

Mannose-binding lectin deficiency associated with neutrophil chemotactic unresponsiveness to C5a

Rosa M. Ten, MD, PhD,^a Eva M. Carmona, MD,^a Dusica Babovic-Vuksanovic, MD,^c and Jerry A. Katzmann, PhD^b Rochester, Minn

Background: Mannose-binding lectin (MBL) plays an important role in host defense by activating the complement cascade.

Objective: Three children with a history of recurrent infections since infancy were found to have MBL deficiency associated with a neutrophil chemotactic unresponsiveness specific to C5a. We have studied the genomic sequence of the C5a receptor (C5aR) in 2 of the subjects to determine whether this unresponsiveness was due to a genetic mutation or to aberrant complement activation associated with the MBL deficiency.

Methods: MBL genotype analysis was performed by PCR-based methods with use of specific primers and restriction enzymes to detect the 3 previously reported mutations. Expression of C5aR was analyzed by flow cytometry. The C5aR gene was amplified from the patient's genomic DNA by PCR and sequenced by standard procedures.

Results: C5aR was found to be expressed normally on the neutrophils of one of the subjects. Sequence analysis of the C5aR gene revealed a point mutation that substituted threonine at position 261 for alanine in one patient but no abnormality in the other, suggesting gene polymorphism. Treatment of 2 patients with granulocyte-colony stimulating factor corrected the neutrophil chemotactic abnormality in vitro and induced a significant clinical improvement.

Conclusion: MBL deficiency can be associated with neutrophil chemotactic unresponsiveness to C5a and it is clinically manifested by recurrent and chronic infections. Treatment of these patients with granulocyte-colony stimulating factor results in normalization of neutrophil chemotaxis against C5a and significant clearing of infections. (*J Allergy Clin Immunol* 1999;104:419-24.)

Key words: Mannose-binding lectin deficiency, C5a, neutrophil chemotaxis, G-CSF

Mannose-binding lectin (MBL) is an acute phase protein, secreted by hepatocytes and involved in host innate immunity. It is a multimeric molecule composed of several identical subunits that associate by collagenous helices in the C-terminus and form globular heads in the N-terminus. MBL binds surface mannose-rich pathogens through its globular heads (lectin domain) as well as collectin receptors in macrophages through its collagenous

Abbreviations used

C5aR: C5a receptor

CT: Computerized tomogram

G-CSF: Granulocyte colony-stimulating factor

MASP: Mannose-binding lectin-associated proteases

MBL: Mannose-binding lectin

tail, acting as an opsonin to induce phagocytosis.¹ In addition, MBL is able to activate the classical pathway of complement by means of its high structural homology with C1q, which allows binding to C1r and C1s and subsequent activation of C4 and C2. MBL can also activate C2 and C4 by the action of the MBL-associated proteases MASP1 and MASP2.^{2,3} In addition, MASP1 can cleave C3 directly, inducing the formation of the membrane attack complex.⁴ MBL deficiency has been associated with an increased incidence of infections, mainly during childhood,^{5,6} as well as with some autoimmune disorders⁷ and recurrent miscarriage.⁸ MBL deficiency is caused by mutations in the MBL gene that alter the structure of the protein, leading to the production of an unstable molecule and a consequent low level in the serum. Three point mutations have been described clustered within exon 1 of the MBL gene (codons 52, 54, and 57), which are responsible for MBL deficiency.¹ The prevalence of variant alleles is high in the general population.⁹ Screening of a healthy population in the Midwest of the United States reported 35.8% individuals with at least one mutant allele, 5.4% being homozygous.¹⁰

The complement system also forms an important part of the host's innate immunity. It is composed of multiple components that are sequentially activated by enzymatic cleavage, leading to the formation of the membrane attack complex, which is responsible for cellular lysis. Several breakdown products of complement components are released during complement activation, which can act as opsonins, chemotactic factors, and anaphylotoxins, playing an important role in inflammation.¹¹ Three independent cascades, the classical, the alternative, and the lectin pathways, can activate the complement system. Binding of C1q to the Fc portion of IgG or IgM triggers the classical pathway. Initiation of the alternative complement pathway can be triggered by bacterial products or it occurs after spontaneous hydrolysis of the C3 component, followed by regulation by factors H and I.¹¹ Complement activation by the lectin pathway is C1q- and

From the Divisions of Allergy and Immunology^a and Clinical Biochemistry and Immunology,^b Mayo Clinic, and the Department of Medical Genetics, Mayo Clinic and Mayo Foundation,^c Rochester, Minn.

