Low mannose-binding lectin (MBL) is associated with paediatric inflammatory bowel diseases and ileal involvement in patients with Crohn disease

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KEYWORDS
Mannose-binding lectin (MBL); MBL deficiency; Inflammatory bowel disease; Crohn disease; Ulcerative colitis; Paediatric

Abstract

Background: Mannose-binding lectin (MBL) is a pattern-recognition molecule of the innate immune system and may be involved in the pathogenesis of inflammatory bowel disease (IBD). Our aim was to assess the prevalence of MBL deficiency in a cohort of patients with paediatric-onset IBD and study whether it is associated with the clinical manifestations, serum antibody formation, or genetic factors.

Methods: This prospective study included 159 paediatric patients (mean age: 14.0 years) with IBD [107 patients with Crohn disease (CD) and 52 patients with ulcerative colitis (UC)]. Furthermore, 95 controls were investigated. Serum samples were determined for MBL by enzyme-linked immunosorbent assay (ELISA) and for serologic markers [autoantibodies against Saccharomyces cerevisiae (ASCA) and perinuclear components of neutrophils (pANCA)] by indirect immunofluorescent
1. Introduction

The inflammatory bowel diseases (IBD), including Crohn disease (CD) and ulcerative colitis (UC), are chronic relapsing and remitting disorders of the gastrointestinal tract. The pathogenesis of IBD is complex and multifactorial. Current evidence suggests that IBD results from aberrant immune response and loss of tolerance to the normal intestinal flora, leading to chronic inflammation of the gut in a genetically susceptible host.1,2 This idea is supported by the occurrence of antibodies directed to microbial antigens and by the identification of NOD2/CARD15 as a gene conferring susceptibility to CD.3,4 Both CD and UC are characterized by intestinal inflammation caused by a disturbance in the balance between cytokines and increased complement activation.5

Mannose-binding lectin (MBL) is an important component of innate immunity. MBL is a pattern-recognition molecule that activates the lectin pathway of the complement system irrespective of an antibody. MBL can directly opsonize microorganisms and enhance their uptake by phagocytic cells.6 MBL is primarily synthesized in the liver and predominantly circulates as a serum protein. In addition, Seyfarth et al. showed intestinal MBL2 gene expression using a commercial cDNA library.7 In contrast, a more recent study could not find meaningful MBL2 gene expression in intestinal tissues.8 Furthermore, MBL binds to apoptotic and necrotic cells and facilitates uptake by macrophages.9,10 Recently, a new role for MBL as a Toll-like receptor (TLR), co-receptor in directing intracellular signalling, has been identified.11

Three different point mutations of exon1 of the MBL2 gene (in codons 52, 54 and 57) have the greatest influence on serum levels and functional activity of MBL, but promoter region polymorphism is also important.12,13 The inter-individual levels of MBL vary from approximately 5 ng/mL to more than 10000 ng/mL, but the level of MBL in each individual is genetically determined, quite stable throughout the life. At birth, the level is about 2/3 of the adult level, which is reached in a month, and there is a minor decline at elderly age.11,14

Low MBL level was observed in up to 40% and the prevalence of MBL deficiency is 8–10% in the normal population.15,16 MBL deficiency has been associated with increased susceptibility and severity of infections, especially in children and in immune-compromised patients.17–19 Furthermore, the MBL pathway may be involved in the development of various autoimmune diseases (e.g., IBD and coeliac disease).20,21

The role of MBL in IBD is still controversial. Rector et al. reported a significant decrease in the frequency of the MBL variants in sporadic adult UC patients.22 In contrast, Sivaram et al. observed a significantly higher frequency of mutations at codon 54 in UC patients than in healthy controls, which was accompanied by low MBL concentrations.23 In other studies, the frequency of MBL polymorphism, MBL deficiency, and the median level of MBL were not significantly different from controls — neither in adult CD nor in UC.24–26 Only one Polish survey was conducted in paediatric IBD, in which the frequency of MBL2 gene variants responsible for MBL deficiency was significantly higher in CD patients than in controls or in children with UC. However, only a small number of patients were involved in this study (CD:30, UC:26).27 Up until now, the prevalence of MBL deficiency has not been investigated in paediatric IBD.

The aim of our study was to determine the serum MBL levels and the prevalence of MBL deficiency in a cohort of patients with paediatric-onset IBD and to evaluate possible associations with the clinical presentation, response to treatment, and extraintestinal manifestations in IBD. Additionally, we studied the relationship between MBL deficiency and serum autoantibodies, and polymorphism of NOD2/CARD15.

