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Differences in MBL levels between juvenile patients newly diagnosed with type 1 diabetes and their healthy siblings

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ABSTRACT

The incidence of type 1 diabetes (T1D) has during the last few decades been increasing in children and juveniles. Multi-factorial courses combining genetic disposition and environmental factors might be in play, and through the years, there has been a mounting interest in the innate immune system's role in the development of T1D.

The aim of this study was to determine mannose binding lectin (MBL) levels in newly diagnosed children with T1D ($n = 481$) over a period of 10 years (1997–2005) and to compare these levels with corresponding levels in their healthy siblings ($n = 479$). Furthermore, the aims were to evaluate if MBL-levels in patients and siblings were influenced by season, age autoimmunity and/or changed over time.

The study found that MBL levels differed between patients and their healthy siblings when adjusted for age, gender, season and period. More patients than siblings had MBL levels above 0.8 μg/ml, associated with high producing MBL genotypes, and the elevated MBL levels were associated with high levels of four T1D related cytokines (IL-1β, IL-12, IL-18 and TNF-α). MBL levels increased during the study period and siblings had seasonal variance in concentrations with the lowest level during wintertime (Dec–Feb).

In conclusion, more patients than siblings had a high MBL level, and high levels of MBL were related to high levels of T1D specific cytokines, supporting a role of the innate immune system and MBL on the risk of developing T1D.

1. Background

Type 1 diabetes (T1D) is an immune-mediated disorder, characterized by an immune-mediated destruction of pancreatic β-cells by T-cells and B-cells. During the last few decades, there has been an increase of T1D incidence in children and juveniles (Svensson et al., 2009). The etiology of T1D is largely unexplained, but properly multi-factorial involving both genetic disposition and environmental factors. The role of the innate immune system in the development of T1D has been of mounting interest (Nerup et al., 1988). The theory is that the innate immune system might prime and promote the adaptive immune system, as it is the first in line in response to environmental factors encountered in the human organism (Beyan et al., 2003; Nerup et al., 1988).

Mannose binding lectin (MBL) is an important component of the innate immune system. Containing multiple carbohydrate recognition domains (CRD), MBL recognizes and binds on the surface of micro-organisms to glycogens including glucose, fructose, mannose, *N*-acetyl-D-glucosamine and *N*-acetyl-mannosamine, in a calcium (Ca2+) dependent manner. MBL is mainly produced by the liver and circulates as a serum protein.

There is evidence that MBL function includes activation of the complement system through the lectin pathway in association with MBL-associated serine proteases (MASPs) (Degn and Thiel, 2013), affecting inflammation by complex modulation of cytokine release (Jack et al., 2001; Wang et al., 2011), facilitating phagocytosis of apoptotic cells by macrophages (Nauta et al., 2003)—and might have a not yet clear role in opsonosphagocytosis (Neth et al., 2002).

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MBL has also been proposed as an acute phase response protein in relation to infection, but this response seems to be highly dependent on genotype (Herpers et al., 2009).

The *MBL2* gene is located on chromosome 10q11.2. Three single nucleotide polymorphisms (SNPs) are located in exon 1, of codons 52, 54 and 57, resulting in three allelic variants and one wild type—respectively, called D, B, C (associated to low serum levels of MBL) and A. Genotype A/A is associated with higher serum MBL levels (Madsen et al., 1994). MBL serum levels are not only dependent on the allelic variant, but also polymorphisms in the promoter region of the gene with the variants *H*/*L* (position −550) and *X*/*Y* (position −221) (Madsen et al., 1995). MBL genotypes are most often divided into low, intermediate and high producing genotypes.

The aim of this study was to compare levels of MBL over a period of nine years in a cohort of Danish children age 0 to 18 years with newly diagnosed T1D, with their healthy siblings. Furthermore, the aims were to monitor the level of MBL over time, the association with pro-inflammatory cytokines and to describe the influence of season, age and/or autoimmunity.

2. Material and method

The study was a population based registry of children with T1D collected from DanDiabKids, formerly The Danish Registry of Childhood Diabetes (The Danish name is listed). The registry was initiated in 1996 and contains 2200 newly diagnosed patients aged 0 to 18 years. It is associated with a Bio-bank, which comprises blood samples from more than 75% of all the children newly diagnosed with T1D in Denmark, and their first degree relative—including more than 1550 blood tests from sibling under the age of 15. For this study blood samples from about 500 patients with T1D were included. Blood samples were collected less than 3 months after onset. For 90% of the siblings the blood sample was taken within 1 month of their relatives with T1D. Date of onset was registered as the date of first insulin-injection administrated. We randomly sampled 500 patients and 500 siblings, with same sample year distribution and with similar age. The material was insufficient in 18 patients and 21 siblings, and therefore they were excluded from the study. Since the patients and siblings were randomly chosen, they were not necessarily related, but all the siblings had a sibling diagnosed with T1D, under the age of 18 years. Blood samples were collected from 1997 to 2005. The serum samples were stored at minus 80 degree Celsius.

