Association of *Chlamydia pneumoniae* With Coronary Artery Disease and Its Progression Is Dependent on the Modifying Effect of Mannose-Binding Lectin

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Background—The possible association between coronary artery disease (CAD) and *Chlamydia pneumoniae (C pneumoniae)* infection is controversial. On the basis of the recent suggestion that mannose-binding lectin (MBL) variant alleles are related to an increased risk of severe atherosclerosis, and on the in vitro interaction of MBL with *C pneumoniae*, we asked whether MBL might contribute to CAD in conjunction with *C pneumoniae*.

Methods and Results—Antibodies to *C pneumoniae* were measured by immunofluorescence and MBL alleles were determined by polymerase chain reaction technique in samples from 210 patients with CAD and 257 healthy subjects from Hungary collected between 1995 and 1996. A higher percentage of patients with CAD were anti-*C pneumoniae* positive as compared with the control group ($P=0.058$). However, at logistic regression analysis adjusted to age, sex, and serum lipid levels, this difference was confined only to subjects carrying MBL variant alleles ($P=0.035$, odds ratio 2.63, [95% CI: 1.07 to 6.45]). In contrast, no significant difference was seen in those homozygous for the normal MBL allele ($P=0.412$). During a 65 ± 5.8-month follow-up period, major outcomes (new myocardial infarction, and/or bypass operation or cardiovascular death) occurred in 11 *C pneumoniae* positive and 3 *C pneumoniae* negative patients. In the *C pneumoniae* positive group, the odds ratio of development of outcomes was 3.27 (95% CI: 1.10 to 9.71, $P=0.033$) in the carriers of the MBL variant alleles compared with the homozygous carriers of the normal MBL allele.

Conclusions—These results indicate that infection with *C pneumoniae* leads mainly to the development and progression of severe CAD in patients with variation in the MBL gene. **(*Circulation*. 2002;106:1071-1076.)**

Key words: coronary disease ■ genetics ■ immunology ■ infection

Since the original study by Saikku et al,[1] several studies have indicated an association between *Chlamydia pneumoniae* (*C pneumoniae*) infection and coronary artery disease (CAD),[2–4] although this field remains highly controversial.[5]

Mannose-binding lectin (MBL) is a complement-activating innate immune defense serum protein which binds to mannose and acetylglucosamine sugar groups on different microorganisms.[6] Recent findings indicate that MBL inhibits infection of HeLa cells by different *Chlamydia* species,[7] suggesting that MBL participates in the protection against *C pneumoniae*. MBL variant alleles that result in decreased serum levels of functional MBL are associated with an increased risk of respiratory infections especially during early childhood,[8] and also with susceptibility and outcome of infections in adults already weakened by a concomitant condition like cystic fibrosis.[9] Furthermore, we[10] and others[11] have reported that MBL variant alleles are associated with accelerated development of severe atherosclerosis.

On the basis of these observations, we asked whether the association between the presence of *C pneumoniae* and CAD and the further progression of severe CAD in patients who underwent coronary bypass surgery might be associated with a modifying effect of MBL variant alleles, which cause low levels of functional MBL in the blood.[12–14]

Methods

Patients and Controls

The study was performed in 210 Hungarian patients with CAD (50 women and 160 men, aged 58.8 ± 8.2 years) with signs of severe stenosis and clinical signs of unstable angina pectoris with typical ECG changes. Diagnosis was confirmed by coronary angiography. All patients underwent bypass surgery. Serum and DNA samples

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TABLE 1. Demographic Variables and Laboratory Data in 210 Patients With Severe CAD and in 257 Healthy Controls

	Patients With Severe CAD (n=210)	Healthy Controls (n=257)	*P*
Age, y	58.8±8.2	46.5±11.9	<0.0001*
Sex, n, male/female	160/50	164/93	0.005†
Total cholesterol, nmol/L	6.20 (5.50–7.25)	5.59 (4.83–6.40)	<0.0001*
Triglyceride, nmol/L	2.00 (1.45–3.10)	1.50 (0.98–2.23)	<0.0001*
Smokers, %	58.5	23.9	<0.0001†
Hypertension, %	33.3	21.2	0.046†
BMI	0.285±0.050	0.267±0.041	0.004*
C pneumoniae, n, positive/negative	157/53	162/95*	0.007†

Values are given as mean±SD, median (interquartile range), number of patients, or percentage of patients.

