Mannose-binding lectin: The Dr. Jekyll and Mr. Hyde of the innate immune system.

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CHAPTER 3

MBL and type I diabetes

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

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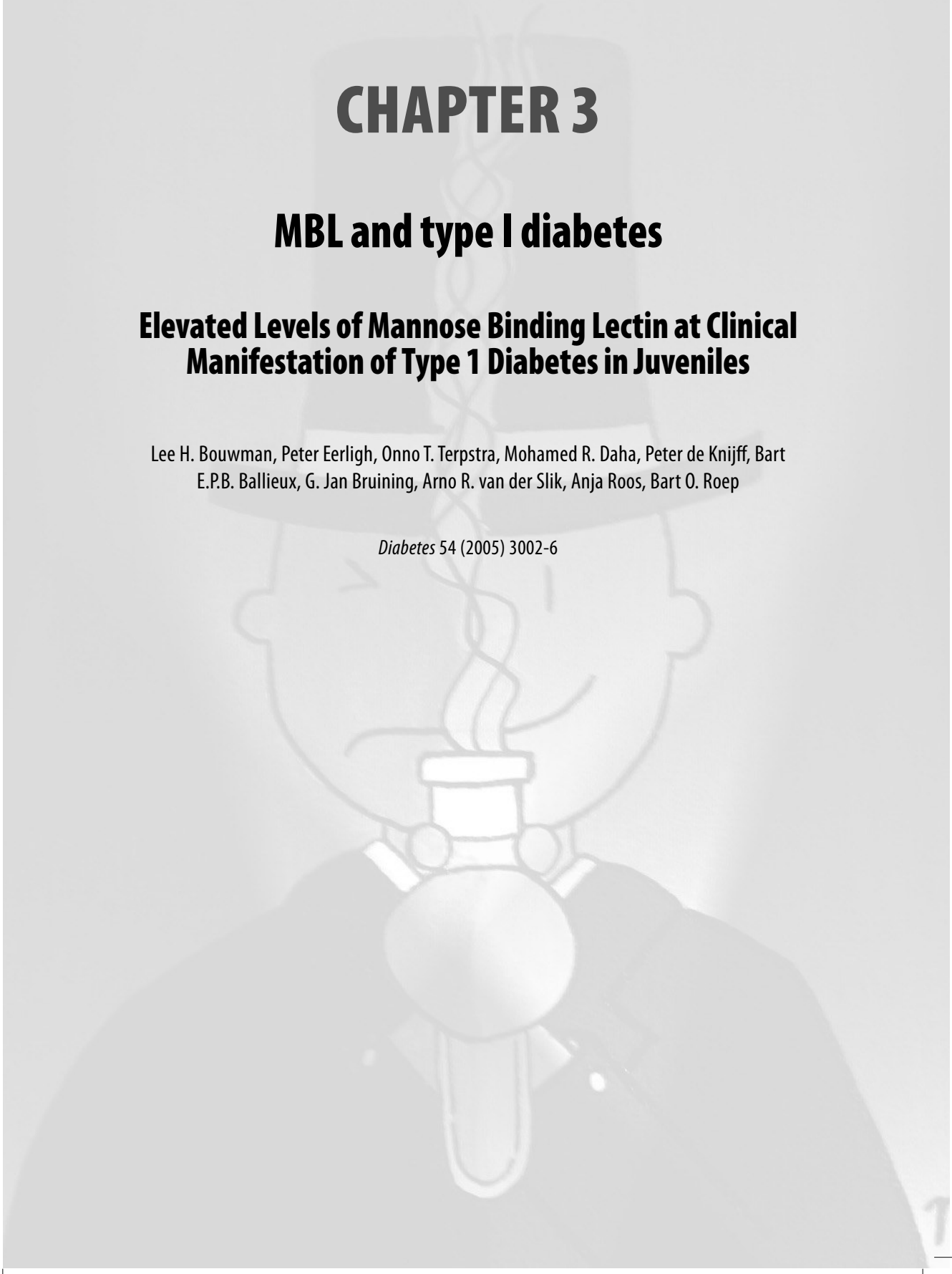

