Mechanisms of disease

MBL genotype and risk of invasive pneumococcal disease: a case-control study

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Summary

Background *Streptococcus pneumoniae* is a major cause of morbidity and mortality in developed and developing countries. No common genetic determinants of susceptibility have been defined. Mannose-binding lectin (MBL) is a key mediator of innate host immunity that activates the complement pathway and directly opsonises some infectious pathogens. Mutations in three codons in the *MBL* gene have been identified, and individuals homozygous for a mutant genotype have very little or no serum MBL. We did a case-control study in the UK to assess whether these mutant genotypes were associated with invasive pneumococcal disease.

Methods The frequencies of genotypes defined by the three mutations in codons 52, 54, and 57, and a functional promoter polymorphism at –221, were compared in a two-stage study of 337 patients with invasive pneumococcal disease and 1032 controls. All individuals were recruited from an ethnically homogeneous white population in Oxfordshire, UK. Patients had *S pneumoniae* isolated from a normally sterile site.

Findings In our initial set of participants, 28 (12%) of 229 patients and 18 (5%) of 353 controls were homozygotes for *MBL* codon variants (odds ratio 2·59 [95% CI 1·39–4·83], p=0·002). Neither heterozygosity for these codon variants nor the promoter polymorphism was associated with susceptibility. In a confirmatory study, 11 (10%) of 108 patients were *MBL* homozygotes compared with 36 (5%) of 679 controls (p=0·046).

Interpretation Homozygotes for *MBL* codon variants, who represent about 5% of north Europeans and north Americans and larger proportions of populations in many developing countries, could be at substantially increased risk of invasive pneumococcal disease.

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Introduction

Infection with *Streptococcus pneumoniae* is a major cause of morbidity and mortality worldwide. In developed countries, it is the main infectious cause of death and the most common cause of community-acquired bacteraemia in adults.[1] In developing countries, it is a leading cause of mortality in children younger than 5 years of age, and probably accounts for over a million deaths annually.[2] Although the organism is commonly found harmlessly colonising the mucosa of the upper respiratory tract, invasion can lead to pneumonia, bacteraemia, and meningitis with reported case-fatality rates of 7%, 20%, and 30%, respectively.[1] The extent to which host genetics might influence susceptibility to invasive pneumococcal disease is unknown, and, unlike other major infectious causes of mortality, no susceptibility or resistance genes have been defined.

A protein belonging to the COLLECTIN group—mannose-binding lectin (MBL, also called mannose-binding protein)—is a calcium-dependent lectin produced in the liver during the acute-phase response to infection.[3] Its function in host defence has been of interest since early studies showed it to bind baker's yeast mannans, opsonise some bacteria, and affect infection of lymphoblasts by HIV-1.[4,5] Two potentially protective functions are well defined. First, binding of MBL-ASSOCIATED SERINE PROTEASES (MASP) leads to activation of the complement cascade independently of antibody;[6] and second, MBL opsonises bacteria by binding to specific surface oligosaccharides, particularly N-acetyl glucosamine and mannose.[4]

The *MBL* gene on chromosome 10 encodes a homotrimeric molecule with a carbohydrate recognition domain and a collagenous tail.[7] Formation of a triple helix in the collagenous tail is impaired by mutation in any one of codons 52, 54, or 57 (denoted variants D, B, and C, respectively), and this impaired helix formation disrupts polymerisation and leads to enzymatic degradation and functional deficiency of MBL.[3,8,9] These three codon variants are very common: heterozygote and homozygote frequencies are about 33% and 5% in most populations.[9] Sequence variation in the promoter region of the gene has also been described, and a single base-pair change at position –221 is common in white individuals.[10] Concentrations of MBL in serum are about 20% lower in heterozygotes for any codon variant than in individuals with none of the mutations, and are very low (typically <2%) or absent in homozygotes or individuals heterozygous for two different codon mutations (hereafter simply termed homozygotes).[9,10] The –221 promoter G→C change, present in about 40% of Europeans, is the most relevant non-coding polymorphism and has a smaller but detectable effect on concentrations of MBL in serum.[10]

Results of clinical studies have shown associations between *MBL* codon mutations and hospital admissions due to infection in children,[11] susceptibility to infection in

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GLOSSARY

ALLELE-SPECIFIC OLIGONUCLEOTIDE HYBRIDISATION
A technique used to detect DNA sequence differences by means of differential binding of DNA probes specific for each genetic variant.

