

BRIEF REPORT

Inherited Deficiency of Mannan-Binding Lectin–Associated Serine Protease 2

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N Engl J Med 2003;349:554-60.

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THE COMPLEMENT SYSTEM IS PART OF THE INNATE IMMUNE SYSTEM and contributes to the establishment of adaptive immune responses.¹ The mannan-binding lectin pathway of the complement system is activated when mannan-binding lectin binds to carbohydrate structures on microorganisms.² This happens through autoactivation of the mannan-binding lectin–associated serine protease 2 (MASP-2), which then cleaves complement factors C4 and C2, generating the C3 convertase C4bC2b.^{3,4} Activation of C3 initiates the alternative pathway and the formation of the membrane-attack complex. Complement fragments deposited on microorganisms facilitate phagocytosis and initiate inflammatory reactions. Like mannan-binding lectin, the recognition molecules L-ficolin and H-ficolin activate complement by a MASP-2–dependent mechanism.⁵ Three other proteins are associated with mannan-binding lectin and ficolins: mannan-binding lectin–associated proteases 1 and 3 (MASP-1 and MASP-3), serine proteases of unknown function, and MAP19, a fragment of MASP-2, with four additional amino acid residues.^{6,7} A deficiency of mannan-binding lectin is associated with susceptibility to infections and with the development of immunologic disease.⁸ We describe a patient with an inherited deficiency of MASP-2.

CASE REPORT

A man who had been born in 1967 was essentially healthy until 1980, when he received a diagnosis of ulcerative colitis. The condition was successfully treated with topical prednisolone. In 1996 erythema multiforme bullosum developed. Systemic lupus erythematosus was suspected because of joint symptoms and myalgia in combination with weakly positive tests for antinuclear antibody. The patient had a favorable response to treatment with prednisolone, and other immunosuppressive drugs were subsequently added to the regimen.

Severe pneumococcal pneumonia was documented at least three times between 1995 and 1997, with one episode of sepsis requiring prolonged intensive care. In 1997, roentgenography and transbronchial biopsy showed progressive lung fibrosis without vasculitis, alveolitis, or granulomas. Hypocomplementemia was found, but a Coomb's test and tests for antibodies against native DNA, cardiolipin, neutrophil cytoplasm, human immunodeficiency virus, and hepatitis A, B, and C virus were negative. Differential leukocyte counts and tests of kidney and liver function were also normal.

The patient's inflammatory disease persisted. Complement analysis in October 2002 showed severe hypocomplementemia with anti-C1q autoantibodies and low C1q levels (see Supplementary Appendix 1, available with the full text of this article at <http://www.nejm.org>). Immunosuppressive treatment is now limited to 5 to 15 mg of prednisolone daily. Without this treatment, the patient's skin symptoms worsen. The pa-

tient's parents, brother, and his two children have no increased susceptibility to infections.

METHODS

REAGENTS AND SAMPLES

We used a monoclonal antibody raised against MAp19 and a monoclonal antibody that reacts only against MASP-2 alone, generated with the use of the three C-terminal domains of MASP-2. Antibody against MASP-1 was generated from clone 12F2.⁹ Rat anti-MASP-3 antiserum was generated with the use of recombinant MASP-3 B-chain. EDTA-treated plasma samples were obtained on three occasions from the patient while his condition was clinically stable and from his parents, brother, and two children. Mannan-binding lectin-deficient serum (<20 ng of mannan-binding lectin per milliliter) was obtained from two healthy volunteers. Serum samples from 17 patients who had hypocomplementemia with low C1q levels (8 with systemic lupus erythematosus, 5 with hypocomplementemic urticarial vasculitis syndrome, and 4 with unexplained hypocomplementemia) were also analyzed. The studies were approved by the appropriate ethics committees, and samples were obtained after each subject or the subject's parent had provided written informed consent.

