CONCISE COMMUNICATION

Mannose-Binding Lectin Gene Polymorphisms as a Susceptibility Factor for Chronic Necrotizing Pulmonary Aspergillosis

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It was investigated whether a deficiency of mannose-binding lectin (MBL), which binds *Aspergillus* species avidly in vitro, could account for chronic necrotizing pulmonary aspergillosis (CNPA), which is seen most commonly in nonimmunocompromised patients. Blood samples were obtained from 11 patients (10 white) with CNPA and were compared with blood samples from 82 white control subjects. MBL haplotype profiles were determined by polymerase chain reaction, using sequence-specific primers and sequence-specific oligonucleotide probing techniques. Seven of the 10 white patients with CNPA had MBL haplotypes that encode for low levels of the protein, compared with 25.6% of the white control subjects ($P = .004$). Presence of the codon 52 mutation was particularly common in patients with CNPA ($P = .015$), which suggests a greater involvement of this mutation.

Chronic necrotizing pulmonary aspergillosis (CNPA) is an uncommon manifestation of aspergillosis. It runs a chronic course (months to years), with increasing damage to the architecture of the lung, and causes substantial morbidity if not treated. Patients are not immunocompromised [1, 2]. The syndrome usually affects middle-aged individuals and has a predilection for men. Chronic productive cough is the usual presenting symptom, often with mild or moderate hemoptysis. Malaise and weight loss are common features. Aspergilloma or fungus ball (a chronic noninvasive lesion) can have presenting symptoms similar to those of CNPA, which makes it difficult to distinguish between the two, especially since many patients have both CNPA and aspergillomas. Indeed, it may be that many patients thought to have complex aspergillomas do in fact have CNPA.

The genus *Aspergillus* comprises several pathogenic species, all of which are large and are killed extracellularly by activated phagocytes. Mannose-binding lectin (MBL) is a major opsonizing protein of the innate immune system [3] and avidly binds to *Aspergillus fumigatus* [4]. Once bound to *A. fumigatus,* MBL causes activation of the complement system via its associations with MBL-associated serum proteases 1 [5] and 2 [6]. However, the role of complement in the killing of *Aspergillus* species is thought to be small.

A mutation in the structurally encoding region of the MBL gene that leads to dramatically reduced serum levels of the functional protein could result in impaired immune clearance of *Aspergillus* species. Five functional single-nucleotide polymorphisms exist within the MBL gene, each affecting serum levels of the protein. Two polymorphisms located within the promoter region of the gene (H or L [G→C] at −550 and Y or X [G→C] at −221) affect transcriptional activity of the basal-promoter complex and reduce levels of circulating MBL [7]. The remaining 3 functional polymorphisms at codons 52 (W/M52 [i.e., wild-type/mutant allele at codon 52], type D), 54 (W/M54, type B), and 57 (W/M57, type C) of the MBL gene result in single amino acid substitutions that dramatically reduce functional levels by causing structural defects in the MBL protein [8, 9].

These structurally encoding mutations have dominant genetic consequences. The presence of one mutant allele (heterozygote) decreases functional MBL to almost the same extent as that in homozygous individuals. A rat model of MBL variant strains recently demonstrated that all mutations affecting the architecture of the MBL molecule significantly affect MBL activity, which implicates a major contribution to an immunodeficient phenotype in these individuals [10]. Low serum levels of MBL are associated with increased rates of bacterial infections in children and adults and with specific infections, such as hepatitis B virus and human immunodeficiency virus [11, 12]. We investigated whether genetic defects in MBL could explain why CNPA develops in otherwise nonimmunocompromised patients.

Patients and Methods

Patients and control subjects. To be enrolled in the study, patients had to have a diagnosis of CNPA, as determined according to established criteria [1], and other pathogens that could account for the symptoms (e.g., mycobacteria) had to be excluded. Eleven patients (10 white) with chronic (>1 month) pulmonary and systemic symptoms were enrolled. No patients had discernible immunocompromising factors, and all had radiologic evidence of a progressive pulmonary cavitating lesion with surrounding inflammation, with or without an intracavitary mass. In addition, all patients had precipitating (IgG) antibody to *Aspergillus* species in serum, persistently elevated inflammatory markers, and a respiratory sample with a culture positive for *A. fumigatus*. Three patients (patients 3, 6, and 10) had had a lung resection, and hyphae had been visualized in cavities with surrounding chronic inflammation. Four additional patients with other forms of aspergillosis also were studied. For comparison, 82 previously described [13] white individuals (control subjects) were recruited from the General Practice registers in the United Kingdom.

MBL typing. DNA was extracted from EDTA-blood samples by use of the DNAceT MaxiBlood Purification System (Bioline). Wild and mutant alleles were detected at MBL codons 52, 54, and 57 by use of polymerase chain reaction (PCR), using sequence-specific primers (SSP), as described elsewhere [14]: 1042 bp of the promoter and most of exon 1 of the MBL gene were amplified. An additional mismatch introduced at the second base at the 3' end of the primer significantly increased the specificity of SSP-based PCRs (SSP-PCRs).