COLLECTIN
A collagenous lectin, a sugar-binding molecule with a collagen-like structural domain.

HARDY-WEINBERG EQUILIBRIUM
A proportionality between the frequencies of heterozygotes and homozygotes for a genetic variant in the population determined by Mendel's laws of inheritance.

LIGATION-DETECTION REACTION
A method of detecting single nucleotide changes in DNA with PCR to amplify the relevant DNA segment. The variants are then detected by use of a labelled conserved oligonucleotide probe and two variant specific oligonucleotide probes of different lengths, the latter terminating at the variant nucleotide. A ligase enzyme will join the conserved probe only to the specific oligonucleotides in the amplified sample and the larger DNA fragment is then detected by gel electrophoresis.

MBL-ASSOCIATED SERINE PROTEASES
Enzymes that bind to mannose-binding lectin in serum and that cleave other proteins to activate complement.

patients with systemic lupus erythematosus,[12] and a heterogeneous group of immunodeficiencies.[13] MBL deficiency has also been associated with increased episodes of acute respiratory infection, but not acute otitis media, in children from Greenland.[14,15] However, there are no established associations between MBL deficiency and susceptibility to diseases caused by specific pathogens, since evidence is conflicting and studies limited.[16–21] In this study, we compare the frequency of the three codon mutations and the –221 promoter polymorphism in a large-scale investigation of cases of invasive pneumococcal disease and controls.

Methods

Patients and controls

Patients with invasive pneumococcal disease were recruited from three hospitals (John Radcliffe, Horton General, and Wycombe General Hospitals) in Oxfordshire, UK, as part of the enhanced active surveillance of the disorder. Controls were selected randomly from adult volunteer donors to the Oxford Blood Transfusion Service and from transplant donors. Individuals with non-UK ancestry were excluded, and all patients and controls were white. Further controls for a confirmatory study were 679 healthy neonates born at the John Radcliffe Hospital, Oxford. For these controls, we used a needle and syringe to obtain blood from the umbilical veins of discarded umbilical cords, after careful cleaning of the cords to minimise the risk of contamination with maternal blood. The samples were taken anonymously, and the ethnic origin of the child was the only information recorded. Subsequent analysis of randomly chosen microsatellite markers in a subset of samples showed no instances of contamination with maternal DNA.

Cases of invasive pneumococcal disease were defined by the isolation of *S pneumoniae* from a normally sterile site (eg, blood, cerebrospinal fluid, joint fluid). Blood was collected into sodium/EDTA tubes, separated, and stored at –80°C until DNA extraction with Nucleon II kits (Scotlab, Coatbridge, UK). Clinical diagnoses were recorded on a standard proforma. Outcomes for cases measured in duration of hospital stays and death were obtained from hospital and general practitioner databases. The research ethics committees of the participating hospitals approved the study.

Genotyping and serotyping

Extracted DNA was genotyped by two PCR-based methods: ALLELE-SPECIFIC OLIGONUCLEOTIDE HYBRIDISATION (ASO) and the LIGATION-DETECTION REACTION (LDR). The primers used in the PCRs are given in table 1.[19] In each case, PCR was done in a 25 μL reaction volume with 2·5 mmol/L $MgCl_2$, 0·32 mmol/L dNTP, 0·1 μmol/L each primer, 1 U Taq enzyme (TaqGold, Perkin-Elmer, Wellesley, MA, USA), and 50 ng genomic DNA.

