CHAPTER SEVEN

Deficient mannose-binding lectin-mediated complement activation despite MBL-sufficient genotypes in an outbreak of *Legionella pneumophila* pneumonia

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ABSTRACT

Polymorphisms leading to deficiency of mannose-binding lectin (MBL) are associated with predisposition to infection. However, MBL deficiency can be protective against intracellular pathogens that use MBL to enter host cells. The role of MBL genotype and activity in infection with the intracellular pathogen *Legionella pneumophila* was studied in a large outbreak of legionellosis at a Dutch flower show.

A total of 141 patients, 65 exposed asymptomatic exhibition staff members and 670 unexposed blood bank donors were included for the study of *MBL2* genotypes and MBL-mediated complement activation.

Genotypic MBL deficiency was equally prevalent in patients and controls. Deficient MBL-mediated complement activation was more prevalent in patients. Even in patients with genotypes that confer MBL sufficiency, 20.6% lacked MBL-mediated complement activation. In most patients with MBL-sufficient genotypes who lacked MBL-mediated activation at the acute phase of disease, lectin pathway functionality was restored at convalescence.
In conclusion, genotypic MBL deficiency was not a risk factor for legionellosis. However, patients with legionellosis displayed deficient MBL-mediated complement activation even with MBL-sufficient genotypes. Together, these genotypical and functional data suggest that the observed deficiency of lectin pathway activation is an effect of legionellosis rather than a risk factor for acquiring it.

INTRODUCTION

Mannose-binding lectin (MBL) is a pattern recognition receptor of the innate immune system that activates complement via the lectin pathway [1]. Functional MBL is a multimeric molecule, with its subunits organized in a bouquet-like structure [2]. In its multimeric form, it binds to a variety of microorganisms, including respiratory pathogens such as influenza A virus, *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Legionella pneumophila* [3,4,5]. Single nucleotide polymorphisms (SNPs) in the *MBL2* gene control the level of MBL in serum. Certain combinations of SNPs can lead to MBL deficiency. Coding polymorphisms in exon 1 ("0" alleles B, C and D vs. wild-type A allele) lead to monomeric non-functional MBL subunits. The X/Y promoter polymorphism determines the serum level of functional MBL multimers by transcriptional control of the wild-type A allele. In healthy individuals, genotypes 0/0 and LXA/0 display MBL levels <0.2 µg/ml and are considered deficient [6]. MBL deficiency is associated with an increased risk of infection, as opsonization by complement is compromised [7]. In infections with intracellular pathogens, the role of MBL deficiency is more ambiguous, as some intracellular pathogens use opsonization by MBL to enter their host cell [7]. Although MBL can bind to the intracellular pathogen *Legionella pneumophila* [5], the role of MBL genotypes and activity as a risk factor for legionellosis is unclear. We determined *MBL2* genotypes and MBL-mediated complement activation in a retrospective case-control study in the setting of a clonal outbreak of Legionnaires’ disease at a flower show in the Netherlands in 1999 [8,9]. Since this clonal outbreak had no pathogen variability, this patient cohort offered a unique opportunity to study the interplay between these host factors and legionellosis. The combination of genetic and functional data in this study allowed us to distinguish the influence of *MBL2* polymorphisms on MBL activity from the effect of legionellosis itself.
MATERIALS AND METHODS

Patients and controls
Patient criteria were described earlier in detail [9]. In brief, *Legionella pneumonia* was diagnosed in 188 visitors or exhibition staff members of a flower show, according to the criteria described by the European Working Group on Legionella Infections (EWGLI) [10]. Informed consent was obtained from 141 hospitalized patients and these were included in the study.

We used two control groups. In the exposed control group, asymptomatic exhibition staff members who had been exposed to *L. pneumophila* as evidenced by seroconversion were included (n=65 for seroconversion controls) [11]. A second, unexposed control group consisted of a group of 670 blood bank donors composed of 223 donors for genotypical analysis and 447 different donors for functional analysis. All participants in both control groups gave informed consent.

Data collection and definitions of clinical parameters were previously described in detail [8,9,11]. DNA extracted from whole blood samples and serum samples collected earlier from these study groups were used to determine *MBL2* genotypes and MBL activity as described below. To study MBL-mediated complement activation over time, we defined acute and convalescent phase sera as follows. Serum samples drawn between day 0 and +3 after presentation at the hospital were considered acute phase samples. Samples drawn at day 20 or later were considered convalescent phase samples.

