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MANNOSE-BINDING LECTIN:
The Dr. Jekyll and Mr. Hyde of the Innate Immune System

Lee H. Bouwman

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MANNOSE-BINDING LECTIN: The Dr. Jekyll and Mr. Hyde of the Innate Immune System

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Lee Hans Bouwman

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The Wisdom of a Bad Memory

Always remember to forget
The things that made you sad.
But never forget to remember
The things that made you glad.
Always remember to forget
The friends that proved untrue.
But never forget to remember
Those that have stuck by you.
Always remember to forget
The troubles that passed away.
But never forget to remember
The blessings that come each day.
Anonymous

Aan mijn ouders
Voor Vivian

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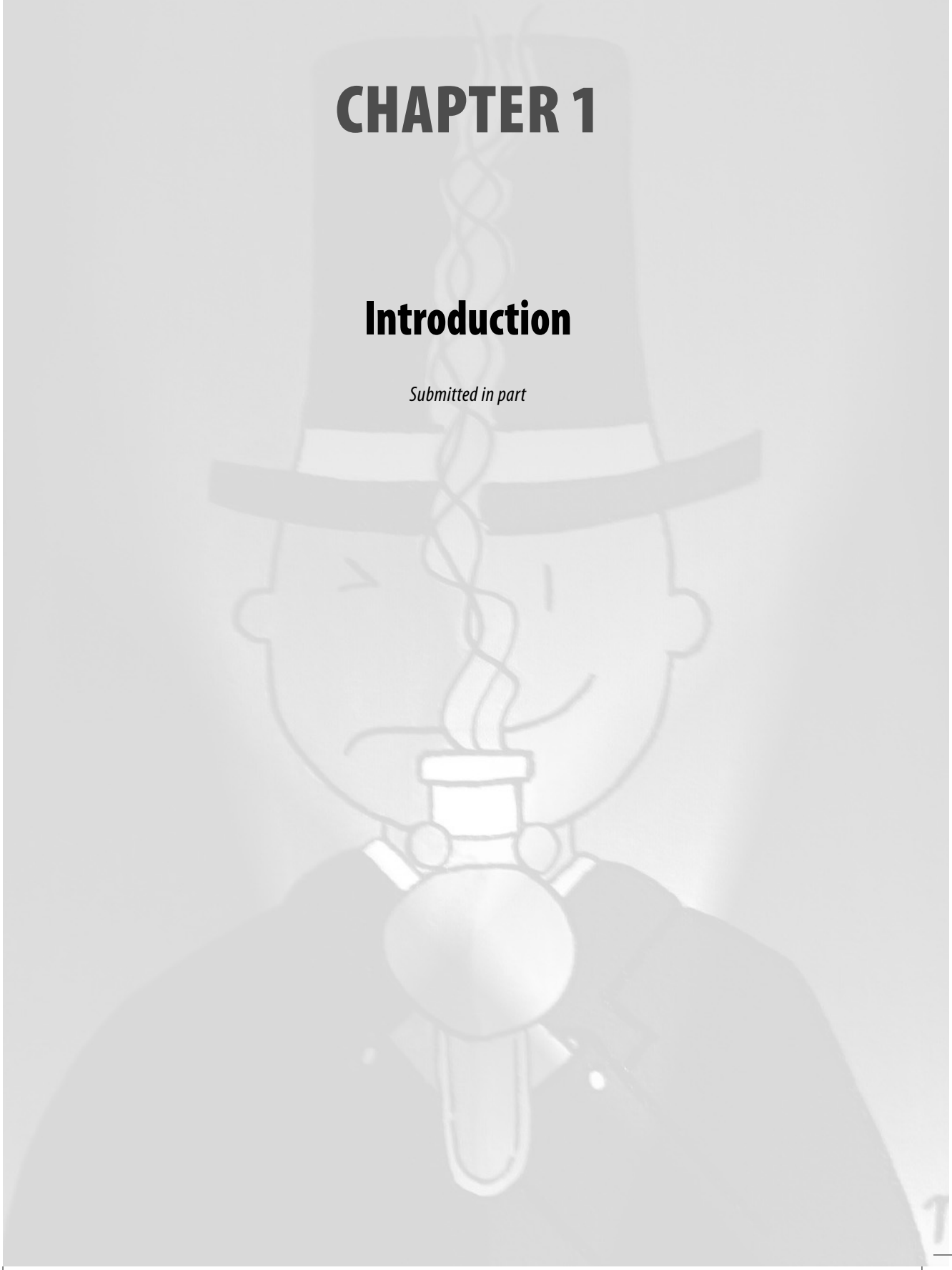
CHAPTER 1

Introduction

Submitted in part



1. INTRODUCTION

The term immunity is derived from the Latin word *immunitas*, which referred to the exemption from various civic duties and legal prosecution offered to Roman senators during their tenures in office. Since Roman times immunity had been taken to mean freedom from infections, which connoted the ability to resist infection. The immune system is a complex assemblage of cells and molecules, which allow the body to function in a habitat crowded with pathogens.

The immune system can be divided in natural or innate immunity and acquired, adaptive or specific immunity. This general and crude division is based on the ability of the adaptive immune system to increase defense mechanisms in magnitude and specificity following exposure to infectious agents, enabling a more rigorous immune response after a second infection. The innate immunity differs from adaptive immunity as it recognizes a restricted array of structures on a broad range of microorganisms, the so-called pathogen-associated molecular patterns. Repeated contact with pathogens always results in a constant innate immune reaction, which is not amplified.

The ability to vastly counteract a great variety of pathogenic microorganisms is of eminent importance for homeostasis. However, profligate functioning of the immune system is disadvantageous under specific circumstances. Two situations in which excessive immunological response is unwanted are autoimmunity and transplantation. The inability of the immune system to distinguish self-tissue and self-proteins from non-self pathogens (i.e. to maintain of self-tolerance) can result in autoimmune disease causing tissue and organ damage. Traditionally, it is assumed that the recognition of self-determinants in autoimmune diseases is confined to the adaptive immune system, ignoring the role of the innate immune system in auto-immunity. More recently it has been argued that, in order to initiate an (auto)immune response, additional signals generated by the innate immune system (i.e. danger signals) are required (1). Evidence is growing that (auto) antigen recognition by the innate immune system, in a certain context, could lead to autoimmunity, either by priming or promoting aggressive immune responses. In transplantation, disproportionate immune responses can cause both acute and chronic graft rejection as the adaptive immune system may consider the graft hazardous.

The present thesis underscores the current concept of collaboration between the innate and adaptive immune system by showing close interactions between both immune systems. This thesis shows that several constituents of the complement system, a core component of innate immunity, are highly allied with the adaptive immune system. Furthermore the involvement of innate immunity in type 1 diabetes, generally accepted as an adaptive immune system-mediated autoimmune disease is addressed.

In the following paragraphs, a general overview will be given of a specific component of the innate immune system, the complement system. The most recently discovered pathway of the complement system, the lectin pathway and particularly its activating molecule mannose binding lectin (MBL) will be discussed in greater detail. After having outlined the current view on association between MBL and various diseases and disease complications, the role of MBL in transplantation will be addressed. Subsequently, the role of the adaptive immune system will be discussed in pancreatic islet transplantation. Finally, the aim and content of the studies in this thesis will be presented.

2. THE COMPLEMENT SYSTEM

The complement system consists of a variety of functionally linked proteins, including complement factors classified as C1 to C9, which act in conjunction and result in many effects in humoral immunity and inflammation. The five principal biological functions of the complement system are: 1) complement-mediated cytolysis; 2) opsonisation of foreign organisms; 3) activation of inflammation; 4) clearance of self and non-self debris; and 5) amplification of adaptive immunity.

The complement system consists of three pathways, the classical, the alternative and the lectin pathway. All pathways are activated differently, however they converge in a shared terminal pathway and result in the same basic effects. The classical pathway is activated when the first classical pathway component C1, binds to the fragment crystalline (Fc) portion of an immune complex. Activation of the alternative pathway occurs when complement factor C3b binds to various activating surfaces, like microbial cell walls. Finally the lectin pathway, a recently discovered complement activation pathway, is triggered when MBL binds to common carbohydrate structures of a variety of microorganisms (including bacteria, viruses and fungi) (2-6).

Central in the complement system is the complement factor C3. All activated pathways result in generation of a C3 convertase, facilitating the proteolytic cleavage of complement factor C3 into C3a and C3b. Subsequent binding of C3b to C3 convertase enzymes allow the formation of C5 convertase, enabling proteolytic cleavage of the complement factor C5. Upon C5 cleavage, all pathways converge and enter the terminal pathway of complement activation, eventually resulting in the formation of a so-called membrane attack complex (MAC), a lipid-soluble pore structure which causes osmotic lysis of cells (figure 1). Cleavage products C3a and C5a are potent chemotactic products amplifying inflammation. C3b facilitates both opsonisation of foreign organisms and clearance of immune complexes.

As the MBL forms a major topic of the present thesis, the lectin pathway will be discussed in greater detail in the next section.

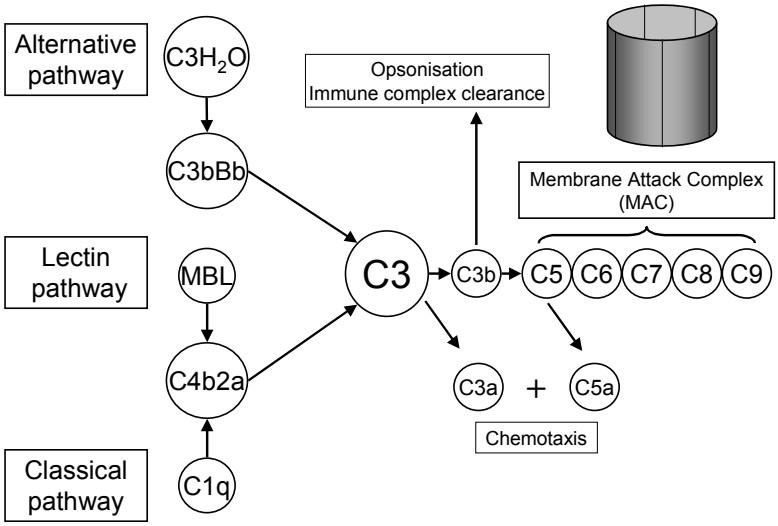


Figure 1: A bird's eye view of the complement system.

3. MANNOSE BINDING LECTIN

THE PLURIPOTENT MOLECULE OF THE INNATE IMMUNE SYSTEM

Mannose binding lectin, also referred to as mannan binding lectin or mannan binding protein, is believed to be a central player in the innate immune response. The first case of an association of MBL deficiency and infectious disease dates back to 1968. A small girl, suffering from severe dermatitis, consisting diarrhea and recurrent bacterial infections indifferent to antibiotic and steroid therapy, was reported. Hematological examination revealed a defect in the phagocytosis of yeast particles from *Saccharomyces cerevisiae*, rice starch and *Staphylococcus aureus* in polymorphonuclear leukocytes. This defect was serum-dependent. Infusion of fresh plasma corrected the phagocytic deficiency. As the same phagocytic defect was observed in several direct relatives of the patient, it was concluded that this condition had a genetic origin (7).

In order to fully appreciate the implication of MBL in clinical settings, biological characteristics of MBL will be discussed prior to focusing on the association of MBL with various diseases.

3a. MBL Characteristics

Mannose binding lectin is a C-type serum lectin and is presumed to be produced by the liver. MBL is build up out of 96 kDa structural units, which in turn are composed of three identical 32 kDa primary subunits. The subunits consist of an N-terminal cross-linking region, a collagen-like domain and a C-terminal carbohydrate-recognition (CRD) domain (8). Circulating MBL is comprised of higher-order oligomeric structures, which include dimers, trimers, tetramers, pentamers and hexamers of the structural homotrimeric unit. The oligomeric configuration of the structural units allows the MBL molecule to have multiple CRDs facilitating multivalent ligand binding (figure 2). Each CRD of MBL is structurally identical and is able to bind a wide range of oligosaccharides including N-acetylglucosamine, mannose, N-acetylmannosamine and fucose (5). Although the various sugars are bound with different affinities, the cluster-like array of multiple binding sites allows activation of the complement activation to be most effective. MBL is considered to play its major role in innate defense against pathogens, involving recognition of arrays of MBL-binding carbohydrates on microbial surfaces. However, more recent studies have shown that MBL is also involved in the recognition of self-targets, such as apoptotic and necrotic cells.

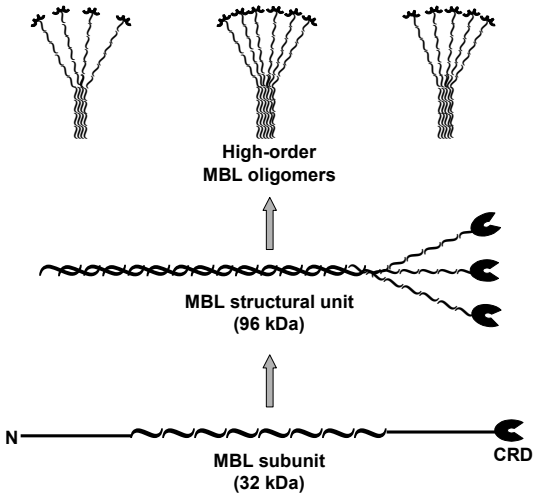


Figure 2: Mannose binding lectin (MBL) structural build-up.
MBL is composed of 32 kDa subunits. The primary subunits combine to form a trimeric MBL structural unit, which in term forms high-order oligomers. (figure modified from D.P. Eisen and R.M. Minchinton (22)).

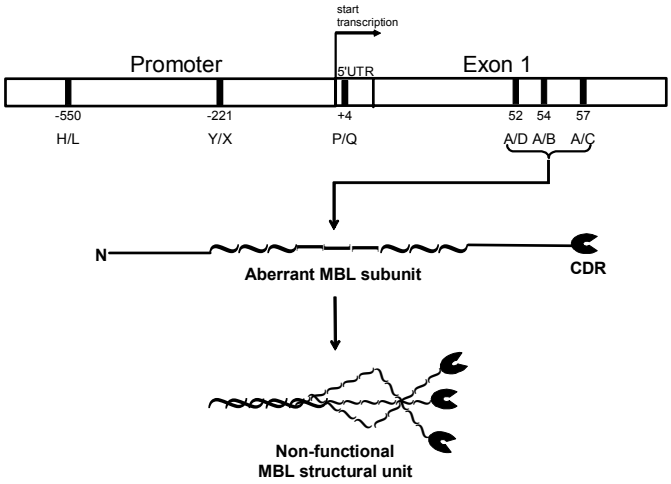


Figure 3: Location of single nucleotide polymorphisms located in the promoter and exon 1 of the *mbl-2* gene.
Promoter SNPs are located at positions -550 (H/L variant), and -221 (X/Y variant), both G to C nucleotide substitutions. Another SNP is located in the 5′-untranslated portion of the MBL gene, at position +4 (P/Q variant). Exon 1 SNPs are located at codon 52 (Arg→Cys; allele 'D'), codon 54 (Gly→Asp, allele 'B') and codon 57 (Gly→Glu, allele 'C').
The SNPs of exon 1 result in aberrant formation of MBL subunits, which are unable to form functional MBL structural units and high-order oligomers.

In plasma, MBL is associated with MBL-associated serine proteases (MASP) (9). Currently, three MASPs have been identified, MASP-1, MASP-2 and MASP-3 (10-12). Although the function of MASP-1 and MASP-3 remains subject to debate, there is a general consensus of the role of MASP-2, which is responsible for cleavage of C4 and C2, generation of the C3 convertase C4b2a and subsequent complement activation.

Exon 1 of the *mbl-2* gene, which is located at chromosome 10, contains three known single nucleotide polymorphisms (SNPs) at codons 52 (CGT to TGT; Arg → Cys), referred to as allele 'D', codon 54 (GGC to GAC; Gly → Asp, allele 'B') and codon 57 (GGA to GAA; Gly → Glu, allele 'C') (8). All SNPs of exon 1 result in altered collagenous regions and as a consequence, interfere with the formation of high-order oligomers. This impairment of polymerization causes low serum levels of high molecular weight MBL and impaired MBL function. 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) (13). 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), both G to C nucleotide substitutions. Furthermore a polymorphic site is located at position +4 of the 5'-untranslated portion of the *mbl-2* gene (P/Q variant, C→T) (14-16) (figure 3). The common allele A of exon 1 is associated with the following haplotypes: HYPA,

LYPA, LYQA and LXPA with high, high-intermediate, intermediate and low promoter activity (17). Although there is great variety of MBL levels between the different haplotypes, it has been advocated in order to ease interpretation, to only show the most significant promoter allele in position –221 (X/Y), which is only found in normal A haplotype background (YA or XA) exhibiting high and low promoter activity and serum MBL levels (14). The structural alleles carry the following haplotypes: LYPB, LYQC and HYPD.

3b. MBL and associated diseases

MBL has been studied in a great diversity of diseases. Both decreased and elevated serum levels of MBL and different SNPs of the *mbl2* gene and its promoter have been associated with a variety of diseases, indicating the Jekyll-and-Hyde character of MBL. In order to structure the discussion of this double-edged sword phenomenon, involvement of MBL in different diseases will be discussed according to the aetiology.

MBL and infectious diseases

When the adaptive immune response is either immature or compromised, the innate immune system constitutes the principle defense against infection. A logical consequence of impaired MBL function would be an enlarged susceptibility to infectious disease. The phenomenon of an increased incidence of infectious disease in MBL-deficient patients has been shown in pediatric patients and in immune compromised patients. However it also has been shown that adult patients with recurrent infectious disease are more likely to have insufficient serum MBL levels.

MBL and bacterial infections

Pediatric patients are still in the developmental stage of the adaptive immune system and rely to a great extent on their innate immune system to counteract infectious pathogens. In support of the theory that MBL has an important protective role in early childhood is a British study amongst 266 pediatric patients (mean age 3.5 years) suffering from meningococcal disease (18). Showing a clinical association between MBL variant alleles and meningococcal disease, the authors of this study suggested that genetic variants of MBL gene might account for a third of all meningococcal disease cases.

Patients undergoing myeloablative bone marrow transplantation or cytotoxic chemotherapy are severely immune-compromised. MBL deficiency has been shown to be associated with severe bacterial infections after chemotherapy and major infections following allogeneic hemopoietic stem cell transplantation (19-21).

In immune-competent Caucasians, it has been suggested that homozygotes for MBL exon 1 codon variants could have an increased risk of invasive pneumococcal

disease (22; 23). Considering post-operative infections a surgical complication, low MBL levels are associated with significantly increased infection rates (24).

In vast contrast to the protective properties of MBL against extracellular bacterial infections is the observation that mycobacterial infections (*Mycobacterium tuberculosis and M. leprae*) occur more frequently in patients with increased serum MBL levels. Complement-mediated enhanced phagocytosis as a result of opsonization has been suggested to facilitate these intracellular infections (25).

MBL and virus infections

MBL has been studied in relation to various viruses. Persistent hepatitis B virus infection has been reported to be associated with the variant alleles located at codons 52 and 54 of the MBL gene, responsible for low MBL serum levels (26; 27). Furthermore it has been suggested that high MBL serum levels are associated with increased survival rates among Japanese patients with hepatitis B (28). MBL in hepatitis C has been studied to lesser extent and appears to be somewhat contradictive (22; 29-31) .

The role of MBL in HIV infection and progression has been a conflict of debate over the last years. The envelope protein gp120 of the HIV-1 virus is highly glycosylated with N-linked carbohydrates, enabling MBL to bind (32; 33). Although it is conceivable that MBL-mediated complement activation could facilitate the immune response directed against HIV infection, reports on the effects of MBL on HIV infection and progression are contradictory.

Although common pediatric virus infection, including RSV and EBV, lack association with MBL (34-36), it has been shown that MBL is able to neutralize the influenza A virus and inhibit the spread of this virus (37; 38).

MBL and autoimmunity

It is generally assumed that the recognition of self-determinants is confined to the adaptive immune system, neglecting the role of the innate immune system in autoimmunity. However, evidence is growing that the innate immune system could lead to autoimmunity, either by priming or promoting aggressive immune responses (39; 40). 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 (41; 42). A major current pathophysiological concept of autoimmunity is impaired apoptotic cell clearance. MBL has been shown to facilitate the clearance of apoptotic cells (43; 44). A result of cells going into apoptosis is alteration of membrane carbohydrates leading to increased expression of fucose and *N*-acetyl-glucosamine (45; 46). Redistribution or clustering of glycoproteins has been suggested to enable MBL to bind to these carbohydrates expressed on apoptotic cells, thereby facilitating clearance (47; 48).

When studying the association between MBL and Rheumatoid arthritis, it has been shown that MBL is able to bind to rheumatoid factor (RF) complexes and as a consequence could assist RF clearance by the reticuloendothelial system (49; 50). Although several other studies have not yet been able to reproduce these findings (51), the observations that MBL insufficiency is associated with both elevated IgM RF and increased joint erosions, inflammation and early disease onset are in support of the MBL RF clearance theory (52-56).

MBL and transplantation

Tissue damage and impaired organ function as a result of ischemia/reperfusion (I/R) injury still remain enormous predicaments in solid organ transplantation. The hypoxic state to which an organ is subjected during organ harvesting, transport and implantation, result in activation of various immunological events (57-60). The complement system plays an important role in mediating tissue injury after oxidative stress. Activation and deposition of complement on the vascular endothelium following oxidative stress has been shown (61-63) and more interestingly, tissue injury after I/R is significantly reduced by complement inhibition (64; 65). Complement activation via the lectin pathway has been shown following oxidative stress, indicating that inhibition of MBL could be a novel approach in reducing ischemia/reperfusion damage (66;

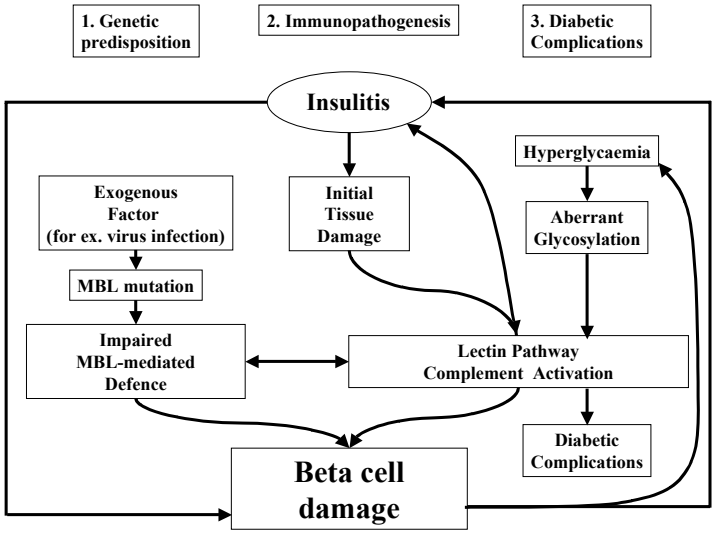


Figure 4: Theoretical association between MBL and type 1 diabetes.
The genetic predisposition (1) of low MBL serum levels could result in an impaired clearance and inactivation of pathogens responsible for beta cell destruction. Contrarily, high MBL serum levels during insulitis could facilitating further beta cell damage and fulminant insulitis (2). Finally, aberrantly glycosylated proteins could result in MBL mediated complement activation and result in subsequent tissue damage (i.e. diabetic complications) (3).

67). In support of the involvement of MBL in I/R injury is the fact that MBL-depositions were observed early after transplantation of ischemically injured kidneys (68). Furthermore, it has been suggested that high MBL levels are associated with a more severe form of rejection leading to graft loss in kidney transplantation (69).

MBL and diabetes

A major source of mortality and morbidity in diabetes is caused by microvascular complications, as a substantial portion of diabetic patients develop diabetic nephropathy and retinopathy. MBL has been associated with diabetic microvasular complications. Several studies have shown the association between an increased risk of developing renal failure and high MBL producing genotypes in diabetic patients (70-72). The involvement of MBL in the pathogenesis of diabetic nephropathy now appears to be appreciated, however the exact immunological process remains to be studied (figure 4). Controversially, it has been suggested that high MBL serum levels may predict a decreased likelihood of myocardial infarction in diabetic patients (73).

4. ADAPTIVE IMMUNITY IN ISLET TRANSPLANTATION

Type 1 (Insulin Dependent) Diabetes Mellitus (T1D) is an autoimmune disease characterized by the specific destruction of beta cells in the pancreas resulting in the loss insulin production. The aetiology of T1D is multifactorial, consisting of genetic predisposition and environmental factors possibly including a variety of viruses and dietary components (74-78). Currently insulin substitution is the most common therapy for patients with T1D. A potential novel therapy for diabetes is transplantation of insulin-producing beta-cells of isolated pancreatic islets. Two major immunological hurdles have to be tackled in order to obtain successful islet transplantation. As the autoimmune response of T1D potentially can destroy the transplanted islets, the recurrence of T-cell autoreactivity against islet determinants, needs to be prevented. In addition, induction of alloimmunity to donor antigens, has to be inhibited (79-81). The attractive and recently successful therapy of transplanting pancreatic islets in T1D is overshadowed by the need for permanent immune suppression. Without the administration of these non-specific and potentially harmful immunosuppressive drugs, graft failure seems inevitable. Islet transplantation is thus limited to diabetic patients already receiving immune suppression for a previous organ transplant, or to patients with severe hypoglycemia unawareness or uncontrollable hyperglycemia. The introduction of a new glucocorticoïd-free immunosuppressive regime, the so-called Edmonton protocol, has improved the outcome of islet transplantation considerably (82). This protocol includes sirolimus, tacrolimus and dacluzimab. All

these immunosuppressive drugs share the same basic quality that they all inhibit T-cell stimulation and proliferation, identifying once again T-cells as key-players in this rejection process (83).

Prediction and prevention of ongoing beta-cell destruction after islet transplantation, resulting in long-term graft survival is of utmost importance. In order to be able to optimise the current islet transplantation, it is essential to study the reaction of T-cells to islets.