ABSTRACT

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 which 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 non-diabetic siblings and healthy unrelated control subjects. Polymorphisms of MBL exon 1 and promoter were determined and serum concentration and MBL-complex activity were measured. While the genetic polymorphisms of MBL were not different between patients and controls, MBL serum concentration as well as MBL complex activity was significantly higher in new onset diabetic patients compared to 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/MBL concentration ratio ($p=0.004$). We conclude that MBL serum concentration and complex activity is 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.

INTRODUCTION

Type 1 (insulin dependent) diabetes mellitus (T1D) is an autoimmune disease characterized by the specific destruction of beta cells in the pancreas. The etiology of T1D 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 T1D is mounting (3-5). It is generally proposed that the recognition of self-determinants is confined to the adaptive immune system, diminishing the role of the innate immune system in auto-immunity. 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 (MASP). 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', codon 54 (allele 'B') and codon 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% (wildtype = 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 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 T1D. 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 auto-antigens, interweaving MBL in the complex autoimmune process of T1D.

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 was included of every diabetic patient as control. 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.

PATIENTS AND METHODS

Patients

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 females). Diabetes was diagnosed according to the criteria set out by the World Health Association (19). Of every patient, a sibling control was included as control for serological assessment of MBL concentration and MBL complex activity (age 10.3 ± 4.8 years; 36 females). In order to avoid a parental selection bias, a control group of 69 healthy blood donors was included for allele frequency analysis. Serum was immediately aliquotted and frozen at -70°C. DNA was routinely isolated from heparinized blood.

MBL genotyping

MBL SNPs at codon 52, codon 54 and codon 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 wildtype allele carriers at all three positions were designated as A/A.

Promoter SNPs located at positions H/L (-550) and Y/X (-221) were typed by polymerase chain reaction (PCR) using sequence-specific priming (SSP). PCR's were performed in a total volume of 10 µl, containing 10 ng genomic DNA, 3 pmol of each primer, 200 µM dNTPs (Amersham Biosciences), 5% glycerol (Gibco), and 0.5 units of *Taq* DNA polymerase (Promega Life Science). The amplification buffer consisted of 50 mM KCl (Merck), 10 mM Tris-HCl (USB) pH 8.4, 1.5 mM $MgCl_2$ (Promega Life Science), and 0.06 mg/ml bovine serum albumin (BSA; Promega Life Science). We included a PCR accounting for a *growth hormone-1* gene fragment as an internal positive control using 2 pmol of each primer. The primer sequences for the MBL promoter genotyping and internal control are shown in table 1. PCR's were carried out in a Peltier Thermal Cycler (PTC-200; MJ Research). After an initial denaturation step at 95°C for 5 min, touchdown PCR was used to increase the specificity of primer annealing during the first five cycles, consisting of denaturation at 94°C for 30 s, annealing at 65°C→60°C for 30 s and extension at 72°C for 20 s, followed by 30 cycles with an annealing step at 60°C for 30 s. Finally, a 2 min extension was performed at 72°C. For visualization, the amplification products were run on a 1.5% (w/v) agarose MP gel (Boehringer Mannheim) prestained with ethidium bromide. Primer sequences are described in table 1.

The conditions for PCR amplification and primer sets that are used in this study are available on the journal's website (online appendix available from 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 (HP). Low MBL producing genotypes (LP) were defined as LXA/LXA, HYA/O and LYA/O. Genotypes LXA/O and O/O were considered MBL deficient (DF).