Genotyping of *MBL2* in whole blood samples
Combined haplotypes of the X/Y promoter and exon 1 SNPs of *MBL2* were determined using a previously described denaturing gradient gel electrophoresis (DGGE) assay with modifications in a nested PCR protocol [12]. Per sample, two PCR assays specific for the promoter X SNP (forward primer ATT TGT TCT CAC TGC CAC C; reverse primer GAG CTG AAT CTC TGT T TT GAG TT; annealing temperature 63°C; 25 cycles; 629 bp) or Y SNP (forward primer TTT GTT CTC ACT GCC ACG; 628 bp) were run. The PCR products were diluted 1:100 in distilled water. *MBL2* exon 1 was amplified from these dilutions with an additional GC-clamp attached to one primer to meet DGGE requirements (forward primer with clamp: CCG CCC GCC GCG CCC CGC GCC CCC GCC CCT CCA TCA CTC CCT CTC TTT CTC; reverse primer: GAG ACA GAA CAG CCC AAC ACG; 241 bp).

The amplified DNA was run overnight at 75 Volts on a 6% polyacrylamide gel containing a denaturing gradient linearly increasing from 35% to 55% formamide and urea. All *MBL2* exon 1 genotypes could be distinguished by their different patterns of migration. The corresponding *MBL2* X/Y promoter haplotype could be inferred from the presence or absence of a product in the two nested PCR assays. Genotypes 0/0 and XA/0 were considered “MBL-deficient”, and genotypes YA/0, XA/XA, XA/YA and YA/YA were considered “MBL-sufficient” [6,13].

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Genotyping of MBL2 in serum samples

To increase the number of patients available for genotypic analysis, genotyping from serum was used, but a stringent protocol for MBL2 genotyping from serum was set up. Genomic DNA was isolated from 100 µl of serum with the MagNAPure LC robot (Roche Diagnostics, Mannheim, Germany) using the MagNAPure DNA Isolation Kit according to the manufacturer’s protocol. To minimize the risk of genotyping errors due to minimal DNA concentrations in these samples, each sample was genotyped in two different nested PCR assays. In the first assay, a 433 bp fragment was amplified (forward primer TAT TTC TAT ATA GCC TGC ACC CA; reverse primer GAG CTG AAT CTC TGT TTT GAG TT; annealing temperature 57°C; 40 cycles) to serve as a template for the consecutive exon 1 PCR. In the second assay, the X and Y SNP-PCR products served as the first step of the nested protocol similar to the whole blood genotyping protocol described above, except for running 40 cycles. In both assays, exon 1 was amplified from 1:100 diluted PCR products and further analyzed as described above.

Serum genotyping was only considered reliable when the two different PCR protocols resulted in the same genotype.

Functional MBL assay

Functional MBL levels were determined in serum samples using a hemolytic assay [14]. In this assay, functional MBL levels were determined by measuring complement mediated bystander-hemolysis evoked by binding of MBL to mannan residues on the surface of Saccharomyces cerevisiae. To ensure that the rate of hemolysis via the lectin pathway was not limited by shortage of complement components down-stream of MBL, MBL-deficient serum (donor genotype 0/0) was added to the test wells to provide an excess of these components. The functional MBL level in the test sample was calculated from comparison with hemolysis by a standard serum of 1.67 µg/ml MBL [14]. Since this assay measured a down-stream effect of MBL activity rather than the protein concentration itself, levels were expressed as microgram equivalents per milliliter (µg.eq/ml).

In healthy adults, levels below 0.2 µg.eq/ml are considered deficient in this hemolytic assay [14]. To confirm this cut-off value for the seroconversion controls, data on MBL2 genotypes and MBL-mediated complement activation from this control group were analyzed using a receiver operating characteristic (ROC) curve. The cut-off value derived from the ROC-curve analysis was used to assess MBL-mediated complement activation as a predictor of MBL-deficient genotypes 0/0 and XA/0 in patients and seroconversion controls.
Patients were compared to controls to assess whether MBL2 genotypes and MBL-mediated complement activation are risk factors for L. pneumophila pneumonia. The highest MBL-mediated complement activation measured in multiple serum samples per patient was used for overall analysis.

