Original Article

Elevated Levels of Mannose-Binding Lectin at Clinical Manifestation of Type 1 Diabetes in Juveniles

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Mannose-binding lectin (MBL) is a recognition molecule of the lectin pathway of complement and a key component of innate immunity. MBL polymorphisms have been described that are associated with MBL serum concentration, impaired function, and diabetic complications. We investigated 86 new-onset juvenile type 1 diabetic patients and compared these with their nondiabetic siblings and healthy unrelated control subjects. Polymorphisms of MBL exon 1 and promoter were determined, and serum concentration and MBL-complex activity were measured. Although the genetic polymorphisms of MBL were not different between patients and control subjects, MBL serum concentration as well as MBL complex activity was significantly higher in new-onset diabetic patients compared with their siblings matched for high-producing MBL genotypes ($P = 0.0018$ and $P = 0.0005$, respectively). The increase in MBL complex activity in high-MBL–producing patients could only partially be explained by high MBL production, as demonstrated by an increased MBL complex activity–to–MBL concentration ratio ($P = 0.004$). We conclude that MBL serum concentration and complex activity are increased in early- onset diabetic patients upon manifestation independently of genetic predisposition to high MBL production, indicating a possible role in the immunopathogenesis of type 1 diabetes, in addition to the adaptive islet autoimmunity. *Diabetes* 54:3002–3006, 2005

Type 1 (insulin dependent) diabetes is an autoimmune disease characterized by the specific destruction of β-cells in the pancreas. The etiology of type 1 diabetes is multifactorial, consisting of genetic predisposition and environmental factors including a variety of viruses and dietary components (1,2). The role of the adaptive immune system in the autoimmune process leading to type 1 diabetes is well established. Presently, the interest for the innate immune system in the immunopathogenesis of type 1 diabetes is mounting (3–5). That the recognition of self-determinants is confined to the adaptive immune system is generally proposed, diminishing the role of the innate immune system in autoimmunity. However, evidence is growing that changes in the innate immune system could lead to autoimmunity, either by priming or promoting aggressive immune responses (6).

Mannose-binding lectin (MBL) is a key molecule of the innate immune system and is able to bind common carbohydrate structures of a variety of microorganisms (including bacteria, viruses, and fungi), resulting in direct opsonophagocytosis and complement activation. In plasma, MBL is associated with MBL-associated serine proteases (MASPs). Upon binding of MBL to its ligand, the subsequent MASP-2 activation is responsible for complement activation via the lectin pathway (7). Exon 1 of the *mbl-2* gene contains three known single nucleotide polymorphisms (SNPs) at codons 52 (referred to as allele "D"), 54 (allele "B"), and 57 (allele "C") (8). These SNPs are associated with low serum concentrations, disturbed polymerization, and impaired function of MBL (9,10). Dependent on ethnicity, the allele frequency of variant alleles B, C, and D, commonly referred to as O-alleles, may be above 40% (wild type = A/A). In addition to the three SNPs in exon 1, there are several other polymorphic sites located in the MBL promoter region, including SNPs located at positions −550 (H/l variant) and −221 (X/Y variant). The common allele A of exon 1 is associated with the following haplotypes: HYA, LYA, and LXA, exhibiting respectively high, intermediate, and low promoter activity and serum MBL levels. The structural alleles carry the following haplotypes: LYB, LYC, and HYD (11,12). Low MBL serum levels and genetic polymorphisms associated with impaired MBL function have been shown to be associated with different autoimmune diseases including celiac disease and systemic lupus erythematosis (13,14). Although the complement system has been studied in diabetes (15), the association between MBL and the immunopathogenesis of diabetes has not yet been investigated to any extent. MBL has been associated with vascular complications in diabetic patients. High-MBL genotypes are significantly more frequent in diabetic patients with nephropathy than in normoalbuminuric diabetic patients. Furthermore, comparing patients with identical MBL genotypes, serum MBL levels were higher in patients with nephropathy than those with normoalbuminuria (16,17). Recently, high MBL levels in the early course of type 1 diabetes were shown to

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CRP, C-reactive protein; mAb, monoclonal antibody; MASP, mannose-binding lectin-associated serine protease; MBL, mannose-binding lectin; SNP, single nucleotide polymorphism.

be associated with development of albuminuria, indicating that MBL may be involved in the pathogenesis of diabetic microvascular complications (18).