2. Patients and methods

2.1. Patients

One hundred and seven consecutive patients with paediatric-onset CD [male/female (m/f) ratio: 64/43, mean age: 14.1 years (range: 5.3–20 years)], 52 patients with paediatric-onset UC [m/f ratio: 22/30, mean age: 14.0 years (range: 6–19.7 years)], and 95 age-and sex-matched controls were included in this study.

Diagnosis of IBD was established according to clinical, radiographic, endoscopic, and histological criteria.28,29 Disease activity was evaluated according to the Paediatric Crohn’s Disease Activity Index (PCDAI) in children with CD and according to the Paediatric Ulcerative Colitis Index (PUCAI) for the patients with UC.30,31 Activity index > 30 is defined as moderate-severe disease, the index between 11
and 30 indicates mild disease, and the index ≤10 refers to inactive disease.

Age, age at onset, presence of extraintestinal manifestation (EIM); arthritis, ocular manifestations, skin lesions, and hepatic manifestations, frequency of flare-ups (frequent flare up: >1/year), therapeutic effectiveness (e.g.: need for steroid and/or immunosuppressive therapy, steroid resistance, as defined in the European Crohn’s and Colitis Organisation Consensus (ECCO) Report, or short-term response to infliximab therapy, need for surgery (resection), the presence of familial IBD were collected by the clinical investigator reviewing the medical charts and completing a questionnaire. In CD, an additional parameter, perianal involvement, was also investigated. Localization and phenotype of disease were based on the Montreal classification criteria. Only patients with a confirmed diagnosis for more than 1 year were enrolled. In UC, disease extent was defined based on the maximum extent during the follow-up.

Prospectively, blood samples were obtained for measurement of mannan-binding lectin (MBL), for serologic markers [anti-Saccharomyces cerevisiae antibodies (ASCA), perinuclear anti-neutrophil cytoplasmic antibodies (pANCA)], complete blood count and C-reactive protein (CRP). When blood samples were taken, patients underwent a clinical assessment, including calculation of clinical disease activity scores. Sera for MBL and serological marker determination were coded to maintain blinding. After serum separation, blood samples were stored at 80 °C until further analysis.

The study protocol was approved by the Ethical and Science Committee of the Semmelweis University. Each parent of the subject, who had been informed about the nature of the study before, signed the informed consent form.

2.2. MBL assay

Serum concentration of MBL was detected by the use of the double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) system adopted from Minchinton et al. using monoclonal mouse antihuman MBL antibody (BioPorto Diagnostics A/S, Gentofte, Denmark). A vial of standard MBL solution (BioPorto Diagnostics A/S) was supposed to have an MBL content of 1000 AU and we accepted that it corresponded to 3200 ng/mL oligomerized MBL, as declared by the manufacturer. The detection limit was 4.86 ng/mL. According to the recommendations of the manufacturer, low MBL concentration was defined as a serum level <500 ng/mL and MBL deficiency as <100 ng/mL. The MBL assay was performed at the Clinical Research Center of the Debrecen University in a blinded manner, without having known the patients’ diagnosis or other clinical information before.

2.3. Antibody assays for ASCA and pANCA antibodies

Presence of ASCA and pANCA antibodies was determined in a commercially available indirect immunofluorescent (IIF) assay (EUROIMMUN AG, Luebeck, Germany) according to the manufacturers’ instructions. IIF coated cover glasses with several biological substrates were cut into millimetre-sized fragments (BIOCHIPs, EUROIMMUN AG) and used side by side in the same reaction field, giving the opportunity to investigate one serum on several tissues or prepared cells: ASCA: Saccharomyces cerevisiae fungal smear, ANCA: ethanol (EOH)-fixed and formalin (HCHO)-fixed human granulocytes. Sera were incubated at a 1:10 dilution (ASCA IgG:1:1000, ASCA IgA: 1:100) in phosphate-buffered saline (PBS)/Tweem for 30 min. After being washed with the buffer, slides were incubated with fluorescein-labelled goat antihuman IgG or IgA antibodies for another 30 min. After another washing process, evaluation and classification of the patterns were performed with the help of a fluorescence microscope (EUROIMMUN LED, EUROStar Bluelight, EUROIMMUN AG, Luebeck, Germany) under ultraviolet light (UV).

