Research article

Innate Immunity

17(1) (2011) 35–40

ISSN 1753-4259 (print)
10.1177/1753425909349759

Low mannose-binding lectin levels and *MBL2* gene polymorphisms associate with *Chlamydia pneumoniae* antibodies

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Objective: Mannose-binding lectin (MBL) has been shown to inhibit infection of host cells by *Chlamydia pneumoniae in vitro*. We studied if MBL levels and *MBL2* polymorphisms associate with the presence of *C. pneumoniae* antibodies *in vivo*.
Materials and Methods: Single nucleotide polymorphisms (SNPs) of the *MBL2* gene (promoter alleles H/L, X/Y and P/Q; and exon 1 variant alleles B, C and D and wild-type allele A) were genotyped and serum MBL concentrations and *C. pneumoniae* IgG, IgA and IgM antibodies were analysed in 889 Finnish military recruits.
Results: An MBL level below the median concentration and the *MBL2* P/P genotype were significant risk factors of IgG or IgA seroconversions or the presence of IgM antibodies during military service (adjusted odds ratio (OR) 1.5; 95% confidence interval (CI) 1.1–2.1 and OR 1.5; 95% CI 1.0–2.2, respectively). In addition, the promoter Y/Y (OR 1.6; 95% CI 1.1–2.3) and exon 1 variant allele genotypes (OR 1.4; 95% CI 1.0–2.0) were possibly associated with elevated antibodies.
Conclusions: These results suggest, for the first time, that low serum MBL levels and *MBL2* polymorphisms may associate with elevated *C. pneumoniae* antibodies and seroconversions and thus support the previous findings *in vitro*.

Keywords: antibody, *Chlamydia pneumoniae*, infection, mannose-binding lectin, military recruits, polymorphism

Introduction

Mannose-binding lectin (MBL) is an important innate immune defence serum collectin that binds to sugar groups of micro-organisms and then activates a complement lectin pathway or acts directly as an opsonin for phagocytosis.[1] Single-nucleotide polymorphisms (SNPs) of the *MBL2* gene have been reported to have a major effect on the MBL protein structure and serum concentration. Three of these SNPs are located in exon 1 at codons 52 (allele D, dbSNP no. rs5030737), 54 (allele B, dbSNP no. rs1800450) and 57 (allele C, dbSNP no. rs1800451), two SNPs are in the 5′-regulatory region at positions –550 (alleles H and L, dbSNP no. rs11003125) and –221 (alleles X and Y, dbSNP no. rs7096206) and one is in the 5′-untranslated region at position +4 (alleles P and Q, dbSNP no. rs7095891).[2,3] Mannose-binding lectin deficiency caused by the polymorphisms has been associated with susceptibility to recurrent infections in several studies;[4,5] in our previous study, MBL deficiency was associated with susceptibility to respiratory tract infections.[6]

Received 16 June 2009; Revised 18 August 2009; Accepted 31 August 2009

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Chlamydia pneumoniae is an obligate intracellular bacterium and a common pathogen in acute upper and lower respiratory tract infections in humans.[7] The high-mannose oligosaccharide glycans of the chlamydial cell surface have been shown to mediate attachment and internalization of the organisms to the host cell,[8] and previous studies have suggested that exogenous oligosaccharide ligands, such as mannan and mannose-6-phosphate, could inhibit the infectivity of *Chlamydia trachomatis* and *C. pneumoniae*, respectively.[9] Earlier findings have also indicated that MBL may play a role in protecting against chlamydiae by inhibiting infection of the host cells *in vitro*.[10] However, an association between MBL and susceptibility to *C. pneumoniae* infection *in vivo* has not been reported. Therefore, in this study, we investigated whether serum MBL levels and *MBL2* gene polymorphisms are associated with the presence of *C. pneumoniae* antibodies in Finnish military conscripts as well as with seroconversion during military service.