5. SCOPE OF THE THESIS

The scope of the current thesis is to obtain insight in immunological aspects of transplantation and diabetes. As previously stated, the present thesis underscores the current concept collaboration between the innate and adaptive immune system by showing close interactions between both immune systems. Mannose binding lectin as a major recognition molecule of the lectin pathway and as a key protein of the immune system was studied in relation to its functional characteristics. Appreciating the Jekyll-and-Hyde character of MBL and the fact that MBL serum levels and functionality are under strict genetic control, MBL was studied under distinct pathological conditions. **Chapter 2** describes molecular and biological aspects of mannose binding lectin and the interaction of MBL with the adaptive immune system. The first part focuses on functional characterizion of the lectin pathway of complement. The second part discusses the possible compensation of MBL deficiency by antibodies and the classical complement pathway as an example of interaction between adaptive and innate immunity. Furthermore, it describes the functional consequences of genetic MBL polymorphisms for the activation of the lectin pathway in serum of healthy individuals. In the last part of this chapter, lectin pathway complement activation via human IgA is described, presenting a novel linkage between innate and adaptive immunity. **Chapter 3** focuses on the involvement of MBL in autoimmunity, by studying juvenile type 1 diabetic patients at disease onset. Prior to starting the study, it was hypothesized that MBL could be associated with type 1 diabetes in three different manners, as shown in figure 4. The genetic predisposition of low MBL serum levels could result in an impaired clearance and inactivation of pathogens responsible for beta cell destruction. Contrarily, high MBL serum levels could contribute to complement activation and inflammation via the recognition of injured tissue, facilitating further beta cell damage and aggravating insulitis progression. Finally, aberrantly glycosylated proteins, as a consequence of the diabetic state, could result in MBL-mediated complement activation resulting in subsequent tissue damage in eyes, kidneys and in various vascular structures (i.e. diabetic complications).

The last theory has been shown in several different studies during the completion of this thesis (70-72; 84). **Chapter 4** addresses the role of the liver in production of serum MBL and to evaluates the effect of MBL variant alleles on the susceptibility to infection after liver transplantation.

Having studied the role of innate immunity in new onset diabetic patients and transplantation, **chapter 5** focuses on the effect of the adaptive immune system on islet transplantation, a novel treatment of type 1 diabetes. The aspects of HLA incompatibility in human pancreatic islet transplantation and the subsequent adaptive allogeneic immune response are discussed. Finally, the results and conclusions of all studies will be summarized and discussed in **chapter 6**.

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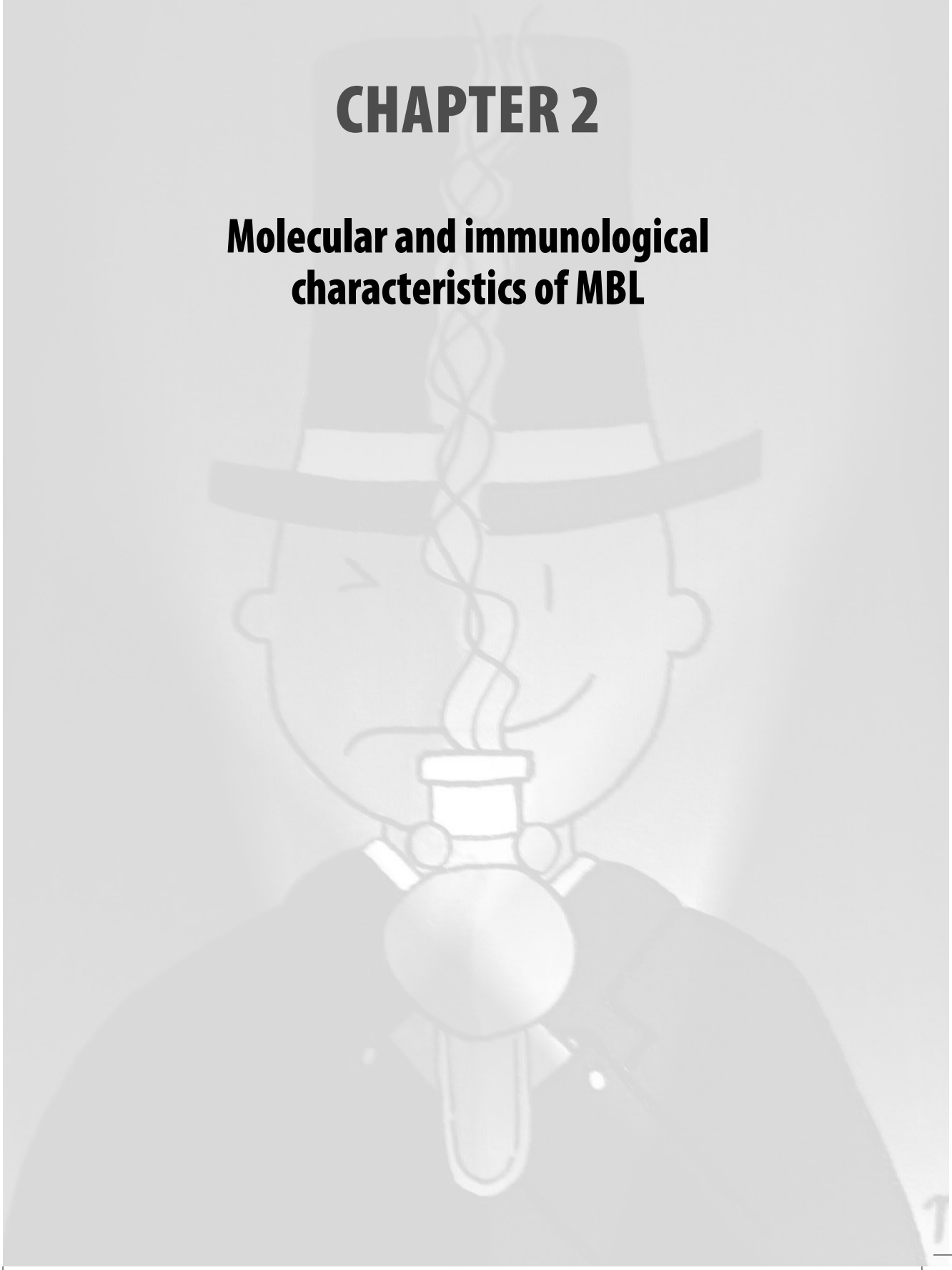
CHAPTER 2

Molecular and immunological characteristics of MBL



CHAPTER 2.1

Functional characterization of the lectin pathway of complement in human serum

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ABSTRACT

Mannan-binding lectin (MBL) is a major initiator of the lectin pathway (LP) of complement. Polymorphisms in exon 1 of the MBL gene are associated with impaired MBL function and infections. Functional assays to assess the activity of the classical pathway (CP) and the alternative pathway of complement in serum are broadly used in patient diagnostics. We have now developed a functional LP assay that enables the specific quantification of autologous MBL-dependent complement activation in human serum.

Complement activation was assessed by ELISA using coated mannan to assess the LP and coated IgM to assess the CP. Normal human serum contains IgG, IgA and IgM antibodies against mannan, as shown by ELISA. These antibodies are likely to induce CP activation. Using C1q-blocking and MBL-blocking mAb, it was confirmed that both the LP and the CP contribute to complement activation by mannan. In order to quantify LP activity without interference of the CP, LP activity was measured in serum in the presence of C1q-blocking Ab. Activation of serum on coated IgM via the CP resulted in a dose-dependent deposition of C1q, C4, C3, and C5b-9. This activation and subsequent complement deposition was completely inhibited by the C1q-blocking mAb 2204 and by polyclonal Fab anti-C1q Ab. Evaluation of the LP in the presence of mAb 2204 showed a strong dose-dependent deposition of C4, C3, and C5b-9 using serum from MBL-wildtype (AA) but not MBL-mutant donors (AB or BB genotype), indicating that complement activation under these conditions is MBL-dependent and C1q-independent. Donors with different MBL genotypes were identified using a newly developed oligonucleotide ligation assay for detection of MBL exon 1 polymorphisms.

We describe a novel functional assay that enables quantification of autologous complement activation via the LP in full human serum up to the formation of the membrane attack complex. This assay offers novel possibilities for patient diagnostics as well as for the study of disease association with the LP.

INTRODUCTION

Activation of the complement system is an important component of host defense. Following infection, triggering of the complement activation cascade via direct binding of complement components to microbial surfaces may lead to opsonization and pathogen elimination via humoral and cellular mechanisms. Furthermore, complement activation may trigger and amplify the acquired immune system. Until now, three different pathways of complement activation have been described, i.e. the classical pathway, the alternative pathway, and the lectin pathway. These pathways converge at the level of C3, leading to activation of the common terminal complement pathway and finally formation of the membrane attack complex (1; 2).

Defects in the complement system may lead to a partial or complete blockade of the complement activation cascade. Depending on the level of the defect, either the induction phase or the effector phase of complement activation may be hampered, and the defect may affect more than one pathway. Impaired function of the complement system may occur due to genetic defects, or due to acquired deficiencies of complement components. Acquired complement deficiencies may occur due to formation of autoantibodies to complement components or due to excessive complement consumption (1-3). Genetic complement deficiencies have been described at all levels of the system, i.e., in the classical pathway, in the alternative pathway, in the lectin pathway, and in the terminal pathway from C3 to C9 (4).

Most complement defects are associated with disease, ranging from a relatively mild increase in the susceptibility to infections to the occurrence of a severe systemic autoimmune syndrome. Furthermore, impaired complement function is associated with the occurrence of flares in patients with systemic lupus erythematosus (SLE) (1; 2; 4). Therefore, functional assays to measure complement activity in human serum have a clear diagnostic and prognostic value.

Complement function in serum is mostly measured using hemolytic assays that enable the functional assessment of the classical complement pathway and the alternative complement pathway, respectively. In these hemolytic assays, the function of the complement pathways is expressed as its ability to generate the C5b-9 complex upon activation. However, such an assay is currently not available for the evaluation of the lectin pathway of complement in serum.

The lectin pathway of complement (LP) is mainly driven by binding of mannan-binding lectin (MBL) to one of its carbohydrate ligands (5). Binding will induce activation of the MBL-associated serine proteases (MASP) leading to formation of the C3 convertase C4b2a (6-8). The MBL-MASP complex, being the recognition complex of the LP, has a strong structural and functional similarity to the C1 complex, the recognition unit of the classical complement pathway (CP). The C1 complex, consist-

ing of C1q and the serine proteases C1r and C1s, is mainly activated by binding of C1q to immune complexes comprising IgG or IgM, also leading to the generation of C4b2a.

The gene encoding human MBL is characterized by a high degree of polymorphisms, both in the promoter region and in exon 1 (9). In the promoter region, various single nucleotide polymorphisms (SNP) have been described that are involved in quantitative gene expression and hence determine the MBL plasma concentration. Furthermore, at least five different SNPs have been discovered in exon 1 of the MBL gene, encoding the collagenous region of MBL (10-13). At codon 52 (D genotype), codon 54 (B genotype) and codon 57 (C genotype), SNPs are frequently present: the allele frequency in the Caucasian population is 5% (D allele), 13% (B allele) and 2% (C allele), respectively (9). These SNPs induce amino acid substitutions that affect the polymerization of the MBL molecule in a dominant way. Accordingly, small-sized MBL molecules are generated with impaired functional properties (14-16).

The presence of MBL-mutant alleles is associated with increased susceptibility to infections, mainly in childhood and in immune-compromised individuals (17-20). Furthermore, the above-described SNPs confer an increased progression of severe chronic diseases such as cystic fibrosis, rheumatoid arthritis, and SLE (21-23). Therefore, since there is such a high inter-individual variability in expression of (functional) MBL, which is determined by multiple variables, functional assessment of LP activity in human serum generates novel and most likely clinically more relevant possibilities for risk assessment for individual patients.

We have now developed an ELISA-based LP assay that enables the functional evaluation of successive steps of autologous complement activation in full human serum without any interference of the CP. Measurement of the activity of the CP and the alternative complement pathway (AP) in a similar ELISA system provides the possibility of parallel quantification of all three complement activation pathways in patient serum using one assay system.

MATERIALS AND METHODS

Human materials

Human serum was obtained from 70 healthy adult volunteers and immediately frozen at -80°C in aliquots. Genomic DNA was isolated from heparinized blood as described below. Human DNA samples with known MBL gene polymorphisms were kindly provided by Dr. P. Garred, (Copenhagen, Denmark). Outdated healthy donor plasma was obtained from the Bloodbank Leiden-Haaglanden, Leiden, the Nether-

lands. From a patient with Waldenström's macroglobulinemia, plasma was obtained that became available after a plasmapheresis treatment.

Anti-C1q and anti-MBL antibodies

Monoclonal antibodies directed against C1q were produced in mice as described before (24). The anti-C1q mAb 2204 (IgG1) is directed against the globular head domain of C1q and is able to inhibit the binding of C1q to IgG, as well as C1q-dependent hemolysis (25). For the purification of mAb 2204, gamma globulins were precipitated from ascites using 50% $(NH_4)_2SO_4$. The precipitate was dialyzed against 10 mM Tris containing 2 mM EDTA (pH 7.8) and subjected to anion exchange chromatography using DEAE-Sephacel (Pharmacia, Uppsala, Sweden). Proteins were eluted using a salt gradient and the fractions that showed binding of mouse IgG to C1q-coated ELISA plates in the presence of 1 M NaCl were pooled, concentrated, dialyzed against PBS and stored at -80°C.

Polyclonal anti-C1q antibodies were produced in rabbits. New Zealand White rabbits were immunized (weekly for four weeks) with 180 µg C1q dissolved in complete Freunds adjuvant, resulting in antisera with a positive titer on C1q-coated ELISA plates beyond 1/25,000. IgG was precipitated from rabbit serum using 40% $(NH_4)_2SO_4$ and purified using DEAE-Sephacel as described above.

Starting from purified rabbit IgG anti-C1q, Fab fragments were generated using papain. IgG was dialyzed against 10 mM phosphate buffer containing 10 mM L-cysteine and 2 mM EDTA (pH 7.0). Subsequently, mercuripapaine (from Sigma, St. Louis, MO) was added (1% w/w of the protein content) followed by incubation for 16 hours at 37°C. After dialysis against PBS, the sample was applied to Sepharose-coupled protein G (from Pharmacia), and the fall through fractions, containing Fab fragments, were pooled, concentrated, and used for experiments. Analysis by non-reducing SDS-PAGE showed a prominent band at approximately 45 kD.

A mouse mAb directed against the lectin domain of human MBL (mAb 3F8) was kindly provided by Dr. G.L. Stahl (Harvard Medical School, Boston, Massachusetts, USA) (26).

Preparation of human C1q and C1q-depleted serum

Human C1q was isolated from human donor plasma exactly as described previously and was stored at -80°C (25). Isolated C1q was able to completely restore the lysis of antibody-coated erythrocytes in the presence of C1q-depleted human serum.

For the preparation of C1q-depleted serum, undiluted normal human EDTA-plasma (obtained from a donor with the MBL/AA genotype) was applied on column consisting of rabbit IgG anti-human C1q coupled to Biogel A5 (from Biorad). The column was washed using Veronal-buffered saline (VBS; 1.8 mM Na-5,5-diethylbarbital,

0.2 mM 5,5-diethylbarbituric acid, 145 mM NaCl) containing 10 mM EDTA. Fractions were tested in a C1q-dependent hemolytic assay in the absence or presence of purified C1q, as previously described (25). Fractions that showed complete erythrocyte lysis in the presence of C1q, but not in the absence of C1q, were pooled and concentrated until the original volume. After recalcification, C1q-depleted serum was stored at -80°C.

Isolation of human IgM

Plasma containing an IgM paraprotein was dialyzed against 10 mM sodium acetate containing 2 mM EDTA (pH 5.0). The precipitated proteins were recovered by centrifugation, dissolved in PBS, dialyzed against Tris/EDTA buffer (10 mM Tris, 2mM EDTA, pH 7.8 and conductivity 5.0 mS), and subjected to anion exchange chromatography using DEAE-Sephacel. IgM that eluted in the salt gradient was pooled, dialyzed against 10 mM sodium acetate (6.0 mS, pH 7.0) and applied to a CM-C-50 Sephadex anionic exchange column (from Pharmacia). Following elution with a salt gradient, fractions containing IgM were pooled, concentrated, and applied to a Superdex 300 gel filtration column. Peak fractions containing IgM and free of IgG were pooled, concentrated, and stored at -80°C.

Assessment of functional lectin pathway activity by ELISA

Functional activity of the lectin pathway was assessed by ELISA using immobilized mannan as a ligand. Mannan was obtained from Sigma (from Saccharomyces Cerevisiae; M7504), dissolved in PBS (10 mg/ml) and stored at -20°C. Nunc Maxisorb plates (Nunc, Roskilde, Denmark) were coated with mannan (100 µg/ml; 100 µl per well) in coating buffer (100 mM Na_2CO_3/ $NaHCO_3$, pH 9.6), for 16 hours at room temperature or for 2 hours at 37°C. After each step, plates were washed three times with PBS containing 0.05% Tween 20. All incubation volumes were 100 µl. Residual binding sites were blocked by incubation with PBS containing 1% BSA for one hour at 37°C. Serum samples were diluted in GVB++ (VBS containing 0.5 mM $MgCl_2$, 2 mM $CaCl_2$, 0.05% Tween-20, and 0.1% gelatin; pH 7.5) in the presence of mAb 2204 (20 µg/ml) as an inhibitor of C1q, unless otherwise indicated. This mixture was pre-incubated for 15 minutes on ice, before addition to the plates. The plates were then sequentially incubated for 1 hour at 4°C and for 1 hour at 37°C, followed by washing. Complement binding was detected using mouse mAb conjugated to digoxygenin (dig) using digoxygenin-3-O-methylcarbonyl-ε-aminocaproic acid-N-hydroxysuccinimide ester (from Boehringer Mannheim, Mannheim, Germany) according to instructions provided by the manufacturer. Detection of MBL, C1q, C4, C3, and C5b-9 was performed using mAb 3E7 (anti-human MBL, kindly provided by Dr. T. Fujita, Fukushima, Japan), mAb 2214 (anti-human C1q), mAb C4-4a (anti-human C4d), RFK22

(anti-human C3) and AE11 (anti-human C5b-9, kindly provided by Dr. T.E. Mollnes, Oslo, Norway), respectively. Binding of mAb was detected using dig-conjugated sheep anti-mouse antibodies (Fab fragments) followed by HRP-conjugated sheep anti-dig antibodies (Fab fragments, both from Boehringer Mannheim). All detection antibodies were diluted in PBS containing 1% BSA and 0.05% Tween 20. Enzyme activity of HRP was detected following incubation with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (from Sigma; 2.5 mg/ml in 0.1 M Citrate/Na_2HPO_4 buffer, pH 4.2) in the presence of 0.01% H_2O_2, for 30-60 min. at room temperature. The OD at 415 nm was measured using a microplate biokinetics reader (EL312e, from Biotek Instruments, Winooski, Vermont, USA).

Assessment of functional classical pathway activity by ELISA

The protocol for the functional activity of the classical pathway was similar to the protocol for the LP assay, as described above, with important modifications. As a ligand for CP activation, human IgM was coated at 2 µg/ml. After blocking of residual binding sites, serum samples, diluted in GVB++, were added to the plate and incubated for 1 hour at 37°C. Complement binding was assessed using dig-conjugated mAb directed against C1q, C4, C3, and C5b-9, followed by the detection of mAb binding using HRP-conjugated sheep anti-dig antibodies.

Assessment of functional alternative pathway activity by ELISA

The protocol for the functional activity of the alternative pathway was similar to the protocol for the LP assay, as described above, with important modifications. As a ligand for AP activation, LPS was coated at 10 µg/ml. LPS from Salmonella Typhosa was obtained from Sigma (L-6386), dissolved in PBS at 1.6 mg/ml and stored at -20°C. Plates were blocked using 1% BSA in PBS. Serum samples were diluted in GVB/MgEGTA (VBS containing 10 mM EGTA, 5 mM $MgCl_2$, 0.05% Tween-20, and 0.1% gelatin; pH 7.5) and incubated in the plate for 1 hour at 37°C. Complement binding was assessed using dig-conjugated mAb directed against C4 and C3, followed by the detection of mAb binding using HRP-conjugated sheep anti-dig antibodies.

Quantification of anti-mannan antibodies in human serum

For the quantification of anti-mannan antibodies in human serum, ELISA plates were coated with mannan and blocked with 1% BSA in PBS. Serum samples were diluted 1/100 for detection of IgG anti-mannan Ab, 1/10 for detection of IgA anti-mannan Ab, and 1/40 for detection of IgM anti-mannan Ab, respectively, unless otherwise indicated. For quantification, pooled human IgG (48 mg/ml IgG), pooled human IgA (41 mg/ml IgA), and pooled human IgM (35 mg/ml IgM) were used as a standard for detection of IgG, IgA and IgM anti-mannan antibodies, respectively (kindly provided

by Biotest Pharma GmbH, Dreieich, Germany). The concentration of anti-mannan antibodies in these preparations was arbitrarily set at 1000 U/ml. All samples were diluted in PBS containing 0.05% Tween 20 and 1% BSA. Antibody binding was detected using biotinylated HB43 (mouse mAb anti-human IgG), biotinylated HB57 (mouse mAb anti-human IgM) and dig-conjugated 4E8 (mouse mAb anti-human IgA), respectively, followed by either HRP-conjugated streptavidin or HRP-conjugated sheep anti-dig antibodies (both from Boehringer).

DNA isolation

Genomic DNA was isolated from heparinized blood according to standard procedures (27). Briefly, 10 ml blood was diluted with 40 ml EL buffer (erythrocyte lysis buffer: 155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA, pH 7.4) and incubated on ice for 20 minutes. After centrifugation (10 minutes at 500 g), the pellet was washed with 25 ml EL buffer, and resuspended in 3 ml of KL buffer (10 mM Tris, 2mM EDTA, 400 mM NaCl, pH 8.4), followed by thoroughly shaking. After addition of 25 µl pronase (20 mg/ml in water, Boehringer Mannheim, Germany) and 150 µl SDS (20% in water), the mixture was incubated in a shaking water bath at 37°C for 18 hours. Finally, the DNA was precipitated with ethanol, dissolved in 0.5 ml TE buffer (10 mM Tris, 0.1 mM EDTA, pH 7.4), heated for 5 minutes at 65°C, and kept at 4°C.

PCR amplification of exon 1 of the MBL gene

Exon 1 of the MBL gene was amplified from genomic DNA by PCR. Starting from 1 µl of genomic DNA (approximately 0.7 µg), a 40 µl PCR reaction was performed, using 0.25 mM dNTP (from Pharmacia Biotech), 0.8 U Amplitaq (from Perkin Elmer,

Table 1. Oligonucleotides used for MBL genotyping

Oligonucleotide	Sequence
PCR forward	5′-ACCCAGATTGTAGGACAGAG-3′
PCR reverse	5′-GTTGTTGTTCTCCTGTCCAG-3′
OLA 52-common	5′-P-CCCATCTTTGCCTGG-bio-3′
OLA 52-wildtype	5′-dig-CCTTGGTGCCATCACG-OH-3′
OLA 52-mutant	5′-dig-CCCTTGGTGCCATCACA-OH-3′
OLA 54-common	5′-P-CATCACGCCCATCTTTG-bio-3′
OLA 54-wildtype	5′-dig-CTTTTCTCCCTTGGTGC-OH-3′
OLA 54-mutant	5′-dig-CCTTTTCTCCCTTGGTGT-OH-3′
OLA 57-common	5′-P-CCTTGGTGCCATCACG-bio-3′
OLA 57-wildtype	5′-dig-TGGTTCCCCCTTTTCTC-OH-3′
OLA 57-mutant	5′-dig-CTGGTTCCCCCTTTTCTT-OH-3′

PCR primers were derived from (9) with slight modifications. OLA primers are labelled with either biotin (bio) or digoxygenin (dig). All primers were obtained from Eurogentec (Seraing, Belgium).

Wellesley, MA), and 12.5 pmol of both PCR primers (from Eurogentec, Seraing, Belgium; Table 1) in PCR buffer (10 mM Tris HCl, 50 mM KCl, 2.5 mM $MgCl_2$, 0.6 mg/ml BSA, pH 8.3). The PCR reaction was performed in a Peltier Thermal Cycler (PTC200, from MJ Research, Waltham, MA) using the following program: denaturation for 5 min at 95°C, followed by 36 cycles of 1 min. 95°C, 1 min. 57°C, and 1 min. 72°C, and a final elongation period for 7 min. at 72°C. Evaluation of the PCR products by agarose electrophoresis showed one specific band of the expected molecular weight (679 bp) with an estimated concentration of about 30 ng/µl.

Oligonucleotide Ligation Assay (OLA) for MBL genotyping

For detection of MBL mutant alleles at codon 52, 54, and 57, three different OLA protocols were developed. For each OLA, two reactions were performed in parallel, using either the wildtype or the mutant primer, both in combination with a common primer (Table 1). PCR products were first heated for 5 min at 99°C. The OLA reaction was performed in a 20 µl reaction mixture consisting of 2 µl of PCR product, 5 pmol common primer, 5 pmol of either the wildtype or the mutant primer (Table 1), and 1.2 U Taq DNA ligase, using the buffer supplied by the manufacturer (from New England Biolabs, Beverly, MA). The following program was run in a PTC 200 Thermal Cycler: denaturation for 2 min. at 94°C, 10 cycles of 10 sec. 94°C and 3 min. 60°C, followed by a final incubation of 5 min. at 99°C. For OLA detection of codon 57 polymorphisms, probe anealing was performed at 54°C instead of 60°C.

For detection of OLA products, ELISA plates were coated with avidin (20 µg/ml, from ICN Biomedicals inc., Aurora, Ohio, USA) and aspecific binding sites were blocked with PBS containing 3% BSA. The OLA reaction mixture was 1/5 diluted in PBS containing 1% BSA, added to the plate, and incubated for 1 hour at 37°C. Plates were washed and dig-conjugated reaction products were detected using HRP-conjugated sheep anti-dig antibodies as described above.

