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Association of *Chlamydia pneumoniae* Infection with HLA-B*35 in Patients with Coronary Artery Disease▽

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The immune system may interplay between *Chlamydia pneumoniae* infection and coronary artery disease (CAD). Major histocompatibility complex genes regulate innate and adaptive immunity. Patients with CAD (n = 100) and controls (n = 74) were enrolled. Human leukocyte antigens (HLA-A, HLA-B, and HLA-DRB1), four lymphotoxin alpha single-nucleotide polymorphisms, and complement C4A and C4B allotypes were typed, and their haplotypes were inferred. The presence of serum *C. pneumoniae* immunoglobulin A (IgA) (titer, ≥40) or IgG (titer, ≥128) antibodies or immune complex (IC)-bound IgG antibodies (titer, ≥2) was considered to be a serological marker suggesting chronic *C. pneumoniae* infection. *C. pneumoniae* IgA antibodies were found more frequently in patients than in controls (P = 0.04). Among the patients, multiple logistic regression analysis showed the HLA-B*35 allele to be the strongest-risk gene for *C. pneumoniae* infection (odds ratio, 7.88; 95% confidence interval, 2.44 to 25.43; P = 0.0006). Markers of *C. pneumoniae* infection were found more frequently in patients with the HLA-A*03-B*35 haplotype than in those without the haplotype (P = 0.007 for IgA; P = 0.008 for IgG; P = 0.002 for IC). Smokers with HLA-B*35 or HLA-A*03-B*35 had markers of *C. pneumoniae* infection that appeared more often than in smokers without these genes (P = 0.003 and P = 0.001, respectively). No associations were found in controls. In conclusion, HLA-B*35 may be the link between chronic *C. pneumoniae* infection and CAD.

Atherosclerosis is a chronic inflammatory process (11) in which infections, especially those caused by *Chlamydia pneumoniae* (24, 35), have been suggested to play a role. *C. pneumoniae* is a common cause of respiratory tract infections. Like all chlamydial species, it has a tendency to cause chronic infections. This may lead to severe sequelae such as chronic obstructive pulmonary disease (42) and cardiovascular diseases (24). Most likely, all individuals get infected with *C. pneumoniae* during their lifetimes, but many of them are capable of resolving the infection, and only some become chronically infected. It is assumed that in order to maintain a persistent infection and to evade host defense mechanisms, chlamydiae have developed specific strategies (9), e.g., by being an obligatory intracellular organism and by having a unique and complicated life cycle (21). An aberrant and persistent form of *C. pneumoniae* can be induced by gamma interferon (31), antibiotics (7), and tobacco smoke (44) in in vitro cell cultures. In vivo, however, immunogenetic factors (22) of the host also contribute to the infection outcome.

The major histocompatibility complex (MHC) region takes part in innate and adaptive immunity (28). MHC molecules present microbial peptides to the appropriate subsets of T cells. Patients with specific HLA genes are susceptible or resistant to certain viral and nonviral pathogens (12, 32). Immunoglobulin A (IgA) and IgG antibodies against *C. pneumoniae* (35) and immune complexes (ICs) (23, 25) have frequently been found to be present in sera of patients with coronary artery disease (CAD). Recently, we showed that HLA-B*35- and DRB1*01-related haplotypes are found more frequently in patients with CAD than in healthy age- and sex-matched controls. Also, smokers who had a complement component C4B null allele together with HLA-DRB1*01 were shown to be prone to cardiovascular disease (30). Therefore, in this study, we assessed MHC genes associated with serological markers of *C. pneumoniae* infection, elevated specific IgA and IgG antibody levels and the presence of specific circulating ICs, in patients with CAD.

MATERIALS AND METHODS

Study subjects. Patients with CAD (n = 148) were recruited from nine different central hospitals in Finland between September 1998 and December 2000, as described previously (39). The inclusion criteria for the patients were as follows. Patients had to have clear symptoms of angina with electrocardiographic evidence of myocardial ischemia. Patients who met the anginal pain inclusion criteria but none of the electrocardiographic criteria were eligible to enter the trial if their cardiac enzymes were consistent with the occurrence of myocardial infarction. Patients with prolonged chest pain with electrocardiogram changes indicating either unstable angina (n = 43) or non-Q-wave myocardial infarction (n = 105) were enrolled. No differences in the *C. pneumoniae* infection markers between the patients with unstable angina and non-Q myocardial infarction were found (data not shown). The original study was a placebo-controlled study on clarithromycin treatment of patients with acute coronary syndrome (ACS) (39). For the MHC gene study, we randomly selected 100 of the patients. No differences in the *C. pneumoniae* infection markers studied were found between the