Subjects and Methods

Study population and specimens

The study population included 889 military recruits from the July 2004 and January 2005 intake groups for compulsory military service in Kajaani Garrison, Kainuu Brigade, in northern Finland (Table 1). All the men who agreed to participate in the study signed an informed consent form. Sera were obtained at the beginning and end of their service and paired sera, during each respiratory infectious episode diagnosed by the physician. The mean age of the participants was 19.6 years (SD = 0.8). Body mass index (BMI) of the conscripts was defined in health examinations at the beginning of their military service. Smoking was classified as smokers and non-smokers, based on a questionnaire at the beginning of the service. Smokers were current daily smokers, and non-smokers had never smoked or had stopped smoking or smoked only occasionally. The information on education was collected from the conscripts' personal data forms. The study population and infectious episodes are described in more detail elsewhere.[6] The study protocol was approved by the Medical Ethics Committee of the Kainuu Central Hospital.

Table 1. Characteristics of the conscripts included in the study ($n = 889$)

Age (years), mean (SD)	19.6 (0.8)
Asthma, % (*n*)	26 (227)
Current smoker ($n = 874$), % (*n*)	43 (378)
Pack-years of smoking, median (range)	2.5 (0.1–-15.8)
Service time, % (*n*)	
180-day	58 (517)
270-day	6 (55)
362-day	28 (245)
Study aborted	8 (72)
Intake group, % (*n*)	
July 2004	47 (418)
January 2005	53 (471)
Infectious episodes (≥ 1 episode), % (*n*)	51 (452)
BMI (kg/m^2), geometric mean (95% CI)	24.2 (23.9–24.4)
Education ($n = 830$), % (*n*)	
Further education after comprehensive school	85 (756)

BMI, body mass index; CI, confidence interval; SD, standard deviation.

Blood samples were collected and the leukocytes and sera were separated and stored at -70°C or -20°C, respectively, for later analysis. The leukocytes were homogenized, DNA was extracted and DNA concentrations were measured as described previously.[6]

MBL2 genotyping by real-time PCR

Genotyping with a LightCycler Instrument (Roche Diagnostics, Mannheim, Germany) was performed as described previously.[6,11]

Serum MBL concentrations

The MBL concentrations of the serum samples obtained at the beginning of service were measured using a Human MBL (Lectin assay) enzyme-linked immunosorbent assay (ELISA) test kit (Hycult Biotechnology, The Netherlands) according to the manufacturer's instructions and as described previously.[6]

Micro-immunofluorescence test for measurement of C. pneumoniae *antibodies*

A micro-immunofluorescence test (MIF) was used to measure *C. pneumoniae* IgG, IgA and IgM antibody levels in serum samples from the military recruits according to a previously published protocol.[12,13] Using Finnish *C. pneumoniae* strain Kajaani 6 (K6) elementary bodies as an antigen, serial 2-fold dilutions of the serum samples, starting from 1 : 8, were done to detect IgG antibodies. For IgA and IgM antibodies, Gullsorb inactivation reagent (Gull Laboratories; Salt Lake City, UT, USA) was used according to the manufacturer's instructions to neutralize IgG. Positive serum controls

with known titres were included in each series. Immunoglobulin G titrations were started from 1 : 32 dilution, and IgA and IgM titrations, from 1 : 10 dilution. The presence of IgM in any serum during service or at departure and/or a ≥ 4-fold antibody rise in IgG or IgA antibodies between any paired serum samples was considered an indication of clinical or subclinical *C. pneumoniae* infection.

Statistical analysis

Statistical analyses were done using SPSS v.15.0. Median MBL levels in different groups and *MBL2* genotypes were analyzed with a Mann–Whitney U-test and a Kruskal–Wallis test, respectively. A χ^2 test was used to evaluate the statistical significance of the association of MBL level and *MBL2* genotypes with *C. pneumoniae* antibodies and seroconversion. Odds ratios (95% CI), adjusted for asthma status, smoking status, service time and intake group, were estimated by logistic regression. Smoking status and service time proved to be clearly non-significant and they were dropped from the final model.