RESULTS

Anti-mannan antibodies in human serum

Mannan is a major ligand of MBL that can efficiently activate the LP of complement. Human serum contains anti-carbohydrate antibodies, probably resulting from previous microbial contacts. Such anti-carbohydrate antibodies may bind to mannan and the resulting immune complex may contribute to complement activation by mannan via activation of the classical complement pathway (28; 29). Mannan-binding antibodies are clearly detectable in human serum as assessed by ELISA (Fig. 1). Incubation of pooled human IgG (Fig. 1A), IgA (Fig. 1B) and IgM (Fig. 1C) on

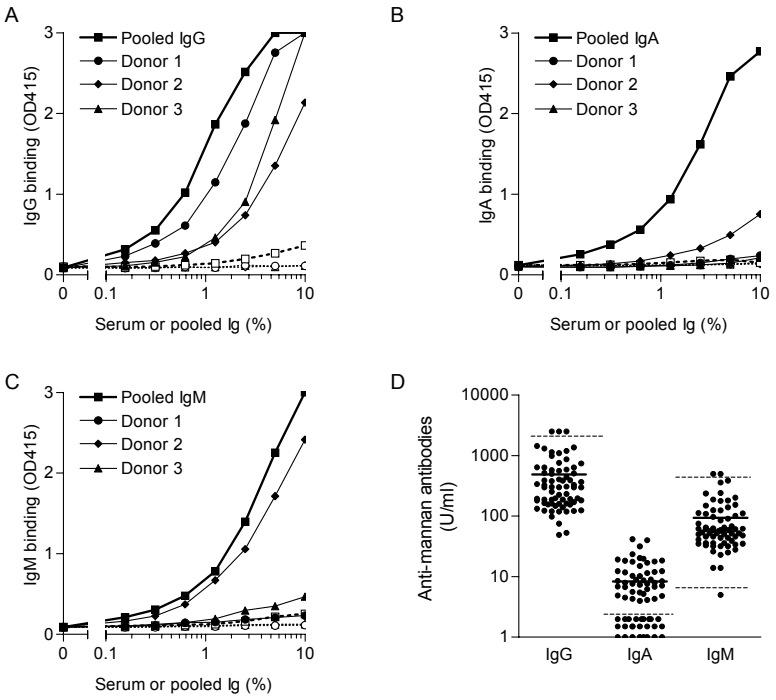

Figure 1. Anti-mannan-antibodies in human serum. **A-C**: Different concentrations of pooled immunoglobulin, as indicated, or human serum from three different healthy donors were incubated on plates coated with either mannan (closed symbols, solid lines) or BSA (open symbols, dashed lines). Binding of IgG (A), IgA (B) or IgM (C) was detected. **D**: Anti-mannan antibodies of the three major Ig classes were quantified in healthy donor serum (N = 70). Solid lines indicate the median concentrations, dashed lines indicate the detection limits.

immobilized mannan resulted in a dose-dependent binding of IgG, IgA, and IgM as detected by isotype-specific mAb. As a control, parallel incubations were performed on immobilized BSA, resulting in low or undetectable background binding of pooled Ig. Incubation of three sera from healthy donors on mannan-coated plates resulted in strong dose-dependent IgG binding in all three sera. In donor 1, IgA and IgM anti-mannan Ab were undetectable, serum from donor 2 contained IgG, IgA and IgM anti-mannan antibodies, whereas in donor 3 some IgM binding was observed but no IgA binding (Fig. 1A-C). Binding of Ig was undetectable following incubation of serum on BSA-coated plates (Fig. 1A-C). Quantification of anti-mannan antibodies in serum from 70 healthy donors is presented in fig. 1D. IgG and IgM anti-mannan Ab were present in nearly all donors, with a large interindividual variation, whereas IgA anti-mannan Ab were detected in 63% of the donors. No significant correlation was observed between the three major isotypes of anti-mannan antibodies, or between anti-mannan antibodies and MBL concentrations (not shown).

Functional characterization of the lectin pathway in the presence of C1q-inhibitory Ab

Both the LP and the CP are calcium-dependent and lead to activation of C4. A distinction between both pathways can be made by selection of a specific ligand that induces activation of either the LP or the CP. In view of the presence of anti-mannan Ab in human serum, mannan is likely to activate both the LP, via MBL, and the CP, via anti-mannan Ab. Therefore, a strategy was developed to inhibit activation of the CP in order to allow solely the activation of the LP by immobilized mannan, by using inhibitory anti-C1q antibodies.

Anti-C1q antibodies were tested for their ability to inhibit the CP of complement using immobilized IgM as a specific activator of the CP. Incubation of 1% normal human serum (NHS) on immobilized IgM induces deposition of C4, which could be dose-dependently inhibited by the anti-C1q mAb 2204, by rabbit IgG anti-C1q antibodies and by Fab fragments prepared from this rabbit anti-C1q antibody preparation (Fig. 2A). Complete inhibition was reached when the antibodies were applied at 5 μg/ml. In contrast, rabbit IgG prepared from non-immunized rabbits did not have an effect on C4 activation via the CP. These antibodies were tested for their effect on complement activation induced by immobilized mannan. Incubation of NHS on mannan induced a dose-dependent deposition of C4, with a maximal activation at a serum concentration of 1% (Fig. 2B). Addition of a fixed concentration of mAb 2204, Fab anti-C1q fragments, or normal rabbit IgG as a control had a slight inhibitory effect on C4 activation. In contrast, rabbit IgG anti-C1q Ab induced complete inhibition of C4 activation by mannan, most likely due to complement consumption via C1q-anti-C1q complexes (Fig. 2B). These data show that C1q-inhibitory antibodies can block CP activation completely whereas mannan-induced activation of the LP can proceed in a C1q-independent way.

To further examine the role of C1q in complement activation by mannan and by IgM, NHS was depleted of C1q. Depletion of C1q from NHS resulted in a complete inhibition of C4 activation by immobilized IgM (Fig. 3A), as previously described (30), whereas C4 activation by immobilized mannan was slightly inhibited by depletion of C1q (Fig. 3B). Reconstitution of C1q-depleted serum with purified C1q resulted in a complete restoration of C4 activation by IgM (Fig. 3C). In contrast, C4 activation by mannan was slightly inhibited by addition of purified C1q to C1q-depleted serum, possibly due to the presence of an inhibitory protein co-isolated with C1q. The contribution of C1q and MBL to C4 activation by IgM and mannan was further studied using blocking mAb against C1q and MBL, respectively (Fig. 3D). C4 activation on IgM-coated plates was completely inhibited by mAb anti-C1q and no inhibition occurred with a blocking anti-MBL mAb. In contrast, C4 activation induced by mannan was partially inhibited by mAb anti-C1q and strongly inhibited by mAb anti-MBL. Complete inhibition of mannan-induced C4 activation was achieved when

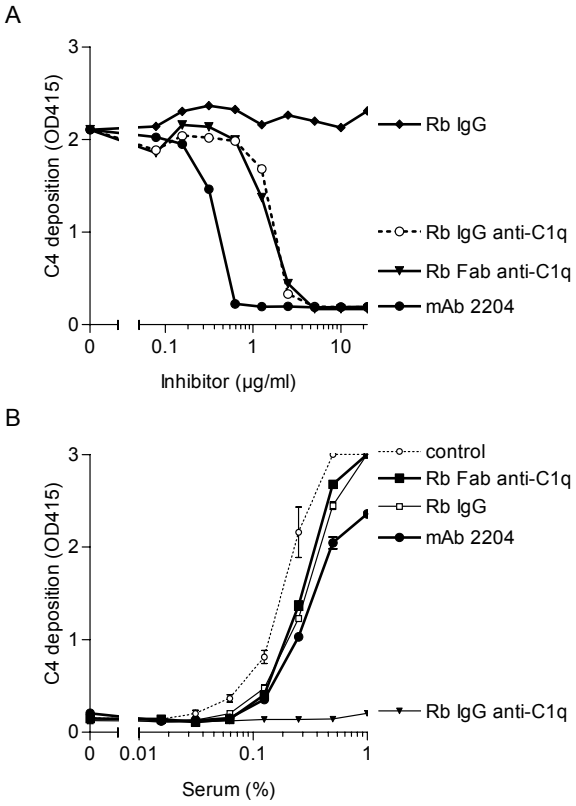

Figure 2. The effect of C1q inhibitory antibodies on complement activation via the CP and the LP. **A**: Normal human serum (diluted 1/100) was pre-incubated in the presence or absence of different antibody preparations (rabbit IgG anti-C1q, rabbit Fab anti-C1q, normal rabbit IgG or mAb 2204 in concentrations as indicated), and added to IgM-coated plates to assess CP activation. Activation of C4 was detected. **B**: Normal human serum in different concentrations was pre-incubated in the presence or absence of a fixed concentration of antibodies (rabbit IgG anti-C1q, rabbit Fab anti-C1q, normal rabbit IgG (10 µg/ml) or mAb 2204 (20 µg/ml)). Activation of C4 via the LP was assessed following addition of the mixture to mannan-coated plates. Results represent mean ± SD from one out of three similar experiments.

a combination of mAb anti-C1q and mAb anti-MBL was used (Fig. 3D). Together, these data indicate that IgM-mediated activation of C4 is completely dependent on C1q and does not involve MBL. In contrast, mannan-induced activation of C4 is mainly mediated by the LP but comprises a minor contribution of the CP. The latter contribution of the CP can be inhibited by C1q-blocking Ab, thus allowing activation of the LP only.

Complement activation and formation of C5b-9 via the CP and via the LP

The complement activation cascade was further studied using mAb to detect binding of specific complement components upon their activation via the CP and the LP, respectively. Incubation of NHS on immobilized IgM resulted in a dose-dependent

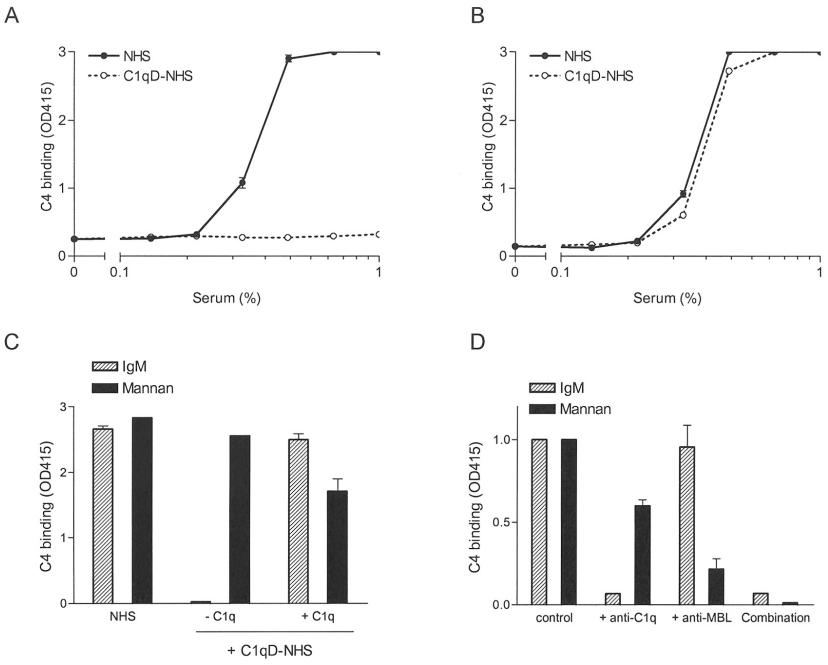

Figure 3. The role of C1q in activation of the CP and the LP. **A, B**: Normal human serum or C1q-depleted serum (C1qD-NHS), diluted in GVB++, was incubated on plates coated with IgM (A) and mannan (B), respectively, followed by detection of C4 binding. **C**: NHS and C1q-depleted NHS (diluted 1/400) were incubated on plates coated with IgM or mannan in the presence or absence of purified C1q (0.5 µg/ml), as indicated. **D**: NHS was incubated on IgM- or mannan-coated plates in the presence or absence of blocking mAb directed against MBL (mAb 3F8, 10 µg/ml) or C1q (mAb 2204, 20 µg/ml), or both (combination). Results represent mean ± SD from one of at least two experiments

deposition of C1q, C4, C3, and C5b-9 to the plate (Fig. 4A). Binding of C1q and subsequent complement activation induced by IgM could be completely inhibited by mAb 2204. Incubation of NHS on immobilized mannan resulted in dose-dependent binding of C4, C3 and C5b-9, whereas binding of C1q was hardly detectable (Fig. 4B). Complement activation by mannan was only slightly inhibited by addition of mAb 2204. Therefore, addition of mAb 2204 in serum allows the specific detection of LP activation using mannan as a ligand, without interference of the CP.

Activation of the alternative pathway

To enable the detection of all complement activation pathways in one assay system, we also studied activation of the alternative pathway in an ELISA system. In contrast to the LP and the CP, activation of the AP is calcium-independent. Therefore, a calcium-free buffer was used, thus excluding involvement of the CP and the LP. As previously described (30), incubation of NHS in a buffer containing EGTA and Mg^{++} on plates coated with LPS resulted in a dose-dependent deposition of C3 (Fig. 5A).

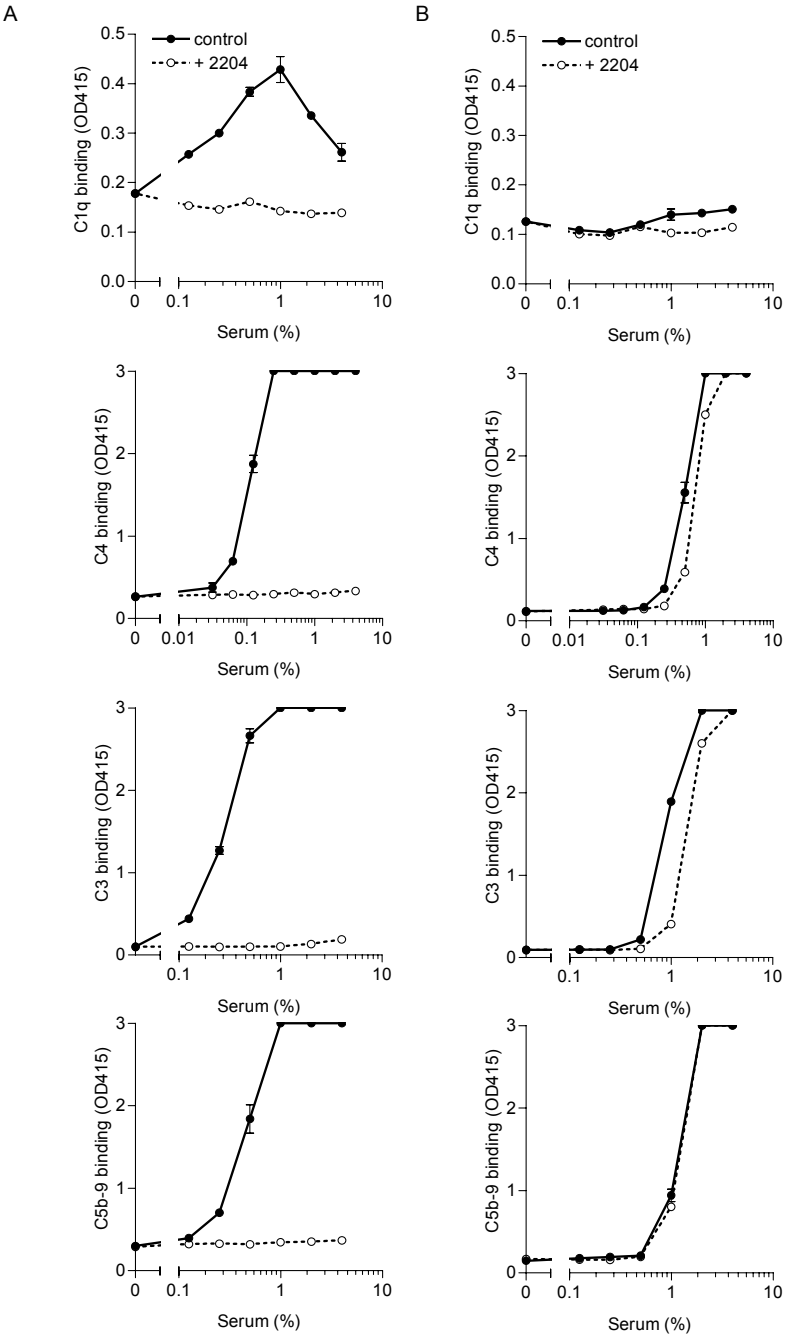

Figure 4. Complement activation via the LP and the CP. Complement activation was induced by incubation of different concentrations of NHS on plates coated with IgM for CP activation (A) or with mannan for LP activation (B), in the presence or absence of mAb 2204 (20 µg/ml). Activation and binding of complement was demonstrated by detection of C1q, C4, C3, and C5b-9 using specific mAb. Results represent mean ± SD from one out of at least three experiments.

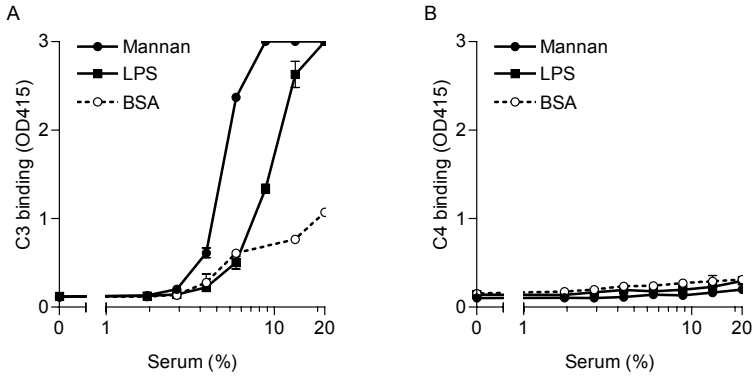

Figure 5. Activation of the alternative pathway. NHS was incubated on plates coated with mannan, LPS, or BSA, in a calcium-free buffer (GVB/MgEGTA) to block activation of the CP and the LP. Binding of C3 (A) and C4 (B) was subsequently assessed. Results are shown as the mean ± SD from a representative experiment; similar data were obtained in at least two experiments.

Some activation of C3 was also observed on plates coated with BSA only. Surprisingly, strong activation of C3 was also observed when NHS was incubated on mannan-coated plates using the same conditions, suggesting that mannan may also support activation of the AP (Fig. 5A). Detection of C3 was reduced until background levels when EDTA was present in the complement source (not shown). As expected from an AP-dependent mechanism, C3 activation in calcium-free buffers required a serum concentration that is about 10-fold higher than that required for C3 activation by mannan in a calcium-containing buffer via the LP (compare fig. 5A with fig. 4B). Although C3 activation was clearly detectable in a calcium-free buffer, no activation of C4 could be established (Fig. 5B), suggesting that under these conditions activation of C3 is independent of MBL binding and C4 activation.

MBL genotyping by oligonucleotide ligation assay

Single nucleotide polymorphisms in exon 1 of the MBL gene are the most important genetic modifiers of MBL function. We developed three oligonucleotide ligation assays (OLA) for the detection of MBL exon 1 SNPs at codon 52, codon 54, and codon 57, respectively. Using this technique, the presence of B (codon 54), C (codon 57), and D alleles (codon 52), as indicated by formation of double-labeled DNA products using the mutant oligonucleotides, can be easily detected with a standard laboratory equipment, both in homozygous and in heterozygous patterns (Fig. 6).

Lectin pathway activation is dependent on the MBL genotype

In the Caucasian population, the B allele is the most frequent exon 1 polymorphism in the MBL gene. It has been previously reported that recombinant MBL with the BB genotype has a strongly reduced ability to support complement activation (14)).

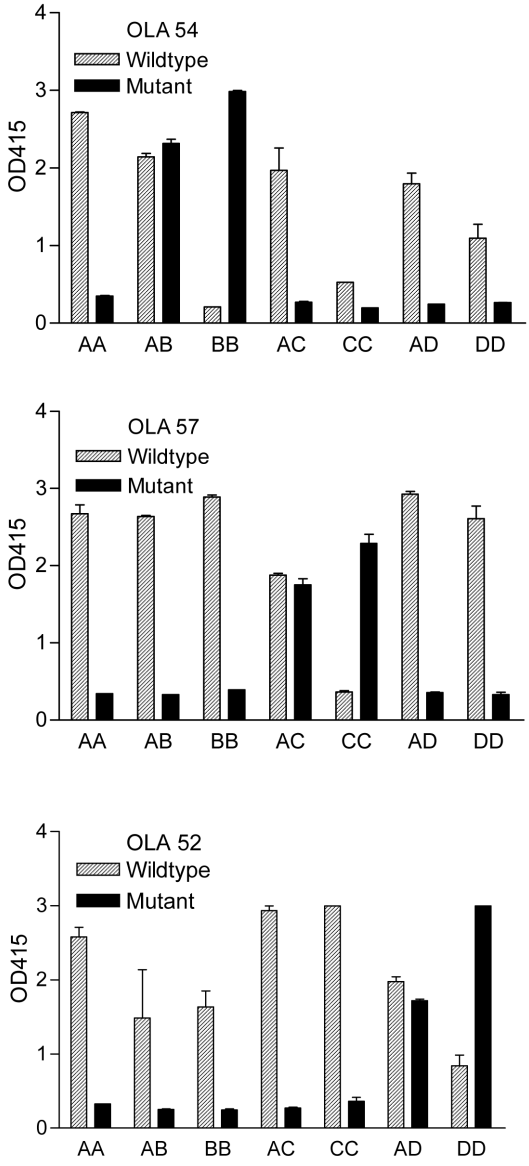

Figure 6. Detection of MBL mutant alleles by oligonucleotide ligation assay. DNA from individuals with different MBL genotypes was used for detection of wildtype or mutant alleles in an OLA for codon 54, codon 57 and codon 52. Results are expressed as the OD at 415 nm of the detection of biotin and dig-labeled OLA products upon ligation using either the wildtype or the mutant allelic probes, and represent mean ± SD of one out of three experiments performed in duplicate. MBL genotypes were confirmed by DNA sequencing.

Using MBL genotyping by OLA, sera from individuals with different MBL genotypes were identified. We compared activity of the CP and the LP in serum obtained from an MBL wildtype donor (AA genotype) with serum from donors with a heterozygous

and a homozygous mutation at codon 54 (AB and BB genotype, respectively). Serum from all three donors showed binding of C1q and strong activation of C4, C3 and C5b-9 via the CP upon incubation on immobilized IgM, in a similar dose-response relationship (Fig. 7A). Upon serum incubation on immobilized mannan, strong dose-dependent binding of MBL to mannan was observed in AA serum, whereas MBL binding in AB serum was about 8-fold less and no binding of MBL to mannan could be established in BB serum (Fig. 7B, upper panel). In parallel, LP activity was assessed in the same sera by their incubation on immobilized mannan, in the presence of mAb 2204 to block the CP. In sharp contrast to the results obtained on coated IgM, only AA serum, but not BB serum nor AB serum, was able to induce detectable activation of C4, C3 and C5b-9 via the LP (Fig. 7B). These results indicate that LP activity is dependent on the presence of functionally active MBL.

DISCUSSION

In the present study, we describe a novel assay for the detection of functional activity of the LP of complement. The assay is based on the detection of various stages of complement activation induced by binding of MBL to immobilized mannan, and involves the addition of inhibitory anti-C1q antibodies to prevent interference of activation of the CP. We demonstrate that in this novel assay system activation of autologous C4, C3, and C5b-9 in full human serum is totally dependent on the presence of functionally active MBL.

Our results show the broad presence of anti-mannan antibodies in the human population. These antibodies may be produced in response to a previous yeast contact and/or may belong to the so-called natural antibodies. Increased levels of antibodies binding to mannan from Saccharomyces Cerevisiae have been described in patients with inflammatory bowel disease (31; 32). Certain anti-carbohydrate antibodies can be present in extremely high levels, as is the case for antibodies directed against the major xenoantigen Galα1-3Gal (33). IgG and IgM anti-carbohydrate antibodies can activate the classical complement pathway, and this mechanism is likely to contribute to anti-microbial defense, in addition to lectin-mediated mechanisms. Indeed it has been described that IgG anti-mannan antibodies contribute to opsonization of Candida albicans with C3 (34). Such a mechanism may especially be important in cases where the function of the lectin pathway of complement is impaired. In our study, we were not able to detect any significant difference in levels of anti-mannan Ab between MBL-wildtype and MBL-mutant individuals (not shown).