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TABLE 1. Characteristics of patients with ACS and controls

Characteristic	Value for patient group[a]	
	ACS (n = 100)	Control (n = 74)
Mean age (yr) ± SD	63.2 ± 9.6	63.9 ± 1.6
Mean body mass index (kg/m^2) ± SD	27.3 ± 3.8	NA
No. of patients (%)		
Female	30 (30)	25 (33.8)
Hypertension	44 (44)	NA[b]
Diabetes	21 (21)	NA[b]
Hypercholesterolemia	74 (74)	NA[b]
Smoking status		
Current smoker	33 (33)	8 (10.8)[c]
Ex-smoker	26 (26)	NA[b]
Never smoker	41 (41)	NA[b]
HLA status[d]		
HLA-A*03	52 (52)	32 (43.2)
HLA-B*35	35 (35)	14 (18.9)
HLA-DRB1*01	38 (38)	13 (17.6)
HLA-A*03-B*35	25 (25)	11 (14.9)
LTA+724C	86 (86)	64 (86.5)
HLA-A*03-B*35-LTA+724C	21 (21)	11 (14.9)

[a] NA, not available; n, number of patients.
[b] Controls filled the questionnaire formatted by the Blood Transfusion Service before blood donation. Any of these clinical conditions are severe enough for the need of medication, and they would have been a contraindication for blood donation.
[c] Current smokers in the control group were detected with cotinine measurement (see the text for details).
[d] Number of patients positive for the MHC allele or haplotype. Differences between ACS patients and controls had a P value of <0.05 for HLA-B*35 and a P value of <0.01 for HLA-DRB1*01; the rest of them were not significant, as described previously (30).

patients receiving study medication and placebo (data not shown). Blood samples from patients were taken at the time of hospitalization (visit 1) and 1 week (visit 2), 3 months (visit 3), and 1 year (visit 4) after hospital admission. Consecutive age- and sex-matched healthy blood donors (n = 74) served as controls and donated a blood sample once. The criteria for blood donation are available at http://www.veripalvelu.redcross.fi/.

Baseline characteristics of the patients and controls are shown in Table 1. All patients and controls gave written informed consent. Study protocols were approved by local ethics committees.

Laboratory procedures. Genomic HLA-A, HLA-B, and HLA-DRB1 tissue typing, four lymphotoxin alpha (LTA) single-nucleotide polymorphisms (positions +253 [a/g], +496 [C/T], +633 [c/g], and +724 [C/A]), and complement C4A and C4B allotyping were performed (lowercase indicates intronic and uppercase indicates exonic variation). MHC haplotypes of the subjects were inferred using all these markers (30).

Sera were tested for *C. pneumoniae*-specific IgA and IgG antibodies by the microimmunofluorescence method using elementary bodies of Kajaani 6 as antigens (43). Fourfold serum dilutions were used, starting from 1/32 for IgG and 1/10 for IgA, as described previously (13, 14). Isolation of *C. pneumoniae*-specific ICs was performed by polyethylene glycol precipitation, and IgG antibodies against *C. pneumoniae* were measured from dissociated ICs by microimmunofluorescence at twofold dilutions starting from a dilution of 1/2, as described previously (13, 25).

Due to the lack of information on controls' smoking statuses, we measured plasma cotinine levels by use of a gas chromatograph equipped with a nitrogen phosphorous detector. The cutoff point for the classification of active smoking was set at 10 ng/ml (1), revealing subjects who had smoked tobacco within a week.

TABLE 2. *C. pneumoniae* markers, gender, and smoking status in patients with ACS (n = 100) and controls (n = 74)[a]

Risk factor	No. (%) of subjects in group:		P value
	ACS	Control	
IgA titer ≥40	37 (37.4)	17 (23.0)	0.04
IgA GMT	14.6	9.4	0.05
IgG titer ≥128	24 (24.2)	14 (18.9)	NS
IgG GMT	34.1	32.3	NS
IC titer ≥2 (%)	31 (31.3)	14 (18.9)	NS
Marker positivity[b]	51 (51.5)	27 (36.5)	0.05
Gender			
Male	38/69 (55.1)	20/49 (40.8)	NS
Female	13/30 (43.3)	7/25 (28.0)	NS
Smoking status			
Never smoked	19/40 (47.5)	NA	—
Ever smoked[c]	32/59 (54.2)	NA	—
Current smoker	19/33 (57.6)	4/8 (50.0)[d]	NS
Ex-smoker	13/26 (50.0)	NA	—