We decided to address the possible association between MBL and the pathogenesis of type 1 diabetes. We hypothesize that as the insulin production diminishes during insulitis, MBL serum concentration will rise as a consequence of the inflammation process. MBL in turn could promote the adaptive immune response, either via enhanced complement activation or increased opsonophagocytosis of autoantigens, interweaving MBL in the complex autoimmune process of type 1 diabetes.

To test our hypothesis, we studied 86 juvenile type 1 diabetic patients at clinical presentation. With the intention to match for age, genetic background, municipality of residence, and other environmental factors, an unaffected sibling of every diabetic patient was included as a control subject. For genetic analysis, a healthy, unrelated control group was included in the study of 69 voluntary healthy blood donors. MBL genotype, concentration, and complex activity were further correlated with diagnostic and predictive parameters as serum fructosamine levels, the presence of islet autoantibodies, and HLA type.

RESEARCH DESIGN AND METHODS

Meeting all legal and ethical criteria set out by the local and ethical committees, fresh peripheral blood samples were obtained from 86 juvenile type 1 diabetic patients at diagnosis (mean ± SD, age 9.3 ± 3.5 years, 34 girls). Diabetes was diagnosed according to the criteria set out by the World Health Association (19). For every patient, a sibling control subject was included as control for serological assessment of MBL concentration and MBL complex activity (age 10.3 ± 4.8 years, 36 girls). To avoid a parental selection bias, a control group of 69 healthy blood donors was included for allele frequency analysis. Serum was immediately aliquoted and frozen at −70°C. DNA was routinely isolated from heparinized blood.

MBL genotyping. MBL SNPs at codons 52, 54, and 57 of the *mbl2* gene were typed by pyrosequencing. The MBL genotype of carriers of one or two variant allele(s) (B, C, or D alleles) was designated as A/O and O/O, respectively, whereas the MBL genotype of only wild-type allele carriers at all three positions was designated as A/A.

Promoter SNPs located at positions H/l (−550) and Y/X (−221) were typed by PCR using sequence-specific priming. The conditions for PCR amplification and primer sets that are used in this study are available in the online appendix (available at http://diabetes.diabetesjournals.org). For analysis, MBL genotypes HYA/HYA, HYA/LYA, LYA/LYA, HYA/LXA, and LYA/LXA were considered high-MBL–producing genotypes. Low-MBL–producing genotypes were defined as LXA/LXA, HYA/O, and LYA/O. Genotypes LXA/O and O/O were considered MBL deficient.

MBL concentration. MBL serum concentrations were measured blinded in all serum samples by sandwich enzyme-linked immunosorbent assay essentially as previously described with some modifications (10). Briefly, plates were coated with monoclonal antibody (mAb) 3E7 (anti-MBL mAb provided by Dr. T. Fujita, Fukushima, Japan) at 5 μg/ml. Sera were diluted in PBS containing 0.05% Tween-20 and 1% BSA. MBL was detected using dig-conjugated mAb 3E7, followed by horseradish peroxidase-conjugated sheep anti-dig antibodies (Boehringer).

MBL complex activity. MBL complex activity was measured blinded in all serum samples as previously described (20). Briefly, mannan-coated plates were incubated with human serum diluted in GVB++ (veronal-buffered saline containing 0.5 mmol/l $MgCl_2$ 2 mmol/l $CaCl_2$, 0.05% Tween-20, and 0.1% gelatin; pH 7.5), containing 1 mol/l NaCl, for 16 h at 4°C. Plates were washed with PBS/Tween containing 5 mmol/l $CaCl_2$, followed by incubation with purified C4 (1 μg/ml), diluted in GVB++ for 1 h at 37°C. Activation of C4 was assessed.