The presence of highly variable amounts of anti-mannan Ab in human serum necessitates a special strategy to prevent involvement of CP activity in mannan-based

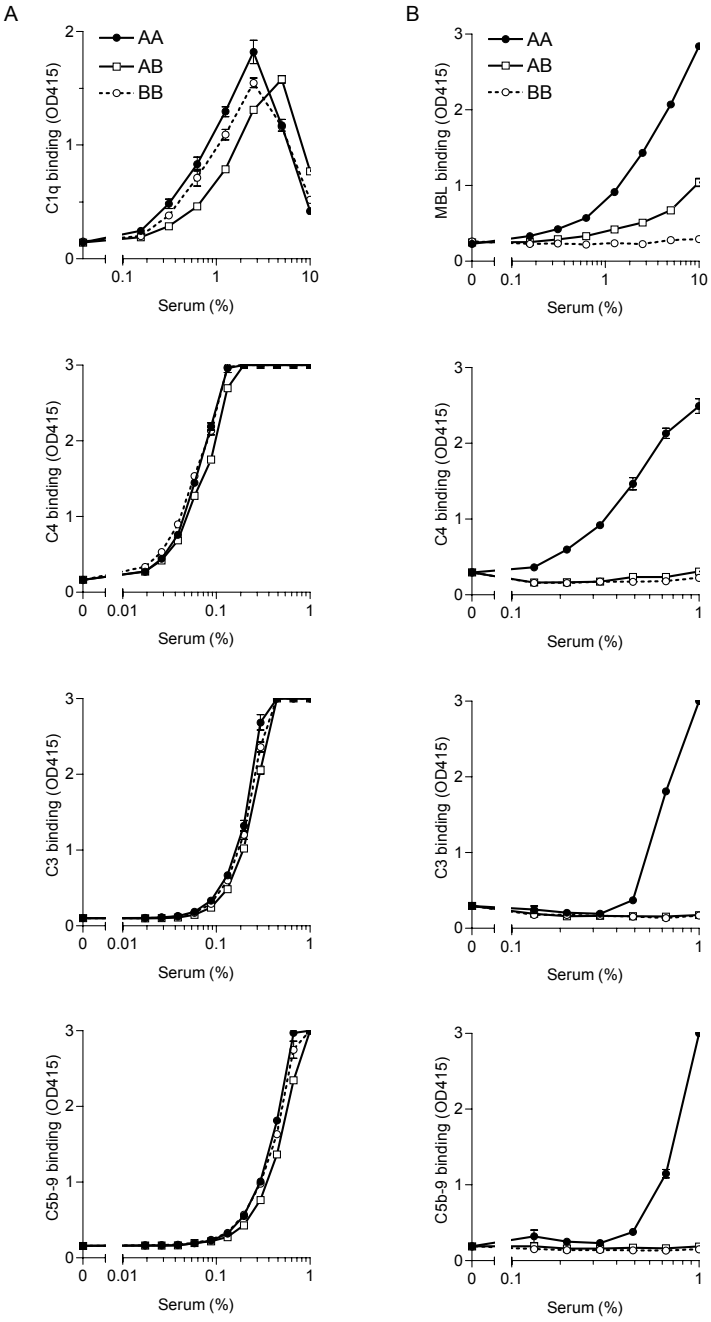

Figure 7. Lectin pathway activity is impaired in serum from MBL-mutant donors. Serum was obtained from three different donors with a wildtype MBL genotype (AA), a heterozygous mutation (AB) or a homozygous mutation (BB) at codon 54, respectively. Different concentrations were applied to plates coated with IgM to assess CP activity, in the absence of mAb 2204 (A), or with mannan to assess LP activity, in the presence of mAb 2204 (B). Deposition of C4, C3, and C5b-9 was assessed. Results represent mean ± SD from one out of at least two similar experiments.

assays for LP activation. The present study therefore includes a specific inhibitor of C1q in the assay, which prevents any activation of the CP in serum. Until now, at least two other groups reported a functional assay for the MBL pathway activity that excluded the interference of the CP. Petersen et al. reported an elegant assay that detects the functional activity of the MBL-MASP complex in serum (28). This assay is based on the difference between the C1 complex and the MBL-MASP complex with respect to its sensitivity to ionic strength. By addition of 1 M NaCl to the serum dilution buffer, C1q binding and CP activation can be completely prevented whereas MBL binding can proceed. In the assay described by Petersen et al. (28), the serum incubation step is performed at 4 °C, thus allowing binding of the MBL-MASP complex but not the subsequent complement activation. Activity of the complex is subsequently assessed by addition of exogenous purified C4. The advantage of this technique is that activity of the MBL-MASP complex is directly detected, without any interference of other variables in donor serum. The major difference with the technique as described in the present study is that we now describe a LP assay that assesses activation of autologous complement, which is more representative for the in vivo situation. Furthermore, our assay enables the detection of the complete complement activaton cascade, up to the formation of the membrane attack complex. In this respect, our assay is comparable to hemolytic assays (CH50, AP50) generally used in clinical practice for the evaluation of CP and AP activity. The functional analysis of all three complement activation pathways in parallel by ELISA, as described in the present study, is potentially useful in routine diagnostic laboratories for a more complete diagnostic evaluation of complement defects.

An alternative functional MBL pathway assay was recently described by Zimmerman-Nielsen et al. (35). This assay also includes 1 M NaCl in the incubation buffer, but analyzes autologous C4 activation. However, activation of C4 in the presence of 1M NaCl is highly inefficient ((28) and our own unpublished observations), which is apparent from the low serum dilutions used in this study. These suboptimal conditions may have a differential effect on C4 activation in serum from various donors, and therefore the C4 activation assessed in this respect is difficult to interpret. Furthermore, also in this assay it is not possible to assess complement activation at a later stage than C4, since formation of C4b2a is strongly dependent on ionic strength (36), and accordingly C3 activation is undetectable in 1 M NaCl (our unpublished observations).

An alternative approach for the analysis of LP activity in serum is the quantification of serum-induced hemolysis of mannan-coated sheep erythrocytes (6; 37). MBL is able to bind to these coated erythocytes (37), leading to complement activation and erythrocyte lysis. Also in such an hemolytic assay, it is important to prevent interference of the classical complement pathway, which may occur both by anti-mannan

antibodies and by anti-erythrocyte antibodies. Suankratay et al. described a method in which mannan-coated erythrocytes were pre-sensitized with purified MBL, followed by incubation with serum in the presence of MgEGTA (37). This hemolytic assay analyzes the activity of the lectin pathway of complement most likely from C4 until C9, and hence does not provide information about the activity of the MBL-MASP complex in the serum source. Therefore, this assay can not be used to detect a functional impairment of LP activity at the level of MBL. We did not succeed to set up an hemolytic assay with mannan-coated erythocytes using full serum and a C1q inhibitor, probably due to unsufficient sensitivity (data not shown).

We show in the present study that both C1q and MBL have a contribution in the activation of complement by mannan-coated ELISA plates, using inhibitory antibodies directed against C1q and MBL. It is likely that the relative contribution of C1q is strongly increased in donor serum containing high levels of IgG and IgM anti-mannan antibodies in combination with low levels of functional MBL. In such a situation, the contribution of C1q may mask the detection of deficiency of the LP unless CP activation is prevented. Therefore, inhibition of CP is crucial for a reliable functional LP assay.

Different strategies are conceivable for the inhibition of C1q-mediated complement activation in human serum. In the present study, we show C1q inhibition with mAb 2204, an anti-C1q monoclonal antibody that binds to the globular heads of C1q and blocks the interaction with immunoglobulins. Furthermore, Fab fragments from polyclonal rabbit anti-C1q antibodies, but not complete IgG, can be used to specifically inhibit CP activation. An alternative option is the use of C1q-inhibitory peptides (25). This option is under investigation in our laboratory.

Our studies indicate that incubation of low serum concentrations on immobilized mannan may also activate the alternative pathway. This may involve stabilization of spontaneously activated C3, in a complex with activated factor B and properdin, by its binding to mannan, in a similar way as is effected with other heavily glycosylated microbial products that activate the AP, such as LPS and zymosan. Activation of C3 in the absence of calcium, as well as the lack of detectable C4 activation, strongly suggest an MBL-independent mechanism. Additional studies indicated that binding of MBL in the presence of EGTA was undetectable (38). However, we can not totally exclude that small amounts of MBL binding under these conditions may trigger complement activation, which is subsequently strongly amplified by the alternative pathway at the level of C3. Since activation of the AP requires a high serum concentration, it is highly unlikely that similar MBL-independent complement activation is involved in the LP activity assay when serum is diluted at least 50-fold. This is clearly demonstrated in experiments showing that activation of C4, C3, and C5b-9 was completely undetectable in serum from an individual with a homozygous mutation

at codon 54 of the MBL-gene (BB genotype), although this serum had an intact AP activity.

Two members of the ficolin family, L-ficolin and H-ficolin (Hakata antigen) have been recently shown to interact with MASP proteins, and thereby activate complement via the lectin pathway (39; 40). Ficolins are multimeric proteins with a carbohydrate-binding fibrinogen-like domain. L-ficolin does not bind to mannan and is therefore not likely to be involved in complement activation induced by mannan. Both L-ficolin and H-ficolin are present in human serum. At present, there is no information available about the activity of ficolin-mediated complement activation in full serum. Development of such an assay is dependent on the identification of ficolin-specific ligands that are able to activate ficolin-MASP complexes.

The activity of the LP in human serum is determined by a number of variables, including the concentration and molecular structure of MBL and MASP proteins, the activity of complement proteins from C4 until C9, as well as the presence and activity of serum inhibitors of complement activation (41; 42). The assay we now describe enables the functional detection of important consequences of LP activation, i.e. opsonization of the target with complement components, and formation of the membrane attack complex. Studies using recombinant MBL molecules clearly showed that structural mutations of the MBL gene lead to an impaired functional activity (14). In agreement with these data, we demonstrate that serum from donors with a mutation at codon 54 of the MBL gene (B genotype) has a defect in activation of the LP, which is in the homozygous mutant serum accompanied by a apparent failure of MBL to bind to the activating ligand mannan. Primarily in heterozygous individuals, the consequences of structural mutations may be highly variable, depending on the relative expression of the mutated and the wildtype gene. Therefore, functional assessment of LP activity most likely provides a more relevant marker for LP defects than analysis of mutations in the MBL gene and promoter region. Further studies are now underway to examine the relation between the different parameters involved in LP function and the resulting LP-mediated complement activation.

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CHAPTER 2.2

Antibody-mediated activation of the classical pathway of complement may compensate for mannose-binding lectin deficiency

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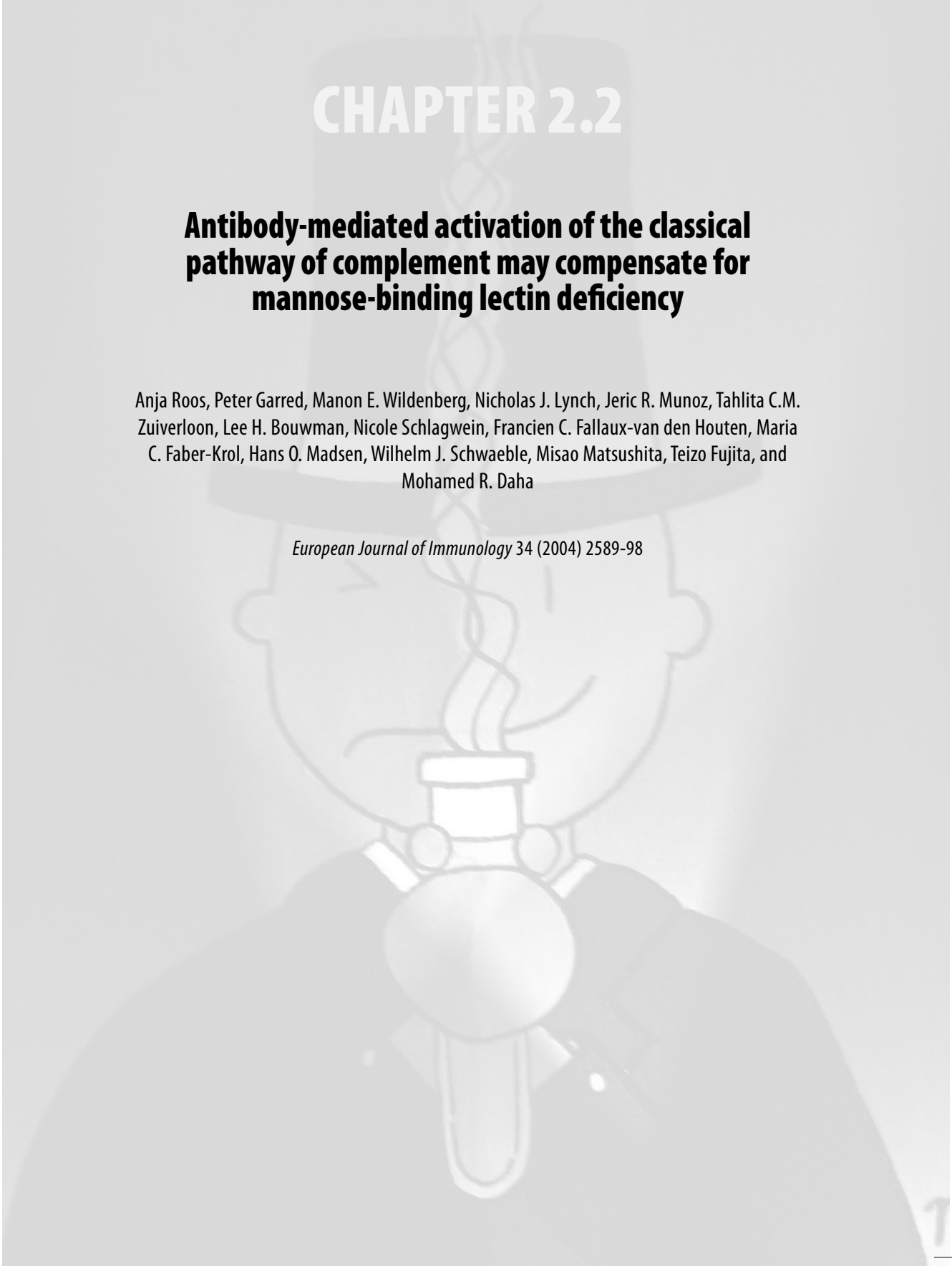

ABSTRACT

Deficiency of mannose-binding lectin (MBL), a recognition molecule of the lectin pathway of complement, is associated with increased susceptibility to infections. The high frequency of MBL deficiency suggests that defective MBL-mediated innate immunity can be compensated by alternative defense strategies. To examine this hypothesis, complement activation by MBL-binding ligands was studied.

The results show that the prototypic MBL-ligand mannan can induce complement activation via both the lectin pathway and the classical pathway. Furthermore, antibody binding to mannan restored complement activation in MBL-deficient serum in a C1q-dependent manner. Cooperation between the classical pathway and the lectin pathway was also observed for complement activation by p60 from *Listeria monocytogenes*.

MBL pathway analysis at the levels of C4 and C5b-9 in the presence of classical pathway inhibition revealed a large variation of MBL pathway activity, depending on *mbl2* gene polymorphisms. MBL pathway dysfunction in variant allele carriers is associated with reduced MBL-ligand binding and a relative increase of low molecular weight MBL.

These findings indicate that antibody-mediated classical pathway activation can compensate for impaired target opsonization via the MBL pathway in MBL-deficient individuals and imply that MBL deficiency may become clinically relevant in absence of a concomitant adaptive immune response.

INTRODUCTION

Recognition of pathogen-associated molecular patterns by molecules of innate immunity can lead to direct and early target elimination as well as to antigen presentation resulting into clearance via adaptive immunity. A number of pattern recognition molecules have been identified, such as lectin receptors, Toll-like receptors, and soluble opsonins including complement factors. The importance of the complement system in innate immune defense is clearly illustrated by a number of genetic complement deficiencies described both in humans and mice.

Activation of the complement cascade can take place via at least three pathways identified until now: the classical pathway (CP), the alternative pathway (AP), and the lectin pathway (LP). Whereas the LP and the AP primarily use a direct target recognition mechanism, the CP is mainly activated via binding of the initiating factor C1q to e.g. antigen-bound IgG or IgM antibodies. The LP can be initiated by mannose-binding lectin (MBL), which in a calcium-dependent way binds to carbohydrate ligands present on a large number of pathogens (reviewed in (1; 2)). Both MBL and C1q are composed of trimers that are assembled into larger structures. The collagenous domains of C1q and MBL bind to related serine proteases, being the serine proteases C1r and C1s for the C1 complex and the MBL-associated proteases MASP-1, MASP-2 and/or MASP-3 for the MBL complex (2; 3). Activation of both pathways leads to formation of the C3 convertase C4b2a. Recently, two members of the ficolin family, i.e. L-ficolin and H-ficolin, have also been shown to bind MASPs and to activate the LP of complement (2). In the present study, therefore, MBL-dependent activation of the LP is called the MBL pathway (MP).

Three MBL gene (*mbl2*) polymorphisms have been identified that are associated with MBL deficiency. These single nucleotide polymorphisms (SNP) are located in codon 54 (B genotype), codon 57 (C genotype), and codon 52 (D genotype) of the first exon, encoding the collagenous region of the MBL molecule (reviewed in (4; 5)). Experiments with recombinant MBL confirmed that these SNP affect the structure and function of MBL (6-8). Furthermore, SNP in the promoter and untranslated region of the *mbl2* gene modify the basal serum level of MBL (9).

MBL deficiency has been identified as the basis of a common defect in opsonization of yeast-derived mannan (10), which is associated with decreased pathogen resistance (11; 12), particularly during early childhood (13). Among the complement deficiencies described in humans, deficiency of MBL has the highest frequency. Depending on ethnicity, the total allele frequency of the B, C and D allele may be above 40% (9). Since apparently these polymorphisms are not subject to a high negative selection pressure, it has been proposed that the polymorphisms are associated with host protection in certain situations (14). Furthermore, the clinical effect of MBL deficiency

is strongly dependent on the immune status of the individuals tested, and most MBL-deficient individuals are apparently healthy. Together, the data suggest that although MBL gene polymorphisms do have important functional consequences for activation of the MP of complement, most affected persons have alternative mechanisms for target recognition to reach a sufficient level of anti-microbial protection.

Recently, assays became available that allow detailed functional evaluation of the MP, thus allowing a thorough examination into the mechanisms involved in complement activation by the prototypic MBL-ligand mannan (15; 16). The present study shows that, although the function of the MP of complement activation can be strongly hampered by MBL gene polymorphisms, recognition of mannan by the complement system may still proceed in an MBL-independent but C1q-dependent manner. Similarly, we show that p60, a protein derived from *Listeria monocytogenes,* can activate the complement system via both MBL-dependent and MBL-independent mechanisms.

RESULTS

Both C1q and MBL can support complement activation by mannan in human serum

To characterize the mechanisms of complement activation by mannan in full human serum, serum samples from healthy donors who were genotyped for SNP of the *mbl2* gene were investigated for their capacity to activate C4 by mannan. C4 activation was observed in all sera examined, with a high inter-individual variability (Fig. 1A). Activation of C4 in *A/B* donors was significantly lower than in wildtype (*A/A*) donors ($p < 0.01$).

In order to assess complement activation by mannan via the MP only, activation of C4 by mannan was assessed in human serum in the presence of a C1q-blocking mAb, as recently described (16). The activation of autologous C4 via the MP was strongly dependent on the MBL genotype (Fig. 1B, $p = 0.0002$ in ANOVA), and was significantly hampered in *A/B* donors ($p < 0.001$) but not in *A/D* donors, as compared to *A/A* donors (Fig. 1B).

Initial strong activation of C4 by mannan could in a number of sera be inhibited to an undetectable level by inhibition of C1q (compare fig. 1A with 1B, for example donor *A/C* and donor *B/D*), indicating a clear contribution of C1q and the CP in complement activation by mannan. The relative contribution of C1q is significantly higher in sera with low activity of the MP than in sera with high activity of the MP (Fig. 1C, ANOVA $p < 0.0001$).

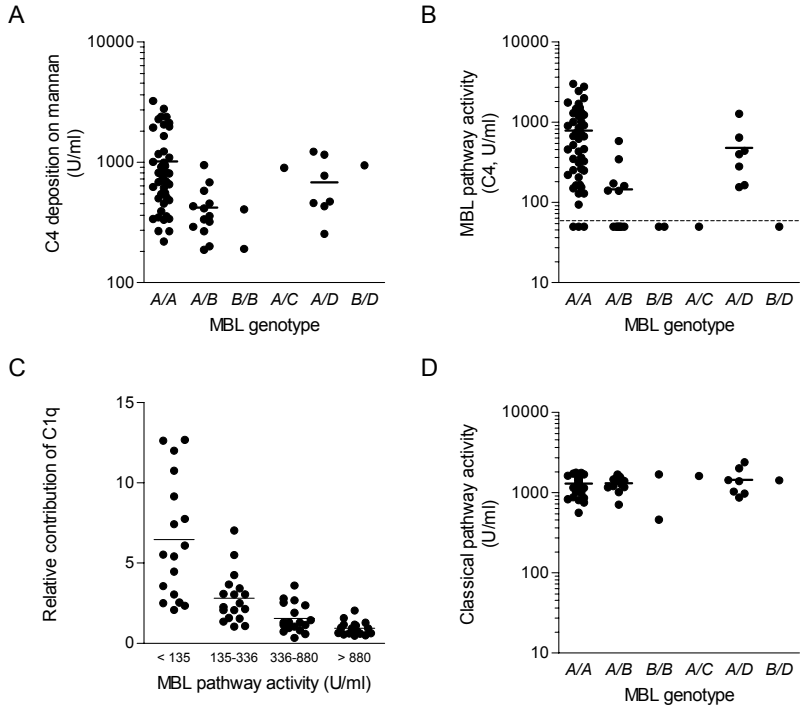

Figure 1. Complement activation via MBL pathway and classical pathway in human serum.

A. Activation of C4 by mannan in sera from donors with different MBL genotypes (serum dilutions starting from 1/100). No C1q inhibition was applied. Horizontal solid lines indicate the median. ANOVA: $p = 0.0066$, *A/A* versus *A/B* $p < 0.01$.
B. MP activity at the level of C4 assessed in the presence of mAb 2204 for C1q inhibition (dilutions starting from 1/100). ANOVA: $p = 0.0002$, *A/A* versus *A/B* $p < 0.001$. The horizontal dashed line indicates the detection limit.
C. The relative contribution of C1q, expressed as the ratio between the activation of C4 without C1q inhibition (Fig. 1A) and with C1q inhibition (Fig. 1B). Values below the detection limit were set at 75 U/ml. The results are shown for 4 quartiles, based on MP activity. ANOVA: $p < 0.0001$.
D. CP activity assessed at the level of C4 (dilutions starting from 1/1500). ANOVA: $p = 0.87$.

The activity of the CP, based on the activation of C4 induced by immobilized IgM (16), was high in all donor sera, with a low variation, and no difference between the sera of different MBL genotypes (ANOVA: $p = 0.87$) (Fig. 1D).

Since activation of C4 by mannan in a number of cases was C1q-dependent, the role of anti-mannan antibodies in complement activation by mannan was further studied. Serum levels of IgG and IgM anti-mannan antibodies are highly variable (16) and did not significantly differ between the MBL genotypes (not shown). Pre-incubation of mannan-coated plates with purified IgG (Fig. 2A) or IgM (Fig. 2B) induced a dose-dependent deposition of C4 on mannan upon addition of MBL-deficient serum. This activation of C4 was completely inhibited by a C1q-inhibitory mAb (Fig. 2), clearly indicating that mannan-binding IgG and IgM can restore complement activa-

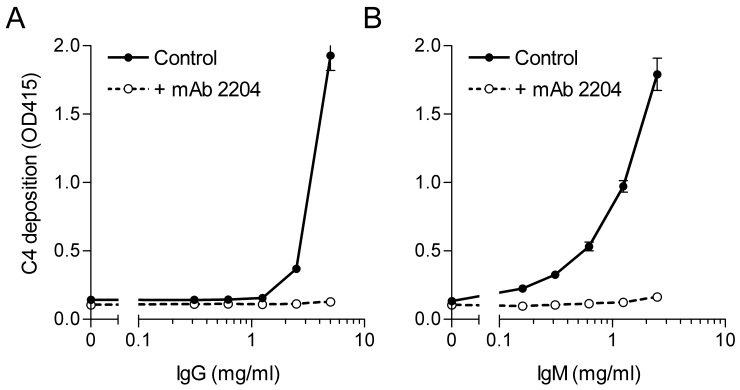

Figure 2. Cooperation between the classical pathway and the MBL pathway in complement activation by mannan. Purified IgG (A) and IgM (B) were incubated on mannan-coated plates followed by incubation with MBL-deficient serum (*B/B* genotype, 1/100), in the absence or presence of mAb 2204 anti-C1q, and assessment of C4 activation.

tion by mannan in MBL-deficient serum via activation of the CP, in the absence of functional MBL.

C1q and MBL cooperate in complement activation by p60 of *Listeria monocytogenes*

Data presented above indicate that mannan, a major yeast antigen, can support activation of the complement system via both the CP and the MP. To extend these observations towards another microbial target, we examined activation of the complement system by protein p60 from *Listeria monocytogenes*. Purified human MBL showed a strong and dose-dependent binding to p60, which was completely inhibited by D-mannose but not L-mannose (Fig. 3A), indicating involvement of the lectin domain of MBL. Interestingly, also purified C1q showed a strong binding to immobilized p60 (Fig. 3B) but not to mannan (not shown). Complement activation was further studied using two groups of sera that were either sufficient or deficient for MP activity. C4 activation by listerial p60 was significantly higher in MBL-sufficient sera than in the MBL-deficient sera (Fig. 3C). In the presence of mAb 2204 for C1q inhibition, only sera with a functional MP activity showed activation of C4 (Fig. 3C). When C1q is inhibited, activation of C4 by p60 is completely blocked by D-mannose, strongly suggesting a complete dependence on MBL under these conditions (Fig. 3D). These data support an important role for both C1q and MBL in complement activation by Listeria p60.

Together, these results provide evidence for a contribution of the CP to complement activation by two different ligands for MBL, which can compensate for MBL dysfunction. In studies presented below the basis of MP dysfunction is further examined using MBL-specific assays that exclude participation of the CP.

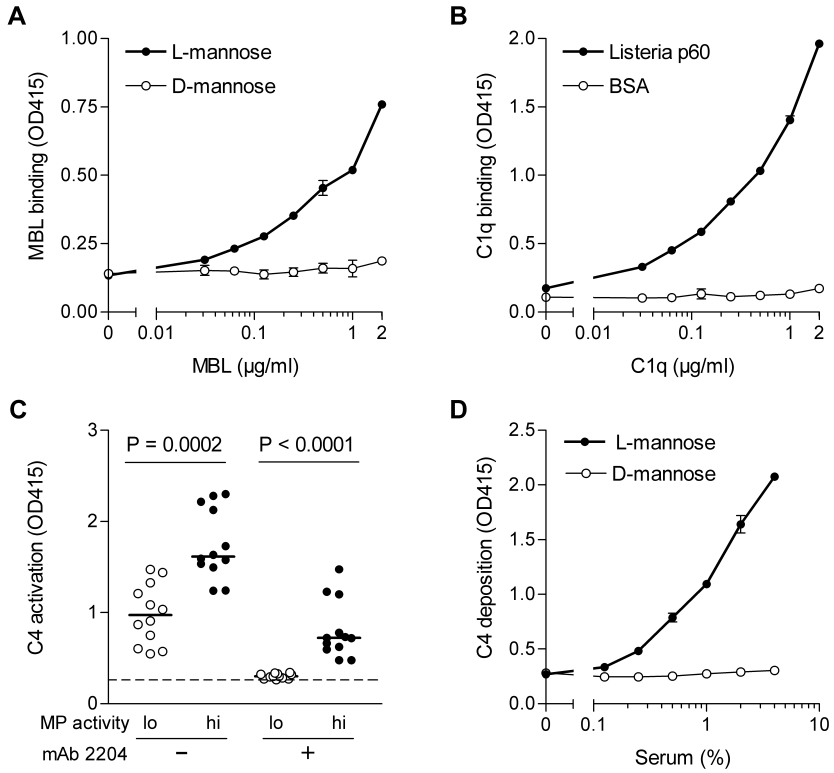

Figure 3. Contribution of the classical pathway and the MBL pathway to complement activation by p60 from *Listeria monocytogenes*.