P calculated with the *Mann-Whitney test and †Fisher's exact test for the continuous and categorical variables, respectively.

were collected from the patients between 1995 and 1996. A questionnaire on the occurrence of different events (death of the patient, new myocardial infarction, necessity of bypass reoperation, coronary angioplasty, stroke, carotid endarterectomy, etc) was mailed to each patient in December 2000. In 4 months, 150 patients sent back filled questionnaires that could be properly evaluated. By contacting the family doctors, information on an additional 27 patients was obtained. Thus, in 177 of 210 patients (84.3%) the number and types of events that occurred during the 65±5.8-month follow-up period were known.

Healthy controls included 93 women and 164 men, all Hungarian (blood donors and volunteers) and aged 46.5±11.9 years. All control subjects were examined and asked about any diseases, including CAD, in their medical history. Only healthy subjects without suspicion of CAD were enrolled in this study. A history of past and current cigarette smoking was obtained for each patient and control. Those who had stopped smoking >20 years ago were considered not to have smoking as a risk factor. Patients were considered to have hypertension if they had received a diagnosis with an arterial pressure >140/90 mm Hg. Table 1 lists the demographic variables and some laboratory data assessed in the patients and control groups. The review committee of Semmelweis Medical University approved the study, and the subjects gave informed consent. The patients and some controls have recently been described.[15]

Titration of IgG-Type Antibodies Against *C pneumoniae*

C pneumoniae-specific IgG antibodies were quantitated in serum samples diluted 1:128 by microimmunofluorescence assay (ServiMif Chlamydia S630, Servibio, Meudon, France) according to the manufacturer's instructions. Sera were designated as positive (titer: ≥1:128) or weakly positive or negative (titer: <1:128), referred to as negative in the text and tables, on the basis of typical immunofluorescence associated with evenly distributed *C pneumoniae* organisms. Sera positive for *Chlamydia trachomatis* or *Chlamydia psittaci* were excluded from the study.

Determination of MBL Alleles

MBL variant alleles *B* (codon 54), *C* (codon 57), and *D* (codon 52) in the *mbl2* gene, which disrupts the structure of the protein and causes a dominant decrease in the functional MBL serum level, were determined by PCR-based techniques as described.[12] The normal allele is named *A*, whereas the common designation of the variant alleles is *O*. MBL promoter alleles were also analyzed.[16]

Other Laboratory Tests

C-reactive protein (CRP) serum concentrations were measured by particle enhanced immunoturbidimetric assay (Roche, Cobas Integra 400). The detection limit of the assay is 0.07 mg/L, the coefficient of variation 3.9% at 108 mg/L mean value. The amount of IgG-type antibodies reacting with human hsp60 (Lionex GmbH, Braunschweig, Germany) was assessed by ELISA, as described previously.[15]

Statistical Analyses

Fisher's exact test was used to compare frequencies. Mann-Whitney test was used to compare quantitative data. In some comparisons, logistic regression analyses were performed. Two-tailed tests were used throughout.

Results

Basic Characteristics of the Patients and Controls

The average age of the patients was markedly higher than that of the healthy control subjects (Table 1). Similarly, serum concentrations of total cholesterol and triglyceride were significantly higher in the group of patients than in the control group. The ratio of men to women was higher in the patient group than among the controls. Body mass index (BMI), as well as the percentage of smokers and subjects with hypertension, was also significantly higher in the patients than in the control group. Additionally, a significantly higher percentage of the patients were *C pneumoniae* seropositives (Table 1).