For ASO, amplified product was blotted onto nylon membranes by means of a dot-blot manifold, and then probed with allele-specific oligonucleotides labelled with digoxigenin (Roche, Lewes, UK). The sequence-specific oligonucleotides used are also listed in table 1. For genotyping of the codon variants by LDR, fluorescently labelled allele specific-probes (table 1) were incubated at optimised temperatures together with Taq DNA ligase (New England Biolabs, Hutchin, UK), and subjected to thermal cycling. The products were visualised with ABI 373 software (Applied Biosystems, Warrington, UK).

Typing of *S pneumoniae* was done by the Neufeld Reaction with standard typing antisera obtained from the Statens Serum Institute, Copenhagen, Denmark.

Statistical analysis

Statistical analysis of genotype frequencies was done with χ^2 tests or logistic regression, by use of the programs EPI and SPSS/PC+. Frequencies of clinical presentations and serotypes in the genotype groups were compared likewise. The distribution of duration of hospital stay was not normally distributed and was compared between genotypes by the Mann-Whitney *U* test. Survival of patients was compared by Kaplan-Meier techniques. In the confirmatory study, a single hypothesis was tested—ie, whether homozygotes were over-represented in patients compared with controls—and a 2×2 χ^2 test was used. Age as a potential confounder was analysed by logistic regression with SPSS/PC+.

Role of the funding source

The design, execution, and reporting of the study were largely the work of university investigators with no undue influence from the original commercial co-sponsor.

Results

229 consecutive patients with invasive pneumococcal disease and 353 controls were initially enrolled in the study. The patients were aged 0–94 years, mean age 59 years (SD 25·2). Eight were younger than 1 year, and 23 were younger than 10 years; 51% were male. 40 patients died in hospital and 46 died during follow up. The median duration of hospital stay for survivors was 10 days. The ages of the controls ranged from 23 to 62 years, and the mean of the 96 for whom age was known was 40 years (SD 8·5). All patients and controls were genotyped for the three *MBL* codon mutations and the single base-pair change at position –221 in the *MBL* promoter region. Allele and genotype frequencies in controls were similar to those previously reported for UK whites,[22] and were in HARDY-WEINBERG EQUILIBRIUM.

Because of the known effects of *MBL* genotypes on serum MBL concentrations, the primary analysis asked whether individuals homozygous or doubly heterozygous for *MBL* codon variants were found at different frequencies in patients and controls. Such individuals, sometimes termed functional mutant homozygotes[19] and

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	Primer pairs and oligonucleotide probes
PCR amplification	
All three codon variants	5′-GCACCCAGATTGTAGGACAGAG-3′
	5′-CAGGCAGTTTCCTCTGGAAGG-3′
Promoter variant	5′-GAAGCTTAGACCTATGGGGCTAG-3′
	5′-AACTGCAGGGAAGGTTAATCGCAGTT-3′
Allele-specific oligonucleotide hybridisation*	
D variant	5′-AAGATGGGCGTGATGG-3′
	5′-AAGATGGGTGTGATGG-3′
B variant	5′-CGTGATGGCACCAAGGA-3′
	5′-CGTGATGACACCAAGGA-3′
C variant	5′-CACCAAGGGACAAAAGGG-3′
	5′-CACCAGGAAGAAAAGGG-3′
Promoter variant	5′-CATTTGTTCTCACTGCCACG-3′
	5′-CATTTGTTCTCACTGCCACC-3′
Ligation-detection reaction*	
D variant	tet-TTCGGCTTCCCAGGCAAAGATGGGC
	fam-AACGGCTTCCCAGGCAAAGATGGGT
	GTGATGGCACCAAGGGAGAAAAGGGGG
B variant	tet-AAAAAAAACCAGGCAAAGATGGGCGTGATGG
	fam-AAAAAAACCCAGGCAAAGATGGGCGTGATGA
	CACCAAGGGAGAAAAGGGGGAACCAGGAATT
C variant	tet-TTATTTAATGGGCGTGATGGCACCAAGGG
	fam-TTATTTGATGGGCGTGATGGCACCAAGGA
	AGAAAAGGGGGAACCAGGCCAAGGGAAAT

tet=tetrachloro-6-carboxyfluorescein. fam=6-carboxyfluorescein. *Probe for more common allele is listed first in each case.