2.4. Detection of NOD2/CARD15 mutations

Genomic DNA was isolated from whole blood using the QIAamp DNA Blood Mini Kit (QIAGEN, Germany). The three NOD2/CARD15 variants, Arg702Trp, Gly908Arg and Leu1007fs, were typed using polymerase chain reaction/restriction fragment length polymorphism as previously described. NOD2/CARD15 variants were detected by denaturing high-performance liquid chromatography (dHPLC, Wave DNA Fragment Analysis System, Transgenomic, UK). Sequence variation, observed in the dHPLC profile, was sequenced on both strands to confirm the alteration. Sequencing reactions were performed with the ABI BigDye Terminator Cycle Sequencing Kit v1.1 (Applied Biosystems, Foster City, CA) and samples were sequenced on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). Genotyping was carried out at the National Haematology and Immunology Institute, Budapest, Hungary.

2.5. Statistical methods

Statistical analysis was carried out using Graph Pad Prism 5 (GraphPad, San Diego CA, USA). T-test with separate variance estimates and Fisher’s exact test were used to evaluate differences between IBD and control patients as well as within subgroups of IBD patients. Results are expressed as odds ratio (OR) with 95% confidence intervals (95% CI). Spearman’s rank order correlation was calculated to test the association between MBL levels, CRP, and clinical activity indexes. A p < 0.05 was considered as significant.

3. Results

3.1. MBL levels and MBL deficiency in patients with IBD and their association with other serological markers

Low MBL levels (defined as < 500 ng/mL) were found in 34 of 107 (31.8%) patients with CD, in 18 of 52 patients (34.6%) with UC, and in 13 of 95 (13.7%) controls. MBL deficiency (defined as <100 ng/mL) was found in 10 (9.4%) patients with CD, 6 (11.5%) patients with UC and 9 controls (9.5%) (Table 1). We noted significantly lower median MBL levels in both CD and UC patients compared to controls (CD, p = 0.04, UC, p = 0.004, IB, p = 0.007). Furthermore, prevalence of low MBL level (<500 ng/mL) was significantly higher in both CD and UC groups than in controls (CD, p = 0.002, UC, p = 0.006, IB, p = 0.001). Nevertheless, prevalence of MBL
deficiency (<100 ng/mL) was similar in IBD patients and in controls. No significant difference was found in the prevalence of MBL deficiency and low MBL level as well as in median MBL levels between UC and CD patients. Moreover, we did not find any significant association between low MBL level or MBL deficiency and ASCA/pANCA in CD or in UC (Figs. 1, 2).

3.2. Association between MBL levels, clinical phenotype, CRP, and actual disease activity in patients with IBD

The clinical phenotype of patients with CD and UC is shown in Tables 2 and 3 according to different MBL levels. Low MBL level (<500 ng/mL) was associated with isolated ileal involvement (p=0.01) in patients with CD (Fig. 3). In addition, MBL deficiency (<100 ng/mL) was related to male gender (p=0.004) in CD. Nevertheless, the MBL level or deficiency was not associated with medical therapy, need for surgery, or extraintestinal manifestations — neither in CD nor in UC. Additionally, MBL level was not associated with CRP and actual PCDAI in CD or with CRP and PUCAI in UC. Moreover, CRP values correlated positively with PCDAI in CD (n=86, R: 0.617, p<0.0001). However, CRP values were not significantly associated with PUCAI in UC.

3.3. Association between MBL levels and NOD2/CARD15 genotype in CD

The prevalence of NOD2/CARD15 genotypes was available in 44 patients. MBL deficiency was not associated with NOD2 variants. The prevalence of NOD2/CARD15 mutations were not statistically different between patients with high (>500 ng/mL, 20.6%) and low (<500 ng/mL, 33.3%) MBL levels.

4. Discussion

This is the largest study to assess MBL levels and MBL deficiency as well as relevant associations with phenotype in paediatric patients with IBD. The MBL serum concentration was significantly lower in IBD (both in CD and UC) compared to controls. Prevalence of low MBL level (<500 ng/mL) was significantly higher in both CD and UC groups compared to controls. Furthermore, low MBL level was associated with isolated ileal involvement and MBL deficiency (<100 ng/mL) with male gender in patients with CD.