RECOMBINANT PROTEINS

MASP-2 complementary DNA was cloned in the pCI eukaryotic expression vector (Promega), and recombinant MASP-2 was produced in a human embryonic-kidney-cell line, HEK293, with use of the Free Style 293-F system (GIBCO). Recombinant mutant MASP-2 was produced after site-directed mutagenesis by an overlapping polymerase chain reaction (PCR). Recombinant mannan-binding lectin was produced as described previously.¹⁰

COMPLEMENT ANALYSIS

C3 and C4 were quantified by turbidometry. C1q, C1 inhibitor, properdin, C3d fragments, terminal component complexes (SC5b-C9), the function of the classic and alternative pathways, and autoantibodies to C1q were determined as described previously.¹¹⁻¹⁵ The function of C1 inhibitor was determined with the use of a commercial kit (Behringwerke), and mannan-binding lectin by means of a time-resolved immunofluorometric assay.¹⁴ The function of the mannan-binding lectin pathway was assessed on the basis of the capacity of the mannan-binding

lectin-MASP complex to induce the deposition of C4b onto a mannan-coated surface.¹⁶ Levels of MASP-2 were measured with the use of a sandwich-type time-resolved immunofluorometric assay on plates coated with antibody against MASP-2 and subsequent incubation with a biotinylated antibody against MAp19-MASP-2 and europium-labeled streptavidin. To identify MASPs bound to mannan-binding lectin with the use of Western blotting, we incubated plasma samples in microtiter wells coated with antibody against mannan-binding lectin. The resulting bound material was eluted with sample buffer for sodium dodecyl sulfate-polyacrylamide-gel electrophoresis and analyzed by Western blotting through incubation with antibodies against MASP.¹⁷

GENE SEQUENCING

DNA was isolated from EDTA-treated blood. The exons of the gene encoding MAp19 and MASP-2 as well as 1100 bp of the promoter region were amplified by PCR and sequenced (Lark). We subsequently used the primer sets 5'GCGAGTACGACTTCGTC-AAGG3' and 5'CTCGGCTGCATAGAAGGCCTC3' to amplify parts of the region encoding the first domain (CUB1) and 5'CCAGACCTTTGGAAAGTTA-GC3' and 5'GGCTCAAGTCCAAGTATTGC3' to amplify part of the region encoding the fifth domain (the complement control protein domain 2) of MASP-2. These PCR products were sequenced. The gene for mannan-binding lectin (MBL) was sequenced after the individual exons had been amplified by PCR.

RESULTS

Analyses of the functional activity of the mannan-binding lectin-MASP complex in the patient revealed severe deficiency, with less than 10 mU of mannan-binding lectin activity per microgram. The 5th to 95th percentile is 300 to 950 mU of mannan-binding lectin per microgram.¹⁸ The patient was heterozygous for the mannan-binding lectin B allotype, and his mannan-binding lectin level (0.7 µg per milliliter) was within the range reported for blood donors with this allotype.¹⁹ In other persons with this allotype, the specific activity of the pathway is within the normal range.¹⁸

The mannan-binding lectin-MASP complexes from the patient contained MASP-1 (Fig. 1B) and MASP-3 (Fig. 1C) but no MAp19 or MASP-2 (Fig. 1A). This result was confirmed by Western blot anal-