Groups were compared first by means of univariate analysis. For normally distributed continuous variables, a t-test was used after correction for inequality of variances (based on Levene tests). Categorical variables were analyzed with a Pearson’s chi-square test or a Fisher’s exact test. To adjust for confounders, multivariate logistic regression models using backward stepwise elimination by likelihood ratio tests were used. Where appropriate, randomly selected seroconversion controls matched by sex and age within 2 years of each legionellosis patient were used in analysis. Mean MBL functionality was compared by genotype between patients and controls using a univariate analysis of variance (ANOVA). Data were analyzed with SPSS software version 15.0 (SPSS, Chicago, USA).

RESULTS

In this study of a clonal outbreak of legionellosis in 188 patients at a flower show, 141 hospitalized patients, 65 exposed asymptomatic exhibition staff members with seroconversion and 670 unexposed blood bank donor controls were included to assess MBL2 genotypes and MBL-mediated complement activation. MBL2 genotypes were determined in whole blood samples available from 78 of 141 patients, 53/65 seroconversion controls and 223/670 blood bank donors. Serum samples for genotyping were available from 111 of 141 patients. With the use of a stringent protocol, 72 patients could be genotyped reliably from these 111 serum samples. Thirty-eight patients were genotyped from both whole blood and serum samples, and no discrepancies were found between the results from these genotyping protocols. By combining whole blood and serum genotyping results, a total of 112 of 141 patients could be included in the final analysis of MBL2 genotypes.

MBL-mediated complement activation could be determined in serum samples available from 125 of 141 patients (a total of 265 samples available), 59/65 seroconversion controls and 447/670 blood bank donors (1 sample per control). Patients and exposed controls that could not be included (because of the absence of samples) did not differ in age, sex or smoking habits from those included in the study (data not shown).

The ROC curve of data from the seroconversion controls on genotypic MBL deficiency and MBL-mediated complement activation confirmed that the previously reported cut-off value for healthy adults of 0.2 µg.eq/ml was also
appropriate in this control group [14]. The ROC curve had an area under the curve of 1, and, using 0.2 µg.eq/ml as cut-off for MBL deficiency, the sensitivity and specificity were 100% in seroconversion controls. The cut-off value of 0.2 µg.eq/ml derived from the ROC-curve analysis was used to categorize MBL-mediated complement activation as sufficient or deficient.

Cases were older than those in both control groups (table 1; t-tests, p<0.05). A non-significant difference in sex was observed, with more patients being male compared to seroconversion controls (2x2 χ² test, p=0.13).

MBL2 genotypes were equally distributed between patients with legionellosis, seroconversion controls and blood bank donors in univariate analysis (table 1; 3x6 χ² test, p=0.27). Also, MBL-deficient genotypes were equally distributed between the three groups (A/A and YA/0 vs. XA/0 and 0/0; 3x2 χ² test, p=0.082). When comparing only patients and seroconversion controls, the frequency of genotypic MBL deficiency also did not differ significantly between the two groups (OR 2.6, 95% CI [0.7-9.3]; 2x2 χ² test, p=0.14). When patients and seroconversion controls were matched by sex and age, genotypic MBL deficiency remained equally distributed between the two groups (3/30 patients with genotypes XA/0 or 0/0 versus 0/30 matched seroconversion controls; 2x2 Fisher’s exact test, p=0.24).

In contrast, deficient MBL-mediated complement activation was more prevalent in patients than in both seroconversion controls and blood bank donors (table 1; 3x2 χ² test, p<0.01). When comparing cases and seroconversion controls, the frequencies of deficient MBL-mediated complement activation differed significantly (OR 7.6, [2.2-25.7]; 2x2 χ² test, p<0.001). Also, smoking (OR 2.1 [1.1-4.1]; p=0.03), age (t-test, p<0.001) and diabetes mellitus (OR 1.7 [1.5-1.9]; p=0.01) were associated with legionellosis, while sex (OR 1.6 [0.9-2.9], COPD (OR 0.4 [0.1-1.2]), rheumatic diseases (Fisher’s exact test; p=0.30) and malignancies (OR 1.1 [0.2-6.4]) were not. When patients and seroconversion controls were matched by sex and age, deficient MBL-mediated complement activation remained more prevalent in patients than in matched controls (8/35 patients with MBL-mediated complement activation <0.2 µg.eq/ml versus 0/35 matched seroconversion controls; 2x2 Fisher’s exact test, p<0.01). All parameters were included in the logistic regression models.