A. Plates were coated with Listeria p60 and incubated with purified MBL in the presence or absence of D-mannose or L-mannose. Binding of MBL was assessed.

B. Plates were coated with Listeria p60 or BSA and incubated with purified C1q. Binding of C1q was assessed.

C. Sera were selected on basis of MP activity (lo < 135, hi > 880 U/ml (N = 12)) and incubated (1/50) on p60-coated wells in the presence or absence of mAb 2204 for C1q inhibition. Activation of C4 was assessed.

D. Serum (*A/A* genotype) was incubated on p60 in the presence of mAb 2204 as well as D-mannose or L-mannose. Activation of C4 was assessed.

Factors involved in the variability of MBL pathway activity in serum

Activation of the MP by mannan was subsequently assessed at its final stage. Formation of C5b-9 via the MP, as assessed in the presence of a C1q inhibitor, is strongly dependent on the MBL genotype (Fig. 4A). A significantly lower activity was observed in *A/B* donors ($p < 0.001$) but not in *A/D* donors, as compared to *A/A* donors. MP-mediated activation of C4 and activation of C5b-9 was strongly correlated (R = 0.89, P < 0.0001).

Next to MBL exon 1 polymorphisms, other factors considered to be involved in the extreme variation of MP activation by mannan in human serum were the MBL serum concentration, the capability of MBL to bind to mannan, the activity of the

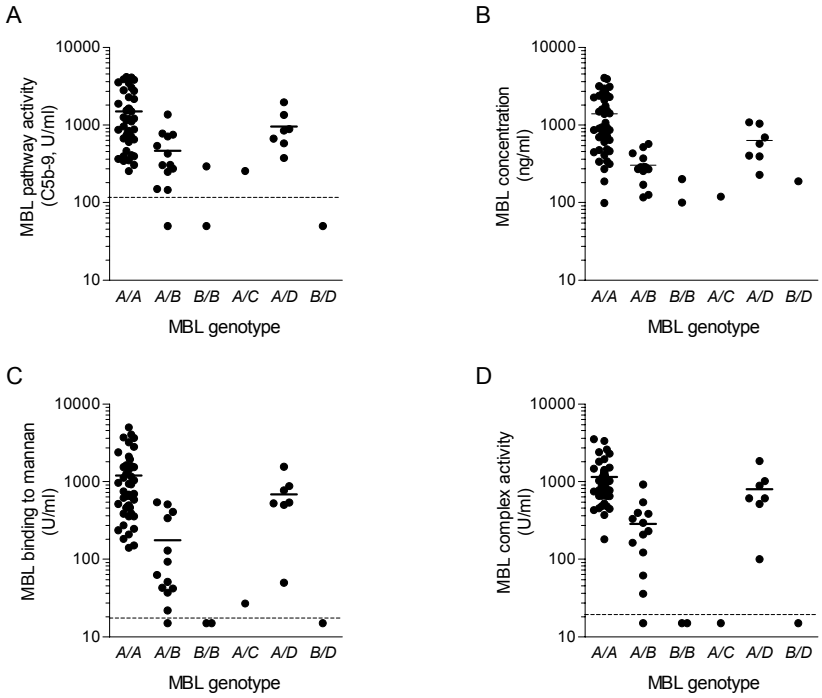

Figure 4. Functional characterization of the MBL pathway
MP activity assessed at the level of C5b-9 in the presence of mAb 2204 (A, dilutions starting from 1/100), MBL concentration (B), MBL binding to mannan (C, dilutions starting from 1/10), and MBL complex activity (D, dilutions starting from 1/50) in different sera. ANOVA: $p < 0.001$, *A/A* versus *A/B* $p < 0.001$

MBL-MASP complex, MBL promoter polymorphisms and MASP-2 polymorphisms. The MBL serum concentration (Fig. 4B), the capacity of MBL to bind to mannan (Fig. 4C) as well as the C4-cleaving activity of the MBL complex, as determined by exogenously added C4 (15) (Fig. 4D), were strongly decreased in carriers of MBL variant alleles. For all three parameters, *A/B* donors but not *A/D* donors showed a significant difference as compared to *A/A* donors.

Both in wildtype and variant serum, MP activity assessed at the level of C5b-9 correlated highly significantly with the MBL concentration (Fig. 5A), the MBL ligand binding activity (Fig. 5B), and the MBL complex activity (Fig. 5C), demonstrating that the availability of functionally active MBL is the major determinant of the activity of the MP in full human serum. Furthermore, MBL complex activity was strongly correlated to both the MBL concentration (Fig. 6A), and the capacity of MBL to bind to mannan (Fig. 6B), suggesting that impaired ligand binding is an important cause of low MBL complex activity in carriers of variant alleles.

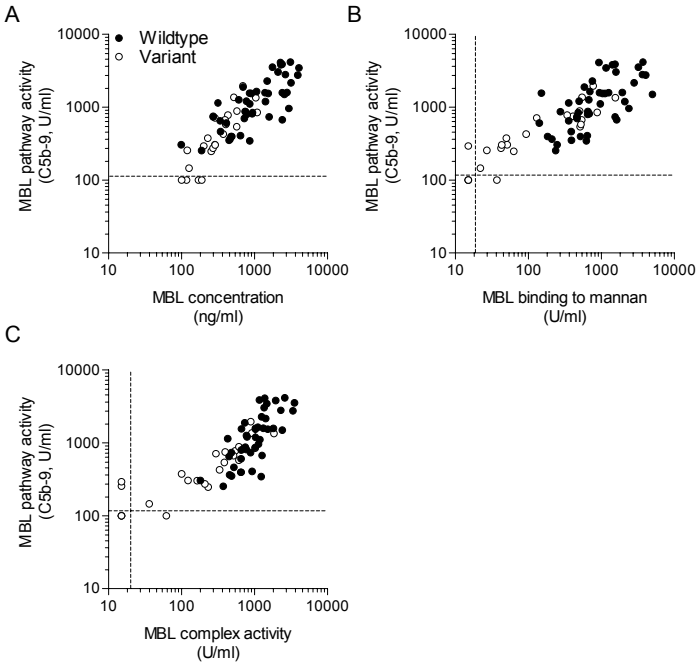

Figure 5. MBL pathway activity is dependent on the presence of functional MBL.
MP activity is plotted against the MBL concentration (A; R = 0.74 (wildtype); R = 0.90 (variant)), MBL binding to mannan (B; R = 0.67 (wildtype); R = 0.90 (variant)) and MBL complex activity (C; R = 0.76 (wildtype); R = 0.91 (variant)), for sera obtained from MBL wildtype and variant individuals, as indicated. $P < 0.0001$ for all correlations.

Since our data indicate a functional MP defect in carriers of the *A/B* genotype, we further characterized the functional properties of MBL in serum from *A/B* donors. Serum samples from *A/B* donors were compared with serum samples from *A/A* donors having a comparable MBL concentration (Table 1). The MBL ligand binding capacity and the MBL complex activity of circulating MBL were significantly decreased in A/B donors as compared to A/A donors. This difference in MBL complex activity between wildtype and variant MBL is also illustrated in fig. 6A.

MBL promoter polymorphisms have been identified that control the MBL serum concentration. Accordingly, A/A donors with the *H/H* promoter genotype show higher MBL levels than A/A donors with the *L/L* promoter genotype (Fig. 7A). Furthermore, MBL complex activity and MP activity were significantly higher in sera obtained from *H/H* donors than in sera obtained from *L/L* donors (Fig. 7A). These functional effects are presumably directly related to the effects of the promoter polymorphisms on MBL gene expression.

MBL promoter polymorphisms are in strong linkage disequilibrium with exon 1 polymorphisms. In this respect, the *B* genotype is always found in haplotypes car-

Table 1. Functional activity of circulating MBL in A/A and A/B serum

Parameter	MBL genotype	Median (range)	*p*
MBL concentration (ng/ml)	*A/A*	342 (99-607)	0.41
	A/B	287 (126-571)	
MBL ligand binding capacity of circulating MBL (U/ng)	*A/A*	1.09 (0.34-2.49)	0.008
	A/B	0.25 (0.15-1.5)	
MBL complex activity of circulating MBL (U/ng)	*A/A*	1.36 (0.99-1.98)	0.003
	A/B	0.83 (0.29-1.77)	

A/B donors ($n = 12$) were compared to *A/A* donors ($n = 11$) matched on basis of similar MBL serum concentrations. The parameters given were calculated by dividing the MBL binding to mannan (U/ml) or the MBL complex activity (U/ml), by the MBL concentration (ng/ml), as indicated. Data were analyzed by using the Mann-Whitney U test.

rying the *LYP* allele, and the *D* genotype is found on the *HYP* haplotype (9). In donors with heterozygous exon 1 SNP, MBL promoter polymorphisms present in the wildtype allele will determine the relative expression of the wildtype and the variant allele. To directly assess the impact of the B and the D allele, sera from *A/B* donors and from *A/D* donors were compared with serum from *A/A* donors with the same promoter genotype, respectively. Sera from *LYQA/LYPB* donors have a significantly lower MBL concentration and MP activity as compared to *LYQA/LYPA* donors. Furthermore, sera from *HYPA/HYPD* donors have a significantly lower MBL concentration than sera from *HYPA/HYPA* donors (Fig. 7B).

Recently, a SNP in the MASP-2 gene was identified that can cause MASP-2 deficiency (17). Although the frequency of this variant allele was described to be 5.5%, all donors included in our study were homozygous carriers of the wildtype allele (assessed by PCR-RFLP, data not shown).

Impaired MBL function is related to impaired MBL polymerization

The molecular structure of MBL was examined in whole human serum from individuals with different genotypes by Western blotting (Fig. 8). Both wildtype and variant MBL showed a doublet between approximately 160 and 200 kDa, at variable amounts correlating with the MBL serum concentration. A number of high molecular weight bands (above ± 200 kDa) were observed only in those sera that show detectable MBL complex activity, also following prolonged exposure. In contrast, a double band was observed around 90 kDa that is predominantly present in carriers of variant alleles. Carriers of two variant alleles contained only low molecular weight MBL (up to ± 200 kDa), whereas a mixture of low and high molecular weight MBL was detected in heterozygous carriers of variant alleles.

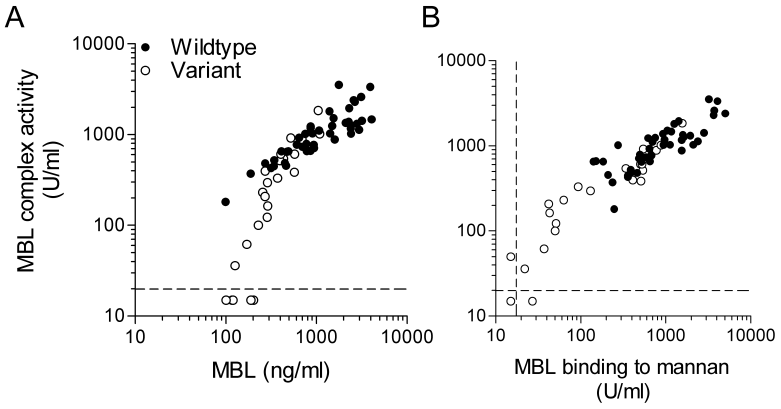

Figure 6. MBL complex activity is determined by the availability of MBL binding to mannan.
MBL complex activity is plotted against the MBL concentration (A; R = 0.88 (wildtype); R = 0.94 (variant)) and the level of MBL binding to mannan (B; R = 0.86 (wildtype); R = 0.96 (variant)) for sera obtained from MBL wildtype and variant individuals. P < 0.0001 for all correlations.

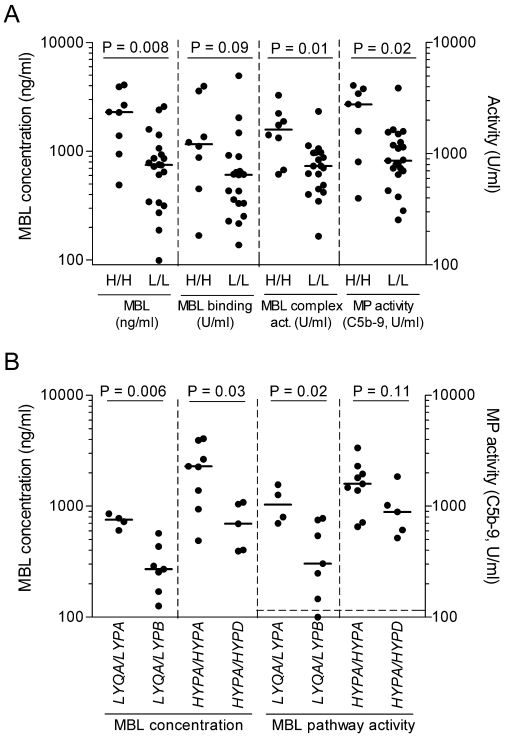

Figure 7. Functional effects of MBL promoter variants.
Sera from *A/A* donors homozygous for the *H* or *L* promoter variant (A) and sera from *A/B* donors, *A/D* donors and *A/A* donors with identical promoter haplotype (B) are compared for different parameters of MBL function, as indicated.

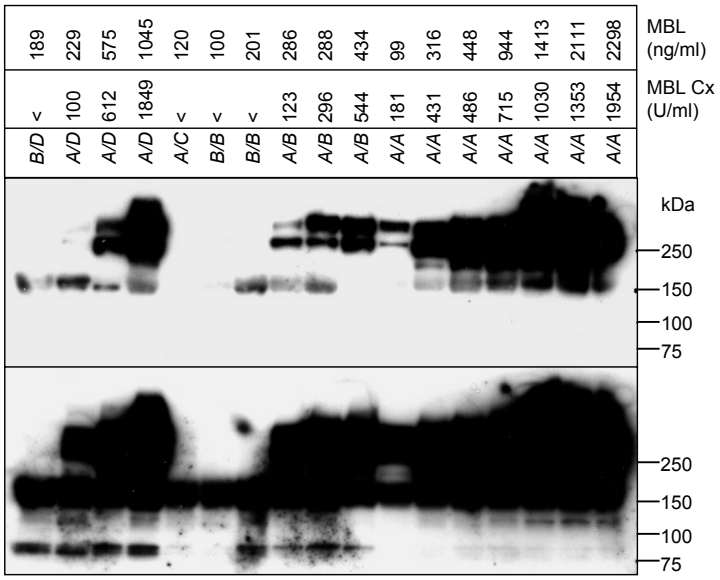

Figure 8. Impaired MBL function is related to the presence of low molecular weight MBL.
Total serum (1.5 µl) was subjected to SDS-PAGE followed by Western blotting and detection of MBL. The position of markers is indicated. For each donor, the MBL exon 1 genotype, the MBL concentration and the MBL complex activity are indicated. The same blot is shown after short exposure (5 minutes, top) and long exposure (30 minutes, bottom) of the film.

DISCUSSION

The present study demonstrates that, although the MP of complement can be severely hampered by MBL deficiency, activation of the CP of complement via C1q and anti-carbohydrate antibodies can compensate for such a defect.

Epidemiological studies clearly indicated that MBL is an important factor of innate immune defense, both as a primary opsonin and as an activator of the complement cascade. In case of MBL deficiency, hampering the opsonization of targets with MBL and complement components, other molecules may support phagocytosis and presentation of carbohydrate antigens to the acquired immune system, thereby compensating for the lack of functional MBL. In this respect, recognition of carbohydrates on pathogens may also involve lectin receptors expressed on phagocytes, other soluble lectins, including collectins and ficolins (1; 18), and anti-carbohydrate antibodies.

Certain anti-carbohydrate antibodies are present at high levels in human serum, such as antibodies directed against the ABO blood group antigens and antibodies against the major xenoantigen, Galα1-3Gal. These anti-carbohydrate antibodies most likely originate from the continuous immune stimulation by bacteria of the gut flora. It is likely that similar mechanisms are responsible for the production of anti-mannan

antibodies in humans, in response to the high mannose structures commonly found on microbial surfaces. In the current study we show that anti-mannan antibodies are able to induce the CP of complement, thereby inducing complement activation in MBL-deficient serum until a level that is comparable to that of MBL-sufficient serum.

The presented data indicate that human sera display a high variability when tested for the activation of C4 by mannan, which is in agreement with findings of Super and Minchinton (19; 20). Our study indicates significant MBL-independent complement activation by mannan, which is dependent on C1q. In case of MBL dysfunction, C4 activation by mannan is dominated by activation of the CP, as is illustrated by an increasing relative contribution of C1q with decreasing MP activity. The contribution of the CP is most likely explained by the presence of anti-carbohydrate antibodies, which are present in human serum with a high inter-individual variation (16). Anti-carbohydrate antibodies may contribute to early host defense, and, when produced at a sufficient level, may compensate for the lack of functional MBL in the protection against at least part of the pathogens recognized by MBL. This could also explain why MBL-deficient young children, who do not yet produce sufficient levels of antibodies, are more prone to acquire infections than MBL-deficient adults (13).

In the present study we investigate the role of MBL and C1q in opsonization of mannan, a component of *Saccharomyces Cerevisiae*, as well as p60, a protein from *Listeria monocytogenes*. The latter microorganism is a facultative intracellular bacterium that can cause severe systemic infections, associated with e.g. septicemia and meningitis, in humans. Previous studies have shown binding of purified MBL (21) and C1q (22) to this microorganism, and C1q binding was shown to be involved in the uptake of *L. monocytogenes* by macrophages. We now show that the p60 protein of *L. monocytogenes* is able to activate the LP of complement via a direct interaction with MBL. Furthermore, C1q is also able to directly bind to this protein, activating the CP. Activation of the CP may be further promoted by antibodies against p60 which are present in human serum (23). In case of MBL deficiency, activation via the CP only results into a significantly lower level of opsonization. Interestingly, the p60 molecule has been implicated in host cell invasion, as well as in the phagocytosis of L. monocytogenes by dendritic cells (23). Our data indicate cooperation between the CP and the LP in complement activation by p60 from *L. monocytogenes*, thereby most likely promoting phagocytosis and bacterial killing. However, a role for complement and complement receptors in the invasion of this bacterium into non-phagocytic host cells can not be excluded.

The difference that we noticed between complement activation by mannan in the absence or in the presence of C1q inhibition confirms previous suggestions that the CP is likely to be involved in complement activation assays using mannan.

Therefore, assays have been developed, which are used in the present study, in which the CP was inhibited, either by the use of high ionic strength buffer (15), or by including a blocking mAb directed against C1q (16). Previous studies have assessed complement activation by mannan without excluding interference of the CP (19; 20). Minchinton et al. recently presented a detailed study concerning the capacity of serum from healthy donors with different MBL genotypes to activate C4 by mannan, using incubation of serum samples at physiologic ionic strength, followed by a second incubation with an MBL-deficient complement source (20). In agreement with our study, this method also revealed that C4 activating capacity was severely hampered in carriers of MBL variant alleles.

In the present study we evaluate specific MP activation of the whole complement cascade, up to C5b-9 formation, using autologous complement components and an inhibitor of C1q. Our results indicate that heterozygous and homozygous expression of the *B* allele is associated with low MBL serum concentrations, low MBL binding to mannan and low MBL complex activity, resulting into hampered activation of C4 and C5b-9 via the MP. These findings raise the question of the primary cause of impaired MBL function in individuals with structural MBL polymorphisms.

The serum level of MBL is a strong determinant of both MBL complex activity (15) and MP activity. In our study, decreased serum concentrations of MBL were observed in donors with two structural mutations as well as in both *A/D* and *A/B* donors, when compared to wildtype donors with the same promoter haplotype.

MBL in serum shows a wide range of molecular weights, and MBL gene mutations are associated with low molecular weight MBL (24). We show that low molecular weight MBL, which may represent MBL oligomers consisting of up to two trimers (8), is present in variant serum and virtually absent in wildtype serum. Furthermore, the presence of MBL with a molecular weight above 200 kDa, presumably representing molecules consisting of at least three trimers, is required for MBL complex activity and activation of the MP of complement in serum, and these bands are lacking in serum from carriers of two variant alleles. These results agree with recently obtained data with human recombinant MBL (8). Furthermore, the MASP-2 content and the ability to cleave C4 were reported to be highest in human MBL complexes of about 345 kDa (3).

Experiments with recombinant human and rat MBL also point to functional defects of the variant molecules, showing impaired complement activation and MASP binding (6-8). Our data show that MBL binding to mannan and MBL-MASP complex activity of circulating MBL are strongly decreased in carriers of MBL variant alleles. This difference is apparent in *A/B* donors and in donors with a double MBL mutation (*B/B* and *B/D*) but not in *A/D* donors, which is in agreement with data from Minchinton et al. (20), and which was also suggested by data obtained with recombinant rat MBL

(7). Taken together, the intrinsic defects that we detect in circulating variant MBL are a relative increase in low molecular weight MBL and an impaired ability to bind to mannan, resulting into reduced MBL complex activity and hampered activation of C4 and C5b-9. Impaired ligand binding most likely results from a lower avidity that is inherent to smaller MBL molecules with less ligand-binding domains. Low molecular weight MBL in carriers of variant alleles, in association with impaired ligand binding, was also recently shown by gel filtration (24; 25).

Associations between MBL deficiency and increased susceptibility to infectious diseases have been frequently reported, in otherwise healthy subjects (10-12) and, more strongly, in patients with additional immunological defects or chronic diseases (26-28). In these studies, MBL deficiency was always defined on basis of the MBL genotype, the MBL concentration, or both, and genetic associations could be observed both for homozygous and heterozygous carriers of MBL variant alleles (10; 11; 28).

The present study shows that, also when the MBL haplotype is taken into account, MP activity shows a much larger variation than CP activity. Sera from healthy donors with an identical MBL haplotype can have a 10-fold difference in MP activity (Fig. 7 and unpublished results). Additional polymorphisms in the genes of MBL and/or MBL-associated molecules could play a role in this variation. The recently characterized variant allele of MASP-2 (17) was not present in the donors examined in our study, suggesting that this variant may be more prevalent in the Scandinavian population.

In conclusion, activation of the complement cascade via the MP is critically dependent on the availability of MBL that is able to bind to its ligands. However, MP dysfunction is not necessarily associated with inadequate opsonization, since anti-carbohydrate antibodies and the CP of complement can take over this function. Antibodies, C1q and MBL can cooperate in early host defense by simultaneous activation of parallel pathways of the complement system. Therefore, MBL deficiency primarily may become clinically relevant in situations without a concomitant adaptive immune response.

MATERIALS AND METHODS

Human materials

Blood was taken from 70 healthy donors. Serum was immediately aliquotted and frozen at -80°C. Heparinized blood was used for genomic DNA isolation (16).

MBL genotyping

MBL exon 1 SNP were identified using an oligunucleotide ligation assay (16) and confirmed by PCR using sequence-specific priming (29). Furthermore, the regulatory genetic variants *H/L* (-550), *X/Y* (-221), and *P/Q* (+4) (9) were typed as described (29).

Functional assessment of complement activation

Functional activity of the MP and the CP were assessed using methods that were previously described in detail (16), using plates coated with mannan and human IgM to assess MP and CP activity, respectively. MP function was assessed in the presence of a blocking mAb directed against C1q (mAb 2204, 20 µg/ml; kindly provided by Dr. C.E. Hack, Sanquin Research, Amsterdam). In some experiments, complement activation on mannan was assessed in the absence of mAb 2204, as indicated. Complement activation was detected using monoclonal antibodies directed against C4 and C5b-9, as described (16). For specific experiments, mannan-coated plates were pre-incubated with IgG and IgM purified from human donor plasma, diluted in PBS containing 1% BSA, 0.05% Tween 20, and 10 mM EDTA.

Binding of MBL to mannan was assessed following incubation with human serum diluted in a Calcium-containing buffer (GVB++), using dig-conjugated mAb 3E7 (anti-human MBL) as described (16).

Complement activation by p60 from Listeria monocytogenes

ELISA plates were coated with p60 (5 µg/ml), a protein from *Listeria monocytogenes* expressed as described before ((30), kindly provided by Dr. S.H.E. Kaufmann, Berlin, Germany) in coating buffer (100 mM Na_2CO_3/ $NaHCO_3$, pH 9.6). Binding sites were saturated with 1% BSA in PBS. Binding of MBL (diluted in GVB++) and C1q (diluted in PBS/ 1% BSA / 0.05% Tween 20) (both purified from normal donor plasma), was assessed using mAb 3E7 and mAb 2214 (anti-human C1q, from Dr. C.E. Hack), respectively, both conjugated to dig. Deposition of C4 in the presence or absence of mAb 2204, D-mannose or L-mannose (both used at 100 mM, from Sigma) was assessed.

MBL concentration

The concentration of MBL in serum was assessed by sandwich ELISA. Plates were coated with 3E7 (mAb anti-MBL) at 5 µg/ml. Sera were diluted in PBS containing 0.05% Tween-20 and 1% BSA. MBL was detected using rabbit IgG anti-MBL (20 µg/ml, prepared by immunization of rabbits with purified human MBL), followed by HRP-conjugated goat anti-rabbit IgG (from Jackson ImmunoResearch Laboratories, West Grove, PA, USA).

MBL complex activity

MBL complex activity was assessed using the method described by Petersen et al. (15) with slight modifications. Mannan-coated plates were incubated with serum, diluted in GVB++ containing 1 M of NaCl, during 16 hours at 4°C. Plates were washed with PBS/Tween containing 5 mM $CaCl_2$, followed by incubation with purified C4 (1 µg/ml), diluted in GVB++, for 1 hour at 37°C. Activation of C4 was assessed.

Western blotting

Human serum (1.5 µl) was subjected to SDS-PAGE using a 4 to 15% polyacrylamide gradient gel (Criterion Pre-cast gel, Tris-HCl, from Bio-Rad Laboratories, Richmond, CA, USA) under non-reducing conditions. Proteins were transferred to polyvinyldene fluoride membranes (Immobilon, Millipore, Bedford, USA) using a semi-dry blotting procedure. Membranes were blocked with PBS/0.05% Tween 20/ 2% Casein followed by incubation with mAb 3E7 (1 µg/ml) for 16 hours at 4°C and HRP-conjugated goat anti-mouse IgG (Dako, Glostrup, Denmark) for 2 hours at room temperature. Development of blots was performed with Supersignal (Pierce Chemical Co., Rockford, IL, USA) and exposure to Hyperfilms (Amersham Pharmacia Biotech).

