This test distinguishes 125 different HLA-DRB1 allele groups, 10 DRB3 groups, 2 DRB4 groups and 7 DRB5 groups. Based on the kit, some oligonucleotide defined groups were sets of multiple alleles. The assay procedure followed the manufacturer's instructions. The HLA-DRB typing is assigned by reading the blue lines on the HLA-DR typing strip to determine which HLA-DR alleles are present in the DNA samples. This homozygous hybridization pattern was read by using the Dynal RELI SSO Pattern Matching Program.

3.5. Statistical Analysis

Comparisons of HLA-DR types in seropositive patients, seronegative patients or healthy donors were made using contingency tables (two-by-two) from which the odds ratio and P value were calculated by Fisher's exact test (two-sided). P values less than 0.05 were considered statistically significant.

4. RESULTS

Forty patients with various manifestations of LD were included in this study. Of the twenty-two seropositive patients, 9 were female and 13 male patients ranging in age from 35 to 83 (mean, 56). Signs and symptoms in this subgroup included the following: 6 (27%) had tick bites, 8

Table 3. Serological and PCR results

Tests	Seropositive	Seronegative	Healthy Donors
Western Blot (+/tested)	22/22	0/18	0/26
ELISA(+/tested)	22/22	0/18	0/26
OspA PCR on CSF(+/tested)	3/10	5/11	0/0
OspA PCR on Mononuclear (+/tested)	2/22	14/18	0/26

(36%) had acute erythema migrans (EM), 6 (27%) had primarily neurological symptoms, 5 (23%) had primarily arthritic complaints and 5 (23%) had a mixed neurological and arthritic picture (Table 1). There were eighteen seronegative patients (14 females and 4 males) ranging in age from 24 to 71 (mean, 47). Of these, 6 had tick bites (33%), 3 (17%) had EM rashes, 9 (50%) had primarily neurological complaints, 6 (33%) predominantly arthritic problems and 4 (22%) a mixed neurological and arthritic picture. Twenty-six healthy volunteers (14 females and 12 males) ranging in age from 21 to 59 (mean, 37) without symptoms or exposure to a Lyme endemic area were enrolled in the study.

Twenty-two patients had positive Western blots and ELISAs. Eighteen were seronegative. Of twenty-two seropositive LD patients, two were positive on OspA PCR of mononuclear cells and 3 were positive for CSF using the same method. Of eighteen seronegative LD patients, 14 were OspA PCR positive on mononuclear cells and 5 were positive on CSF. None of twenty-six healthy donors tested positive on any of the tests (Table 3).

The frequency of HLA-DRB1*0701, *0703, *0704 which determine serological antigen HLA-DR7 was significantly higher in seropositive LD patients than in seronegative LD patients (40.9% vs. 5.6%, OR=11.8, P=0.0126). There was a similar finding on the alleles shown on Table 4, which encode serologically determined antigen DR6. These alleles frequently represented HLA type in seropositive LD patients (40.9% of seropositive vs. 5.6% of seronegative, OR=11.8, P=0.0126). Taken all together, 16 of 22 seropositive patients had alleles which determine either DR7 antigen or the alleles which determine DR6 antigen, or the alleles which determine both antigens compared with only 2 out of 18 seronegative patients (72.7% vs. 11.1%, OR=21.3, P=0.0001) (Table 4).

HLA-DRB1*01021 and HLA-DRB1*0101, *0104, *0105 which carry serologically determined antigen HLA- DR1 was present more frequently in seronegative than in seropositive LD patients (38.9% vs. 4.5%, OR=13.4, P=0.0138).

The frequency of HLA-DR7 alleles in healthy donors was similar to seropositive LD patients, but was significantly different from seronegative LD patients (34.6% vs. 5.6%, OR=9.0, P=0.0311). However, the frequency of DR1 alleles and the frequency DR6 alleles were not significantly different between healthy donors and LD patients. The frequency of DR4 alleles was found in 44.4% of seronegative and 22.7% of seropositive patients (OR=2.7, P=0.185). There was no significant difference in the frequency of DRB3, DRB4 and DRB5 between the three groups (data not shown).

5. DISCUSSION

The presence of certain HLA alleles with seronegativity to disease has been reported in malaria (10), HIV (16,17), rheumatoid arthritis (RA) (18) and spondylarthropathies (SpA) (19). HLA-DR1 was associated with seronegative/weakly seropositive RA and HLA-DR4 was associated with seropositive RA. An HLA-B27 correlation with seronegative SpA is another example of disease associated with a hereditary marker (19). In addition to disease related antibody production, HLA-B70 and HLA-B35 correlate with non-responsiveness to the hepatitis B surface antigen (HBs) vaccine and, HLA-B46 and HLA-B7 are related to HBs antibody production (20). HLA-DR4 and HLA-DR7 are also involved in the presentation of apple and pollen antigens (21).

Our results provide evidence of a correlation between certain HLA genotypes and the ability to mount an antibody response to *Bb*. In this study, 9 of 22 (40.9%) seropositive LD patients and only 1 out of 18 (5.6%) seronegative LD patients had HLA-DR7 alleles. There was a similar finding for the HLA-DR6 alleles. The percentages of DR7 alleles and DR6 alleles present in normal controls were somewhere between that of seronegative and seropositive patients (34.6% and 23.1% respectively). Taken together, 16 of 22 seropositive had DR7 alleles or DR6 alleles while 2 patients had both. This suggests that DR7 and DR6 may be independently involved in antibody production to *Bb* infection. It was also found that the presence of DRB1*01021 and DRB1*0101,0104,0105 was associated with non-responsiveness to *Bb* antigen and poor to absent antibody production. Seven of 18 (38.9%) seronegative LD patients had these alleles while only 1 out of 22 (4.5%) seropositive LD patients carried these alleles.