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Reprint requests: R. M. Ten, MD, PhD, Mayo Clinic, 200 2nd St SW, Rochester, MN 55905.

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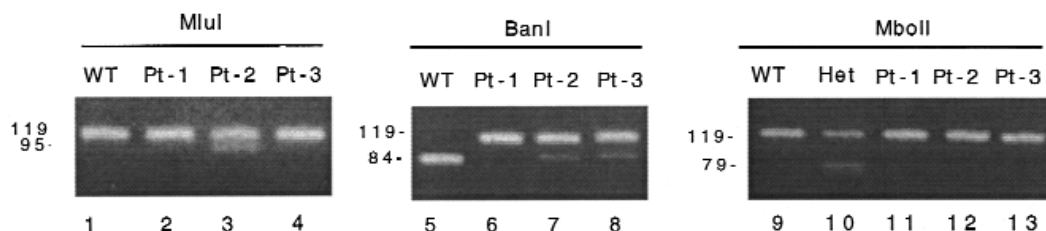


FIG 1. Analysis of MBL gene mutations by PCR. Amplified DNA was digested with *Mlu* I (lanes 1-4), *Ban* I (lanes 5-8), or *Mbo* II (lanes 9-13). WT, Wild type; Het, heterozygote; Pt, patient. Numbers at left of each gel indicate fragment size (in base pairs). Presence of mutation in codon 52 introduces *Mlu* I cut site and produces 2 fragments (95 bp and 24 bp, lane 3), as opposed to single 119-bp fragment in wild-type allele (lanes 1, 2, and 4). Presence of mutation in codon 54 eliminates restriction site for *Ban* I present in wild-type DNA and therefore PCR product runs as single 119-bp band (lanes 6-8) as opposed to 2 bands (84 and 35 bp in wild type, lane 5). Presence of mutation in codon 57 introduces *Mbo* II restriction site and therefore PCR product is seen as 2 bands (79 and 40 bp, lane 10) as opposed to single 119-bp fragment in wild-type DNA (lanes 9, 11, 12, and 13).

antibody-independent and has only been reported with MBL, as described above. During complement activation C5a, a potent anaphylotoxin and chemotactic factor, is released. At low concentrations C5a stimulates chemotaxis of granulocytes, which migrate toward the site of inflammation. At higher concentrations C5a stimulates granulocyte cytotoxic responses. The receptor for C5a (C5aR), present in the granulocyte cytoplasmic membrane, has been shown to induce chemotaxis, granule enzyme release, and superoxide anion production¹²⁻¹⁴; up-regulate the expression and activity of the adhesion molecules MAC-1 and CR1; and decrease cell surface glycoprotein 100mel-14.¹⁵⁻¹⁷ C5aR is a single 350-amino acid polypeptide that belongs to the guanosine triphosphate-binding rhodopsin superfamily of proteins.¹⁸ It initiates signal transduction through G proteins to activate phospholipase C.¹⁹ The protein has 7 hydrophobic regions that span the cell membrane. The N-terminus and the region between the third and fourth transmembrane domains, which loops out on the extracellular site, may be part of the C5a binding site.²⁰ In vitro mutagenesis studies have also implicated the third and fourth loops of the C5aR as possible C5a binding sites.²¹ Granulocyte activation by chemotactic factors is transient and is terminated by a mechanism of cellular desensitization to the chemotactic factors. Three types of desensitization have been described for neutrophils containing G protein-coupled receptors: homologous, heterologous, and receptor class desensitization.²² No congenital deficiency of C5aR has been described to date in humans.

METHODS

Serum MBL was measured with an ELISA method by Specialty Laboratories (Santa Monica, Calif).