In the multivariate logistic regression model analyzing genotypic MBL deficiency between patients and seroconversion controls, only smoking (OR 13.7 [3.8-48.5]) and age (exp(B) 0.9 [0.8-0.9]) were significantly associated with Legionnaires’ disease. Genotypic MBL deficiency was not associated with Legionnaires’ disease. When the ability to activate complement via MBL, rather than the MBL genotype, was analyzed in the multivariate logistic regression model, deficiency of MBL-mediated complement activation was significantly associated with Legionnaires’ disease (OR 11.2 [1.6-76.9]), as were smoking (OR 13.5, [3.9-46.4]) and age (exp(B) 0.9 [0.8-0.9]).
Table 1. MBL2 genotypes and MBL-mediated complement activation in patients with legionellosis, exposed seroconversion controls and unexposed blood bank donors.

<table>
<thead>
<tr>
<th>MBL2 genotype:</th>
<th>Patients</th>
<th>Exposed seroconversion controls</th>
<th>Unexposed blood bank donors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total nr tested</td>
<td>112</td>
<td>53</td>
<td>223</td>
</tr>
<tr>
<td>Age ± SD (y)</td>
<td>63.8 ± 10.5*</td>
<td>45.0 ± 14.3</td>
<td>45.2 ± 12.0</td>
</tr>
<tr>
<td>M:F ratio</td>
<td>1:4:1</td>
<td>1:0:1</td>
<td>1:5:1</td>
</tr>
<tr>
<td>Sufficient:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YA/YA</td>
<td>32 (28.6%)</td>
<td>19 (35.8%)</td>
<td>61 (27.4%)</td>
</tr>
<tr>
<td>XA/YA</td>
<td>27 (24.1%)</td>
<td>18 (34.0%)</td>
<td>45 (20.2%)</td>
</tr>
<tr>
<td>YA/0</td>
<td>30 (26.8%)</td>
<td>12 (22.6%)</td>
<td>66 (29.6%)</td>
</tr>
<tr>
<td>XA/XA</td>
<td>8 (7.1%)</td>
<td>1 (1.9%)</td>
<td>12 (5.4%)</td>
</tr>
<tr>
<td>Subtotal</td>
<td>97 (86.6%)</td>
<td>50 (94.3%)</td>
<td>184 (82.5%)</td>
</tr>
<tr>
<td>Deficient:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XA/0</td>
<td>10 (8.9%)</td>
<td>3 (5.7%)</td>
<td>26 (11.7%)</td>
</tr>
<tr>
<td>0/0</td>
<td>5 (4.5%)</td>
<td>0 (0.0%)</td>
<td>13 (5.8%)</td>
</tr>
<tr>
<td>Subtotal</td>
<td>15 (13.4%)</td>
<td>3 (5.7%)</td>
<td>39 (17.5%)</td>
</tr>
</tbody>
</table>

MBL-mediated complement activation:

| Total nr tested | 125      | 59                             | 447                         |
| Age ± SD (y)    | 64.6 ± 10.4* | 46.0 ± 14.3                    | 45.0 ± 17.2                 |
| M:F ratio       | 1.5:1    | 0.8:1                          | 1.7:1                       |
| Sufficient (>0.2 µg.eq/ml) | 89 (71.2%) | 56 (94.9%)                     | 434 (97.1%)                 |
| Deficient (<0.2 µg.eq/ml) | 36 (28.8%) | 3 (5.1%)                       | 13 (2.9%)                   |

* t-test, p<0.05
b 3x2 χ² test, p<0.01

Analyzing the combined genotypical and functional data, the mean level of MBL-mediated complement activation did not differ between patients and seroconversion controls compared by genotype (figure 1; ANOVA, p=0.974). However, of 97 patients with MBL-sufficient genotypes, MBL-mediated complement activation was sufficient in 77 (79.4%) and deficient in 20 (20.6%). None of the seroconversion controls with MBL-sufficient genotypes showed deficient MBL-mediated complement activation (figure 1; 2x2 χ² test, p=0.001). The deficiency of MBL-mediated complement activation in patients with MBL-sufficient genotypes was observed during the acute phase of legionellosis (11/41 (26.8%) patients with MBL-sufficient genotypes) as well as the convalescent phase (14/55 (25.5%) patients). Paired serum samples were available from 35 patients with MBL-sufficient genotypes. Ten of these patients (28.5%) had deficient MBL-mediated complement activation in the acute phase. In the
convalescent phase, MBL-mediated activation was restored to sufficient levels in 8/10 patients. Two patients continued to show deficient activation, at day 21 (genotype YA/YA) and day 81 (genotype YA/0) respectively.