Calculations and statistical analysis

All sera were analyzed in at least two dilutions in duplicates. Functional activity was expressed in units per ml, based on serial dilutions of a human pool serum used as a standard (set at 1000 U/ml). The activity was calculated using parameters of linear regression following log-transformation of arbitrary units and logit-transformation of OD values. Statistical analysis was performed with GraphPad Prism 3.03 using non-parametric tests. Differences were evaluated by the Mann Whitney U test or by analysis of variance (ANOVA), using the Kruskal Wallis test and Dunn's correction for multiple comparisons. Correlation was evaluated using the Spearman Rank correlation coefficient (R). Results were considered as statistically significant when p values were below 0.05.

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CHAPTER 2.3

Human immunoglobulin A activates the complement system via the mannan-binding lectin pathway

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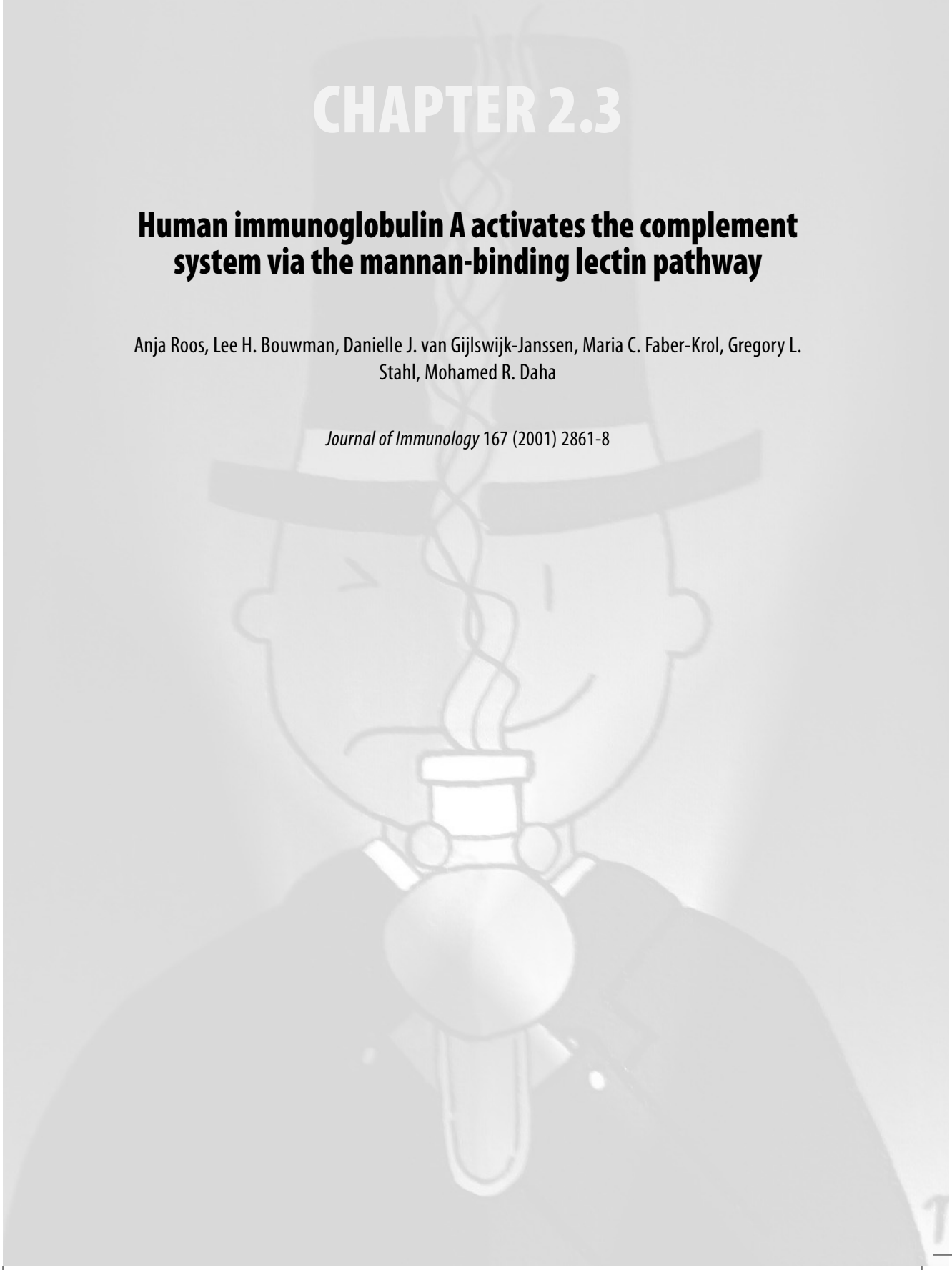

ABSTRACT

The recently identified lectin pathway of the complement system, initiated by binding of mannan-binding lectin (MBL) to its ligands, is a key component of innate immunity. MBL-deficient individuals show an increased susceptibility for infections, especially of the mucosal system. We examined whether IgA, an important mediator of mucosal immunity, activates the complement system via the lectin pathway. Our results indicate a dose-dependent binding of MBL to polymeric but not monomeric IgA coated in microtiter plates. This interaction involves the carbohydrate recognition domain of MBL, since it was calcium-dependent and inhibited by mannose and by mAb against this domain of MBL. Binding of MBL to IgA induces complement activation, as demonstrated by a dose-dependent deposition of C4 and C3 upon addition of a complement source. The MBL concentrations required for IgA-induced C4 and C3 activation are well below the normal MBL plasma concentrations. In line with these experiments, serum from individuals having mutations in the MBL-gene showed significantly less activation of C4 by IgA and mannan than serum from "wildtype" individuals. We conclude that MBL binding to IgA results in complement activation, which is proposed to lead to a synergistic action of MBL and IgA in antimicrobial defense. Furthermore, our results may explain glomerular complement deposition in IgA nephropathy.

INTRODUCTION

The complement system is an important component of host defense. Activation of the complement cascade takes place upon the interaction of complement components with a variety of pathogens, either directly or via antibodies bound to pathogen antigens. Three different pathways for complement activation have been described, the classical pathway, the alternative pathway and the lectin pathway, of which the latter is most recently identified. The lectin pathway is mainly driven by mannan-binding lectin (MBL).

MBL, a member of the collectin family (1), is a C-type lectin present in serum as a part of a large pro-enzymatic complex. The MBL protein consists of three to six identical homotrimeric subunits. Each trimer is composed of a collagen-like tail part and a globular head part containing a carbohydrate recognition domain (CRD). The collagen-like part of MBL interacts with the MBL-associated serine proteases MASP-1 (2), MASP-2 (3), and MASP-3 (4). These enzymes are responsible for the complement-activating properties of the MBL complex, by the cleavage of C4, C2 and C3 (5). The CRD of MBL is able to bind in a calcium-dependent way to a number of saccharides, such as D-mannose, L-fucose, and N-acetyl-glucosamine (1). MBL binding to a ligand induces activation of the MASP enzymes, leading to complement activation up to the terminal pathway. Both the structural and functional properties of MBL are strikingly similar to those of C1q, the recognition unit of the classical complement pathway.

Genetic mutations in the MBL gene are present with a high frequency in the human population (6). Until now, three different point mutations have been described which lead to the production of MBL with structural aberrations and impaired complement-activating properties (6; 7). Heterozygous and homozygous expression of these mutant alleles is associated with an enhanced incidence of a range of infections, in both children and adults (8; 9). In this spectrum of diseases, mucosal infections, occurring in the respiratory tract and the gastrointestinal tract, are common. Furthermore, mutations in the MBL gene have a significant negative impact on chronic diseases such as rheumatoid arthritis, systemic lupus erythematosus (SLE) and cystic fibrosis, resulting in an increased incidence of complicating infections and/or a worse outcome (10-12). These studies indicate the importance of the lectin pathway of complement activation in anti-microbial defense. In line of this function of the lectin pathway, MBL is able to bind directly to a number of microorganisms, via the carbohydrates expressed on their surface (1; 13). Upon binding, complement activation takes place, leading to either direct elimination via the terminal complement pathway, or opsonization and phagocytosis.

In the mucosal immune system, a major factor of defense is immunoglobulin A. It is present in plasma at a concentration of about 2 mg/ml, and it is secreted at mucosal surfaces throughout the body where it is postulated to play an important role as a defense mechanism against invading microorganisms (14; 15). Upon interaction of IgA with pathogens, the IgA molecule can have diverse effector functions, including the direct prevention of invasion of microorganisms, the interaction with the phagocytic IgA Fc receptor CD89, and complement activation.

Complement activation by IgA has been previously shown to involve the alternative but not the classical complement pathway (16; 17). No data are available concerning the possible involvement of the lectin pathway. A classical disease involving IgA and complement activation is primary IgA nephropathy, a common renal disease involving mesangial deposition of IgA and complement components, resulting in end stage renal failure in about 30% of the patients (18). Recent studies indicate the presence of MBL in association with IgA in the mesangial area of patients with IgA nephropathy (19; 20).

Therefore, we examined whether IgA induces complement activation via a possible interaction with MBL. Our results demonstrate activation of the lectin pathway by IgA. This novel interaction in the immune system is proposed to contribute to the roles of MBL and IgA in host defense, as well as to the pathogenesis of IgA nephropathy.

MATERIALS AND METHODS

Purification of MBL/MASP complexes

MBL and its associated proteases were purified from pooled plasma obtained from healthy human donors, essentially as described by Tan et al. (21). In brief, a precipitation step was performed using polyethylene glycol 3350 (Sigma, St. Louis, MO; 7% (w/v)). The precipitate was dissolved in TBS-T/Ca^{2+} (50 mM Tris, 0.15 M NaCl, 0.05% Tween 20, 20 mM $CaCl_2$, pH 7.8) and incubated for 18 hours at 4°C with mannan-agarose (Sigma; equilibrated with TBS-T/Ca^{2+}). After extensive washing with TBS-T/Ca^{2+}/1M NaCl, bound proteins were eluted using TBS-T containing 10 mM EDTA. Fractions containing MBL, as determined by ELISA, were pooled and concentrated. To remove contaminating immunoglobulins, the MBL preparation was absorbed using a mixed absorbent consisting of 4E8 (mAb anti-IgA, produced in the laboratory of Nephrology, Leiden, the Netherlands) coupled to Biogel A5 (Biorad, Richmond, CA, USA), HB57 (mAb anti-IgM, hybridoma obtained from the American Type Culture Collection (ATCC)) coupled to Biogel A5, and protein G coupled to Sepharose (from Pharmacia, Uppsala, Sweden). The resulting MBL preparation con-

tained negligible amounts of IgA (< 0.5%) whereas IgG and IgM were undetectable. Furthermore, the MBL preparation did not contain any detectable C1q, as determined by singe radial immunodiffusion and by a sensitive C1q-specific hemolytic assay. This purification method results in co-purification of MASP proteins, as shown by Western blotting using rabbit anti-MASP-1 antibodies, prepared as described (3), and rabbit anti-MASP-2 antibodies (kindly provided by Dr. R. Sim, Oxford, UK) as well as by a C4 consumption assays (not shown). The resulting MBL-MASP preparation was subjected to ELISA to determine the MBL concentration (as described below) and subsequently used in all experiments.

Purification of human IgA

IgA was purified from pooled normal human serum (NHS) or recalcified donor plasma as described by Hiemstra et al. (16) with minor modifications. In brief, the majority of serum proteins were removed by dialysis against H_2O, and precipitation by $ZnSO_4$. Proteins in the supernatant were precipitated using glycine and $(NH_4)_2SO_4$, dialyzed against TE buffer (10 mM Tris, 2 mM EDTA, pH 7.8) and loaded on a DEAE-Sephacel column (Pharmacia). IgA was eluted with a linear salt gradient (conductivity 1-20 mS). IgA-containing fractions, as determined by ELISA, were pooled, concentrated and further purified by gelfiltration, using a Sephacryl S-300 column (Pharmacia). Veronal-buffered saline (VBS, 1.8 mM Na-5,5-diethylbarbital, 0.2 mM 5,5-diethylbarbituric acid, 145 mM NaCl) containing 2 mM EDTA was used as a running buffer. Fractions were tested for IgA by ELISA; IgA of different molecular sizes, i.e. monomeric, dimeric, and polymeric IgA were pooled separately on basis of its position in the elution profile. Reanalysis of these different IgA preparations indicated that this molecular size is stable: typically > 90% runs at the same position in the elution profile. Contamination with IgG and IgM was below 0.4% on a weight basis, as determined by ELISA. Experiments were performed with polymeric IgA unless otherwise indicated.

Purification of human IgG and IgM

Human IgG was purified from outdated plasma obtained from healthy donors as described previously (22). Human IgM was purified from human serum by euglobulin precipitation and anion exchange chromatography, as described (22), followed by cation exchange chromatography, using CM-Sephadex C-50 (Pharmacia), and gelfiltration, using a Sephacryl S-300 column (Pharmacia). The IgG preparation was free of any detectable IgA and IgM, and the IgM preparation was free of any detectable IgA and IgG.

Purification of functionally active C4

Freshly obtained NHS (120 ml) was adjusted with TEB-buffer (10 mM Tris, 2 mM EDTA, 1 mM benzamidine hydrochloride hydrate (Sigma)) to pH 7.8 and conductivity of 4.0 mS. This sample was loaded on a DEAE-sephacel column (5 x 10 cm) which was previously equilibrated with TEB buffer at pH 7.8 and mS 4.0. The column was extensively washed with the same buffer, followed by elution with a linear salt gradient with a conductivity from 4.0 mS to 25 mS. Fractions were tested for the presence of C4 using a hemolytic assay. In this assay, serum from C4-deficient guinea pig is used as a complement source and sheep red blood cells (SRBC) sensitized with rabbit anti-SRBC antibodies as targets. Lytic activity of an excess amount of C4-deficient serum can be restored by addition of a limiting amount of C4. Peak fractions containing C4 (at 15 mS) were pooled and concentrated. The concentration of C4 was determined by single radial immunodiffusion.

Human serum containing wildtype or mutated MBL

Serum samples were obtained from 5 healthy donors having the wildtype genotype at codon 52, 54, and 57 from the first exon of the MBL gene (A-genotype) as well as from 6 healthy donors with a homozygous (n = 2) or heterozygous (n = 4) mutation at codon 54 (B-genotype) (6). These genotypes were identified by an oligonucleotide ligation assay (A. Roos et al., manuscript in preparation) and were confirmed by DNA sequencing.

ELISA protocol

For all ELISA assays, Nunc Maxisorb plates (Nunc, Roskilde, Denmark) were coated using coatingbuffer (100 mM Na_2CO_3/ $NaHCO_3$, pH 9.6), for 16 hours at room temperature or for 2 hours at 37°C. After each step, plates were washed three times with PBS containing 0.05% Tween 20. Residual binding sites were blocked by incubation with PBS containing 1% BSA. Unless otherwise indicated, all subsequent steps were incubated in PBS containing 0.05% Tween 20 and 1% BSA, for one hour at 37°C. Detection antibodies were conjugated to digoxygenin (dig) using digoxygenin-3-O-methylcarbonyl-ε-aminocaproic acid-N-hydroxysuccinimide ester (from Boehringer Mannheim, Mannheim, Germany) according to instructions provided by the manufacturer. Detection of binding of antibodies conjugated to dig was performed by HRP-conjugated rabbit anti-dig antibodies (Fab fragments, from Boehringer Mannheim). Enzyme activity of HRP was detected using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma). The OD at 415 nm was measured using a microplate biokinetics reader (EL312e, from Biotek Instruments, Winooski, Vermont, USA).

MBL detection ELISA

Plates were coated with 3E7 (mAb anti-MBL (mouse IgG1, kindly provided by Dr. T. Fujita, Fukushima, Japan) at 5 µg/ml. Samples containing MBL were incubated, followed by detection with dig-conjugated 3E7. A calibration line was produced using pooled human serum from healthy donors with a known concentration of MBL (kindly provided by Dr. P. Garred, Copenhagen, Denmark).

MBL binding to IgA or mannan

IgA was coated at 5 µg/ml, unless otherwise indicated. Mannan (from Saccharomyces Cerevisiae; Sigma (M7504)) was coated at 100 µg/ml. As a negative control, BSA (Sigma) was coated at 10 µg/ml. After blocking with BSA, MBL was incubated in BVB++ (VBS, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.05% Tween-20, 1% BSA, pH 7.5) for one hour at 37°C. MBL binding was detected using dig-conjugated 3E7. In some experiments, MBL was pre-incubated (30 minutes, 20°C) in a calcium-free buffer (BVB/MgEGTA: VBS, 5 mM $MgCl_2$, 0.05% Tween-20, 1% BSA, 10 mM EGTA) or in BVB++ containing D-mannose, L-fucose, N-acetyl-glucosamine, or N-acetyl-galactosamine (from Sigma), followed by addition of the mixture to the plates. Saccharides were applied at a concentration of 100 mM unless otherwise indicated. Additional inhibition studies were performed using purified mouse mAb directed against MBL (1C10 and 3F8 (23)), which were incubated together with MBL at concentrations between 2 and 20 µg/ml.

Analysis of complement activation by MBL

Activation of complement via MBL was assessed as follows. Plates were coated with IgA, mannan, or BSA, blocked by BSA and, in some cases, incubated with MBL as described above. Subsequently, plates were incubated with 2% NHS as a complement source, diluted in BVB/MgEGTA, for one hour at 37°C. Deposition of C3 was detected by dig-conjugated RFK22 (mAb anti-human C3 (mouse IgG1) produced at the laboratory of Nephrology, Leiden, the Netherlands). Alternatively, C4 activation was assessed using a method adapted from Vorup-Jensen et al. (24). For these experiments, MBL, diluted in BVB++, was incubated for one hour at 37°C and for 16 hours at 4°C. Plates were washed with PBS / 5 mM $CaCl_2$ / 0.05% Tween-20, and C4 was added (1 µg/ml, diluted in BVB++ containing 1 mM $MgCl_2$ and 2 mM $CaCl_2$). C4 binding was detected with affinity-purified goat anti-human C4 antibodies conjugated to dig, or with dig-conjugated C4-4A (mAb anti-C4, kindly provided by Dr. C.E. Hack, Amsterdam, the Netherlands). In some experiments, activation of C4 was assessed directly in human serum. For this assay, all washing and incubation steps were performed in the absence of Tween 20, which reduced non-specific staining. Plates were coated with IgA, mannan, IgG (5 µg/ml) or IgM (5 µg/ml), washed

with PBS, and blocked by 1% gelatin in PBS. Serum was diluted in VBS containing 2 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 0.1% gelatin and incubated for 1 hour at 37°C. Subsequently, C4 binding was detected, using PBS / 1% BSA as a dilution buffer for antibody conjugates.

Statistical analysis

Differences in C4 activation between sera from two groups of donors (i.e. either MBL-wildtype or MBL-mutant genotype) were analyzed using a *t* test and are considered statistically significant when *p* values are less than 0.05.

RESULTS

Interaction of MBL with IgA

The binding of MBL to IgA was studied using microtiterplates coated with purified human IgA. Addition of MBL resulted in a dose-dependent binding to IgA, but not to a control coating with BSA only (Fig. 1A). Binding was clearly detectable at an MBL concentration of 20 ng/ml. Coating of different concentrations of IgA followed by incubation with a fixed concentration of MBL revealed that MBL binding was maximal at an IgA coating concentration of 5 µg/ml (Fig. 1B).

The characteristics of the interaction between MBL and IgA were studied by pre-incubating MBL in the presence of various inhibitors. Pre-incubation with D-mannose, L-fucose, or N-acetyl-glucosamine (GlcNAc), but not N-acetyl-galactosamine (GalNAc) blocked the binding of MBL to IgA and to its major ligand mannan (Fig. 1C). This inhibition by saccharides was dose-dependent: IC50 values for mannose and GlcNAc were between 5 and 10 mM for binding of MBL to IgA (Fig. 1D) and to mannan (not shown). Furthermore, incubation of MBL in a calcium-free buffer containing MgEGTA prevented binding of MBL to IgA and to mannan (Fig. 1C). Binding of MBL to IgA is calcium-dependent and reaches a plateau at 1 mM $CaCl_2$ (Fig. 1E). This concentration was chosen for further assays. These results indicate a calcium-dependent interaction of the CRD of MBL with human IgA.

IgA activates the complement system via the lectin pathway

In order to assess whether the interaction of MBL with IgA induces complement activation, activation of C4 and C3 was studied by ELISA. For experiments studying activation of C3, NHS diluted in a MgEGTA-containing buffer was used as a complement source. The use of MgEGTA prevented activation of the classical pathway and the lectin pathway in the complement source that may occur irrespective of the MBL that was previously bound to the coating, resulting in low background levels.

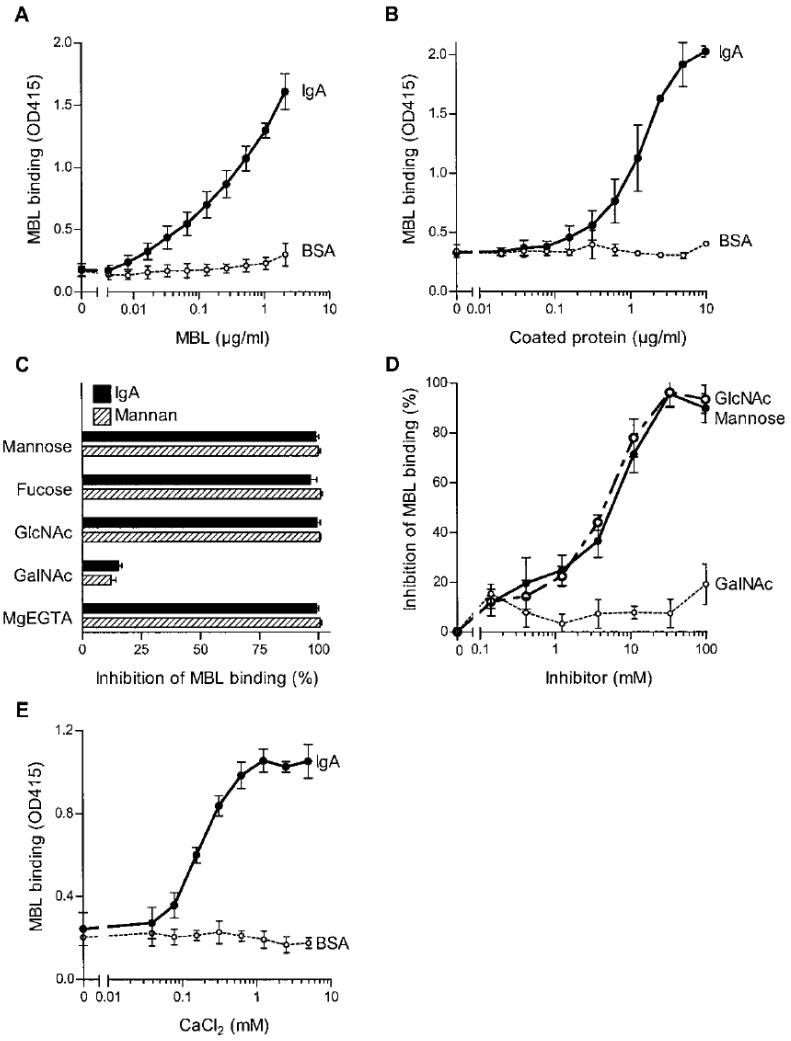

Figure 1. Binding of MBL to immobilized IgA. Microtiterplates were coated with either IgA or BSA, as indicated. MBL binding was detected by ELISA. **A**, MBL was added to coated IgA (5 µg/ml) or BSA at concentrations as indicated. Data represent mean ± SD from 4 independent experiments. **B**, IgA was coated at various concentrations, and binding of a fixed concentration of MBL (2 µg/ml) was detected. Data represent mean ± SD from two independent experiments. **C**, Wells were coated with mannan or IgA, and incubated with MBL (0.1 µg/ml on mannan; 1 µg/ml on IgA) either under standard assay conditions (control) or in the presence of MgEGTA, D-mannose, L-fucose, N-acetyl-glucosamine (GlcNAc) or N-acetyl-galactosamine (GalNAc) as indicated in Materials and Methods. The percentage inhibition of MBL binding to IgA or mannan was calculated using the following formula: 100 – (100 * (MBL binding (+ inhibitor) – MBL binding to BSA) / (MBL binding (control) - MBL binding to BSA)), using OD values measured at 415 nm. None of the inhibitors affected the background binding of MBL to BSA. Mean and SD are shown of triplicate wells of a representative experiment. Similar results were obtained in 5 independent experiments. **D**, Similar experiment as shown in C, but the inhibitors were applied at different concentrations, as indicated (mean ± SD from two independent experiments). **E**, Wells were incubated with MBL (1 µg/ml) in the presence or absence of different concentrations of $CaCl_2$, as indicated. Data represent mean ± SD from two out of three similar experiments.