Frequencies of MBL Variant Alleles in Patients With CAD and Healthy Controls in Relation to *C pneumoniae* Status

Antibodies to *C pneumoniae* and the frequency of various MBL alleles were assessed in the 210 patients with CAD and in the 257 control subjects (Table 2). No significant difference between *C pneumoniae*–seropositive and -seronegative individuals in the frequency of MBL variant alleles was revealed ($P=0.75$, odds ratio 0.94 [95% CI 0.63 to 1.39]). However, in *C pneumoniae*–seropositive subjects, we found that the carriers of variant MBL alleles (*A/O* and *O/O*) occurred more frequently among patients than controls ($P=0.091$, odds ratio: 1.48 [0.94 to 2.31]), whereas no difference between patients with CAD and controls in MBL allele distribution was observed in the group of *C pneumoniae*–seronegative subjects. This observation prompted us to use multiple logistic regression analysis to study if *C pneumoniae* infection is associated with CAD more frequently in carriers of MBL variant alleles.

Dependence of the Association of *C pneumoniae* Seropositivity With Coronary Artery Disease on the MBL Polymorphisms

Patients were stratified according to MBL alleles, and homozygous carriers of normal allele (*A/A*) were compared with heterozygous and homozygous carriers of the variant MBL alleles (*A/O* and *O/O*) (Table 3). Odds ratios adjusted for variables that were found to be significantly different at univariate analysis are shown in Table 1.

For the total number of patients and controls, after adjustment, *C pneumoniae* positivity was associated with CAD only with a marginal significance. When only subjects

TABLE 2. Occurrence of MBL Alleles Stratified According to *C pneumoniae* Antibody Status in 210 Patients With Coronary Heart Disease and 257 Controls

	C pneumoniae Positive		*C pneumoniae* Negative		All Subjects	
MBL Genotype*	Patients	Healthy Controls	Patients	Healthy Controls	Patients	Healthy Controls
A/A	83 (52.9)	101 (62.3)	35 (66.0)	48 (50.5)	118 (56.2)	149 (58.0)
A/B	45 (28.7)	30 (18.5)	9 (17.0)	27 (28.4)	54 (25.7)	57 (22.2)
A/C	5 (3.2)	6 (3.7)	0 (0.0)	2 (2.1)	5 (2.4)	8 (3.1)
A/D	19 (12.1)	17 (10.5)	6 (11.3)	8 (8.4)	25 (11.9)	25 (9.7)
Total *A/O*	69 (43.9)	53 (32.7)	15 (28.3)	37 (38.9)	84 (40.0)	90 (35.0)
B/B	2 (1.3)	3 (1.9)	0 (0.0)	2 (2.1)	2 (1.0)	5 (1.9)
B/C	1 (0.6)	0 (0.0)	0 (0.0)	3 (3.2)	1 (0.5)	3 (1.2)
B/D	1 (0.6)	3 (1.9)	0 (0.0)	2 (2.1)	1 (0.5)	5 (1.9)
C/C	0 (0.0)	1 (0.6)	0 (0.0)	1 (1.1)	0 (0.0)	2 (0.8)
C/D	0 (0.0)	0 (0.0)	0 (0.0)	2 (2.1)	0 (1.9)	2 (0.8)
D/D	1 (0.6)	1 (0.6)	3 (5.7)	0 (1.2)	4 (1.9)	1 (0.4)
Total *O/O*	5 (3.2)	8 (4.9)	3 (5.7)	10 (10.5)	8 (3.8)	18 (7.0)
A/0 + 0/0	74 (47.1)	61 (37.7)	18 (34.0)	47 (49.5)	92 (43.8)	108 (42.0)
Sum	157 (100.0)	162 (100.0)	53 (100.0)	95 (100.0)	210 (100.0)	257 (100.0)
O allele frequency	0.25	0.21	0.20	0.30	0.24	0.25

Values are given as n (%).