The total number of tests performed included the 15 comparisons shown in Table 4 as well as 9 comparisons on DRB3, DRB4 and DRB5 (not shown), for a total of 24 tests. If the null hypothesis that the three groups did not differ were true, then we would expect an average of about one significant result at the 5% level in groups of 24 tests ($24 \times 0.05 = 1.2$). The binomial expansion also shows that if the null hypothesis were true, no more than 2 statistically significant results would occur in 88% of groups of 24 tests. Since our data yield 6 significant results (Table 4**),** it seems reasonable to conclude that at least some of them identified true differences between the three groups.

Interestingly, there are 2 seropositive LD patients who were OspA PCR positive on their mononuclear cells. Patient #7 and patient #13 were seropositive for 3 and 6 months respectively and then became seronegative. Patient #13 was OspA PCR positive for 7 months, on all 4 OspA PCR tested during that period. On HLA testing, patient #7 had DR7 alleles, and #13 did not. The remainder of the seropositive LD patients remained seropositive for years

Table 4. Serological equivalent of PCR-SO DRB allele typing (8)

HLA-DRB1* Alleles	Serological equivalent	Seropositive (N=22)	Seronegative(N=18)	Healthy Donor (N=26)
01021	DR1	0	3	0
0101,0104,0105	DR1	1	5	3
Total of DR1 (%)		1(4.5)	7(38.9)[a,b]	3(11.5)
04011,04012,04013	DR4	1	4	0
0402,0414	DR4	2	2	1
0403,0406,0407,0420	DR4	1	1	3
0404,0408,0419,0423,0432	DR4	1	2	3
Total of DR4 (%)		5(22.7)	8(44.4)	7(26.9)
1301,1302,1316,1328,1331	DR6	2	0	0
13031,13032,1333	DR6	0	0	2
1305	DR6	1	0	1
13071,13072,1311,1314	DR6	0	0	1
1309	—	2	0	1
1401,1407,1426	DR6	4	0	1
1409,1417,1430	DR6	1	1	1
Total of DR6 (%)		9(40.9)[c]	1(5.6)	6(23.1)
0701,0703,0704	DR7	9(40.9)[d]	1(5.6)	9(34.6)[e]
Total of DR7 (%)				
Total of DR7 or DR6 (%)		16(72.7)[f]	2(11.1)	13(50.0)[g]

a. Compared with seropositive group, odds ratio (OD)=13.4; P=0.0138, b. Compared with healthy donors, OD=4.9; P=0.0644, c. Compared with seronegative group, OD=11.8; P=0.0126, d. Compared with seronegative group, OD=11.8; P=0.0126, e. Compared with seronegative group, OD=9.0; P=0.0311, f. Odds ratio of seropositive patients carrying DR7 or DR6 or both alleles compared with seronegative=21.3; P=0.0001, g. Odds ratio of healthy donors carrying DR7 or DR6 or both alleles compared with seronegative=8.0; P=0.01

suggesting that there are other factors involved in the regulation of antibody production.

Researchers have shown that different manifestations of LD and persistent arthritic symptoms may be associated with HLA class II (22,23,24). Others have described a correlation between HLA-DR4 (DRB1*0401, DRB1*0403 and DRB1*0404) and non-responsiveness to antibiotic therapy in Lyme arthritis (14).

The genes encoding HLA class II are highly polymorphic. Several alleles may be present at one locus. Each functional allele determines at least one HLA antigen defined by serologic or cellular assay. Several closely related alleles, which encode different molecules, can determine the same serologic antigen (8). The HLA molecular typing of LD patients was carried out by a Dynal RELI SSO test kit. According to the kit, some oligonucleotide-defined groups are sets of multiply related alleles. In LD patients, DR7 is encoded by one set of alleles; DR1, 2 sets; DR4, 4 sets, and DR6, 7 sets (Table 4).

The HLA class II molecule is a transmembrane heterodimeric glycoprotein. The $\alpha 1$ and $\beta 1$ domains of the class II molecule forms a peptide-binding groove open at both ends allowing a 10-25 amino acid peptide to bind restricting the recognition of peptide by $CD4^+$ T cells (25,26,27). HLA class II restricted antigens are primarily exogenous antigens that are first internalized either by endocytosis or phagocytosis, then processed in endosomes or lysosomes (28). An *in vitro* study has shown that *Bb* is degraded in lysosomes of mouse macrophages soon after being ingested (29). Filgueira *et al* (30) demonstrated that human dendritic cells were able to engulf and process live *Bb*. By using double staining, they found that *Bb* antigen and HLA class II molecules co-localized in the lysosomal compartment. The outcome of the interaction between class II molecules and peptides was peptide sequence dependent (31,32). Different types of class II selectively bind different sets of peptides (27,33). Lack of certain types of class II antigens may result in *antibody* deficiency in LD patients. Our study provides evidence that HLA alleles are involved in antibody responsiveness or non-responsiveness to *Bb* infection. A low frequency of HLA-DR7 alleles and HLA-DR6 alleles and a high frequency of HLA-DR1 alleles may contribute to non-responsiveness of antibody production in LD patients. Thus, genetic predisposition may be a critical factor in the regulation of the host immune response and the diagnosis and prognosis of Lyme disease.

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