A 464-bp segment of the gene for human growth hormone was used as an internal control. Positive and negative controls were included in each run. All SSP-PCRs were done in the presence of 1.5 mM MgCl$_2$, 0.2 mM each dNTP, and 0.5 mM each primer in NH$_4$SO$_4$ buffer (Bioline) with 1 U polymerase (BioTaq) and 1 M betaine. PCR products were electrophoresed in 2% (wt/vol) agarose gels and were stained with ethidium bromide.

Two milliliters of each PCR product was blotted by use of an automatic dot blotter (Robbins), was alkaline denatured, and was fixed onto Hybond N+ nylon membranes (Amersham). Biotinylated sequence-specific oligonucleotide probes (described by Madsen, et al. [7]) were incubated with the PCR product for 2 h at 42°C in hybridization buffer (5× standard saline citrate [SSC; 0.15 M NaCl and 0.015 M sodium citrate], 0.1% *N*-lauroyl sarcosine, 0.5% blocking reagent [Amersham], and 0.02% SDS). Hybridization was followed by stringent washes in 1× SSC/0.01% SDS at the following temperatures; L probe at 48.5°C, H probe at 50.5°C, and X/Y probes at 60°C. Enhanced chemiluminescence detection was performed, followed by autoradiography.

Using these methods alone, we could not determine the haplotype for a number of subjects because their samples had only structurally encoding wild-type alleles (as determined by SSP-PCR) and were positive for X, Y, L, and H polymorphisms. In such cases, *cis/trans* orientation of the promoter alleles was resolved by performing further SSP-PCRs with H/L primers (priming forward 5'→3') and X/Y reverse primers. The primer sequences and reaction conditions used were as described elsewhere [14]. A number of samples were cloned and sequenced (MBL gene exon 1 and promoter region).

Statistical analysis. To determine the significance of a frequency difference between the 2 groups (significance level of 5%), χ^2 analysis (using Yates's correction) was done. Odds ratios were calculated for significant associations and were expressed with 95% confidence intervals.

Results

MBL genotyping and clinical data for the 11 patients with and 4 patients without CNPA are shown in table 1. Eight (73%) of the 11 patients with CNPA had MBL-deficient genotypes, and each of the patients without CNPA had genotypes encoding for wild-type levels of and normal structure of MBL.

The genotype encoding for heterozygosity of the MBL codon 52 mutant allele was genotypically more frequent in white patients with CNPA ($n = 10$) than in control subjects ($n = 82$; $P = .015$; table 2). The MBL codon 52 mutant allelic frequencies also were increased significantly in the CNPA group ($P = .022$). No statistically significant difference was observed in the frequency of the codon 54 mutant phenotype between patients with CNPA and control subjects (20% and 16%, respectively). However, genotypes encoding for heterozygosity of any mutant MBL allele were genotypically more frequent in patients with CNPA than in control subjects (odds ratio, 6.8; 95% confidence interval, 1.7–26.3; $P = .004$). Genotype, haplotype, and allele frequencies for codon 54 and 57 mutations of the CNPA patients were all statistically similar to those for control subjects. A statistically significant difference in haplotype frequency was observed between these 2 populations for haplotype HY D, which encodes a codon 52 mutation as the only variant allele ($P = .022$).

Discussion

To our knowledge, this study is the first report of a noncellular, immunogenetic defect in aspergillosis in humans. Of our 10 white patients with CNPA, 7 have a mutation of the MBL gene that would disrupt the structure of MBL, drastically reducing the amount of active protein available for clearance of pathogens. Only 25.6% of the ethnically matched control subjects had similar mutations, which is consistent with published control frequencies in other white populations. This suggests that MBL deficiency is a genetic factor that predisposes individuals to develop CNPA. Other patients with aspergillosis did not have MBL deficiency, which suggests that CNPA is a distinct clinical group; however, a larger sample of cases of aspergillosis other than CNPA would be important to confirm that this is the case.

Patient 11 is also MBL deficient but is of Gujerati origin (Gujarat is a state in western India) and therefore was excluded from statistical comparisons between patients with CNPA and the control group, to ensure that the groups were ethnically matched. MBL mutant alleles are distributed differently among disparate

Table 1. Mannose-binding protein (MBL) genotypes and underlying diseases in patients with various manifestations of aspergillosis.