*A indicates normal MBL allele; allele *O*, the variant alleles *B* (codon 54), *C* (codon 57), and *D* (codon 52).

carrying MBL variant *O* alleles were considered, however, a high (2.62 [1.07 to 6.44]) and significant ($P=0.035$) adjusted odds ratio of the patients with *C pneumoniae* positive was found. Similar results were obtained when the results were adjusted for age, sex, serum triglyceride concentration, BMI, as well as for the percentage of smokers and subjects with hypertension (Table 3). Because there was a significant ($P<0.001$) interaction between BMI and serum cholesterol concentrations, it was not possible to put these 2 variables in the same model. In contrast, no association between *C pneumoniae* positivity and CAD was seen in subjects homozygous for the normal *A* allele (Table 3).

To further minimize the effect of potentially confounding factors, 147 patients with severe CAD and 147 controls were matched for age and sex, and results for *C pneumoniae* antibodies in carriers of normal or variant MBL alleles were

TABLE 3. Logistic Regression Analysis for the Association Between the Prevalence of Anti-*C pneumoniae* Antibodies and CAD in 210 Patients With Severe CAD and 257 Healthy Controls Stratified According to MBL Genotypes

C pneumoniae Status	Patients, n (%)	Healthy Controls, n (%)	Odds Ratio (95% CI)	*P*	Model 1,* Adjusted Odds Ratio (95% CI)	*P*	Model 2,† Adjusted Odds Ratio (95% CI)	*P*
All subjects								
Positive	157 (74.8)	162 (62.3)						
Negative	53 (25.2)	95 (37.7)	1.74 (1.14–2.65)	0.0067	1.78 (0.98–3.12)	0.058	1.80 (0.55–5.93)	0.336
Sum	210 (100.0)	257 (100.0)						
Subjects homozygous for the normal (*A/A*) MBL alleles								
Positive	83 (70.3)	101 (64.9)						
Negative	35 (29.7)	48 (35.3)	1.13 (0.65–1.97)	0.65	1.39 (0.64–3.02)	0.412	0.64 (0.09–4.53)	0.640
Sum	118 (100.0)	149 (100.0)						
Subjects heterozygous and homozygous for the variant (*A/0* and *0/0*) MBL alleles								
Positive	74 (80.4)	61 (58.5)						
Negative	18 (19.6)	47 (41.5)	3.34 (1.69–6.66)	0.0002	2.63 (1.07–6.45)	0.035	7.98 (1.07–59.24)	0.043
Sum	92 (100.0)	108 (100.0)						

*Adjusted for age, sex, serum cholesterol, and triglyceride concentration.

†Adjusted for age, sex, serum triglyceride concentration, BMI, percentage of smokers, and hypertension.

TABLE 4. Logistic Regression Analysis of the Association Between the Prevalence of Anti–*C pneumoniae* Antibodies and CAD in 147 Patients and 147 Age and Sex-Matched Healthy Controls Stratified According to MBL Genotypes

C pneumoniae Status	Patients, n (%)	Healthy Controls, n (%)	Odds Ratio (95% CI)	*P*	Adjusted* Odds Ratio (95% CI)	*P*
All subjects						
Positive	115 (78.2)	93 (63.2)				
Negative	32 (21.8)	54 (36.7)	2.09 (1.25–3.50)	0.005	1.93 (1.10–3.39)	0.021
Sum	147 (100.0)	147 (100.0)				
Subjects homozygous for the normal (*A/A*) MBL allele						
Positive	63 (75.0)	54 (67.5)				
Negative	21 (25.0)	26 (32.5)	1.44 (0.73–2.83)	0.289	1.44 (0.68–3.06)	0.343
Sum	84 (100.0)	80 (100.0)				
Subjects heterozygous and homozygous for the variant MBL allele (*A/0* and *0/0*)						
Positive	52 (82.5)	39 (58.2)				
Negative	11 (17.5)	28 (41.8)	3.94 (1.51–7.64)	0.003	2.88 (1.10–3.39)	0.016
Sum	63 (100.0)	67 (100.0)				

*Adjusted to the serum total cholesterol and triglyceride levels.

compared (Table 4). The average age (54.9±6.3 years for the patients and 53.6±7.7 years for controls) was almost identical ($P=0.119$), and the distribution of men and women did not differ significantly ($P=0.1$) in the groups of patients (110/37) and matched controls (96/51). Similar to the results obtained in the whole study population, CAD was associated with *C pneumoniae* positivity only in subjects carrying the MBL variant alleles, even when matched pairs were compared by logistic regression analysis adjusted to the serum cholesterol and triglyceride concentrations (Table 4).