The role of MBL in IBD is still controversial. A strong correlation exists between MBL2 genotypes and serum MBL levels, however the current knowledge of the genetics of MBL2 is inadequate to predict serum MBL concentration.33,35
Rector et al. reported a highly significant decrease in the frequency of the MBL variants (mutations in codons 52, 54 and 57) in sporadic adult UC patients (36/124, 29%) compared to CD (93/216, 43.1%) and controls (126/308, 40.9%), suggesting a protective effect of functional MBL mutations in UC. In contrast, in UC patients, Sivaram et al. observed a significantly higher frequency of mutations at codon 54 than in healthy controls, accompanied by low MBL concentrations. In other studies, the frequency of MBL deficiency and the median level of MBL in adult CD patients and in UC patients were comparable. Only one Polish survey has been conducted in paediatric IBD with a small number of patients, in which the frequency of MBL2 gene variants responsible for MBL deficiency was significantly higher in CD patients than in controls or in children with UC. In accordance with the results of this report, the median MBL concentration in our present study was significantly lower in CD than in controls.

Furthermore, our results were similar to the above-mentioned adult data concerning the prevalence of MBL deficiency in IBD patients and in controls. However, in contrast to these studies conducted in adults, we found that the prevalence of low MBL levels was more common and median MBL levels were significantly lower in paediatric patients with IBD than in the control paediatric group. We cannot explain satisfactorily the reason for the differences between paediatric and adult MBL levels in IBD. Different genetic and/or immunologic factors could be responsible for this finding. Nevertheless, further simultaneous studies conducted in paediatric and adult IBD patients are necessary to answer this question.

In the present study, we found no association of MBL deficiency with ASCA and pANCA antibodies.

### Table 2
Clinical phenotype, serological status, and NOD2/CARD15 genotype in patients with Crohn’s disease (CD, n = 107) according to different mannose binding lectin (MBL) categories.

<table>
<thead>
<tr>
<th>MBL deficiency (&lt;100 ng/mL)</th>
<th>Low level of MBL (&lt;500 ng/mL)</th>
<th>MBL competence (&gt;500 ng/mL)</th>
<th>Total n = 107</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13.5 (range:8.7–19.2)</td>
<td>13.8 (range:5.8–20)</td>
<td>14.2 (range:5.3–19.6)</td>
<td>14.1 (range:5.3–20)</td>
</tr>
<tr>
<td>Age at presentation (y)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.1 (range:5.5–16.2)</td>
<td>12.4 (range:5.5–17.6)</td>
<td>12.8 (range:2.3–18)</td>
<td>12.7 (range:2–18)</td>
</tr>
<tr>
<td>Duration (y)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.4 (range:0–3.2)</td>
<td>1.3 (range:0–7)</td>
<td>1.4 (range:0–9)</td>
<td>1.4 (range:0–9)</td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ileum (L1)</td>
<td>3/10 (30%)</td>
<td>11/34 (32.4%)</td>
<td>8/73 (11%)</td>
</tr>
<tr>
<td>Colon (L2)</td>
<td>4/10 (40%)</td>
<td>10/34 (29.4%)</td>
<td>19/73 (26%)</td>
</tr>
<tr>
<td>Ileocolon (L3)</td>
<td>3/10 (30%)</td>
<td>13/34 (38.2%)</td>
<td>46/73 (63%)</td>
</tr>
<tr>
<td>Upper Gl (+L4)</td>
<td>2/10 (20%)</td>
<td>5/34 (14.7%)</td>
<td>17/73 (23.3%)</td>
</tr>
<tr>
<td>Behaviour</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-stricturing, non-penetrating (B1)</td>
<td>6/10 (60%)</td>
<td>22/34 (64.7%)</td>
<td>41/73 (56.1%)</td>
</tr>
<tr>
<td>Stricture (B2)</td>
<td>1/10 (10%)</td>
<td>5/34 (14.7%)</td>
<td>16/73 (22%)</td>
</tr>
<tr>
<td>Penetrating (B3)</td>
<td>3/10 (30%)</td>
<td>7/34 (20.6%)</td>
<td>22/73 (30.1%)</td>
</tr>
<tr>
<td>Perianal disease</td>
<td>2/10 (20%)</td>
<td>5/34 (14.7%)</td>
<td>17/73 (23.3%)</td>
</tr>
<tr>
<td>Frequent relapse</td>
<td>3/10 (30%)</td>
<td>14/34 (41.2%)</td>
<td>27/73 (37%)</td>
</tr>
<tr>
<td>ASCA (IgA or IgG) positive</td>
<td>5/9 (55.5%)</td>
<td>18/27 (66.6%)</td>
<td>48/62 (77.4%)</td>
</tr>
<tr>
<td>NOD2/CARD15 carrier</td>
<td>2/3 (66.6%)</td>
<td>5/15 (33.3%)</td>
<td>6/29 (20.6%)</td>
</tr>
</tbody>
</table>

### Table 3
Clinical phenotype and serological status of patients with ulcerative colitis (n = 52) according to different mannose binding lectin (MBL) categories.