Table 1: Primers of Mannose Binding Lectin (MBL) promoter polymerase chain reaction (PCR) sequence specific priming

SNP	Forward primer	Reverse primer	PCR product (bp)
-550			
H	5′-AGGCTGCTGAGGTTTCTTAG-3′	5′-GCTTCCCCTTGGTGTTTTAC-3′	253
L		5′-GCTTCCCCTTGGTGTTTTAG-3′	253
-221			
Y	5′-CATTTGTTCTCACTGCCACG-3′	5′-CTACAATCTGGGTGCAGGC-3′	228
X	5′-CATTTGTTCTCACTGCCACC-3′		228
Control	5′-CAGTGCCTTCCCAACCATTCCCTTA-3′	5′-ATCCACTCACGGATTTCTGTTGTGTTTC-3′	485

H: G variant of the SNP at position -550 of the MBL promoter.
L: C variant of the SNP at position -550 of the MBL promoter.
Y: G variant of the SNP at position -221 of the MBL promoter.
X: C variant of the SNP at position -221 of the MBL promoter.

MBL concentration

MBL serum concentrations were measured blinded in all serum samples by sandwich ELISA essentially as previously described with some modifications (10). Briefly, plates were coated with mAb 3E7 (anti-MBL mAb kindly 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 HRP-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++, containing 1M NaCl, for 16 hours at 4°C. Plates were washed with PBS/Tween containing 5 mM $CaCl_2$, followed by incubation with purified C4 (1µg/ml), diluted in GVB++ for 1 hour 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 (GAD) and insulinoma antigen 2 (IA2) antibodies were determined in all serum samples by radiobinding assay as described in great detail (21).

C-reactive protein concentration

C-reactive protein was measured by use of a sandwich enzyme immunoassay (Kordia) that was based on two polyclonal rabbit antibodies against C-reactive protein. 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 1 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 Nitrobluetetrazolium 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 Chi-square analyses with Fisher exact tests, and corrected for the number of comparisons. Correlation was evaluated using the Spearman rank correlation coefficient (r). 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 controls. No significant difference in allele frequency of the promoter SNPs could be observed between patients and healthy controls (table 2). Allele C located at position 57 of exon 1 of the MBL gene showed a borderline significant increase in frequency in T1D patients before correction (p=0.05, Fisher's exact test, table 2), that was lost after correction for the number of comparisons. We did not observe any significant difference in allele frequencies of the other polymorphic sites of exon 1. Comparing high MBL producing (HP) genotypes, low MBL producing (LP) genotypes and MBL deficient (DF) genotypes between patients and healthy controls, no dissimilarity was observed.

MBL serum concentration

MBL serum concentration was compared between patients and sibling controls. Groups not stratified according to MBL genotype, showed no significant differences (p=0.25, Mann-Whitney test, figure 1). When patients and sibling_controls were divided according to HP, LP and DF genotypes, patients in the high MBL producing group had a significantly higher serum MBL level (p=0.0018, Mann-Whitney test, figure 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 controls, MBL complex activity was strongly elevated in diabetic patients (p=0.01, Mann-Whitney test, figure 2). Stratifying both patients and sibling controls according to HP, LP and DF genotypes, revealed that MBL complex activity is strongly dependent on MBL genotype in both patients and in sibling controls (p<0.0001, ANOVA). However, MBL complex activity was approximately 2-fold higher in dia-

betic patients with a high producing MBL genotype than in the sibling controls ($p<0.00005$, Mann-Whitney test, figure 1). No difference between patients and sibling controls was observed for the LP and DF genotypes.

Table 2: Frequency, phenotype and nomenclature of MBL Single nucleotide Polymorphisms.