[a] Data are number of subjects (percentage) or number of subjects/total number of subjects studied (percentage). Numbers of subjects indicate the subjects who were positive for *C. pneumoniae* infection markers. For gender and smoking status, the number of subjects indicates the subjects who were positive for the any of the markers of *C. pneumoniae* infection. GMT, geometric mean titer; NA, not available; NS, not significant; —, not calculated. The value for *C. pneumoniae*-specific IgA, IgG, and IC was missing in one patient with ACS.
[b] Positive with any of the three markers of *C. pneumoniae* infection: IgA titer of ≥40, IgG titer of ≥128, or IC titer of ≥2.
[c] Current smokers and ex-smokers.
[d] Current smokers (n = 8) in controls were detected with cotinine measurement (see the text for details), and four of them were positive with any of the markers of *C. pneumoniae* infection.

Statistical analysis. For this study, elevated levels of antichlamydial IgA (titer, ≥40) or IgG (titer, ≥128) or the presence of IC-bound antichlamydial IgG antibodies (titer, ≥2) at any visit was considered to be a marker of *C. pneumoniae* infection, suggesting persistent infection (14). Positivity with any of these three titers was considered to be positivity for *C. pneumoniae* infection. The correlation between *C. pneumoniae* infection and CAD was examined by comparing *C. pneumoniae* infection markers in patients and controls. The potential association between MHC genes and *C. pneumoniae* infection markers was examined as follows.

Forward stepwise multiple-logistic regression analysis was used to study the association between alleles (HLA-A, HLA-B, LTA+253a/g, LTA+496C/T, LTA+633c/g, LTA+724C/A, C4A, C4B, and HLA-DRB1) and any of the markers of *C. pneumoniae* infection. Categorical data were compared by the chi-square or Fisher's exact test to study whether the frequencies of *C. pneumoniae* infection markers differed in patients with and those without the MHC alleles.

Data were analyzed using SPSS 12.0.1 (SPSS Inc., Chicago, IL). For all statistical tests, a P value of <0.05 was considered to be statistically significant.

RESULTS

Baseline characteristics of patients with ACS (n = 100) and controls (n = 74) are shown in Table 1. *C. pneumoniae* titers for both patients and controls ranged between 5 and 640 for IgA, 16 and 2,048 for IgG, and 1 and 32 for IC. Patients were *C. pneumoniae* seropositive more often than controls (Table 2).

In univariate analyses, any single marker (IgA titer of ≥40, IgG titer of ≥128, or IC titer of ≥2) of *C. pneumoniae* infection was found more frequently in the patients with the haplotypes HLA-A*03-B*35 and HLA-A*03-B*35-LTA+724C

TABLE 3. Significant association of MHC genes and haplotypes with a single marker of *C. pneumoniae* infections in patients with ACS[a]

Patient group, gene(s), and infection marker	No. (%) of patients with gene[a]:		OR (95% CI)	*P* value
	Present	Absent		
Diseased				
HLA*03-B*35				
IgA	15 (60)	22 (29.7)	3.55 (1.38–9.10)	0.007
IgG	11 (44)	13 (17.6)	3.69 (1.37–9.93)	0.008
IC	14 (56)	17 (23)	4.27 (1.64–11.12)	0.002
LTA+724C				
IgA	35 (41.2)	2 (14.3)	4.20 (0.88–19.95)	0.054
IgG	23 (27.1)	1 (7.1)		NS
IC	31 (36.5)	0 (0.0)	Undefined	0.004
HLA-A*03-B*35-LTA+724C				
IgA	12 (57.1)	25 (32.1)	2.83 (1.05–7.58)	0.03
IgG	8 (38.1)	16 (20.5)		NS
IC	11 (52.4)	20 (25.6)	3.19 (1.18–8.64)	0.02
HLA-B*35				
IgA	17 (48.6)	20 (31.3)		NS
IgG	12 (34.3)	12 (18.8)		NS
IC	16 (45.7)	15 (23.4)	2.75 (1.14–6.64), 0.02	0.02
Controls				
HLA*03-B*35				
IgA	4 (36.4)	13 (20.6)		NS
IgG	4 (36.4)	10 (15.9)		NS
IC	3 (27.3)	11 (17.5)		NS
LTA+724C				
IgA	14 (21.9)	3 (30.0)		NS
IgG	13 (20.3)	1 (10.0)		NS
IC	14 (21.9)	0 (0.0)		NS
HLA-A*03-B*35-LTA+724C				
IgA	4 (36.4)	13 (20.6)		NS
IgG	4 (36.4)	10 (15.9)		NS
IC	3 (27.3)	11 (17.5)		NS
HLA-B*35				
IgA	4 (28.6)	13 (21.7)		NS
IgG	5 (35.7)	9 (15.0)		NS
IC	3 (21.4)	11 (18.3)		NS