HLA typing. All subjects were HLA typed at class 1 using a PCR sequence–specific oligonucleotide probe (Dynal Biotech) and typed at HLA class 2 using standard PCR for sequence-specific polymorphisms.

Autoantibody typing. Glutamic acid decarboxylase and insulinoma antigen 2 antibodies were determined in all serum samples by radiobinding assay as described in great detail previously (21).

C-reactive protein concentration. C-reactive protein (CRP) was measured by use of a sandwich enzyme immunoassay (Kordia) that was based on two polyclonal rabbit antibodies against CRP. The between-assay coefficient of variation was 5.3% at 0.82 mg/l and 5.1% at 8.9 mg/l. The sensitivity of the assay was 1.1 μg/l in our laboratory. All samples were assayed in one batch. Normal values are <20 mg/l.

Fructosamine concentration. In 53 patients, sufficient amounts of serum were available for routine fructosamine serum concentration quantification. Fructosamine was determined on a Roche Integra analyzer, using nitroblue tetrazolium reagent (Roche Diagnostics, Mannheim, Germany).

Statistical analysis. Statistical analysis for group comparison was performed using a Mann-Whitney test. Allele frequency distribution was analyzed using χ^2 analyses with Fisher's exact tests and corrected for the number of comparisons. Correlation was evaluated using the Spearman rank correlation coefficient (r_s). All statistical analyses were performed using GraphPad Prism (GraphPad Software), and $P < 0.05$ was considered significant.

RESULTS

MBL genotype. The allele frequency of SNPs located in exon 1 and the promoter region of the *mbl-2* gene were compared between patients and healthy unrelated control subjects. No significant difference in allele frequency of the exon 1 or promoter SNPs could be observed between patients and healthy control subjects. Full MBL genotype characterization of all patients and sibling control subjects are available in the online appendix.

MBL serum concentration. MBL serum concentration was compared between patients and sibling control subjects. Groups not stratified according to MBL genotype, showed no significant differences ($P = 0.25$, Mann-Whitney test; Fig. 1). When patients and sibling control subjects were divided according to high-MBL–producing, low-MBL–producing, and MBL-deficient genotypes, patients in the high-MBL–producing group had a significantly higher serum MBL level ($P = 0.0018$, Mann-Whitney test; Fig. 1).

MBL complex activity. Next to the MBL concentration, we also examined MBL function by measuring MBL complex activity. When we compared MBL complex activity between patients and sibling control subjects, MBL complex activity was strongly elevated in diabetic patients ($P = 0.01$, Mann-Whitney test; Fig. 2). Stratifying both patients and sibling control subjects according to high-MBL–producing, low-MBL–producing, and MBL-deficient genotypes revealed that MBL complex activity is strongly dependent on MBL genotype in both patients and in sibling control subjects ($P < 0.0001$, ANOVA). However, MBL complex activity was approximately twofold higher in diabetic patients with a high-producing MBL genotype than in the sibling control subjects ($P < 0.00005$, Mann-Whitney test; Fig. 1). No difference between patients and sibling control subjects was observed for the low-MBL–producing and MBL-deficient genotypes.

Comparing MBL concentration and MBL complex activity. The MBL concentration was related to the MBL complex activity in both diabetic patients and the sibling control subjects. MBL concentration was strongly correlated to MBL complex activity in both groups ($P < 0.0001$, $r_s = 0.87$, Spearman test; table available in online appendix). Because the MBL complex activity shows a stronger elevation in patients compared with sibling control subjects than the MBL concentration (Fig. 1), we normalized the amount of MBL by calculating a ratio. The MBL complex activity–to–MBL concentration ratio was compared between patients and sibling control subjects in accordance with the MBL genotype. Patients with a high-MBL–producing genotype showed a significantly increased ratio (mean 1.6) compared with sibling control subjects (mean 1.1) ($P = 0.004$, Mann-Whitney test; Fig. 2*A*).