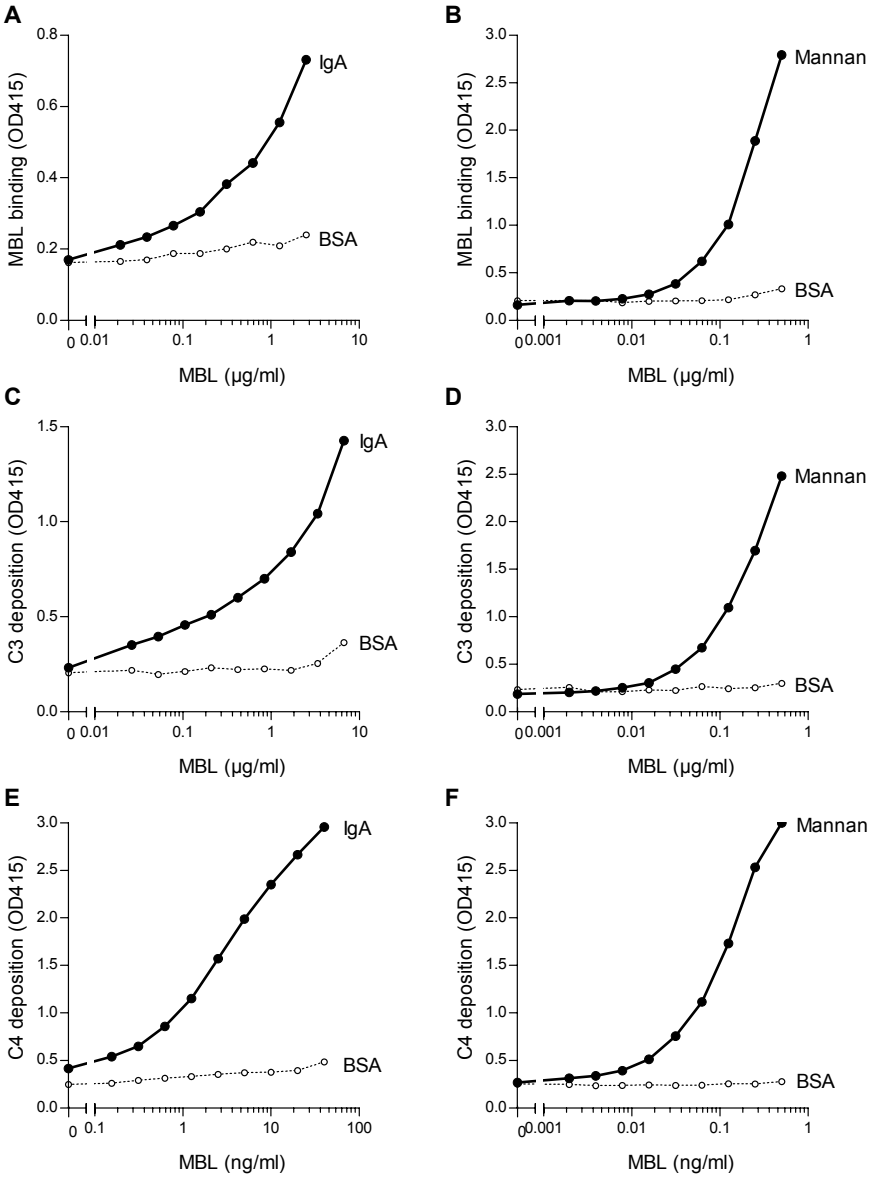

Figure 2. Binding of MBL to IgA induces complement activation. Wells were coated with IgA, mannan or BSA and in the first step incubated with MBL-MASP complexes in various concentrations, as indicated, followed by addition of a complement source in the second step. For **A-D,** the second step consisted of NHS (2% in VBS/BSA/Tween/MgEGTA), which was followed by detection of binding of MBL (**A, B**) or deposition of C3 (**C, D**). Alternatively, purified C4 was added in the second step, followed by detection of C4 binding (**E, F**). Please note that MBL concentrations on the X-axis in **E** and **F** are in *nano*gram per ml. The results are representative for at least three independent experiments.

Binding of MBL to IgA and to mannan, which was first achieved in the presence of calcium, was clearly detectable after a one-hour incubation with serum in the presence of MgEGTA (Fig. 2A, B), as has been previously reported for the binding of MBL to mannan-coated erythrocytes (25). Using these conditions, binding of purified MBL-MASP complexes induced a concentration-dependent deposition of C3 on coated IgA (Fig. 2C) and on coated mannan (Fig. 2D) upon addition of serum. Coated mannan requires about 10 times less MBL than coated IgA to induce the same level of MBL binding and C3 deposition (Fig. 2A, C versus 2B, D).

MBL binding to IgA and mannan also resulted in activation of C4. After binding of MBL-MASP complexes to either IgA (Fig. 2E) or mannan (Fig. 2F), addition of purified C4 resulted in a dose-dependent deposition of C4 on the coating. C4 activation

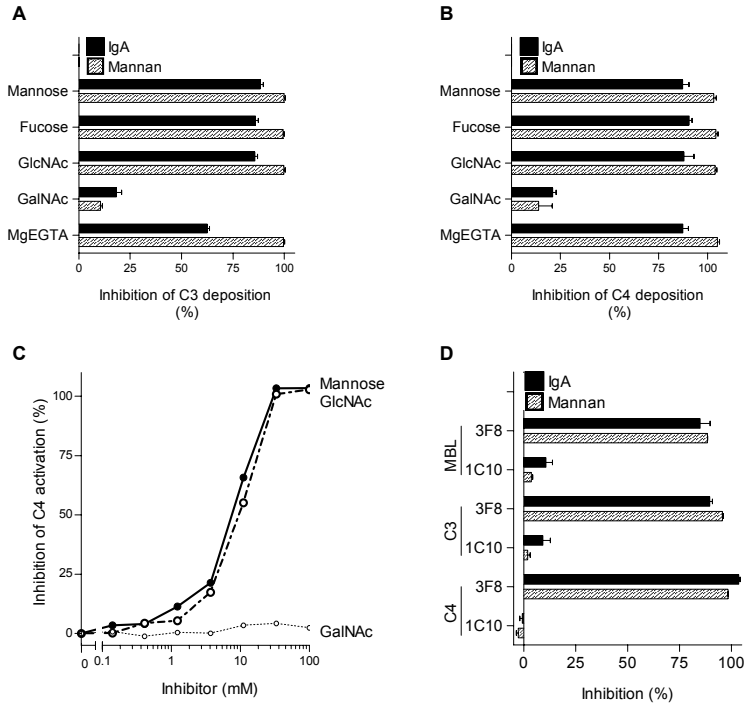

Figure 3. Complement activation is dependent on MBL binding. Plates were coated with mannan or IgA, followed by incubation with MBL in the presence of inhibitors as described at figure 1C. **A**, MBL was incubated at 1 µg/ml and 0.1 µg/ml on IgA and mannan, respectively, followed by addition of NHS (2% in VBS/BSA/Tween/MgEGTA) and assessment of C3 deposition. **B**, MBL was incubated at 2 ng/ml and 0.1 ng/ml on IgA and mannan, respectively, followed by addition of C4 and assessment of C4 binding. Results in A and B are mean ± SD of one out of two or three triplicate experiments. **C**, Similar experiment as shown in fig. 3B, but the saccharides were applied at different concentrations, as indicated. **D**, MBL was incubated on IgA and mannan in the presence or absence of mAb anti-MBL (3F8 and 1C10), as indicated. Binding of MBL and activation of C3 and C4 was assessed as described at fig. 1 and at fig. 3A and B, respectively. Anti-MBL antibodies were used at 20 µg/ml (MBL binding experiments) or at 2 µg/ml (complement activation experiments). Results represent the mean ± SD of triplicate experiments. Inhibition was calculated as described at fig. **1C**.

was detectable at MBL concentrations between 0.01 and 1 ng/ml, depending on the coating used. MASP enzymes required for activation of C4 are present in the MBL preparation as demonstrated by its direct ability to induce C4 consumption in the fluid phase (not shown).

The results presented above strongly suggest that activation of C3 and C4 is induced by the binding of MBL and associated MASPs to IgA or mannan. To further establish this, the MBL preparation was pre-incubated on coated IgA or mannan in the presence of inhibitors, followed by addition of a complement source and analysis of deposition of C3 (Fig. 3A) and C4 (Fig. 3B, C). As expected for an MBL-dependent mechanism, pre-incubation of MBL with mannose, fucose and GlcNAc, but not GalNAc, blocked the activation of C3 and C4, both on IgA and mannan. Inhibition of C4 activation by mannose and GlcNAc was dose-dependent, and 50% inhibition was reached at saccharide concentrations between 5 and 10 mM, both on IgA (Fig. 3C) and on mannan (not shown). Similar dose-response relationships were observed for saccharide inhibition of C3 activation (not shown).

Incubation of MBL on mannan performed in the presence of MgEGTA completely inhibited subsequent C3 and C4 activation (Fig. 3A, B). On IgA, similar results were obtained for C4 deposition (Fig. 3B), whereas deposition of C3 was inhibited for a major part, but not completely (Fig. 3A).

To further prove the MBL-dependence of C3 and C4 activation on IgA and mannan, additional blocking studies were performed using mAb anti-MBL. Two different MBL-specific mAb were used (Fig. 3D): 3F8 that blocks MBL-mediated complement activation and, as a control, 1C10 that binds to MBL but does not block its function

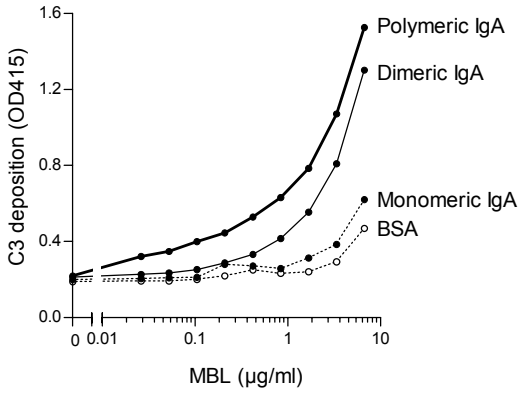

Figure 4. Lectin pathway activation is mainly a function of di- and polymeric IgA. Plates were coated with monomeric, dimeric, and polymeric IgA. MBL in different concentrations and NHS (2% in VBS/BSA/Tween/MgEGTA) were successively incubated and C3 binding was detected. One out of two similar experiments is shown.

(23). The mAb 3F8 totally inhibited the binding of MBL as well as the activation of C3 and C4 on IgA and on mannan, whereas mAb 1C10 did not have any effect.

Complement activation is known to be predominantly a function of polymeric IgA (26). We tested the different molecular sizes of IgA for their ability to activate the lectin pathway. Polymeric IgA is superior to dimeric IgA in activation of the lectin pathway (Fig. 4). No significant activation could be detected by monomeric IgA.

Previous studies have shown that IgA can activate the alternative pathway in serum in the presence of MgEGTA (16). Only the alternative pathway, but not the classical pathway nor the lectin pathway, can proceed in the absence of Ca^{2+}. To examine the combined contribution of the lectin pathway and the alternative pathway to activation of C3 by IgA, complement activation was studied with or without a pre-incubation with MBL. Serum incubated in the presence of MgEGTA, but in the absence of MBL, induced a clear deposition of C3 on IgA (Fig. 5), in agreement with previously published data (27). Pre-incubation with MBL in a calcium-containing buffer enhanced the deposition of C3 dose-dependently. Deposition of C3 was reduced to background levels when EDTA was present in the complement source (not shown).

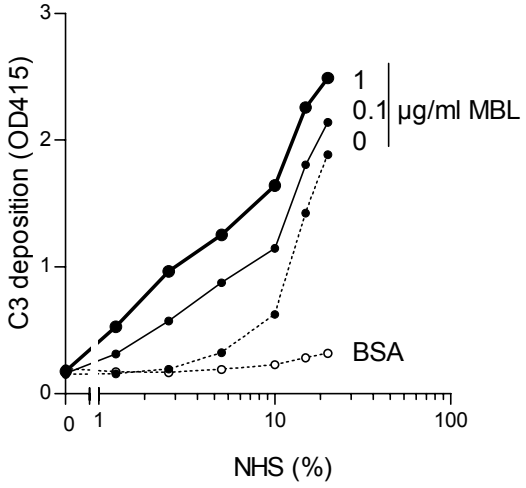

Figure 5. IgA activates both the lectin pathway and the alternative pathway. Plates were coated with IgA or BSA, as indicated, and incubated in the presence or absence of MBL, followed by NHS in different concentrations in VBS/BSA/Tween/MgEGTA. C3 deposition was detected. Similar results were obtained in two experiments.

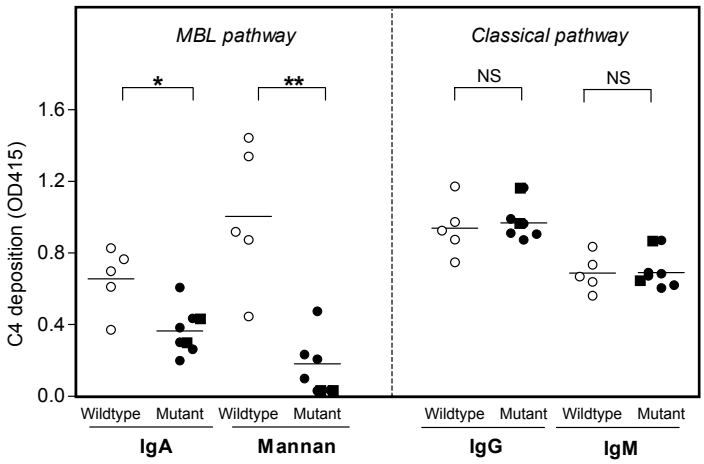

Figure 6. C4 activation in serum with wildtype or mutated MBL. Plates were coated with IgA, mannan, IgG or IgM. Sera obtained from donors with a wildtype (N = 5) or mutant MBL genotype (heterozygous (N = 4; circles) or homozygous (N = 2; squares)) were incubated in a calcium and magnesium-containing buffer (dilution 1/80 (IgA, mannan), 1/160 (IgG) or 1/40 (IgM)), and C4 activation was assessed. Background OD values obtained in the absence of serum were subtracted. The mean of each group is indicated by a horizontal line. Differences between mutant and wildtype sera were evaluated by a *t* test. * $P = 0.015$; ** $p = 0.001$.

Serum containing mutated MBL has a partial defect in the activation of C4 by mannan and by IgA

MBL derived from individuals with mutations in exon 1 of the MBL gene has an impaired ability to activate the complement system (7). In order to examine whether this defect also hampers complement activation by IgA, serum from MBL wildtype donors (n = 5) was compared with that from donors with a mutant genotype (homozygous (n=2) or heterozygous (n=4) point mutations at codon 54) in a C4 activation assay. In comparison to the control group, serum from donors having MBL gene mutations induced significantly less C4 activation both on IgA (p = 0.015) and on mannan (p = 0.001) (Fig. 6). However, when plates were coated with human IgG of IgM, as activators of the classical complement pathway, both groups of sera induced a similar level of C4 activation.

DISCUSSION

The present study demonstrates that the CRD of MBL can bind to IgA and thereby activate the complement system via the lectin pathway. We propose that this interaction between the lectin pathway and IgA may function as a novel link between the innate and the adaptive immune system. Furthermore, the interaction between

MBL and IgA is expected to contribute to mesangial complement deposition in IgA nephropathy.

Our results demonstrate a calcium-dependent interaction of the CRD of MBL with IgA. This binding was evident at concentrations well below the mean MBL plasma concentration in healthy individuals, which is approximately 1.5 µg/ml. The carbohydrate specificity of the MBL-IgA interaction is similar to that of the interaction of MBL with mannan, and is consistent with the known specificity of MBL (1). Binding of MBL to IgA induced lectin pathway activation, as demonstrated by activation of C4 and C3. Complement activation on mannan and IgA-coated plates was inhibited by incubating the MBL preparation in the presence of mannose, in the absence of calcium, or in the presence of an MBL-blocking mAb, which is fully consistent with an MBL-dependent mechanism.

Although it has been demonstrated that MASP-1 can directly activate C3 in the fluid phase (5), we could not detect deposition of activated C3 when mannan-coated plates were incubated consecutively with MBL/MASP complexes and purified C3 (not shown), in agreement with data reported by Vorup-Jensen et al (24). This discrepancy is most likely due to differences in the experimental settings, including the method of detection of C3 activation and the concentration and activity of MASP-1 present. In contrast, activation of C4 was readily detectable after incubation with purified C4 under similar conditions, both on IgA and on mannan. Activation of C3 was demonstrated when NHS was used as a complement source. In the latter experiment, we show that MBL binding to plate-coated IgA or mannan is preserved during a one-hour incubation step with a calcium-free buffer, although calcium is required to establish the primary interaction of MBL with its ligands. Similar characteristics are known for the binding of MBL to mannan-coated erythrocytes (25). The MBL-MASP interaction, which is required for complement activation, is also stable in a calcium-free environment (24).

C3 activation by IgA was also demonstrated when serum was used as a complement source in the absence of calcium and without pre-incubation with MBL. Calcium-independent C3 activation is consistent with activation of the alternative pathway by IgA, which is in agreement with previously published data (16; 27). Apparently, different complement activation pathways cooperate to induce activation of C3 by IgA.

To further establish a role for the lectin pathway in complement activation by IgA in whole serum, we compared sera obtained from donors having either a wildtype or a mutant MBL genotype for their ability to activate C4. Our data indicate that sera from donors having heterozygous or homozygous mutations in the first exon of the MBL gene are partially deficient in activation of C4 both by IgA and by mannan. Since C4 activation by the classical pathway is similar in both groups, the observed

differences in mannan and IgA-induced C4 activation can not be based on differences in the classical pathway activity or the concentration of active C4. Therefore, these data are strongly suggestive for the involvement of the lectin pathway in C4 activation by IgA in whole serum.

Complement activation by IgA has been subject of investigation already during several decades. It is generally agreed that IgA cannot activate the classical complement pathway (14). Activation of the alternative complement pathway by IgA is supported by both in vitro (27-29) and in vivo observations (30), as well as by the present study. It has been argued that complement activation by IgA has to rely on studies using artificially modified or presented IgA (14). However, complement activation has also been demonstrated upon binding of IgA to its natural antigen. For example, xenoreactive human IgA antibodies can induce complement-mediated lysis of pig endothelial cells in a calcium-independent way (28). Furthermore, binding of human serum IgA to Streptococcus pneumonia induces neutrophil-mediated bacterial killing which was complement-dependent and proceeded in the presence of MgEGTA (29).

A strong suggestion for the activation of complement by human IgA in vivo is present in patients with IgA nephropathy. IgA nephropathy is a common glomerular disease characterized by mesangial deposition of IgA and complement components (31). Furthermore, deposition of C4 and C4-binding protein was shown in 30% and 60% of cases, respectively, whereas only 6% showed the presence of C1q (31). Alternative pathway activation by IgA may explain the deposition of C3 but not that of C4 in IgA nephropathy. Therefore, activation of C4 by IgA via the lectin pathway, as demonstrated in the present study, may very well be the mechanism of C4 activation in IgA nephropathy. This hypothesis is strongly supported by the deposition of MBL in association with IgA in the mesangial area of patients with IgA nephropathy (19; 20) and patients with Henoch-Schönlein purpura (32). The latter disease is also characterized by mesangial deposition of IgA and complement.

Lectin pathway activation by IgA was most prominent for polymeric IgA, followed by dimeric IgA and monomeric IgA. Similar differences have been previously reported for activation of the alternative pathway by rat and human IgA (16; 26; 29). In addition, polymeric IgA shows enhanced binding to the phagocytic IgA Fc receptor CD89 (33) and to human mesangial cells (34). The stronger effector functions of polymeric IgA have a beneficial role for the defense functions of IgA (29; 35; 36). In accordance, circulating antigen-specific IgA produced upon primary pathogen contact predominantly consists of polymers (29). The polymeric nature of mesangial IgA in IgA nephropathy (37) will most likely contribute to the development of renal damage, involving complement activation and mesangial cell activation.

At present it is unknown which part of the IgA molecule is involved in binding to the CRD of MBL. IgA is a heavily glycosylated molecule (reviewed in (15)). Several variants in the sugar composition have been described, among which high mannose type N-linked glycan chains (38). Especially the latter glycosylation variant may be a likely candidate to serve as a ligand for MBL. Interestingly, circulatory IgA in patients with IgA nephropathy shows an abnormal glycosylation, characterized by a decreased galactosylation of O-linked sugar chains (39). Patients with rheumatoid arthritis produce increased levels of a certain glycoform of IgG that lacks the terminal galactose moieties on the N-terminal glycan chains. This so-called G0-IgG has been shown to bind MBL (40). The hypothesis that altered glycosylation of IgA contributes to complement activation in IgA nephropathy is presently under investigation.

Binding of IgA to microorganisms enables its interaction with phagocytes via the phagocytic Fcα receptor CD89 (29). Together with complement activation, this may result in pathogen elimination, involving CD89 and complement receptors. MBL binding may directly contribute to phagocytosis via MBL receptors (41; 42). In this respect it is conceivable that complement receptors and IgA receptors act together in pathogen elimination (29). In a similar way, classical pathway activation via IgG antibodies works in concert with Fcγ receptors (43).

Based on a number of studies in individuals with MBL gene mutations, the prominent role of MBL in innate immunity has been well appreciated. The protective role of MBL against infection can be explained by the direct binding of MBL to microorganisms (1; 13). MBL binding to IgA may be an additional protective mechanism against microorganisms to which MBL does not bind directly. In situations in which pre-exposure to a pathogen has taken place, such as after vaccination or during chronic infection, MBL may act in concert with IgA to maintain host integrity. Such a mechanism is conceivable for example in cystic fibrosis, in which chronic lung infections often lead to irreversible pulmonary damage and death. Expression of MBL variant alleles in patients with cystic fibrosis is associated with a severely reduced life span (12).

Although MBL is an important defense factor of the immune system, it may also play an unfavorable role in disease progression. This is proposed for rheumatoid arthritis, and is suggested by the presence of MBL in renal biopsies from patients with IgA nephropathy, Henoch-Schönlein purpura, SLE, and post-streptococcal glomerulonephritis (19; 20; 32; 40; 44). IgA nephropathy is the leading cause of end stage renal disease worldwide. Therefore, the IgA-binding function of MBL described in the present study is also likely to play a dual role in immunity. On the one hand, it may link the innate and the adaptive immune system and thereby protect the individual against invading pathogens. In this respect, antibody-mediated complement activation can be considered as an additional parallel between MBL and C1q. On

the other hand, it may enhance the pro-inflammatory effects of IgA deposition in the glomerulus, ultimately leading to renal injury.

Acknowledgments

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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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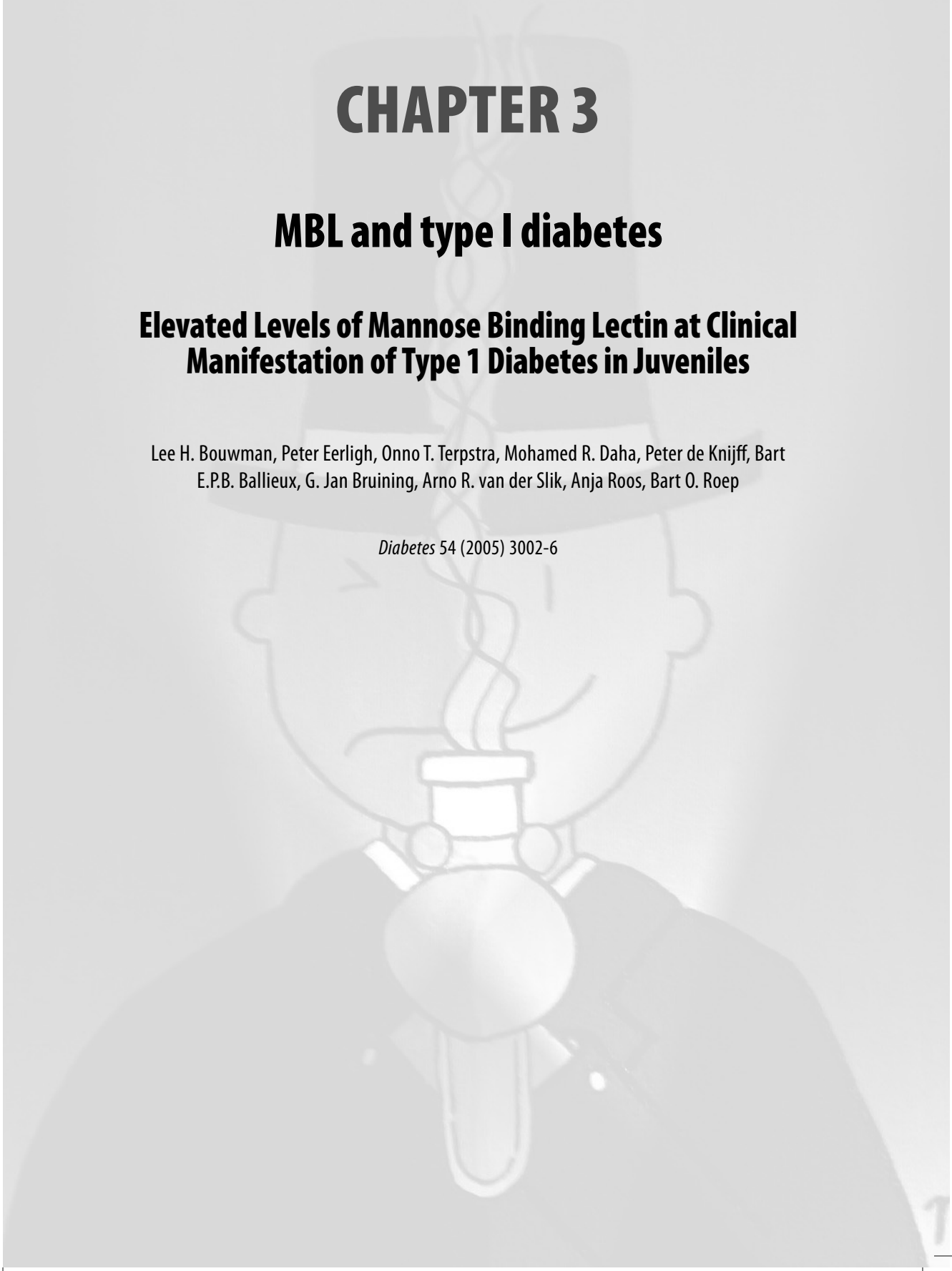

ABSTRACT

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

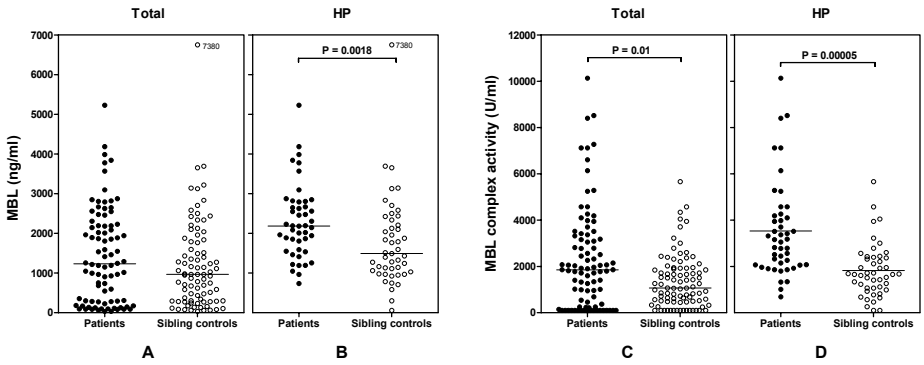

Figure 1. MBL serum concentration in the total group of diabetic patients (A) and diabetic patients with high producing MBL genotypes (HP) (B). Patients with an HP genotype showed an increased MBL serum concentration compared to their sibling controls (p=0.0018, Mann-Whitney test).
MBL complex activity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

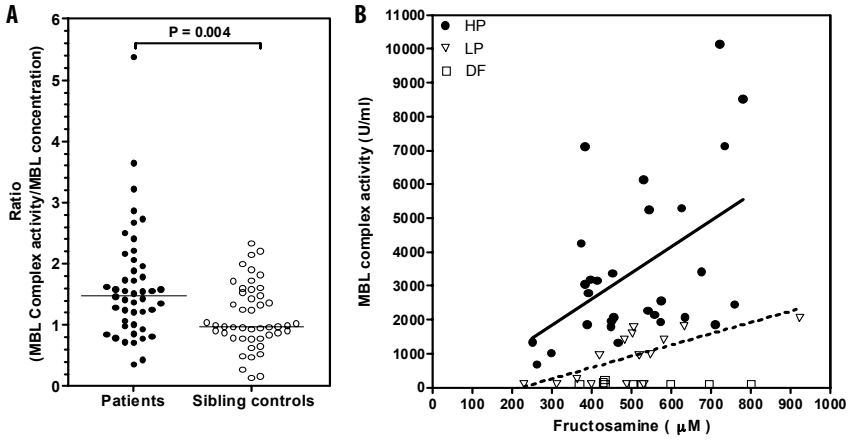

Figure 2. **A**: Ratio MBL complex activity / MBL serum concentration. Patients displayed a significantly increased ratio compared to sibling controls, signifying greater activity per molecule MBL (p=0.004, Mann-Whitney test). **B**: Correlation between MBL complex activity and serum fructosamine level, 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).