Table 1: **Primers and probes for allele-specific oligonucleotide hybridisation and ligation-detection reaction**

here simply homozygotes, were significantly more frequent among patients than controls (table 2). Individuals heterozygous for one of the codon variants were not more common among patients.

We further analysed the genotype frequencies of the –221 promoter variant, previously shown to cause a modest reduction in MBL concentrations,[10] with lower concentrations associated with the C allele than the G allele (also denoted X and Y variants, respectively). The genotypes at this position did not differ significantly between patients and controls (72/229 [31%] and 130/353 [37%] heterozygotes, and 9/229 [4%] and 14/353 [4%] homozygotes, respectively; p=0·36). Furthermore, we did a subgroup analysis of the frequencies of individuals doubly heterozygous for a codon variant on one chromosome and the promoter position –221 C allele to search for any increased risk. Individuals with this genotype have been found to have somewhat lower concentrations of MBL than other heterozygotes.[10] Again, no significant association was

MBL genotype	Patients (n=229)	Controls (n=353)	Odds ratio (95% CI)	p
Normal				
A/A	128 (55%)	213 (60%)	..	..
Heterozygous				
A/D	27	42	..	..
A/B	42	73	..	..
A/C	4	7	..	..
Total	73 (32%)	122 (35%)	1·00 (0·68–1·46)	0·98
Homozygous				
D/D	1	1	..	..
B/B	13	10	..	..
C/C	2	0	..	..
D/B	10	6	..	..
B/C	1	1	..	..
D/C	1	0	..	..
Total	28 (12%)	18 (5%)	2·59 (1·39–4·83)	0·002

A=normal or wild-type allele, D=codon 52 variant, B=codon 54 variant, C=codon 57 variant. Double (or compound) heterozygotes are grouped with homozygotes because of their similar concentrations of MBL in serum.

Table 2: ***MBL* genotype in patients with invasive pneumococcal disease and in controls**

	Other genotypes (n=201)	Variant homozygotes (n=28)	p
Number male	102 (51%)	14 (50%)	0·9
Median (IQR) age (years)	68 (49–77)	65 (34–73)	0·2
Diagnosis			
Isolated bacteraemia	19 (11%)	4 (16%)	0·5
Pneumonia	125 (71%)	17 (68%)	0·7
Meningitis	23 (13%)	3 (12%)	0·9
Other	8 (5%)	1 (4%)	0·9
Median (IQR) duration of hospital stay (days)	11 (6–21)	14 (6–18)	0·4
Median (IQR) survival (years)	2·18 (0·31–3·49)	2·23 (0·32–3·57)	0·9

Median duration of hospital stay included only those surviving until discharge (excluding 38 patients who died). Information on diagnosis relates to 25 variant homozygous patients and 175 with other genotypes. Meningitis was defined as isolation of *S pneumoniae* from cerebrospinal fluid or isolation of *S pneumoniae* from blood with raised white-cell count in cerebrospinal fluid. Pneumonia was based on clinical findings and bacteraemia as isolation of organism from blood with no apparent source. "Other" includes septic arthritis (n=5), peritonitis (1), ophthalmitis (1), cellulitis (1), and abscess (1).

Table 3: **Comparison of clinical data in patients with invasive pneumococcal disease with and without homozygous *MBL* variant genotypes**

detected (21/229 [9%] of patients and 32/353 [9%] of controls had this genotype, p=0·97).

The serotype of *S pneumoniae* was determined for 223 patients. The serotype distribution was similar to that of previous UK surveys,[23] and the serotypes of 94% of patients were present in the current 23-valent vaccine. The commonest serotype—type 14—was the only serotype over-represented in the homozygote group compared with the other *MBL* genotypes (8/25 [32%] *vs* 26/200 [13%], p=0·013).