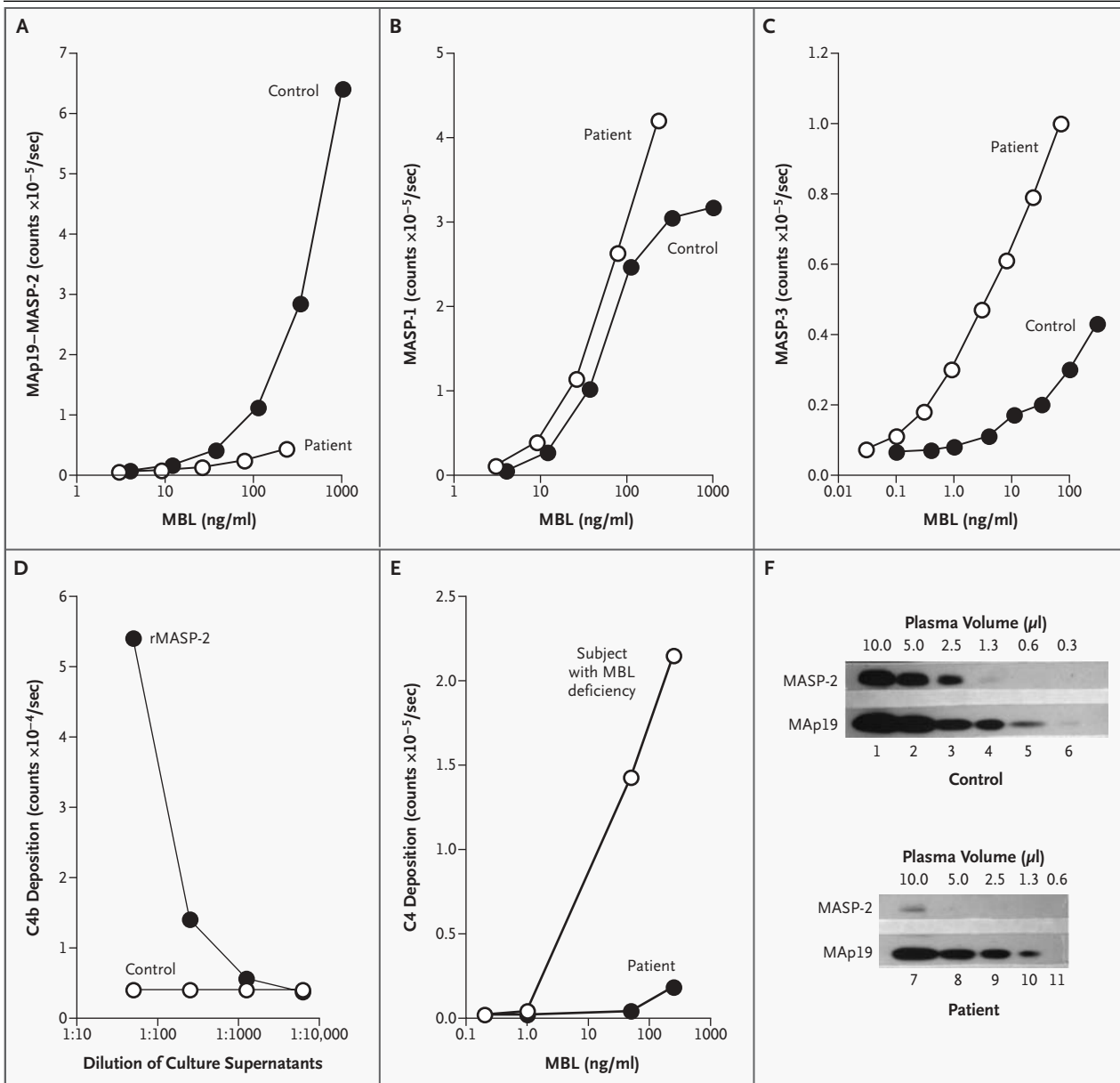


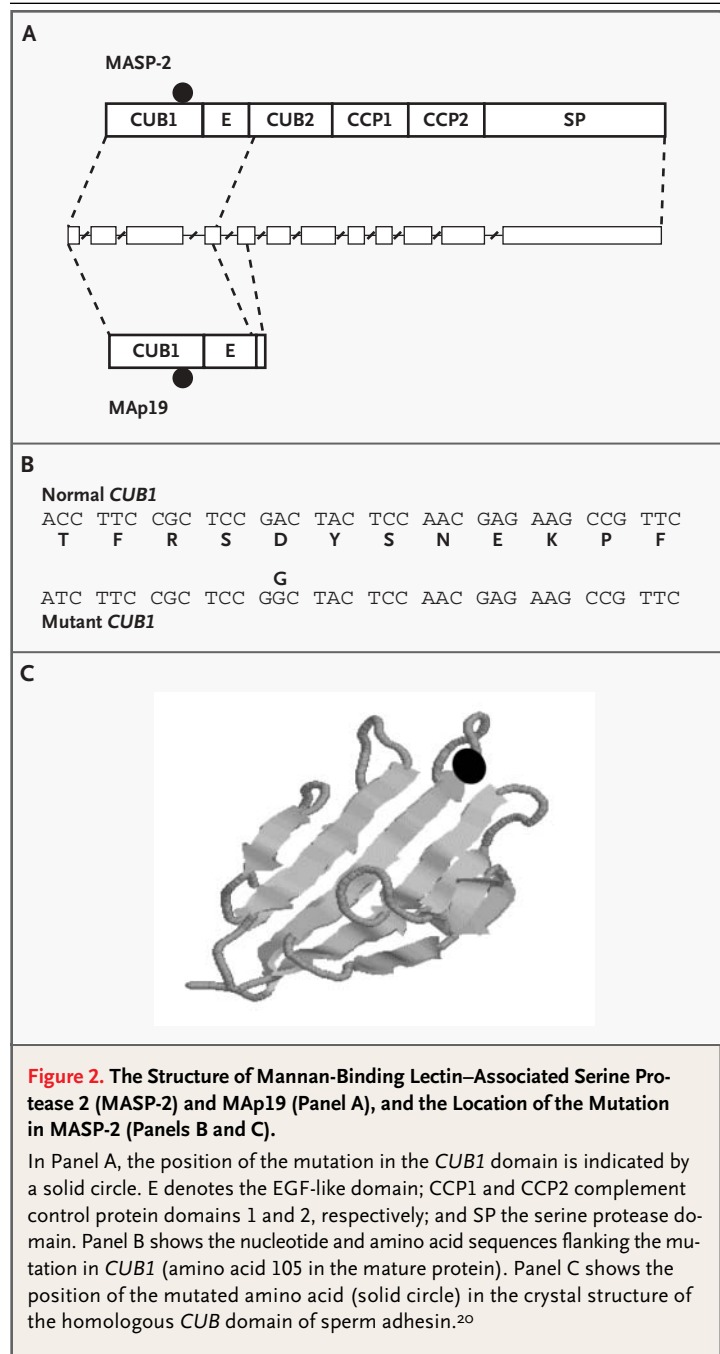
Figure 1. Analyses of the Mannan-Binding Lectin (MBL) Pathway for Complexes of MASP-2 (Panel A), MASP-1 (Panel B), MASP-3 (Panel C), C4b Deposition (Panel D), and C4 Deposition (Panel E), and the Plasma Volume of MASP-2 and MASP-19 (Panel F).

Plasma from one control subject and the patient was diluted in hypertonic buffer and incubated in mannan-coated microtiter wells, and the bound complexes were analyzed with antibody against MASP-2 (Panel A), antibody against MASP-1 (Panel B), and antibody against MASP-3 (Panel C). Bound antibody was detected with anti-immunoglobulin antibody labeled with europium. Other plasma samples from control subjects were tested and yielded results similar to those shown for the control subject in Panels A, B, and C. Panel D shows the ability of recombinant MASP-2 (rMASP-2) to restore the activity of the MBL pathway in the patient. Culture supernatant from an rMASP-2 cell culture or from a control cell culture was added to a serum sample from the patient. The serum was then incubated in mannan-coated wells, allowing MBL-MASP complexes to bind and C4b to be deposited in the wells. The amount of C4b deposited was estimated with the use of an antibody against C4. Panel E shows the lack of effect of adding recombinant MBL to plasma from the patient as compared with plasma from a subject with MBL deficiency, after incubation in mannan-coated wells and washing. C4 was added and the deposition of C4b was measured. With regard to plasma from the patient, the MBL values are the sum of endogenous and added MBL. Panel F shows the quantification of MASP-2 and MASP-19 in plasma. Plasma from a control subject (lanes 1 through 6) and the patient (lanes 7 through 11) at several dilutions was incubated with beads coated with antibody against MASP-2, and bound material was eluted and analyzed with the use of Western blotting.

ysis of complexes eluted from wells coated with antibody against mannan-binding lectin, whereas the same analyses showed all four components in mannan-binding lectin–MASP complexes in plasma from control subjects (see Supplementary Appendix 2, available with the full text of this article at <http://www.nejm.org>).