MBL genotype analysis

Genomic DNA was extracted from peripheral blood leukocytes with use of the Puregene extraction procedure (Gentra Systems). A PCR was used to amplify a 119-bp region of exon 1 containing codons 52, 54, and 57. The upstream primer was a mutated oligonucleotide (5'-CATCAACGGCTTCCCAGGCAAAGACGCG-3'), as described by Madsen et al,²³ which introduced a new restriction

enzyme cut site in the presence of the codon 52 mutation. The downstream primer was the same as that described by Lipscombe et al⁹ (5'-CAGGCAGTTTCCTCTGGAAGG-3'). PCR was performed in a reaction volume of 75 μ L containing 0.4 ng of genomic DNA, 5 pmol of the specific primers, and standard reaction buffer with 200 mmol/L deoxyribonucleoside triphosphate, 2 mmol/L magnesium chloride, and 1.25 U of Taq Gold polymerase (Perkin Elmer). With use of a Perkin Elmer 9600 thermocycler, PCR reactions were 95°C for 10 minutes; 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds; and 72°C for 10 minutes. PCR products were digested with the restriction enzymes *Ban* I, *Mbo* II, and *Mlu* I (New England Biolabs), followed by separation in a 4% agarose gel and ethidium bromide staining (Fig 1).

Neutrophil chemotaxis

Neutrophil chemotaxis was assessed by migration under agarose.²⁴ In this assay the purified neutrophils are placed into a well cut into agarose. The neutrophils adhere to the plastic at the bottom of the well and migrate out of the well. Chemoattractant is placed in an adjacent well to set up a concentration gradient in the agarose. The migration out of the well is measured in response to buffer (random migration), an *Escherichia coli* culture supernatant that contains N-formyl-L-methionyl-L-leucyl-L-phenylalanine capped proteins, as well as C5a that is generated by activating serum with opsonized zymosan.

C5aR expression

C5aR expression was measured by flow cytometry and the anti-C5aR antibody CD88 labeled with FITC (Serotec, Oxford, UK). The cells were stained by mixing 100 μ L of whole blood with 10 μ L of antibody, incubating 15 minutes, lysing the red blood cells, and analyzing a normal and patient sample on a Becton Dickinson FACScan (San Jose, Calif).

C5aR gene analysis

Genomic DNA, extracted from peripheral blood leukocytes as above, was used for amplification of the C5aR gene by PCR. The primers used were 5'-CTCCTTCAATTATACCACC-3' (sense) and 5'-ATGACGTGTCGCTACACT-3' (antisense). The PCR was performed in a reaction volume of 75 μ L containing 0.8 ng of genomic DNA, 50 pmol of the specific primers, and standard reaction buffer with 10 mmol/L deoxyribonucleoside triphosphate and 2.5 U/ μ L Taq polymerase (Boehringer). With a Perkin Elmer 2400 Thermocycler, PCR reactions were 95°C hold for 5 minutes; 35 cycles of 95°C for 30 seconds; 60°C for 30 seconds and 72°C for 30 seconds; and 72°C hold for 7 minutes and 4°C indefinitely. PCR

products were cloned in the pGEM-T easy vector (Promega) and sequenced by standard procedures in both orientations with use of an automated Perkin Elmer ABI PRISM™ 377 DNA Sequencer (PE Applied Biosystems, Foster City, Calif). The obtained sequence was compared with the previously published C5aR gene sequence¹⁸ with use of the MacVector computer program.

CASE REPORTS

Case 1

An 8-year-old white girl with a history of chronic pansinusitis since infancy and no other significant history of infections was referred for immunologic evaluation. Her symptoms included chronic mucopurulent nasal drainage and facial pressure. She had undergone 3 sinus surgeries and had been treated with multiple antibiotics without improvement of the symptoms. Physical examination was normal except for an inflamed nasal mucosa with mucopurulent drainage. A computerized tomogram (CT) of the sinuses revealed complete opacification of most of the paranasal sinuses (Fig 2, A).