RESULTS

MBL2 genotype and MBL concentration

Serum MBL concentrations were determined from 888 recruits. The median MBL level was 1151.6 ng/ml (IQR 431.7–1981.9 ng/ml; range 0–7342.0 ng/ml). There was no difference between the median MBL levels of those with asthma and those without ($P = 0.938$).

H/L polymorphism data were available from 883, X/Y data from 884 and P/Q and exon 1 variant data from 885 participants. The *MBL2* genotype distributions and the MBL serum concentrations of the different genotypes are shown in Table 2. The structural genotypes were categorized as A/A, A/O and O/O genotype groups, where O stands for any of the exon 1 variant alleles B, C or D. There was no difference between the genotype distributions of subjects with asthma and those without (data not shown). Allele frequencies were in Hardy–Weinberg equilibrium. The findings were in line with the previous results in the part of the same military population.[6]

MBL and C. pneumoniae *seroconversions*

We studied the association of MBL levels and *MBL2* genotypes with seroconversions to *C. pneumoniae* during 180-, 270- and 362-day military service (Table 3). The presence of IgM antibodies (seropositivity on arrival was not included) and IgG and IgA seroconversions between arrival, acute and departure sera were all considered a marker of being exposed to *C. pneumoniae* during service. During the follow-up, 13% (112/795) of the men had an IgG seroconversion, 8% (73/794) had an IgA seroconversion and 2% (15/795) had IgM antibodies present; altogether (any of the above), 21%

Table 2. *MBL2* genotype frequencies and serum MBL levels in different *MBL2* genotypes in the study population ($n = 889$)

MBL2 genotype	Frequency (%)	MBL concentration (ng/ml)	*P*-value[a]
H/H	164 (18)	1776	
H/L	427 (48)	1209	
L/L	292 (33)	582	< 0.001
X/X	37 (4)	778	
X/Y	280 (32)	1213	
Y/Y	567 (64)	1131	0.134
P/P	608 (68)	1066	
P/Q	245 (28)	1299	
Q/Q	32 (4)	1592	< 0.001
A/A	569 (64)	1664	
A/O[b]	274 (31)	359	
O/O	43 (5)	1	< 0.001

[a]Pearson chi-squared test of MBL levels between *MBL2* genotypes.
[b]O stands for any of the exon 1 variant alleles B, C or D.
MBL, mannose-binding lectin.

Table 3. The association of MBL levels and *MBL2* genotypes with the *C. pneumoniae* seroconversion during the 180-, 270- and 362-day service

	IgG rise, IgA rise or IgM ≥ 10[a]		
	Percent (*n*)	*P*-value[b]	OR (95% CI)[c]
< Median[d]	24 (95/397)		1.5 (1.1–2.1)
≥ Median	17 (68/398)	0.017	1
X/X+X/Y	22 (63/282)		
Y/Y	20 (99/508)	0.341	
H/H	17 (26/149)		
H/L+L/L	21 (137/640)	0.283	
P/P	23 (122/541)		1.5 (1.0–2.2)
P/Q+Q/Q	16 (40/250)	0.034	1
A/A	21 (108/514)		
A/O+O/O	20 (55/277)	0.701	

[a]Seroconversions between arrival, acute and departure sera. Immunoglobulin M seropositivity in the acute or in the departure sera.
[b]Pearson chi-squared test.
[c]Odds ratio (95% confidence interval), adjusted for asthma status and intake group.
[d]Median MBL concentration was 1151.6 ng/ml.
MBL, mannose-binding lectin.

(163/795) of the men were considered to have been exposed to *C. pneumoniae* during service.