Analysis for MASP-2 and MASP-2 and MASP-2 were not bound to mannan-binding lectin in plasma was carried out with the use of affinity purification on beads coated with antibody against MASP-2 and Western blotting. The results showed the presence of trace amounts of MASP-2 (less than 10 percent of that in normal serum), whereas the amount of MASP-2 was about 50 percent of the normal level (Fig. 1F). The mannan-binding lectin pathway in the patient was restored by reconstitution with recombinant MASP-2 (Fig. 1D) or mannan-binding lectin–deficient plasma (data not shown). This demonstrated that mannan-binding lectin from the patient was fully active. Accordingly, the addition of recombinant mannan-binding lectin to plasma from a subject with mannan-binding lectin deficiency restored the mannan-binding lectin pathway, whereas it had no effect when added to the patient's plasma (Fig. 1E). The activity of the mannan-binding lectin pathway in plasma from the patient's mother (300 mU of mannan-binding lectin per microgram), his two children (200 and 220 mU per microgram), and a brother (233 mU per microgram) was at or below the 5th percentile. The activity could not be directly evaluated in plasma from the patient's father owing to the low level of mannan-binding lectin. However, on the addition of recombinant mannan-binding lectin, the activity was increased to one third of the activity in two reference samples of mannan-binding lectin–deficient serum.

The patient had low levels of C1q, C4, and C3; increased levels of C3dg; and anti-C1q antibodies (see Supplementary Appendix 1). These findings prompted studies in other patients with evidence of pronounced activation of the classic pathway. Eleven of the 17 patients studied had anti-C1q autoantibodies. No patient had reduced activity of the mannan-binding lectin pathway. Autoantibody against MASP-2 did not appear to be the cause of the MASP-2 deficiency in our patient, since antibodies against MASP-2 could not be detected on Western blotting of mannan-binding lectin–MASP preparations, in microtiter wells coated with mannan-binding lectin–MASP complex, or bound to mannan-binding lectin–MASP complex in plasma.



The promoter region and all the exons encoding MASP-2–MAP19 were sequenced and compared with known sequences. GenBank presents two different codons for the amino acid at position 371 in the first part of complement control protein 2: a GAT encoding aspartic acid and a TAT encoding tyrosine. The patient and all his family members were homozygous for TAT. Of 15 unrelated control

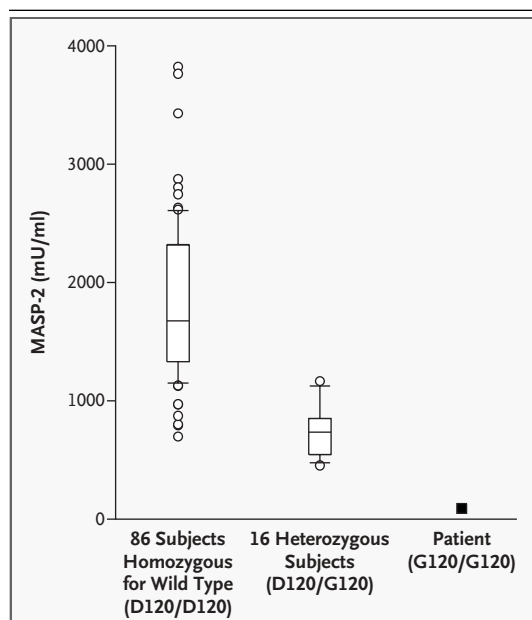


Figure 3. Mannan-Binding Lectin–Associated Serine Protease 2 (MASP-2) Levels in Plasma from 86 Control Subjects Who Were Homozygous for the Wild Type (D120/D120), 11 Heterozygous (D120/G120) Control Subjects and the 5 Heterozygous Members of the Patient’s Family, and the Patient, Who Was Homozygous for the Mutant Sequence (G120/G120).

The levels of MASP-2 were estimated by means of a double-antibody assay. Standard curves were constructed with the use of a pool of normal plasma arbitrarily assigned the value of 1 U of MASP-2 per milliliter. The mean levels (horizontal lines), the 10th and 90th percentiles (I bars), and outliers (open circles) are shown. $P < 0.001$ for the difference in MASP-2 values between the patient and the other two groups.

subjects, 10 were homozygous for TAT, 3 were heterozygous (TAT/GAT), and 2 were homozygous for GAT. Hence, these variants represent common allotypes of no discernible consequence with respect to the function of MASP-2.