Patient	Clinical symptoms	Previous disease(s)	MBL genotype[a]	Effect on MBL
1	CNPA	TB	HY A/HY D	Low serum levels
2	CNPA	Alcoholism, emphysema	HY D/LY A	Low serum levels
3	CNPA	Scoliosis, bronchiectasis	HY A/LX A	Wild-type levels
4	CNPA	Emphysema, atypical TB	LY A/LY B	Low serum levels
5	CNPA	Alcoholism, atypical TB	HY D/LY A	Low serum levels
6	CNPA	None	LY A/LY B	Low serum levels
7	CNPA	Emphysema, TB	HY A/LY A	Wild-type levels
8	CNPA	Lobectomy for *Legionella* pneumonia, alcoholism	LY A/LY A	Wild type levels
9	CNPA	Childhood pneumonia, pneumothorax, marfanoid	HY D/LY A	Low serum levels
10	CNPA	Ankylosing spondylitis, atypical TB	HY D/LY A	Low serum levels
11	CNPA	TB, smoke inhalation	LY B/LY B	Extremely low levels
12	Chronic *Aspergillus* sinusitis	MELAS	LY A/LY A	Wild-type levels
13	Chronic *Aspergillus* sinusitis	Chronic sinusitis	HY A/LX A	Wild-type levels
14	Aspergilloma	TB	LY A/LY A	Wild-type levels
15	Acute invasive aspergillosis	Lung fibrosis, steroids	LY A/LY A	Wild-type levels

NOTE.   Patient 11 is of Gujerati origin (Gujarat is a state in western India) and was not included in statistical comparison with the control subjects, who were white. CNPA, chronic necrotizing pulmonary aspergillosis; MELAS, syndrome of mitochondrial encephalopathy, lactic acidosis, and strokelike episodes; TB, tuberculosis.

[a] MBL genotypes: A, W52 W54 W57; B, W52 M54 W57; C, W52 W54 M57; and D, M52 W54 W57.

ethnic groups and within ethnic groups of different geographical origin. Epidemiologic studies have shown that the codon 52 mutant allele has a constant allele frequency of ~5% in almost all populations studied [7]. Structural MBL mutations occur frequently in all ethnic groups. Codon 54 mutant alleles occur most frequently in white individuals (11%–17%), whereas ≤27% of individuals of African origin carry the codon 57 mutant MBL gene. Patient 11 is a homozygous mutant individual for the codon 54 mutation. This finding further strengthens the argument that MBL mutations, which result in a reduction of serum levels of the protein, are associated with CNPA susceptibility.

If CNPA were caused by MBL deficiency alone, it would be expected that most MBL mutations defined in the CNPA population would be at codon 54. However, on further analysis of the MBL mutations present in patients with CNPA, the frequency of the codon 54 mutant allele was not significantly increased, compared with healthy control subjects. The codon 52 mutant allele, all of which were found in the haplotype HY D, was present in 50% of the patients with CNPA and in 17% of the control subjects, which suggests a greater involvement for this particular mutation in susceptibility to CNPA. It is possible that possession of a codon 52 mutation might be a causative factor for the susceptibility to develop CNPA, possibly because of defective immune clearance of *Aspergillus* species. Alternatively, it is possible that this may be a founder haplotype, carrying another disease susceptibility gene further along chromosome 10. Potential nearby susceptibility loci include the structurally and functionally related lung surfactant proteins A and D, an hypothesis

that our current clinical data and others' experimental data [15] support. Most patients have significant pulmonary damage before the development of CNPA [1, 2], which is consistent with a multifactorial susceptibility to CNPA. This multifactorial element could explain why relatively few individuals with these mutant alleles develop CNPA.

Table 2. Mannose-binding lectin (MBL) genotypes and alleles of the codon 52 polymorphism and MBL polymorphism haplotypes in 10 white patients with chronic necrotizing pulmonary aspergillosis (CNPA) and in control subjects.

Polymorphism	Patients with CNPA (n = 10)	Control subjects (n = 82)	OR (95% CI)	P
Codon 52				
Genotype				
WW	5 (50.0)	68 (82.9)		
WM	5 (50.0)	14 (17.1)	4.9 (1.3–18.0)	.015
MM	0 (0.0)	0 (0.0)		
Allele				
W	15 (75.0)	150 (91.5)		
M	5 (25.0)	14 (8.5)	3.6 (1.2–10.9)	.022
MBL haplotype[a]				
HY D	5 (25.0)	14 (8.5)	3.6 (1.2–10.9)	.022
LY B	2 (10.0)	20 (12.2)		
LX A	1 (5.0)	30 (18.3)		
LY A	9 (45.0)	61 (37.2)		
HY A	3 (15.0)	39 (23.8)		

NOTE.   Data are no. (%) of MBL genotypes, alleles, or haplotypes. CI, confidence interval; OR, odds ratio.

[a] MBL haplotypes: A, W52 W54 W57; B, W52 M54 W57; C, W52 W54 M57; and D, M52 W54 W57.

Although the promoter polymorphisms −550 H/L and −221 X/Y affect serum levels of MBL, they are not thought to reduce levels to the same extent as structurally encoding mutations. H→L at −550 causes a less-substantial reduction in MBL concentration than does Y→X at −221 [7]. Although the −550 L promoter variant was found in almost all the patients we studied, it has little effect on circulating MBL levels [7]. However, even the lowest-producing promoter haplotype, LX, does not have nearly such a substantial effect as any of the structurally encoding mutations, unless present in homozygous form. Within our cohort of patients with CNPA, none had samples that were homozygous for the LX-promoter haplotype.

Acknowledgments

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