Genotyping for MBL promoter alleles (not shown) was not additionally informative.

Predictive Value of Polymorphisms of the MBL of the Major Outcomes With Severe CAD in the *C pneumoniae*–Positive and *C pneumoniae*–Negative Patients

One hundred and seventy seven of 210 patients (84.3%) could be monitored up for 65±5.8 months. We calculated the predictive value of the MBL polymorphisms on the development of major outcomes of CAD (new myocardial infarction and/or new bypass operation and/or cardiovascular death). Altogether, 12 patients developed myocardial infarction, and bypass operation was performed on an additional patient who did not develop myocardial infarction in the meantime. An additional patient died of heart attack without previous myocardial infarction or bypass operation. Therefore, major outcomes occurred in 14 patients during the follow-up period. These patients were divided according to MBL polymorphisms and *C pneumoniae* serostatus measured in the baseline serum samples and predictive values of these variables were determined with the use of multiple logistic regression analysis. We also adjusted the analysis to other variables that may affect the outcomes (Table 5). Major outcomes occurred more frequently in the carriers of the variant MBL alleles (adjusted odds ratio: 2.40 [95% CI 0.96 to 5.96], $P=0.060$). This association was, however, restricted to the patients positive with *C pneumoniae*. In this group, 9 events occurred in 11 carriers of the variant alleles. The MBL variant allele carriers had a 3.27 (CI: 1–10–9.71, $P=0.033$) higher adjusted odds ratio to develop major outcomes than noncarriers. By contrast MBL polymorphisms did not predict the development of events in patients negative for *C pneumoniae* (Table 5).

Lack of Association Between MBL Polymorphisms and Serum Concentration of CRP and IgG Antibodies to hsp60

We compared serum concentrations of CRP and IgG anti-hsp60 in homozygous carriers of the wild MBL allele with carriers of the variant MBL alleles. We did not find significant differences between the 2 groups: CRP concentrations (median (25^{th}-75^{th} percentile) was 4.06 (1.88 to 7.30) mg/L and 3.14 (1.83 to 6.79) mg/L, respectively ($P=0.572$). Serum concentrations of anti-hsp60 antibodies were 103 (46 to 192) AU/mL and 87 (39 to 204) AU/mL, respectively ($P=0.576$). Similar results were obtained when subjects both positive and negative for *C pneumoniae* were analyzed separately.

Discussion

This study supports the association between *C pneumoniae* infection and CAD; however, the association was solely dependent on individual variation in the MBL gene (*mbl2*). No increased risk was seen in individuals homozygous for the normal MBL allele, although a significant risk characterized individuals with MBL variant alleles, even after adjustment for age, sex, serum lipid concentrations, BMI, as well as for the percentage of smokers and occurrence of hypertension. Moreover, during a follow-up period of 5.5 years, a 3-fold increased risk of a major cardiovascular event was observed

TABLE 5. Predictive Value Calculated Logistic Regression of MBL Genotypes Influence on the Major Outcomes in the *C pneumoniae*—Positive and—Negative patients (at Baseline) With Severe CAD

MBL Alleles	New Myocardial Infarction and/or Bypass Operation and/or Cardiovascular Death		Odds Ratio (95% CI) (*P*)	Adjusted* Odds Ratio (95% CI) (*P*)
	Yes	No		
C pneumoniae positive				
Normal *A/A*†	2	68	5.28 (1.10–25.4) (0.029)	3.27 (1.10–9.71) (0.033)
Variant *A/O* or *O/O*†	9	58		
Total	11	126		
C pneumoniae negative				
Normal *A/A*†	2	24	0.92 (0.08–11.20) (1.00)	0.86 (0.08–8.89) (0.896)
Variant *A/O* or *O/O*†	1	13		
Total	3	37		
All patients				
Normal *A/A*†	4	92	3.24 (0.97–10.70) (0.053)	2.40 (0.96–5.96) (0.060)
Variant *A/O* or *O/O*†	10	71		
Total	14	163		

*Adjusted to extent of stenosis of coronary arteries, occurrence of previous myocardial infarction, as well as baseline serum total cholesterol and triglyceride levels by logistic regression.