Another difference in the patient’s sequence was in exon 3 in the codon for the amino acid at position 120 (position 105 in the mature protein) — that is, in the CUB1 domain — where codon GGC was found instead of GAC. The patient was homozygous for this variant, which resulted in the substitution of glycine for aspartic acid (Fig. 2). Amplification by PCR and sequencing of the region around the mutation in CUB1 demonstrated that the patient’s parents, brother, and two children were heterozygous (GAC/GGC). Of 100 control subjects, 89 were ho-

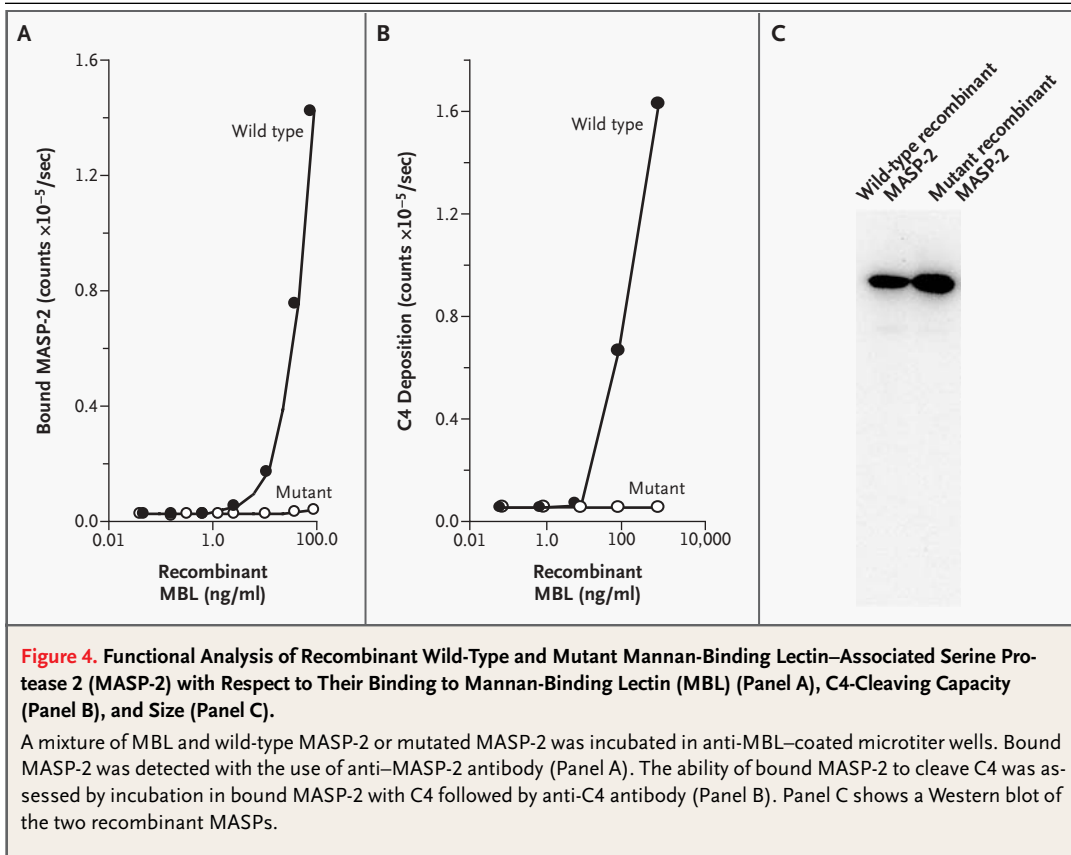
mozygous for GAC and 11 were heterozygous (GAC/GGC), yielding a gene frequency of 0.055 for the mutant allotype. Heterozygotes had significantly lower MASP-2 levels (by 45 percent) than those who were homozygous for the wild-type MASP-2 (Fig. 3). The patient’s MASP-2 level was about 5 percent of the levels in these homozygous subjects.

To determine the importance of the substitution in the CUB1 domain, we examined recombinant wild-type and mutant MASP-2 in functional assays. Mutant MASP-2 could not associate with mannan-binding lectin (Fig. 4A) and thus could not form an active mannan-binding lectin–MASP complex (Fig. 4B). Western blotting showed that the size of the mutated recombinant MASP-2 was identical to that of the wild type, and no degradation fragments were observed (Fig. 4C).