[a] Value for *C. pneumoniae*-specific IgA, IgG, and IC were missing ($n = 1$). Analyses were at the univariate level. NS, not significant. The total numbers of subjects who had HLA*03-B*35 in the diseased and control groups were 25 and 11, respectively (74 and 63 subjects did not have the allele, respectively); the total numbers of subjects who had LTA+724C in the diseased and control groups were 85 and 64, respectively (14 and 10 subjects did not have the allele, respectively); the total numbers of subjects who had HLA-A*03-B*35-LTA+724C in the diseased and control groups were 21 and 11, respectively (78 and 63 subjects did not have the allele, respectively); and the total numbers of subjects who had HLA-B*35 in the diseased and control groups were 35 and 14, respectively (64 and 60 subjects did not have the allele, respectively).

than in patients without these haplotypes (Table 3). Also, any of the markers of *C. pneumoniae* infection were found significantly more frequently in patients with the HLA-A*03-B*35 or the HLA-A*03-B*35-LTA+724C haplotype than in patients with the HLA-DRB1*01 allele ($P = 0.044$ and $P = 0.048$, respectively). In subgroup analyses, when gender and smoking were also considered, any of the markers of *C. pneumoniae* infection were significantly associated with males having the HLA-A*03-B*35 ($P = 0.008$) or HLA-A*03-B*35-LTA+724C ($P = 0.02$) haplotype as well as with current smokers with these haplotypes or the HLA-B*35 allele alone ($P = 0.001$, $P = 0.009$, and $P = 0.003$, respectively) (Table 4). Among the controls, no associations between *C. pneumoniae* markers and MHC genes (Table 3), smoking status, or gender were found.

In multiple-logistic regression analysis, any of the markers of *C. pneumoniae* infection were associated with HLA-B*35 (odds ratio [OR], 7.88; 95% confidence interval [CI], 2.44 to 25.43; $P = 0.0006$) as well as with HLA-A*02 (OR, 4.15; 95% CI, 1.42 to 12.10; $P = 0.009$) and C4B2 (OR, 3.60; 95% CI, 1.19 to 10.86; $P = 0.02$). However, the latter two did not associate in univariate analyses.

DISCUSSION

This study shows for the first time that markers of *C. pneumoniae* infection are associated with HLA-B*35-related haplotypes in patients with ACS. Furthermore, among these patients, the prevalence of *C. pneumoniae* infection markers was

TABLE 4. Significant correlations between any of the markers of *C. pneumoniae* infection and the known risk factors for *C. pneumoniae* infection in patients with ACS[a]

MHC gene	Risk factor	No. of subjects/total no. of subjects studied (%) with combined markers of *C. pneumoniae*[b]:		*P* value
		Present	Absent	
HLA-A*03-B*35	Male	16/20 (80.0)	22/49 (44.9)	0.008
	Current smoker	12/13 (92.3)	7/20 (35.0)	0.001
	Ever smoked[c]	16/19 (84.2)	16/40 (40.0)	0.001
HLA-A*03-B*35-LTA+724C	Male	13/16 (81.3)	25/53 (47.2)	0.02
	Current smoker	10/11 (90.9)	9/22 (40.9)	0.009
	Ever smoked	14/16 (87.5)	18/43 (41.9)	0.002
HLA-B*35	Male	18/28 (64.3)	20/41 (48.8)	NS
	Current smoker	14/17 (82.4)	5/16 (31.3)	0.003
	Ever smoked	18/25 (72.0)	14/34 (41.2)	0.02
HLA-A*03	Male	24/40 (60.0)	14/29 (48.3)	NS
	Current smoker	15/18 (83.3)	4/15 (26.7)	0.001
	Ever smoked	22/33 (66.7)	10/26 (38.5)	0.03
LTA+724C	Male	36/59 (61.0)	2/10 (20.0)	0.03
	Current smoker	19/30 (63.3)	0/3 (0.0)	NS
	Ever smoked	30/50 (60.0)	2/9 (22.2)	NS

[a] Analyses were at the univariate level. NS, not significant.
[b] Positive with any of the three markers of *C. pneumoniae* infection: IgA titer of ≥40, IgG titer of ≥128, or IC titer of ≥2. Values for *C. pneumoniae*-specific IgA, IgG, and IC were missing ($n = 1$).
[c] Current smokers and ex-smokers.

more pronounced in smokers and males. In the controls, HLA-B*35 was not associated with *C. pneumoniae* infection. HLA-B*35 alone or together with the other functionally important genes on the A*03-B*35-LTA+724C haplotype may provide one possible link between CAD and *C. pneumoniae* infection.