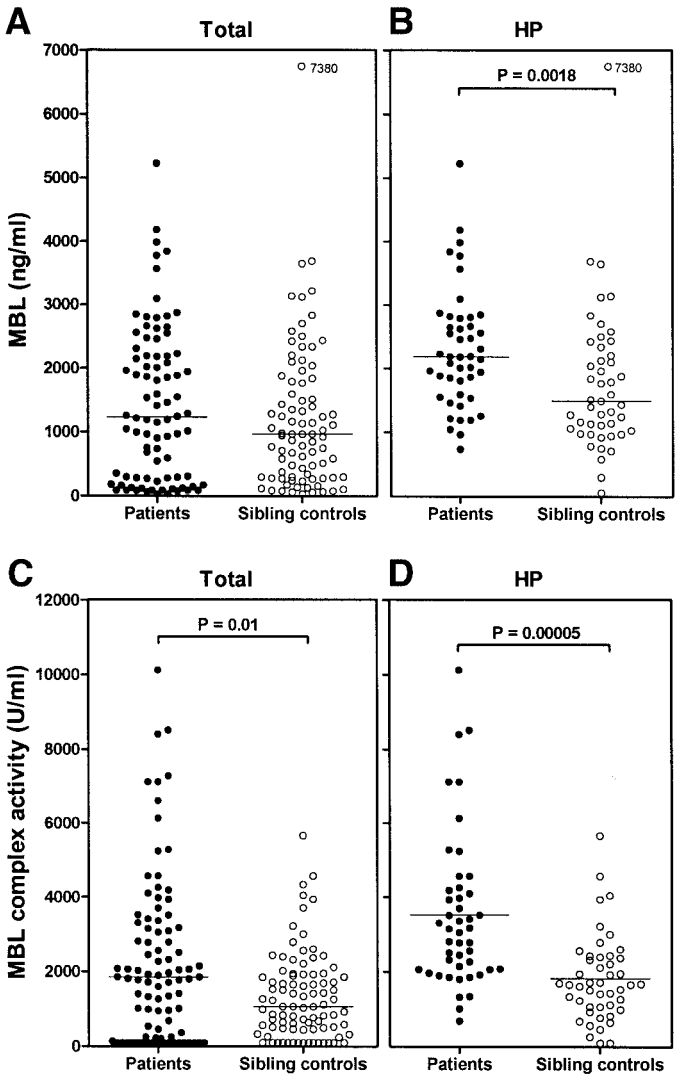

FIG. 1. MBL serum concentration in the total group of diabetic patients (*A*) and diabetic patients with high-producing MBL genotypes (HP) (*B*). Patients with a high-MBL–producing genotype showed an increased MBL serum concentration compared with their sibling control subjects ($P = 0.0018$, Mann-Whitney test). MBL complex activity in the total group of diabetic patients (*C*) and diabetic high-producing MBL genotypes (*D*). When analyzed as a total group, diabetic patients displayed an increased MBL complex activity ($P = 0.01$, Mann-Whitney test), which after stratification according to MBL genotype could be attributed entirely to patients in the high-MBL–producing group ($P = 0.00005$, Mann-Whitney test).

HLA, autoantibodies, and fructosamine concentration. No significant relation was observed between MBL genotype, MBL serum concentration, and MBL complex activity when comparing with the presence of autoantibodies or high-risk HLA types (22) (data not shown). Fructosamine serum concentration correlated with MBL complex activity but not MBL serum levels (overall MBL producers [high MBL producing and low MBL producing] $P = 0.0075$, $r_s = 0.66$; high MBL producing: $P = 0.03$, $r_s = 0.40$; low MBL producing: $P = 0.0076$, $r_s = 0.66$, Spearman test; Fig. 2*B*).