Mannose-binding lectin levels were categorized into two groups, using a median MBL concentration of 1151.6 ng/ml as a cut-off. Mannose-binding lectin levels under the median concentration were significantly associated with IgG or IgA seroconversions or the presence of IgM antibodies during service, compared with over-median MBL levels (24% vs 17%, respectively; $P = 0.017$). Furthermore, in a binary logistic regression analysis, the risk was significant (OR 1.5; 95% CI: 1.1–2.1) when adjusted for asthma status and intake group. In addition, 23% of those who had the P/P genotype and only 16% of those with the P/Q or Q/Q genotype were considered to have been exposed to *C. pneumoniae* during service ($P = 0.034$), and the risk was up to 1.5-fold (95% CI 1.0–2.2) when adjusted for asthma status and intake group (Table 3). When the below-median MBL level and the P/P genotype were combined and the presence of zero risk factors was compared with one and two factors, a significant linear trend was detected (13% vs 19% vs 26%, respectively; $P = 0.002$). The risk also grew when only one factor was compared with two factors (OR 1.5; 95% CI 0.9–2.7 and OR 2.1; 95% CI 1.2–3.7, respectively; data not shown).

Mannose-binding lectin and C. pneumoniae *antibodies*

We studied the association of MBL levels and *MBL2* genotypes with elevated *C. pneumoniae* IgG (titre ≥ 128) and IgA (titre ≥ 40) antibodies on arrival of military service (Table 4). The presence of elevated IgG (titre ≥ 128) or IgA (titre ≥ 40) antibodies or both was considered a marker of past exposure to *C. pneumoniae*. The prevalences of IgG (≥ 128), IgA (≥ 40) or IgG and/or IgA antibodies were 13% (113/889), 8% (72/888) and 16% (144/888).

The Y/Y genotype (OR 1.6; 95% CI 1.1–2.3) and exon 1 variant allele genotypes (A/O and O/O; OR 1.4; 95% CI 1.0–2.0) were significantly associated with the presence of elevated IgG and/or IgA antibodies when adjusted for asthma status and intake group. Furthermore, a borderline significant association was detected between the antibodies and the H/H genotype (OR 1.5; 95% CI 0.9–2.3; Table 4).

Table 4. The association of MBL levels and *MBL2* genotypes with the *C. pneumoniae* antibodies in military recruits on arrival

	IgG ≥ 128 and/or IgA ≥ 40		
	Percent (*n*)	P-value[a]	OR (95% CI)[b]
< Median[c]	18 (74/444)		
≥ Median	16 (70/443)	0.727	
X/X+X/Y	13 (40/317)		1
Y/Y	18 (104/566)	0.026	1.6 (1.1–2.3)
H/H	20 (33/164)		1.5 (0.9–2.3)
H/L+L/L	16 (111/718)	0.145	1
P/P	17 (101/608)		
P/Q+Q/Q	16 (43/276)	0.700	
A/A	15 (83/568)		1
A/O+O/O	19 (61/316)	0.070	1.4 (1.0–2.0)

[a]Pearson chi-squared test.
[b]Odds ratio (95% confidence interval), adjusted for asthma status and intake group.
[c]Median MBL concentration was 1151.6 ng/ml.
MBL, mannose-binding lectin.

Discussion

This is the first time a direct association between MBL and *C. pneumoniae* infection *in vivo* has been reported, and we present here the associations of MBL level and six known *MBL2* gene polymorphisms with *C. pneumoniae* antibodies in healthy Finnish military recruits and with seroconversions that indicate clinical or subclinical infections during military service. The exon 1 variant allele genotypes (A/O and O/O) and Y/Y genotype were associated with the presence of elevated *C. pneumoniae* IgG or IgA antibodies. In addition, an MBL level below the median concentration and the P/P genotype were associated with *C. pneumoniae* seroconversions during military service. The results of these two different analyses are not completely comparable with each other. However, both analyses are in agreement with previous studies on MBL and respiratory tract infections. We previously reported results where the exon 1 variant allele genotypes (A/O and O/O), Y/Y genotype and a below-median MBL level were associated with respiratory infectious episodes in general during military service, based on a part of the same military population (only those from the January 2005 intake group who served 6 months).[6] In addition, several other studies have found an association between exon 1 variant allele genotypes and susceptibility to respiratory tract infections.[4,14,15]

The presence of IgG and/or IgA serum antibodies is a marker of past exposure to *C. pneumoniae*. It has been shown earlier that both IgG and IgA antibodies against *C. pneumoniae* decrease rapidly after an acute infection;[16,17] thus, in the present study, the presence of elevated IgG and IgA antibody levels at arrival to the service is suggestive of a possible chronic infection. Interestingly, low serum MBL levels were significantly associated with susceptibility to *C. pneumoniae* (seroconversions) infection during military service, but not with the presence of elevated antibodies, suggesting that low

MBL levels do predispose to infection susceptibility, but possibly not to development of chronic infection.

