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Research article

Deficient mannose-binding lectin-mediated complement activation despite mannose-binding lectin–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.

1. 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* [3–5].

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 nonfunctional 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

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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.

2. Subjects and methods

2.1. 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 (Yzerman, submitted).

2.2. 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 TTT 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 CGG CCC GCC GCC CCC GCC CCT CCA TCA CTC CCT CTC CTT 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].

2.3. 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.

2.4. 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.

2.5. Statistical analysis

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 χ^2 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, Inc., Chicago, IL).

3. 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.