Immunologic evaluation demonstrated a leukocyte count of $6.5 \times 10^9/L$ with a normal lymphocyte subset distribution, as determined by lymphocyte surface markers (CD3, CD4, CD8, CD19, CD16). Total complement hemolytic activity was 54 U/ml (normal 30-75 U/ml). Serum immunoglobulins (normal range) were as follows: IgA 32.3 mg/dL (33-258 mg/dL), IgM 122 mg/dL (56-242 mg/dL), total IgG 556 mg/dL (598-1379 mg/dL), IgG1 358 mg/dL (390-927 mg/dL), IgG2 39 mg/dL (111-507 mg/dL), IgG3 28 mg/dL (18-122 mg/dL), IgG4 4 mg/dL (1-133 mg/dL). Serologic studies to *Pneumococcus* and tetanus demonstrated protective antibody titers. Serum MBL was 1.4 ng/mL (normal >5 ng/mL) and MBL genotype analysis revealed a homozygous codon 54 mutation (Fig 1, lane 6). A neutrophil chemoluminescence assay was normal, and a neutrophil chemotaxis study revealed normal random migration, normal directed migration to *E coli* chemoattractant, and significantly diminished migration to C5a, which was verified in 2 different assays. Expression of the C5aR was 70% of a normal control (Fig 3), which is considered within the normal variation. Nucleotide sequencing of the C5aR gene demonstrated a G to A mutation at position 781, resulting in exchange of threonine in position 261 by alanine. Treatment with G-CSF was initiated at 5 $\mu\text{g/kg/d}$ subcutaneously for 1 week, on alternating weeks. The neutrophil chemotactic assay with both *E coli* chemoattractant and C5a repeated after 3 weeks of initiating the G-CSF therapy was found to be normal. In a follow-up visit 3 months later the patient reported complete resolution of her symptoms and her physical examination was normal. A CT scan of the sinuses 6 months after the initiation of G-CSF showed significant resolution of the pansinusitis (Fig 2, B).

Case 2

A 17-month-old white boy with a history of recurrent infections was referred for immunologic evaluation. He had recurrent otitis media that did not improve after myringotomy tubes and prophylactic antibiotics; he had had 3 episodes of pneumonia and multiple episodes of gastroenteritis. In addition, he had a history of asthma with frequent exacerbations requiring systemic steroid bursts and constant prophylactic trimethoprim-sulfamethoxazole, prednisone, and nebulization therapy with cromolyn sodium and albuterol. Physical examination was normal. The immunologic evaluation, performed as described above, was significant for normal CH50, IgA 16.3 mg/dL (normal 22-178 mg/dL), serum MBL 0.1 ng/mL, and selective neutrophil chemotactic unresponsiveness to C5a. MBL genotype analysis revealed a combined 52/54 heterozygous status (Fig 1, lanes 3 and 7). C5aR gene sequencing did not demonstrate any mutations compared with the previously reported



FIG 2. CT of sinuses of first patient reported, before (A) and 6 months after initiation of granulocyte colony-stimulating factor (G-CSF) therapy (B).

sequence.¹⁸ The patient was started on G-CSF treatment at 5 $\mu\text{g/kg/d}$ subcutaneously, 4 days per week, on alternating weeks. The selective neutrophil C5a chemotactic unresponsiveness was found to correct to normal values 3 weeks after initiation of G-CSF therapy. At a 3-month follow-up visit the patient reported significant clinical improvement and all his prophylactic antibiotic and asthma therapy was discontinued. Six months after the initiation of G-CSF he continued to do well and G-CSF was discontinued.

Case 3

A 2-year-old white boy with a history of recurrent sinopulmonary infections was referred for immunologic evaluation. He had undergone sinus surgery for drainage of chronic sinusitis and had a history of recurrent pneumonia. His physical examination was significant for mucopurulent nasal drainage and cervical lymphadenopathy. Laboratory evaluation demonstrated normal blood cell count, lymphocyte surface markers, CH50, serum Ig, IgG subclasses, and sweat test. Neutrophil functional assays revealed nor-