Biomarkers: Serum samples were analyzed for MBL, IL-1β, IL-12, IL-18, TNF-α and C-reactive protein (CRP). Using the high-capacity Luminex xMAP technology with an in-house developed 15-plex for simultaneous determination of 15 biomarkers, as described in Skogstrand et al. (2005), Skogstrand (2012). Results concerning cytokines has been published elsewhere (Svensson et al., 2012) and results for chemokines have been accepted for publication. Analysis of MBL has not been described before, but was performed using an in-house made antibody (SSI), as both capture and detection antibody. The detection antibody was in-house biotinylated, as described for CRP (Skogstrand et al., 2005). Working range for MBL was 0.0097-0.5 μg/ml. Seven siblings and 26 patients had MBL levels above the upper working range. CRP was analyzed in a 3-plex analysis of further diluted serum samples, due to the high concentrations of this biomarker. The highest concentration in the working range was for CRP 5.12 μg/ml. The working range for the 15-plex assay was defined as the concentration range for each biomarker, within which the coefficient of variation (standard deviation of repeated measurements divided by the mean) was <20%. The mean intra-assay and inter-assay variation was 11 and 17%, respectively.

MBL levels are determined mainly by the genetic allelic variant, where the variant A/A predominate with high levels of MBL. Previous studies have shown, that MBL serum levels above 0.8 μg/ml identifies individuals, with the A/A genotype with a sensitivity of 91% and a specificity of 95% in healthy Caucasians (Hansen et al., 2003a; Steffensen et al., 2000). Since genetic analysis were not available, we chose to use this cut-off level, to identify individuals with a high producing MBL genotype, well aware that the cut-off was found with other analytical methods and might not be directly transferred to our methods.

Autoantibodies against glutamic acid decarboxylase (GAD65A) and insulinoma associated antigen-2A (IA-2A) were measured in standard radioligand binding assays see previous publication for more details (Svensson et al., 2012).

Statistics: A linear mixed model was used to analyze the impact of different factors on the MBL levels. Since some of the patients and siblings were from the same family, a random effect of family was included. Initially all variables were tested in a univariate model, secondly all variables as fixed effects, were included in one model, except autoantibodies (GAD65A and IA-2A). In the last model adjustment for autoantibody status was included. The co-variates included were gender, age at sampling, autoantibody status (positivity for GAD65A and/or IA-2A), season and date of sampling (approximately the date of diagnosis for patients). MBL was log-transformed before analysis. This implies that the results are reported as relative differences, rather than absolute differences. The season of sampling was divided into four seasons: winter (December, January, February); spring (March, April, May); summer (June, July, August); and autumn (September, October, November). A given effect estimate of e.g. sample date was the difference between children with the same patient status, age, gender, autoantibody status and same season.

The study protocol was approved by The Danish Ethical Committee H-KA-20070009. It was in accordance with the Helsinki-2 Declarations Informed consent was given by the patients, their parents or guardian.

3. Results

3.1. Demographics

The results of this study were based on blood samples from 481 patients and 479 healthy siblings. The gender of 255 patients (53%) and 266 siblings (56%) were male. The mean age was 9.83 years (1.0–18.3 years) for patients and 10.2 years (0.4–16.0 years) for the siblings. There was a positive antibody status in 88% of the patients versus 7% of the siblings. Sibling and patients were statistically comparable for age, gender, season and period. When extracting the 29 sibling with positive antibody status, from the material, it did not alter the statistically results, and therefore we here present the results for the whole group of siblings. Of the patients 18 were immigrants and 13 of unknown origin among. Ethnicities of siblings were unknown, but likely to be similar to patients, since only biologic siblings were included in the register.

3.2. MBL levels in patients versus siblings

Comparing the unadjusted levels for MBL between patients and siblings, shows that patients had a higher MBL concentration ($p < 0.0001$) (Table 1, Fig. 1). This was still significant, when adjusted for age, gender, season and period ($p < 0.0001$) (Table 1). When patients and siblings were sub-divided, according to an MBL concentration >0.8 μg/ml, as a proxy for A/A genotype, we found a higher percentage of patients with MBL >0.8 μg/ml ($p < 0.002$). This was still highly significant, when adjusting for age, gender, period and season ($p = 0.001$). The risk (OR) of having diabetes with MBL levels >0.8 μg/ml was 1.61 (CI95% 1.21; 2.14).