CRP. In all but two diabetic patients, CRP levels were within the normal range (mean ± SD, 1.67 ± 4.73 mg/l; normal range below 20 mg/l). There was no correlation between CRP and either MBL concentration or MBL complex activity (total patient group, CRP vs. MBL concentration: $P = 0.82$, $r_s = -0.024$; CRP vs. MBL complex activity: $P = 0.97$, $r_s = -0.004$; high MBL producing, CRP vs. MBL concentration: $P = 0.32$, $r_s = 0.150$, CRP vs. MBL complex activity: $P = 0.76$, $r_s = 0.046$, Spearman test).

DISCUSSION

Our study demonstrates that serum MBL levels and MBL complex activity are elevated at clinical manifestation in juvenile type 1 diabetic patients with high-MBL–producing genotype compared with sibling control subjects. The complex activity was higher within the group of high-MBL–producing genotypes of type 1 diabetic patients, suggesting that the increase was associated with the immunopathogenesis of type 1 diabetes, rather than genetic variation. Interestingly, the ratio between MBL concentration and MBL complex activity was also significantly higher in the high-MBL–producing patient group, signifying a greater activity per molecule MBL. This indicates that MBL function in new-onset diabetic patients is increased in addition to elevated MBL protein concentration.

The increase in functional MBL activity in diabetic patients could be a result of immune hyperactivity. Although MBL has been suggested to act as an acute-phase protein (23), several studies have been unable to show an association between MBL and CRP as an acute-phase reactant (16,17,24). Our studies confirm the absence of an association between both MBL concentration and MBL complex activity and CRP. This renders acute-phase reactivity as an unlikely explanation for the increased MBL levels and activity in new-onset type 1 diabetes. Alternatively, it could be argued that the significant association of MBL complex activity, but not MBL serum concentration, with fructosamine serum levels implies that this is a consequence of poor glycemic control, rather than a surrogate of immune hyperactivity. Nonetheless, in view of a lack of association of MBL serum levels in either subpopulation of patients with fructosamine levels, we favor the interpretation that the increased serum levels are associated with the immunopathogenesis of type 1 diabetes, while the actual MBL complex activity is affected by glycemic control. Elevated glucose levels resulting in high MBL complex activity could facilitate the adaptive autoimmune response by means of direct opsonophagocytosis of aberrantly glycosylated autoantigens. Finally, it should be appreciated that in plasma, MBL function is dependent on its association with serine proteases (MASPs). Currently, there are three known MASPs, MASP-1, MASP-2, and MASP-3, all of which have a different function. Among these, MASP-2 is responsible for cleavage of C4 and C2 and generation of the C3 convertase C4bC2a (25). It could be hypothesized that an increase in the MBL complex activity on top of an increased MBL serum concentration is a result of preferential binding of MASP-2 to MBL, resulting in a higher C4 splicing ability. Furthermore, in addition to increased MBL serum concentration in high-MBL–producing genotypes, MASP-2 levels could be elevated and result in more prominent MBL complex activity. Finally, it could be hypothesized that the increase in MBL complex activity could be the result of reduced inhibition. Fluid-phase complement inhibitors like C1 esterase inhibitor have been shown to inhibit MASP activity (26). Impairment of complement inhibitors as a result of increasing hyperglycemia could clarify an increased complement activating capacity of MBL with poor glycemic control.