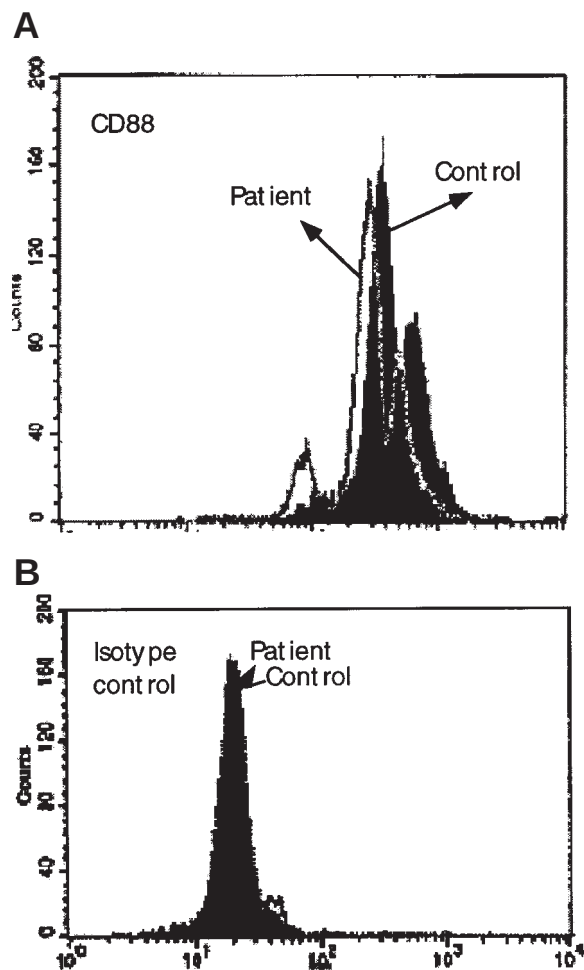


FIG 3. Flow cytometric analysis of expression of C5aR on neutrophils of first case reported (open histogram) compared with normal control (closed histogram). **A**, Cells stained with CD88 antibody. **B**, Cells stained with isotype-matched control.

mal chemoluminescence, random migration, and chemotaxis toward *E coli* chemoattractant but absent migration to C5a. The serum MBL concentration was 6 ng/mL. MBL genotype analysis demonstrated heterozygous 54 mutation (Fig 1, lane 8). A chest roentgenogram showed bilateral basilar infiltrates and a CT scan of the sinuses demonstrated opacification of the maxillary sinuses.

RESULTS

We present here 3 cases of children with life-long histories of recurrent infections. All 3 subjects had mutations in the MBL gene (Fig 1), 1 being homozygous (case 1), 1 combined heterozygous (case 2), and 1 heterozygous (case 3). The MBL genotype correlated with very low (cases 1 and 2) or borderline (case 3) serum MBL levels.

All 3 subjects had a selective neutrophil chemotactic unresponsiveness selective for C5a. In 1 subject (case 1) C5aR expression was studied and was found to be similar to that of a healthy control subject (Fig 3). In 2 subjects (cases 1 and 2) the gene encoding C5aR was entire-

ly sequenced, and no significant mutations that potentially could affect the function of this receptor were found. Two of the subjects (cases 1 and 2) received treatment with G-CSF and a beneficial clinical response was found (Fig 2) that correlated with normalization of neutrophil chemotactic responsiveness to C5a *in vitro*.

DISCUSSION

We report here 3 cases of MBL deficiency associated with selective neutrophil chemotactic unresponsiveness to C5a. The receptor for C5a was found to be expressed normally in one of the patients. Therefore we studied the complete genomic sequence of the C5aR from 2 patients to determine whether mutations were present that could account for a functional abnormality of this receptor. Deficiency or functional abnormality of the C5aR has never been reported in humans. However, C5aR-deficient mice exhibit decreased neutrophil chemotaxis and abnormal cytokine production.²⁵ The anti-CD88 antibody used to detect expression of C5aR does not distinguish between functional or nonfunctional receptors or neutrophils that have or have not been desensitized.

The gene encoding MBL is located in chromosome 10, whereas the C5aR gene is mapped to chromosome 19. Therefore the association of mutations in such distant genes is unlikely. We have found the presence of a mutation in the C5aR gene from one of our subjects, whereas the other subject studied was normal. That mutation was responsible for an amino acid exchange within the sixth transmembrane region of the receptor, which is not thought to be involved in ligand binding nor receptor signal transduction. Therefore we believe that this mutation reflects gene polymorphism and that it is not clinically relevant.