C. pneumoniae infection has been linked to CAD by several methodological approaches, e.g., by seroepidemiological studies and by demonstrating the presence of *C. pneumoniae* in atherosclerotic lesions by culture, immunocytochemistry, PCR, and electron microscopy. In the present study, we used elevated antichlamydial IgA, IgG, and ICs as markers of chronic *C. pneumoniae* infection. Thus, antibodies are only indirect evidence of chronic *Chlamydia* infection; however, IC and IgA levels will not stay high without a continuous infection (25, 36). The prevalence of these markers was lower than that reported previously (35) but significantly higher in patients than in controls. Smoking and male gender are established risk factors for CAD and also predispose one to *C. pneumoniae* infection (19, 22). Also, in this study, male gender and smoking, when related to HLA-B*35, were definite risk factors for *C. pneumoniae* infection in patients with CAD. When HLA-B*35 was absent, any of the markers of *C. pneumoniae* infection were almost threefold lower in current smokers. No such relation was found in controls. Thus, suggested chronic *C. pneumoniae* infection in patients with CAD seems to be the result of multiple factors, and among them, the HLA-B*35-positive haplotype may play an important role.

Associations between immunity and genes regulating inflammation with CAD and *C. pneumoniae* infection have been studied only occasionally (29, 34). Dahlén et al. (5) previously studied class II HLA genes and showed that *C. pneumoniae* antibodies and high Lp(a) levels were associated with HLA-DRB1*03 and HLA-DRB1*13, but the association was not later confirmed by the same group (5, 15). We have recently shown an association between CAD and HLA-B*35, DRB1*01-related haplotypes. The results in this study may indicate that the elevated markers of *C. pneumoniae* infection associate especially with the presence of the HLA-B*35 allele on the haplotype HLA-A*03-B*35-LTA+724C. Eighty-one percent of HLA-A*03-B*35 and HLA-A*03-B*35-LTA+724C haplotype-positive patients had any of the markers of *C. pneumoniae* infection, while on the contrary, 55% of HLA-DRB1*01-positive patients had any of the markers of *C. pneumoniae* infection.

Both the incidence of CAD (16, 17) and the prevalence of *C. pneumoniae* markers (18) are elevated in the eastern part of Finland. The HLA-B*35 allele is one of the most frequently isolated HLA alleles in the Finnish population and has an even higher prevalence in the eastern parts (40), suggesting an accessible association between CAD, *C. pneumoniae*, and the HLA-B*35 allele.

In addition to *C. pneumoniae*, an infectious etiology of atherosclerosis and CAD has been implicated with regard to *Helicobacter pylori*, herpes simplex virus, and cytomegalovirus (27). Several case control studies suggested that vaccination against influenza virus may reduce the risk of myocardial infarction (8, 26). One of the preferred alleles in the influenza A virus-specific cytotoxic-T-lymphocyte response is HLA-B*3501; preferential HLA usage is dependent on the type of the virus and determines differential cytokine expression (2, 3). The HLA-B*35 allele has also been associated with chronic hepatitis B virus, hepatitis C virus, and herpes simplex virus infections; Epstein-Barr virus seropositivity; and cytotoxic responses against *Aspergillus* and *Mycobacterium tuberculosis* (10, 20, 33, 45). In human immunodeficiency virus infection, the rapid progression of the disease has been correlated to the interaction of a virus peptide with B*35 (4). Recently, it has been shown that *C. pneumoniae*-infected host cells can induce genes involved in apoptosis (6), while HLA-B35 influences the apoptosis rate (37).

The results of our study indicate that HLA-B35 presents *C. pneumoniae* peptides and may further provide an environment that predetermines the establishment of persistent *C. pneumoniae* infection. The HLA-B*35 allele has been shown to confer a significant proapoptotic influence to different kind of cells. It may promote pathological cell activation and proliferation and gene transcription through the control of the intracellular cation concentration (38). *C. pneumoniae*-infected monocyte cells are shown to up-regulate many genes including the vasoconstrictor endothelin 1 (41). HLA-B*35 influences the upregulation of endothelin 1, providing an explanation for the epidemiological association existing between isolated pulmonary hypertension and HLA-B*35 (38).

In conclusion, our results show that the HLA-B*35-positive haplotypes confer a *C. pneumoniae*-related risk for CAD.

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