<table>
<thead>
<tr>
<th>MBL deficiency (&lt;100 ng/mL)</th>
<th>Low level of MBL (&lt;500 ng/mL)</th>
<th>MBL competence (&gt;500 ng/mL)</th>
<th>Total n = 52</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13.6 (range:10–17.5)</td>
<td>14.4 (range:8.5–19)</td>
<td>13.7 (range:6–19.7)</td>
<td>14.0 (range:6–19.7)</td>
</tr>
<tr>
<td>Age at presentation (y)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.3 (range:2–15.7)</td>
<td>11.6 (range:1.2–17.8)</td>
<td>11.6 (range:4.6–18)</td>
<td>11.6 (range:1.2–18)</td>
</tr>
<tr>
<td>Duration (y)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.4 (range:0–8)</td>
<td>2.7 (range:0–13.4)</td>
<td>2.1 (range:0–7)</td>
<td>2.3 (range:0–13.4)</td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proctitis (E1)</td>
<td>–</td>
<td>5/34 (14.7%)</td>
<td>5/52 (9.6%)</td>
</tr>
<tr>
<td>Left sided (E2)</td>
<td>3/6 (50%)</td>
<td>4/18 (22.2%)</td>
<td>11/34 (32.3%)</td>
</tr>
<tr>
<td>Extensive (E3)</td>
<td>3/6 (50%)</td>
<td>14/18 (77.8%)</td>
<td>18/34 (52.9%)</td>
</tr>
<tr>
<td>Frequent relapse</td>
<td>2/6 (33.3%)</td>
<td>8/18 (44.4%)</td>
<td>11/34 (32.3%)</td>
</tr>
<tr>
<td>pANCA (IgA or IgG) positive</td>
<td>5/6 (83.3%)</td>
<td>14/16 (87.5%)</td>
<td>20/31 (64.5%)</td>
</tr>
</tbody>
</table>

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Deficiency was associated with ASCA positivity, 39 bial antigens, such as those which had been found on the cell ment of the normal processing of mannan-expressing micro-
also proposed to contribute to ASCA production after impair-
can bind to
intestinal involvement.39 In addition, in previous studies, MBL
tients with a low MBL phenotype and genotype had small in-
associated with ileal involvement in paediatric patients.
MBL2 gene expression in intestinal tissues.8 Although, Müller et al. showed that MBL deficiency resulted in enhanced ASCA production and excessive experimental colitis in response to mannose-expressing milk gut pathogens in MBL deficient mice.6 It is conceivable that MBL2 polymorphisms, resulting in lower MBL levels, may be associated with an increased number of bacteria in the small intestine. It is possible that MBL is responsible for maintaining the rela-
tive sterility of the small bowel as a protective factor by en-
hancing phagocytosis64 or by inhibiting the attachment of microorganisms on intestinal cell surfaces.45 Furthermore, MBL deficiency impairs the normal removal and clearance of apoptotic cells that may subsequently reveal previously hidden self-antigen, causing loss of self-tolerance and spreading of autoimmunity.9,10,21,46
In concordance with the results of the study by Papp et al.,40 MBL level was not associated with CRP level and ac-
tual disease activity index in CD or in UC in the present study. CRP concentration may increase rapidly up to 1000-
fold, compared to modest (2–3 fold) and slow (1–2 weeks after the inducing event) increase of MBL levels during inflam-
47,48 The elevated CRP levels in patients with IBD indicate disease activity rather than bacterial complications caused by the enhanced production of pro-inflammatory cyto-
mes in the mesenteric fat.26,49
In accordance with the results of Tilakaratne et al., CRP
correlated positively with PCDAI in CD in our present study. Children with active disease had significantly higher CRP
values compared to children with inactive disease.50 Furthermore, in concordance with our findings, Henriksen
et al. described that patients with CD had a stronger CRP
response than those with UC.49
In addition, the prevalence of a low MBL level was not related to the presence of major NOD2/CARD15 mutations26
in our study, which is in agreement with the results of the
study of Papp et al.
In conclusion, MBL levels were lower in paediatric pa-
tients with IBD than in controls. Nevertheless, MBL levels in
patients with CD and UC were similar. Furthermore, low
MBL level was associated with ileal involvement in paediatric
patients with CD. We found no association between MBL and
different serological markers or genetic polymorphism
of NOD2/CARD15.

Conflict of interest

There was no conflict of interest in this study.

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