A possible explanation to link MBL deficiency and selective neutrophil C5a unresponsiveness is that patients with MBL deficiency have recurrent infections, in part because of their inability to activate complement through the lectin pathway. In contrast, the continuous presence of bacterial products derived from the chronic infections could activate the alternative pathway of complement in a persistent manner, resulting in increased steady-state levels of C5a. Chronic exposure to excessive amounts of C5a would then cause granulocyte desensitization, manifested by a chemotactic defect specific against C5a (Fig 4). However, levels of C5a were not measured in these patients and therefore further studies need to be done to prove this hypothesis. In addition, neutrophil chemotactic unresponsiveness could be the result of coreceptor internalization, resulting from stimulation of leukocytes by other activating factors derived from chronic infections.

G-CSF is a cytokine currently used in the treatment of several hematologic disorders. It is a growth factor for the myeloid lineage that induces the production and mobilization of PMNs from the bone marrow. In addition, G-CSF acts as a modulator of neutrophil behavior, enhancing phagocytosis, superoxide anion generation, chemiluminescence, bacterial killing, and antibody-

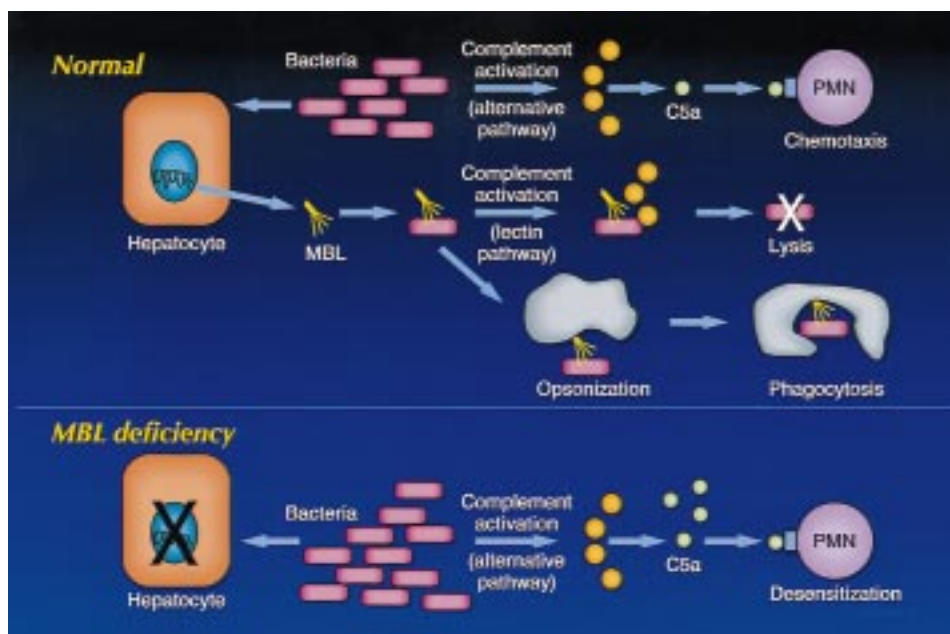


FIG 4. Diagram of proposed hypothesis linking MBL deficiency and neutrophil chemotactic unresponsiveness to C5a. *Upper panel*, Normal state; *lower panel*, MBL deficiency state.

dependent cell-mediated cytotoxicity.²⁶ G-CSF also induces expression of CD14, CD32, and CD64 and decreases expression of CD16 on PMNs. However, neutrophil chemotaxis is reduced after G-CSF treatment.²⁶

Neutrophils from the cases presented here had severely impaired chemotaxis in response to C5a, which normalized after treatment with G-CSF in the 2 patients treated. The third subject was recently identified, and G-CSF therapy has not yet been initiated. This *in vitro* response correlated with a significant clinical improvement in both treated subjects. Additional data need to be collected to determine whether G-CSF affects recovery by decreasing neutrophil desensitization to C5a or by inducing neutrophilia, increased adherence, and phagocytic properties of PMNs, overcoming the chemotactic defect associated with MBL deficiency. Alternatively, G-CSF could induce maturation of pre-PMNs, which replace the desensitized cells. Therefore we propose inclusion of neutrophil functional studies and measurement of MBL levels in the immunologic evaluation of children with suspected immunodeficiency. If MBL deficiency is found to be associated with impaired neutrophil chemotaxis against C5a, administration of G-CSF should be considered. However, the length of treatment remains to be established and should probably be based on the individual response of each patient.

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