Haplotype	**Common reference**	**Phenotype (MBL production)**	**Haplotype frequency**				
			Patients (n=86)		**Controls (n=69)**		
			n	**(%)**	**n**	**%**	**p**
HYA	A	High	53	(30.8)	42	(30.4)	1.00
LYA	A	High/intermediate	46	(26.7)	46	(33.3)	0.21
LXA	A	Low	32	(18.6)	23	(16.7)	0.77
HYD	O	Deficient	12	(7.0)	8	(5.8)	0.82
LYB	O	Deficient	21	(12.2)	18	(13.0)	0.86
LYC	O	Deficient	8	(4.7)	1	(0.7)	0.05
Genotype	**Common reference**	**Phenotype (MBL production)**	**Genotype frequency**				
			Patients (n=86)		**Controls (n=69)**		
			n	**(%)**	**n**	**%**	**p**
HYA/HYA HYA/LYA LYA/LYA HYA/LXA LYA/LXA	HP	High	46	(53.4)	41	(59.4)	0.63
LXA/LXA HYA/O LYA/O	LP	Low	26	(30.2)	22	(31.9)	0.86
LXA/O O/O	DF	Deficient	14	(16.3)	6	(8.7)	0.23

A: MBL wildtype for SNPs located in exon 1 of the mbl-2 gene
O: Varient for SNPs located in exon 1 of the mbl-2 gene
HP: High producing MBL genotype
LP: Low producing MBL genotype
DF: Deficient MBL genotype

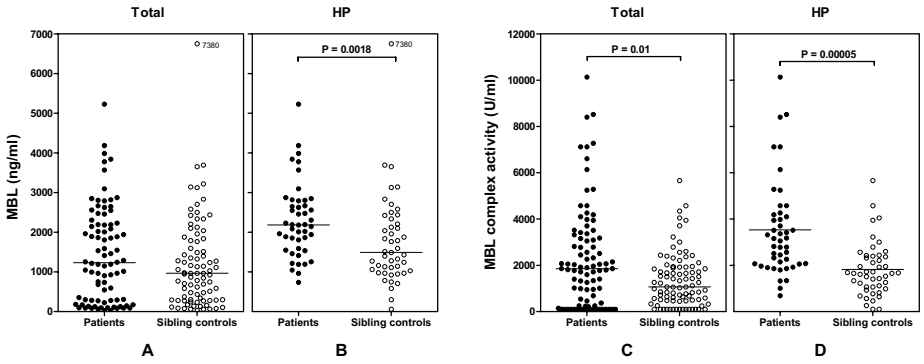

Figure 1. MBL serum concentration in the total group of diabetic patients (A) and diabetic patients with high producing MBL genotypes (HP) (B). Patients with an HP genotype showed an increased MBL serum concentration compared to their sibling controls (p=0.0018, Mann-Whitney test).
MBL complex activity in the total group of diabetic patients (C) and diabetic high producing MBL genotypes (HP) (D). When analyzed as 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 HP group (p=0.00005, Mann-Whitney test).

Comparing MBL concentration and MBL complex activity

The MBL concentration was related to the MBL complex activity in both diabetic patients and the sibling controls. MBL concentration was strongly correlated to MBL complex activity in both groups ($p<0.0001$, $R = 0.87$, Spearman test, figure 3). Since the MBL complex activity shows a stronger elevation in patients as compared to sibling controls than the MBL concentration (figure 1), we normalized the amount of MBL, by calculating a ratio. The MBL complex activity/MBL concentration ratio was compared between patients and sibling controls in accordance to the MBL genotype. Patients with a HP producing genotype showed a significantly increased ratio (mean: 1.6) as compared to sibling controls (mean: 1.1) (p=0.004, Mann-Whitney test, figure 2A).

HLA, autoantibodies and fructosamine concentration

No significant relation was observed between MBL genotype, MBL serum concentration and MBL complex activity when comparing to 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 (HP and LP) p=0.0075, r=0.66; HP: p=0.03, r=0.40; LP: p=0.0076, r=0.66, Spearman test, figure 2B).