In view of the overall association between homozygotes for the *MBL* codon mutations and invasive pneumococcal disease, we assessed the frequency of particular clinical presentations in patients with these genotypes compared with other genotypes (table 3). Information on clinical presentation was available for 200 patients. There were no significant differences in frequencies of the three major clinical presentations—pneumonia, isolated bacteraemia (blood culture yielding *S pneumoniae* without a clinically apparent focus), and meningitis—between these genotypes. Also, the median duration of hospital stay in the patients who did not die in hospital and mean overall probability of survival did not differ significantly between genotype groups (data not shown).

To reassess the main finding of this study—ie, increased susceptibility of *MBL* homozygotes to invasive pneumococcal disease—on a further sample set, 108 new cases and 679 controls matched for area of residence and ethnic origin were recruited and genotyped as previously. Among the patients, there were 35 (32%) *MBL* heterozygotes and 11 (10%) homozygotes, and among the controls there were 234 (34%) heterozygotes (p=0·68) and 36 (5%) homozygotes (p=0·046). Thus, the frequency of these genotypes among patients and controls was similar to that in the initial study. Analysis of the overall dataset to provide the best estimate of the increased risk associated with *MBL* homozygosity indicated an odds ratio of 2·37 (95% CI 1·51–3·73, p=0·0001). Adjustment by regression for age (available for all patients and 75% of controls) made little difference to this estimate (3·48 [1·51–8·01], p=0·003).

Discussion

This study has shown an association between *MBL* genotype and risk of invasive pneumococcal disease in a population of white individuals. The primary analysis was a comparison of genotype frequencies defined by

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mutations in the first exon of the *MBL* gene in patients with invasive pneumococcal disease and controls. Functional mutant homozygotes—ie, individuals who are either homozygous for one *MBL* codon variant or doubly heterozygous for two different codon variants and have little or no serum MBL—were significantly more frequent among patients than controls. Heterozygotes for these codon variants have a lesser reduction in serum MBL concentrations and were not at increased risk of invasive pneumococcal disease. Furthermore, the X/Y promoter polymorphism at position –221, which also influences serum MBL concentrations, was not significantly associated with this disease.

In this study, many of the patients with invasive pneumococcal disease had various non-genetic risk factors for this disorder such as malignancies and cardiac disease. The controls did not share these non-genetic risk factors. There is no need to match patients and controls for such additional risk factors in an assessment of susceptibility genes unless variants of the gene under investigation are associated with those other risk factors. There is no evidence that *MBL* genotype influences any of these heterogeneous risk factors. In particular, we found no evidence that *MBL* genotype changes with age, but because most of the patients studied were relatively old, further studies are required to determine whether the increased risk associated with *MBL* genotype is affected by age. An additional check on the validity of the control group used here was provided by a comparison of the measured genotype frequencies with those of other white UK populations.[22] The frequency of homozygotes measured here, 5%, does not deviate from the expectation of Hardy-Weinberg equilibrium and is very similar to that reported by Mead and colleagues[22] for English whites.

Since the first description of an opsonic defect in immunodeficient children, there has been much interest in the potential role of MBL deficiency in susceptibility to infection.[8] The high prevalence of individuals in the general population homozygous for codon variants rendered the possibility that this gene could be the sole determinant of susceptibility in severe infections unlikely. Several case-control studies have suggested that *MBL* genotype might influence susceptibility to various specific infections, but these results have been equivocal due to one or more factors such as small sample size, marginal statistical significance, genotyping complexity, an unexpectedly low frequency of homozygotes in the control group, ethnic heterogeneity in the study groups, and, often, a lack of Hardy-Weinberg equilibrium in the control genotypes.[11,17,18,20] Other studies of individual infectious diseases, particularly those with a large sample size,[19] have failed to find clear *MBL* associations. However, individuals with both cystic fibrosis and genotypes associated with marked deficiency of MBL have significantly greater impairment of lung function than patients with other *MBL* genotypes, and seem to be more susceptible to *Burkholderia cepacia* infection.[24–26]