DISCUSSION

In our patient with a history of infections and chronic inflammatory disease, the mannan-binding lectin pathway was nonfunctional despite the presence of a normal level of immunoreactive mannan-binding lectin. This malfunction was caused by the absence of MASP-2 in the mannan-binding lectin–MASP complex. The activity of the mannan-binding lectin–MASP complex was restored by the addition of recombinant MASP-2. The mannan-binding lectin–MASP complex contained no MASP-2 but a normal level of MASP-1 and an elevated level of MASP-3, results that are consistent with our previous finding that MASP-3 competes with MASP-2 for binding to mannan-binding lectin.⁶ The mannan-binding lectin–MASP complex also contained no MAP19. MASP-2 and MAP19 could be detected in plasma at low levels.

The gene encoding MASP-2 had a mutation in the CUB1 domain, causing substitution of glycine for aspartic acid in the loop connecting beta strands 8 and 9 (i.e., D120G) (Fig. 2).²⁰ The change from an acidic to a neutral amino acid may have profound effects on the function of the domain. The aspartic acid at this position is conserved in all MASPs as well as in the similar serine proteases C1r and C1s of the classic complement pathway. The CUB1–epidermal growth factor domains are essential for the association of MAP19 and MASP-2 with mannan-binding lectin.^{21,22} Analysis of the recombinant wild-type and mutated MASP-2 showed that the mutation prevents the formation of functional mannan-binding lectin–MASP-2 complexes.



The patient's parents, brother, and two children had a low level of activity of the mannan-binding lectin pathway, and they were all heterozygous for the CUB1 mutation. Likewise, the level of MASP-2 in control subjects who were heterozygous for the CUB1 mutation was about half of that in subjects with the wild type, suggesting a dominant effect of an autosomally inherited mutant allotype. Recombinant wild-type and mutant MASP-2 were secreted at similar levels by transfected cells, indicating that the mutation had no effect on intracellular processing. The failure of the mutant MASP-2 to bind to mannan-binding lectin may enhance its catabolism. MASP-2 forms dimers,^{21,22} which in heterozygous persons may be a mixture of wild-type and mutated MASP-2 and therefore may be more susceptible to degradation. It is not clear why the level of MAP19 was less strongly affected than the level of MASP-2.

The frequency of the gene containing the CUB1 mutation was determined to be 0.055. One would expect about 0.3 percent of the population to be homozygous for the mutation, making MASP-2 deficiency a fairly common complement deficiency.

Complement deficiencies are usually associated with susceptibility to invasive infections caused by encapsulated bacteria or with the development of immunologic diseases such as systemic lupus erythematosus.¹ Mannan-binding lectin deficiency has been described as a factor in susceptibility to infections and may also contribute to the development of immunologic disease.⁸ The consequences of MASP-2 deficiency might be more severe, since MASP-2 also mediates the activation of complement through the ficolins.⁵

It seems likely that the MASP-2 deficiency was at least partly responsible for the manifestations of disease in our patient, even though the clinical findings in a single patient must be interpreted with caution. The absence of serious infections during childhood is consistent with the findings in some patients who have a deficiency of C4 or C2.²³ An impairment in the function of the mannan-binding lectin pathway predisposes patients to invasive pneumococcal disease,²⁴ supporting the assumption that our patient's severe pneumococcal infections were due to a MASP-2 deficiency. The patient's

hypocomplementemia may also have contributed to his impaired immune defense. Low C1q levels and the presence of anti-C1q autoantibodies are characteristics of hypocomplementemic urticarial vasculitis syndrome^{15,25} and severe systemic lupus erythematosus.²⁶ These diagnoses were not justified in our patient, and we found normal MASP-2 levels in patients with hypocomplementemic urticarial vasculitis syndrome and systemic lupus ery-

thematosus. Identification of the prevalence of the CUB1 mutation should facilitate the determination of its clinical effect on immune defense and the development of inflammatory disease.

Supported by grants from the Danish Medical Research Council, the Karen Elise Jensens Foundation, King Gustaf V's 80th Birthday Fund, and the Novo Nordisk Foundation.

We are indebted to Drs. Gerard Arlaud and Nicole Thielens, Grenoble, France, for donating the recombinant MASP-3 B chain, and MASP-2 C-terminal fragment.

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