In vitro studies by Swanson *et al*.[10] have shown that MBL inhibits cell culture infection by chlamydia. They reported that MBL protein inhibited by at least 50% at 98 ng/ml for *C. trachomatis* C/TW-3/OT and E/UW-5/Cx and at 6250 ng/ml for *C. trachomatis* L2/434/Bu, *C. pneumoniae* AR-39 and *Chlamydophila psittaci* 6BC. Those results indicate that MBL has an influence on immunity against *C. pneumoniae*. In addition, several other studies have investigated the structure of the carbohydrates of the outer membrane of chlamydia and their role in the infectivity of host cells[8,18,19] and also the ligands that inhibit infection of host cells by chlamydia.[9,20] Swanson and Kuo[19] reported that the carbohydrates in glycoproteins are mannose, galactose, fucose and *N*-acetyl glucosamine. Later, they showed that the carbohydrate moieties of the major outer membrane protein of chlamydia are involved in the attachment and infectivity of the organisms to HeLa cells.[18] Furthermore, Kuo *et al*.[8] showed that major outer membrane protein is glycosylated with high mannose oligosaccharides, and they indicated that these structures can inhibit infectivity. Supposedly, MBL binds to the mannose structures located on the outer membrane of chlamydia, blocking attachment and entry of the organism into the host cell. This was also investigated and suggested by Swanson *et al*.[10]

To our knowledge, a direct association between MBL and *C. pneumoniae* antibodies has not been indicated before. Rugonfalvi-Kiss *et al*.[21] studied the role of MBL in the association between *C. pneumoniae* and coronary artery disease. They concluded that *C. pneumoniae* infection leads to the development of severe coronary artery disease mainly in those with the *MBL2* exon 1 variant allele. The association was found only among those carrying *MBL2* variant alleles and not in those homozygous for the wild-type allele. Furthermore, Nagy *et al*.[22] have reported that *MBL2* exon 1 variant alleles have an important role in susceptibility to asthma in children infected with *C. pneumoniae*. They did not observe a direct association between *MBL2* genotypes and *C. pneumoniae* antibodies. Both of these studies found an association between *C. pneumoniae*, the *MBL2* variant allele and a chronic inflammatory disease. Therefore, these studies suggest that MBL plays an important role in the immune response against *C. pneumoniae* infection.

Military service in Finland is mandatory and all men aged 18–19 years are called up for the service. Ninety-eight percent of them attend a call-up examination to establish their fitness for military service[23] and 80–85% of all men, including over 80% of asthmatic men, complete their service.[24] Thus, military conscripts can be considered to represent the young Finnish male population. This material also gave us a good opportunity to do a follow-up analysis during the service period, which enabled the study of *C. pneumoniae* exposure during that time. Furthermore, the follow-up was quite frequent, as the samples were obtained at the beginning and end of service as well as during each infectious episode.

Conclusions

Mannose-binding lectin has been shown to inhibit the infection of host cells by *C. pneumoniae in vitro*. In this study, we reported a direct association of low MBL levels and *MBL2* polymorphisms with elevated *C. pneumoniae* antibodies and seroconversions. The results suggest that MBL is associated with susceptibility to *C. pneumoniae* infection, and support the need for prospective association studies as well as *in vitro* studies to confirm the previous and present findings on the role of MBL in protecting against *C. pneumoniae*.

Acknowledgements

The authors thank Leena Kuisma, Anu Ojala and Seija Liikanen for their technical assistance. This study was supported by the Scientific Advisory Board for Defence (MATINE) of the Ministry of Defence of Finland.

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