The observation that serum concentration and complex activity were not increased in either the low-MBL–produc-

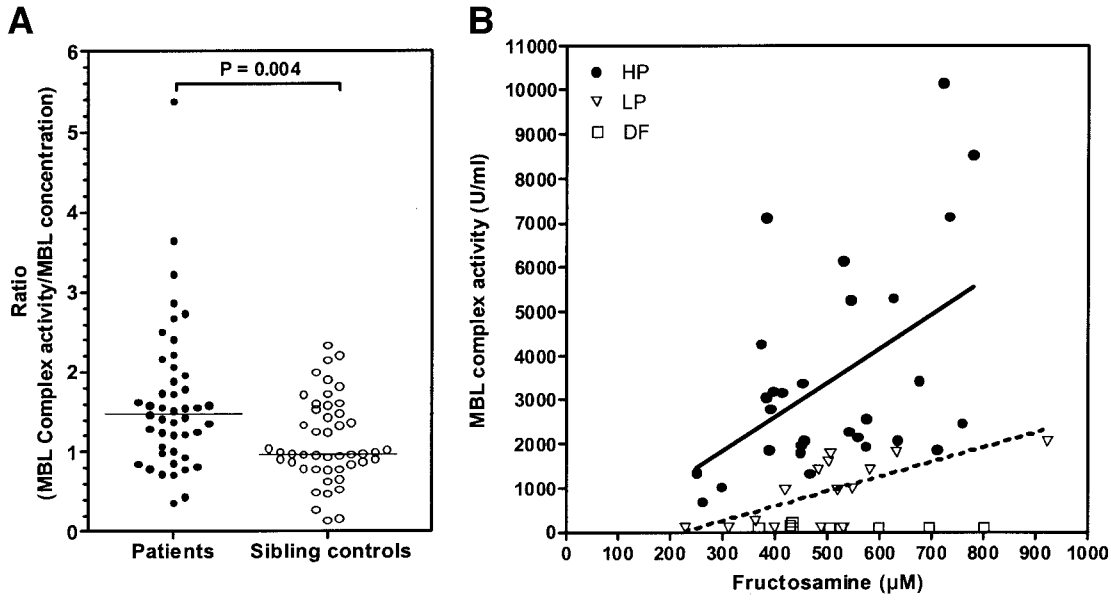

FIG. 2. *A*: Ratio of MBL complex activity to MBL serum concentration. Patients displayed a significantly increased ratio compared with sibling control subjects, signifying greater activity per molecule MBL ($P = 0.004$, Mann-Whitney test). *B*: Correlation between MBL complex activity and serum fructosamine level, stratified according to MBL genotype. Diabetic patients with an MBL-producing genotype (high MBL producing and low MBL producing) showed a significant correlation ($P = 0.0075$, $r = 0.66$). The high-MBL–producing and low-MBL–producing subgroups individually showed a significant correlation ($P = 0.03$, $r = 0.40$; $P = 0.0076$, $r = 0.66$, respectively). Linear regression analysis showed significance in both high-MBL–producing group (solid line) and low-MBL–producing group of diabetic patients (dotted line) ($r^2 = 0.24$, $P = 0.007$; $r^2 = 0.52$, $P = 0.003$, respectively).

ing or MBL-deficient genotypes of MBL in type 1 diabetic patients confirmed our expectation that these genotypes are unable to facilitate a sufficient MBL response in type 1 diabetic patients and in nondiabetic control subjects. In concurrence with our conclusion, previous studies have shown a lack of association between MBL serum levels in diabetic patients and poor glycemic control (27). Interestingly, it has been suggested that an increase in MBL serum concentration as an acute-phase response can be suppressed by intensive insulin therapy, which fortifies our conclusion of the contribution of MBL in the pathogenesis of type 1 diabetes (24). A direct implication would be that low-MBL–producing and MBL-deficient MBL genotypes could have a beneficial effect on type 1 diabetes, because the onset may be less fulminant. In any case, low-MBL–producing genotypes and MBL-deficient genotypes are favorable for diabetic patients, in addition to a potential role of MBL in the pathogenesis, because high MBL serum levels have been shown to be associated with vascular complications (17).

In conclusion, we suggest that elevated MBL levels, resulting in increased complement activation, could assist the autoimmune process of insulitis, pathognomonic for early stages of type 1 diabetes, and act as a marker for ongoing insulitis. This effect may be enhanced by an increased MBL complex activity as a result of poor glycemic control.

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