†See Table 2 for explanation.

in MBL variant alleles carriers (provided that they were positive for *C pneumoniae*) compared with homozygous carriers of the normal MBL allele.

Thus, *C pneumoniae* infection may lead to the development of severe CAD in genetically predisposed individuals carrying MBL variant alleles. These findings are consistent with previous observations indicating that MBL variant alleles are associated with severe CAD[10] and with increased carotid plaque areas.[11] *C pneumoniae* status was not assessed in either study. Moreover, if reproduced, the present findings should also help to clarify much of the inconsistency in literature about *C pneumoniae* and CAD.[5] A relatively small odds ratio because of the high frequency of infected individuals in the background population might undoubtedly lead to miscellaneous results in different studies because of variations in additive parameters leading to vascular pathology.

MBL deficiency has been implicated in *C pneumoniae* infection because the bacteria carry the sugar structures relevant for MBL binding.[7] Moreover, MBL inhibits the uptake of the bacteria to target cells in vitro.[7] We did not observe a direct protective effect of MBL against *C pneumoniae* per se, which is not unexpected because the seroprevalence in healthy adults exceeds 60%. Almost all individuals are probably infected with *C pneumoniae* during their lifetime and many are reinfected.[17] Moreover, MBL is not present in the normal lung.[9,18] Therefore, MBL may act to modulate the severity of the infection, rather than protect against it, as previously suggested.[10] For example, MBL might inhibit the dissemination of *C pneumoniae* from the lungs by monocytes/macrophages to the blood stream and subsequently to the vascular wall. Alternatively, or in addition, MBL might modulate the inflammatory response initiated by chronic infection with *C pneumoniae* or other stimuli in the atherosclerotic plaque. Experimental studies support such a view in which it has been shown that MBL may modulate cytokine responses.[19] We have previously shown that MBL in vivo can exert a striking modulating effect on inflammation associated with rheumatoid arthritis.[20] Nevertheless, the present results indicate that *C pneumoniae* in general may be a rather harmless bacteria in relation to the atherosclerotic process and that an additional component, such as MBL deficiency, is necessary for clinically relevant pathology. This conclusion is in line with the evidence from animal models indicating that it is difficult to induce atherosclerosis with *C pneumoniae* alone, but that the microorganism acts in conjunction with other atherosclerotic risk factors.[21] Some studies suggest a direct inflammatory role of complement in atherogenesis mediated through the binding of CRP to degraded, nonoxidized LDL, enhancing complement activation associated with vascular pathology.[22,23] According to the present study, however, interaction of MBL polymorphisms and *C pneumoniae* infection does not exert its effect on CAD development either through CRP or anti-hsp60 antibodies.

A dual role of MBL in CAD has been suggested, on the basis of the exposure of MBL ligands through oxidative stress of endothelial cells, a model of ischemia/reperfusion injury.[24] Moreover, MBL and complement factor C3 immunostaining was found throughout an ischemic area after rat myocardial reperfusion.[24] Thus, MBL might partly protect against severe atherosclerosis in conjunction with *C pneumoniae* or other related conditions, but this relative advantage may be turned in to a disadvantage during revascularization procedures.

This study indicates that MBL deficiency may represent an important factor in connection with *C pneumoniae* infection in the development of CAD and disease progression. However, the relatively small size and lack of supportive biological data make it necessary that these observations are

confirmed in additional prospective studies before definitive conclusions can be drawn. If confirmed, the results suggest that determination of the MBL genetic status in *C pneumoniae*–seropositive CAD patients may serve to identify those patients more likely to benefit from antibiotic therapy.

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