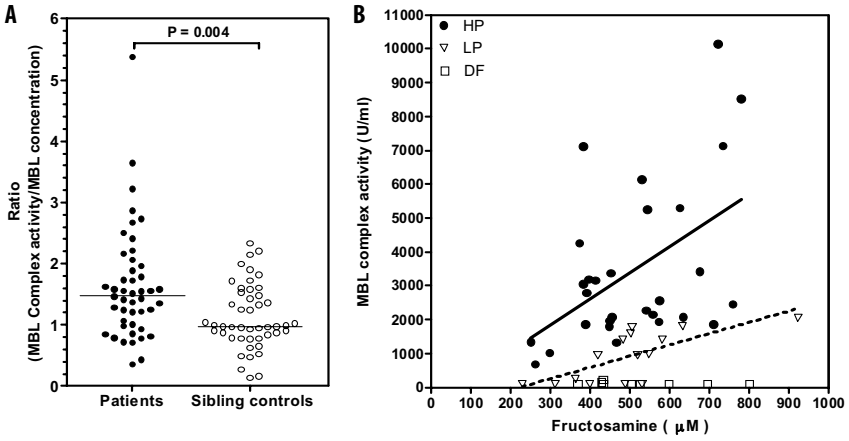

Figure 2. **A**: Ratio MBL complex activity / MBL serum concentration. Patients displayed a significantly increased ratio compared to sibling controls, signifying greater activity per molecule MBL (p=0.004, Mann-Whitney test). **B**: Correlation between MBL complex activity and serum fructosamine level, stratified according to MBL genotype. Diabetic patients with an MBL producing genotype (HP and LP) showed a significant correlation (p=0.0075, r=0.66). The HP and LP 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 HP group (solid line) and LP group of diabetic patients (dotted line) (r^2=0.24, p=0.007; r^2=0.52, p=0.003, respectively).

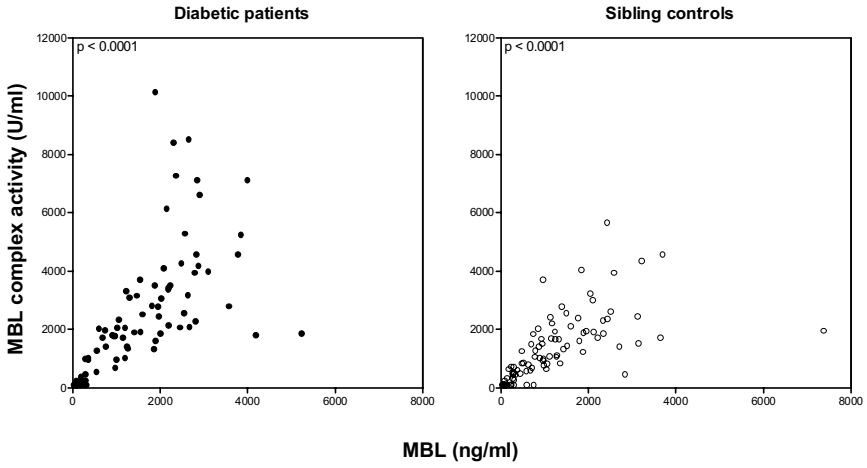

Figure 3. Correlation of MBL complex activity and MBL serum concentration.

C-reactive protein

In all but two diabetic patients, CRP levels were within the normal range (mean: 1.67 mg/L, SD: 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=-0.024, CRP vs. MBL complex activity p=0.97, r=-0.004; HP: CRP vs. MBL concentration: p=0.32, r=0.150, CRP vs. MBL complex activity p=0.76, r=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 HP MBL genotype compared to sibling controls. The complex activity was higher within the group of HP genotypes of T1D 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 HP 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 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 glycosilated autoantigens. Finally, it should be appreciated that in plasma, MBL function is dependent of 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 HP 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 LP or DF genotypes of MBL in T1D patients confirmed our expectation that these genotypes are unable to facilitate a sufficient MBL response both in T1D patients and non-diabetic 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 T1D (24). A direct implication would be that LP and DF MBL genotypes could have a beneficial effect on T1D, as 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, as 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 T1D 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.

Acknowledgments

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