The finding that, in patients with pneumococcal invasive disease, serogroup 14 was more frequent among *MBL* homozygotes than among heterozygotes, suggests that these individuals might be more susceptible to particular capsular types of pneumococcus. However, the multiplicity of serotypes studied makes this interpretation preliminary, and potential capsular-type associations require further study. The surface polysaccharide of serotype 14 has a repeating subunit of N-acetyl glucosamine[27]—the sugar that, along with mannose, has the highest affinity for MBL. One in-vitro study of MBL and various bacterial pathogens found higher levels of binding of radiolabelled lectin to *S pneumoniae* than to *Neisseria meningitidis* of non-A serogroups or to *Haemophilus influenzae* type b,[28] but another found very low levels of binding of *S pneumoniae*.[29]

The relatively high prevalence of homozygotes for *MBL* mutations in most world populations underscores the likely influence of this gene on overall pneumococcal morbidity and mortality. The attributable risk of the *MBL* codon variant homozygotes is 6·7% in the population studied—a high value for a recessive susceptibility genotype, and similar to the effects of most common susceptibility variants for other infectious diseases such as malaria, HIV/AIDS, and tuberculosis.[30] The high prevalence of *MBL* mutations in most parts of the world is intriguing and unexplained. Some have suggested that individuals with these alleles might have been selected through protection against mycobacterial or some autoimmune diseases, but supportive data are lacking.[3,19,21,31]

The mechanism by which MBL deficiency is associated with susceptibility to invasive pneumococcal disease requires investigation. MBL was originally thought to be most important in innate immunity during early childhood before an infant is able to mount an effective humoral response to polysaccharides. Subsequently, MBL deficiency was described in some adults with severe infections.[32] The presentation and outcome of invasive pneumococcal disease depends on the population studied, and, although a small number of infants were included in this study, the increased risk associated with MBL deficiency was evident in adults. We did not investigate an influence of *MBL* genotype on pneumococcal carriage rates, and therefore cannot distinguish between *MBL* genotype effects on carriage, invasion, and susceptibility to disease.

Individuals with MBL deficiency genotypes showed a non-significant trend towards higher rates of isolated bacteraemia rather than pneumonia (table 3). MBL can activate complement using an evolutionarily ancient pathway involving MBL-associated serum proteases[6] that might lead to direct bacterial damage and opsonisation via complement receptors. Additionally, MBL might opsonise bound bacteria using the C1q receptor on macrophages without the involvement of complement.[33] One speculative interpretation of our results is that the mutations in the structural gene that lead to a disruption of the formation of higher order oligomers and thus low concentrations of MBL, might result in reduced opsonisation, with or without the involvement of complement, and favour the survival of *S pneumoniae* early in the course of invasion.

The increased susceptibility of *MBL* homozygotes to invasive pneumococcal disease could have implications for clinical practice. Replacement MBL therapy has been used in Scandinavian patients with MBL deficiency and repeated infections, and a possible beneficial effect was seen.[34] The usefulness of this intervention in severe cases of pneumococcal bacteraemia might merit assessment. A potentially more cost-effective intervention is the use of pneumococcal vaccination. Most of our patients were infected with pneumococcal serotypes included in the licensed 23-valent vaccine, and newer conjugate vaccines will probably be more effective in high-risk groups. The efficacy of pneumococcal vaccines in individuals with MBL deficiency is unknown and requires assessment. If vaccination is equally effective in individuals of all *MBL* genotypes, homozygotes should derive greater benefit from vaccination. Finally, the usefulness of general screening of individuals for MBL deficiency

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genotypes has been debated.[19,35] Our finding of an association with susceptibility to a major infectious pathogen provides support for this measure. *MBL* will probably be included in future genotype profiling to assess susceptibility to common diseases, and the result could influence both prophylaxis and management of pneumococcal disease.

Contributors

S Roy did genotyping, collected samples, analysed data, and prepared the paper. K Knox designed the study, analysed data, and prepared the paper. S Segal did genotyping and analysed data. D Griffiths serotyped *S pneumoniae*. C E Moore did genotyping and collected data. K I Welsh, A Smarason, and N P Day collected samples. W L McPheat designed the study. D W Crook and A V S Hill designed and supervised all aspects of the study.

Conflict of interest statement

None declared.

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