Figure 1. MBL-mediated complement activation by genotype in patients with legionellosis and exposed seroconversion controls. The threshold for sufficient MBL-mediated complement activation is plotted (0.2 µg.eq/ml, dashed line). In patients with legionellosis, deficient lectin pathway activation was observed even in subjects with MBL-sufficient genotypes. This was not observed in seroconversion controls (2x2 χ² test, p=0.001).

DISCUSSION

In this case-control study in a large clonal outbreak of legionellosis, we determined MBL2 genotypes and MBL-mediated complement activation as potential risk factors for acquiring legionellosis. Using a stringent protocol for genotyping from serum samples, genotypes could be determined reliably in a large proportion of patients, for whom no whole blood samples were available. Genotypic MBL deficiency could not be shown to be significantly associated with legionellosis in this cohort. In contrast, deficiency of MBL-mediated complement activation was observed more frequently in patients than in both control groups. The association of legionellosis with functional lectin pathway deficiency was independent of age, sex or smoking, as demonstrated by the univariate matched analysis and multivariate logistic regression models. When genotypes were allocated to alternative categories, considering genotypes XA/XA and YA/0 as producing intermediate levels of MBL, or grouping them with the MBL-deficient genotypes [15], some differences were found between seroconversion controls and blood bank donors (data not shown). However, no differences were found between patients and both control groups. Therefore, legionellosis could also not be shown to be significantly associated with alternative genotype classifications in this study.
The combination of genetic and functional data in our study allowed us to distinguish the influence of MBL2 polymorphisms on the MBL-mediated complement activation from the effect of legionellosis itself. Recently, reduced MBL-mediated C4 deposition was reported in patients with legionellosis in an outbreak in Melbourne. The authors concluded that deficiency of MBL-mediated complement activation appeared to predispose to Legionnaires’ disease [16]. In contrast to the present study, data on MBL2 genotypes were not available in the Melbourne cohort. Since MBL-deficient genotypes were not associated with legionellosis in our study, the observed deficiency of lectin pathway activation in both our patients and the Melbourne patients more likely is an effect of legionellosis rather than a risk factor for acquiring it.

In a large number of patients, deficiency of MBL-mediated complement activation was observed even though they carried a MBL-sufficient genotype. The lack of lectin pathway activity in patients with MBL-sufficient genotypes could not be explained by exhaustion or deficiency of downstream complement factors, since complement factors other than MBL were exogenously added in the functional assay.

Lectin pathway functionality was restored at convalescence in most patients with MBL-sufficient genotypes who showed absent lectin pathway activation in the acute phase. The recovery of lectin pathway functionality supports the hypothesis that the observed functional deficiency is an effect of legionellosis rather than a risk factor.

The discrepancy between MBL-sufficient genotypes and deficient MBL-mediated complement activation was observed in patients only. The normal relationship between MBL genotypes and complement activation in seroconversion controls in our study suggests that deficient lectin pathway activation does not result from asymptomatic infection.

The MBL-mediated complement activity could have been overestimated in the hemolytic assay used in this study due to MBL-independent complement activation by immune complexes, e.g., those formed by antibodies to S. cerevisiae [17]. However, such an effect would also occur in individuals with a MBL-deficient genotype, and we did not observe high levels of hemolysis in these individuals in this study. Instead, classical pathway activity could have led to an underestimation of the frequency of absent MBL-mediated complement activation in individuals with MBL-sufficient genotypes. In the blood bank donors, MBL-deficient genotypes were found more frequently than deficient lectin pathway functionality. This could be due to a sampling error between the two different cohorts used for genotypical and functional analysis. Another possibility is that due to classical pathway activation, deficient lectin pathway activation was underestimated in the cohort used for functional analysis. The potential effects of classical pathway interference described above would not change the observation that in patients with legionellosis, deficient MBL-mediated complement activation
was observed even in those with MBL-sufficient genotypes. Therefore, we believe that this potential interference would not affect the main conclusions of this study. In conclusion, genotypical MBL deficiency could not be shown to be associated with legionellosis in this large clonal outbreak. However, a large number of patients with legionellosis displayed deficient MBL-mediated complement activation even though they carried MBL-sufficient genotypes. In most patients with MBL-sufficient genotypes who showed functional deficiency at the acute phase of disease, the lectin pathway functionality was restored at convalescence. Together, these genotypical and functional data suggest that the observed deficient lectin pathway activation is an effect of legionellosis rather than a risk factor for acquiring it.

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