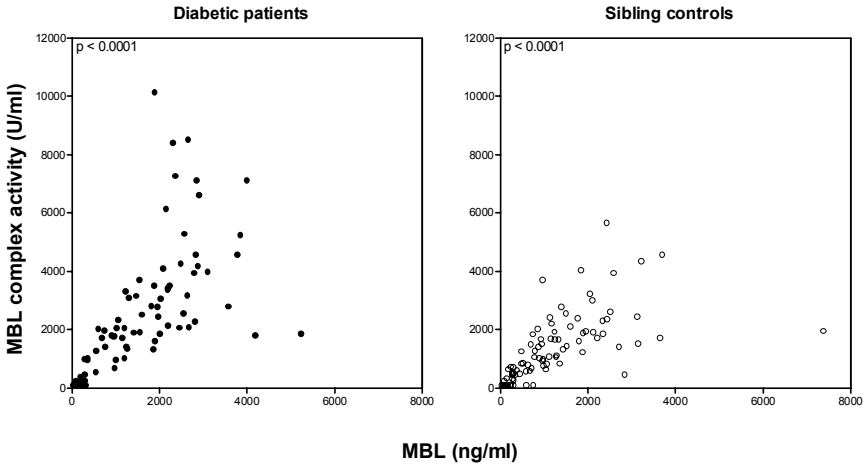

Figure 3. Correlation of MBL complex activity and MBL serum concentration.

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.

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CHAPTER 4

MBL and liver transplantation

Mannose Binding Lectin Gene Polymorphisms Confer a Major Risk for Severe Infections after Liver Transplantation

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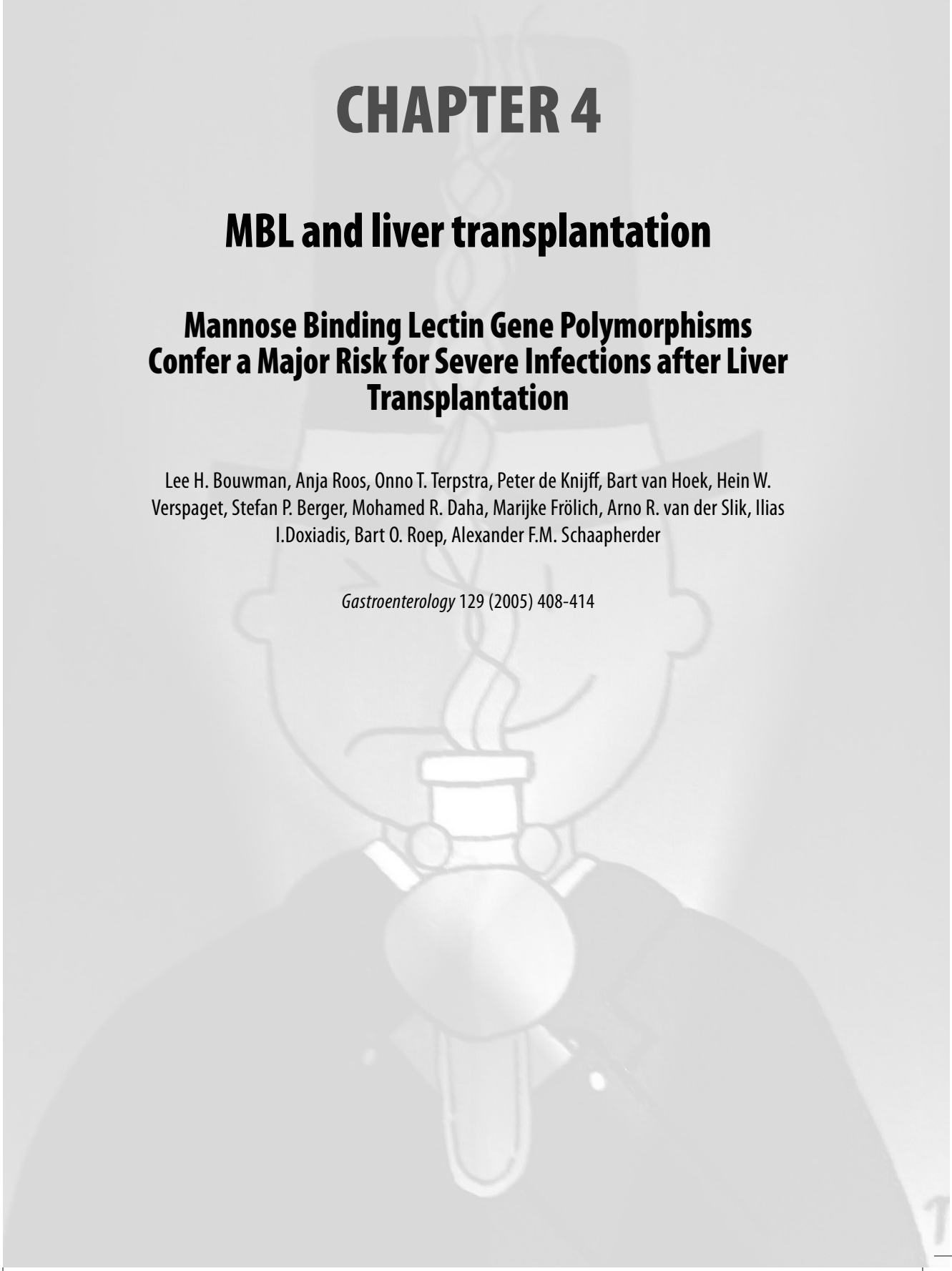

ABSTRACT

Background & Aim: Infection is the primary cause of death after liver transplantation. Mannose Binding Lectin (MBL) is a recognition molecule of the lectin pathway of complement and a key component of innate immunity. MBL variant alleles have been described in the coding region of the MBL gene, which are associated with low MBL serum concentration and impaired MBL structure and function. The aims of our study were to establish the role of the liver in production of serum MBL and to evaluate the effect of MBL variant alleles on the susceptibility to infection after liver transplantation.

Methods: We investigated 49 patients undergoing orthotopic liver transplantation. MBL exon 1 and promoter polymorphisms were determined in patients and in liver donors. MBL serum concentration was determined before and during one year after transplantation. The incidence of clinically significant infections during this period was assessed.

Results: Transplantation of MBL-wildtype recipients with donor livers carrying MBL-variant alleles resulted in a rapid and pronounced decrease of serum MBL levels. This serum conversion was associated with the disappearance of high molecular weight MBL. No indication for extrahepatic production of serum MBL could be obtained. The presence of MBL variant alleles in the MBL gene of the donor liver, but not in the recipient, was associated with a strongly increased incidence of clinically significant infections following transplantation.

Conclusions: Serum MBL is produced by the liver under strong genetic control. Following liver transplantation, the MBL genotype of the donor liver is a major risk determinant for life-threatening infections.

INTRODUCTION

Infection is the Achilles heel in liver transplantation constituting the most common cause of death at all time points, representing 28.4% of all deaths (1). Immune suppressive drugs causing inhibition of the adaptive cellular immune system are generally considered to be the primary cause of high infection rates in this patient group. This notion underscores the significance of the innate immune system in liver transplant patients.

Mannose Binding Lectin (MBL) is a key molecule of the innate immune system. The MBL molecule is composed of homotrimers, containing collagenous domains and C-type lectin domains that are organized into higher order multimers. Via its lectin domains, MBL is able to bind common carbohydrate structures of a variety of micro-organisms (including bacteria, viruses and fungi) resulting in direct opsonophagocytosis and complement activation via the lectin pathway (2).

Exon 1 of the *mbl-2* gene, which is located at chromosome 10, contains three known single nucleotide polymorphisms (SNPs) at codons 52 (Arg→Cys), referred to as allele 'D', codon 54 (Gly→Asp, allele 'B') and codon 57 (Gly→Glu, allele 'C') (3). These SNPs are associated with low serum concentrations, disturbed polymerization and impaired function of MBL (2, 4). 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), both G to C nucleotide substitutions. 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 HXD (5, 6).

The clinical implication of low MBL serum levels in relation to infection has been shown in children and immune-compromised patients (2, 7-9). Variant alleles conferring low MBL concentrations are associated with a doubling of the risk of acquiring infection in early childhood when the adaptive immune system is not yet fully developed (10-12).

Based upon animal models and tissue studies, it is generally assumed that the liver is the main producer of MBL, however direct evidence is lacking (2, 13, 14). Production of MBL by the liver implies that liver transplantation is distinguished from other solid organ transplantations, since it determines functionality of the innate immune system after transplantation. We postulate that MBL deficiency as a result of the liver donor's MBL genotype, contributes to the risk for developing life-threatening infections in liver transplant patients.

PATIENTS AND METHODS

Patients

Meeting all legal and ethical criteria set out by the local and ethical committees, we investigated 49 patients undergoing orthotopic liver transplantation (OLT) in our transplant center for cirrhosis or hepatocellular carcinoma. All patients received deceased donor whole orthotopic liver transplantations and standard immune suppressive therapy consisting of corticosteroids, cyclosporine or tacrolimus with or without mofetil mycophenolate or azathioprine. Furthermore, all patients received 24 hours i.v. antibiotics and 3 weeks of selective bowel decontamination after OLT. To study the correlation between MBL genotype and serum concentration, serum samples were collected of 25 patients at eight time points: twice prior to transplantation (I/II) and at 2 days (III), 1 week (IV), 1 month (V), 3 months (VI), 6 months (VII) and 1 year after OLT (VIII).

MBL genotyping

DNA from all 49 liver donors and from 25 recipients was routinely isolated from blood or tissue samples. MBL SNPs at codon 52, codon 54 and codon 57 of the *mbl2* gene were typed by pyrosequencing (P. de Knijff and A. Roos, submitted). 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. For analysis, carriers of A/O and O/O MBL genotypes were considered as one group (MBL-variant).

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.

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

MBL concentration

MBL serum concentrations were measured blinded in all serum samples by sandwich ELISA essentially as previously described with some modifications (4). 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).

Western blotting

The molecular structure of MBL was examined by Western blotting, essentially as previously described (4). Human serum (1.2 µl) was subjected to SDS-PAGE using a 6% polyacrylamide gradient gel under nonreducing conditions. Proteins were transferred to polyvinyldene fluoride membranes (Immobilon; Millipore, Bedford, MA) using a semi-dryblotting procedure. Membranes were blocked with PBS/0.05% Tween-20/2% Casein followed by incubation with mAb 3E7 (1 µg/ml) for 16 h at 4°C and HRP-conjugated goat anti-mouse IgG (Dako, Glostrup, Denmark) for 2 h at room temperature. Development of blots was performed with Supersignal (Pierce Chemical Co., Rockford, Il) and exposed to Hyperfilms (Amersham Pharmacia Biotech).

Cholinesterase concentration

Cholinesterase was routinely measured in all sera with a fully automated Cobas Integra 800 (Roche, Almere, The Netherlands).

Clinical data

Patients who contracted clinically significant infections (CSI) within the first year after transplantation were identified using a retrospective computer search of the

general patient database. CSI was defined as bacteremia, peritonitis or pneumonia (i.e. positive blood, ascites or sputum culture with accompanying symptoms).

Statistical analysis

Statistical analysis for group comparison of MBL serum concentration between patients receiving an MBL wildtype liver and patients receiving an MBL variant liver was performed using a Mann-Whitney test. Differences in the occurrence of CSI in patients receiving either an MBL wildtype or an MBL variant liver were analyzed using Chi-square analyses with Fisher exact tests. Statistical significance was defined as $P < 0.05$.

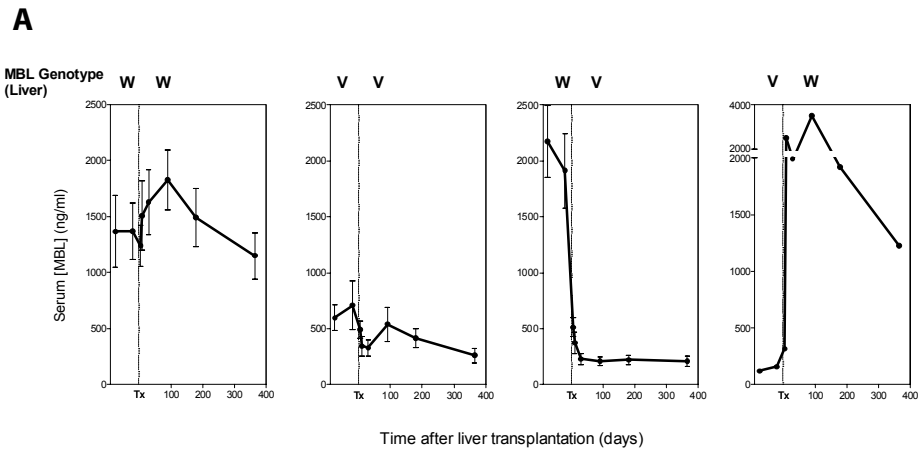

Figure 1: Hepatic production of serum MBL.

A. MBL serum concentration in liver transplant recipients stratified according to the MBL genotype of the liver [W: MBL wildtype (A/A); V: MBL-variant (A/0 or 0/0)]. Error bars show the standard error of the mean. (W→W: Recipients with wildtype MBL genotype transplanted with an MBL wildtype liver, n=10; V→V: Recipients with MBL-variant genotype transplanted with an MBL variant liver, n=5; W→V: Recipients with wildtype MBL genotype transplanted with an MBL variant liver, n=9; V→W: Recipient with MBL variant genotype transplanted with an MBL wildtype liver, n=1)

B. Serum samples before and 1 year following transplantation from liver transplant patients with serum conversion were subjected to SDS-PAGE (6% non-reducing conditions) followed by Western Blotting using mAb 3E7. The MBL genotype of the liver pre and post transplantation is indicated.

RESULTS

MBL serum concentrations were compared with the MBL genotype combination of liver donor and recipient (figure 1A). Patients receiving a liver with an MBL genotype equivalent to their own, showed similar MBL serum concentrations prior and post transplantation. However, MBL genotype mismatches between liver donor and recipient, resulted in a rapid and pronounced serum concentration change that was compatible with the MBL genotype of the liver. Reduction in MBL serum levels was seen in recipients with wildtype MBL genotype transplanted with a liver of donors with MBL-variant genotype, as rapid as 2 days post transplantation. Conversely, in a recipient with MBL-variant genotype receiving a liver with wildtype MBL genotype, the MBL serum concentration increased strongly following transplantation (figure 1 A).

Serum MBL from patients before and one year after transplantation was further characterized by Western blotting. Conversion of serum MBL could be observed from wildtype high molecular weight oligomers to variant low molecular weight mono- and multimers and vice versa, depending on the genetic background of the liver donor and recipient (figure 1B). Serum MBL with a molecular weight above 250 kD was only detectable in carriers of a liver expressing one or two wildtype alleles, also after prolonged exposure of the film (figure 1B and data not shown). No evidence was obtained for extra-hepatic production of serum MBL.

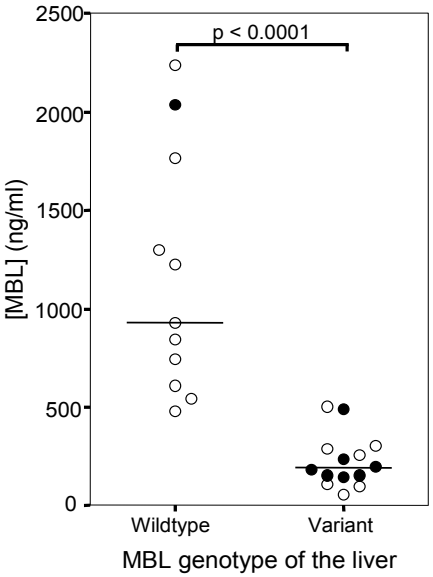

Figure 2: Donor MBL genotype determines MBL serum concentration after transplantation.
MBL serum concentration at 1 year following transplantation in patients stratified according to the MBL genotype of the donor liver. Patients with clinically significant infections (CSI) are represented by black dots.

Table 2: Occurrence of clinically significant infections (CSI) in 49 liver transplant patients, stratified according to donor liver MBL haplotype

Patients receiving an MBL wildtype liver				
number	**Haplotype donor liver**	**CSI**	**Micro-organism**	**Weeks after Tx**
1	HYA/HYA	Sepsis	*Escherichia coli, Enterococcus faecalis*	28
2	HYA/LYA			
3	HYA/LYA			
4	HYA/LYA			
5	HYA/LYA			
6	HYA/LYA			
7	HYA/LYA			
8	HYA/LYA			
9	HYA/LYA			
10	HYA/LYA			
11	HYA/LYA			
12	HYA/LYA			
13	HYA/LXA			
14	HYA/LXA			
15	HYA/LXA			
16	HYA/LXA			
17	LYA/LXA			
18	LYA/LYA			
19	LYA/LYA			
20	LYA/LYA			
21	LYA/LYA			
22	LYA/LYA	Sepsis	*Escherichia coli, Enterococcus faecalis*	6
23	LYA/LXA			
24	LYA/LXA			
25	LYA/LXA	Pneumonia	*Streptococcus pneumoniae, Haemophilus influenzae*	12
Patients receiving an MBL variant liver				
26	LXA/LYB	Sepsis	*Klebsiella oxytoca*	10
27	LYA/LYB	Pneumonia	*Streptococcus pneumoniae*	19
28	HYA/LYB	Sepsis	*Streptococcus mutans, Klebsiella oxytoca*	8
		Sepsis	*Gram-negative coccobacilus, Group D streptococci*	20
29	LYA/LYB			
30	LXA/LYB	Sepsis	*Escherichia coli* *Enterococcus faecalis*	11
31	LYA/LYB			
32	LXA/LYB			
33	LYA/LYB			
34	HYA/LYB			
35	HYA/LYB			
36	LXA/LYB			
37	HYA/LYB			
38	HYA/LYC			
39	LYA/HYD	Peritonitis	*Coagulase-negative staphylococci*	1
40	LXA/HYD	Peritonitis	*Coagulase-negative staphylococci*	4
41	LYA/HYD			
42	LYA/HYD	Sepsis/peritonitis	*Enterococcus faecalis*	3
43	LXA/HYD			
44	LYB/LYB	Sepsis/peritonitis	*Coagulase-negative staphylococci, Pseudomonas aeruginosa*	4
45	LYB/LYB	Sepsis	*Enterococcus faecalis* *Lysteria monocytogenes*	16
46	LYB/LYC	Sepsis	*Streptococcus oralis*	3
47	LYB/HYD	Sepsis	*Enterococcus faecalis*	7
48	HYD/HYD			
49	HYD/HYD			

One year following liver transplantation, recipients of an MBL wildtype liver showed up to 40-fold higher MBL serum concentrations than recipients from an MBL-variant liver ($p<0.0001$, Mann-Whitney test, figure 2). The function of the liver was evaluated at all time points using cholinesterase as a common marker. In all patients, with the exception of one, serum cholinesterase concentration increased after transplantation to normal levels, indicating good graft function (normal range 5.3-13 U/ml). The patient that did not sustain normal cholinesterase levels, was an MBL wildtype patient who received a donor liver with an MBL variant genotype. This patient died shortly after the follow-up period of a sepsis. No difference in liver function could be observed between patients receiving a wildtype or an MBL variant genotype liver (figure 3).

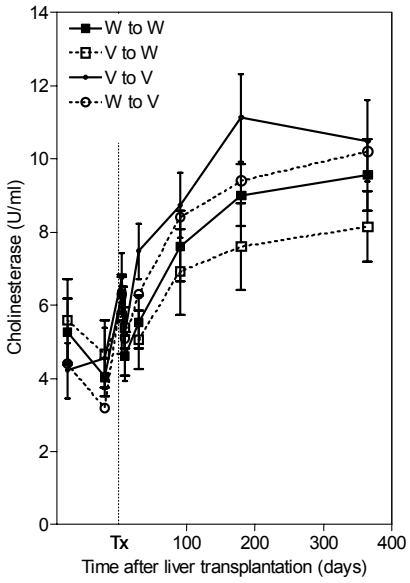

Figure 3: Cholinesterase as a marker for liver function.
Cholinesterase serum concentration in liver transplant recipients stratified according to the MBL genotype of the liver [W: MBL wildtype (A/A); V: MBL-variant (A/0 or 0/0)]. Error bars indicate the standard error of the mean. (W to W: Recipients with wildtype MBL genotype transplanted with an MBL wildtype liver, n=10; V to V: Recipients with MBL-variant genotype transplanted with an MBL variant liver, n=5; W to V: Recipients with wildtype MBL genotype transplanted with an MBL variant liver, n=9; V to W: Recipient with MBL variant genotype transplanted with an MBL wildtype liver, n=1)
Figure 4: Donor MBL genotype is a risk factor for clinically significant infections (CSI).
Percentage of CSI in patients stratified according to the genotype of the donor liver.

Clinical evaluation of all 49 patients showed that the incidence of clinical significant infections was 3.8-fold higher in the recipients of MBL-variant livers, as compared to recipients of MBL-wildtype livers ($p=0.01$, Fisher's exact test; table 2). The incidence

of CSI was highest in recipients of livers with an O/O MBL genotype (4/6), followed by recipients of livers with an A/O MBL genotype (7/18). Patients receiving a MBL wildtype liver had the lowest incidence of CSI (3/25), (p=0.01, Chi-square test, figure 4). We could not detect an association between recipient MBL genotype and incidence of CSI (p=0.34, Fisher's exact test). No significant relation was observed between the different MBL promoter SNPs and the occurrence of clinically significant infections.

DISCUSSION

The present study is the first to investigate directly a change of MBL status in liver transplant patients. We describe 49 liver transplant patients receiving standard immune suppressive therapy. As the cellular adaptive immune system is suppressed, the role of the innate immune system is essential in preventing life-threatening infections. Moreover, surveys of bacteria have shown that MBL binds to a wide range of microbes, including microorganisms that cause severe infections in liver transplant patients (15).

We conclude that the liver is the pivotal source of serum MBL, whereas extrahepatic production is undetectable. After liver transplantation, the donor liver determines the MBL serum concentration and molecular size, as evidenced by rapid MBL serum conversion. Moreover, the MBL genotype of the donor, not the recipient, determines the risk for potential life-threatening infections. Therefore, hepatic production of functional MBL is of major importance for the host defense against infection following liver transplantation. Accordingly, liver MBL genotypes resulting in low levels of serum MBL as well as predominance of low molecular weight oligomers, was associated with a strongly increased risk for infection following liver transplantation. The increase in CSI appeared to be gene dose-dependent, being most prominent in recipients of livers with two MBL-variant alleles. However, as the number of patients in the latter group was limited, further studies are warranted.

A recent study showed that a wildtype MBL genotype of the donor strongly protects against infections in recipients of haemopoietic stem cell transplants, suggesting production of MBL by bone marrow-derived cells (8). However, successful haemopoietic stem cell transplantation from MBL-sufficient donors does not result into a detectable reconstitution of serum MBL in MBL-deficient recipients ((16) and R.G. Bredius and A. Roos et al. unpublished results). Although the present study clearly indicates that serum MBL is produced by the liver, it is conceivable that MBL-production by extrahepatic cells may play a local role in host defense.

The ability to unambiguously identify a group of patients severely prone to infection post transplantation is of significant clinical value. In an era of donor shortage, donor selection based upon MBL genotype is inconceivable. However, our study suggests that patients receiving an 'MBL-variant' liver could benefit from MBL replacement therapy similar to that presently being studied in phase I/II and III studies (17, 18). Furthermore, prophylactic approaches including intensified clinical follow-up, preemptive antimicrobal therapy and prolonged selective digestive decontamination could be considered dependent on the MBL genotype of the liver donor.

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CHAPTER 5

Adaptive immunity in pancreatic islet transplantation

HLA Incompatibility and Immunogenicity of Human Pancreatic Islet Preparations Co-Cultured with Blood Cells of Healthy Donors

Lee H. Bouwman, Zhidong Ling, Gaby Duinkerken, Daniel G. Pipeleers, Bart O. Roep

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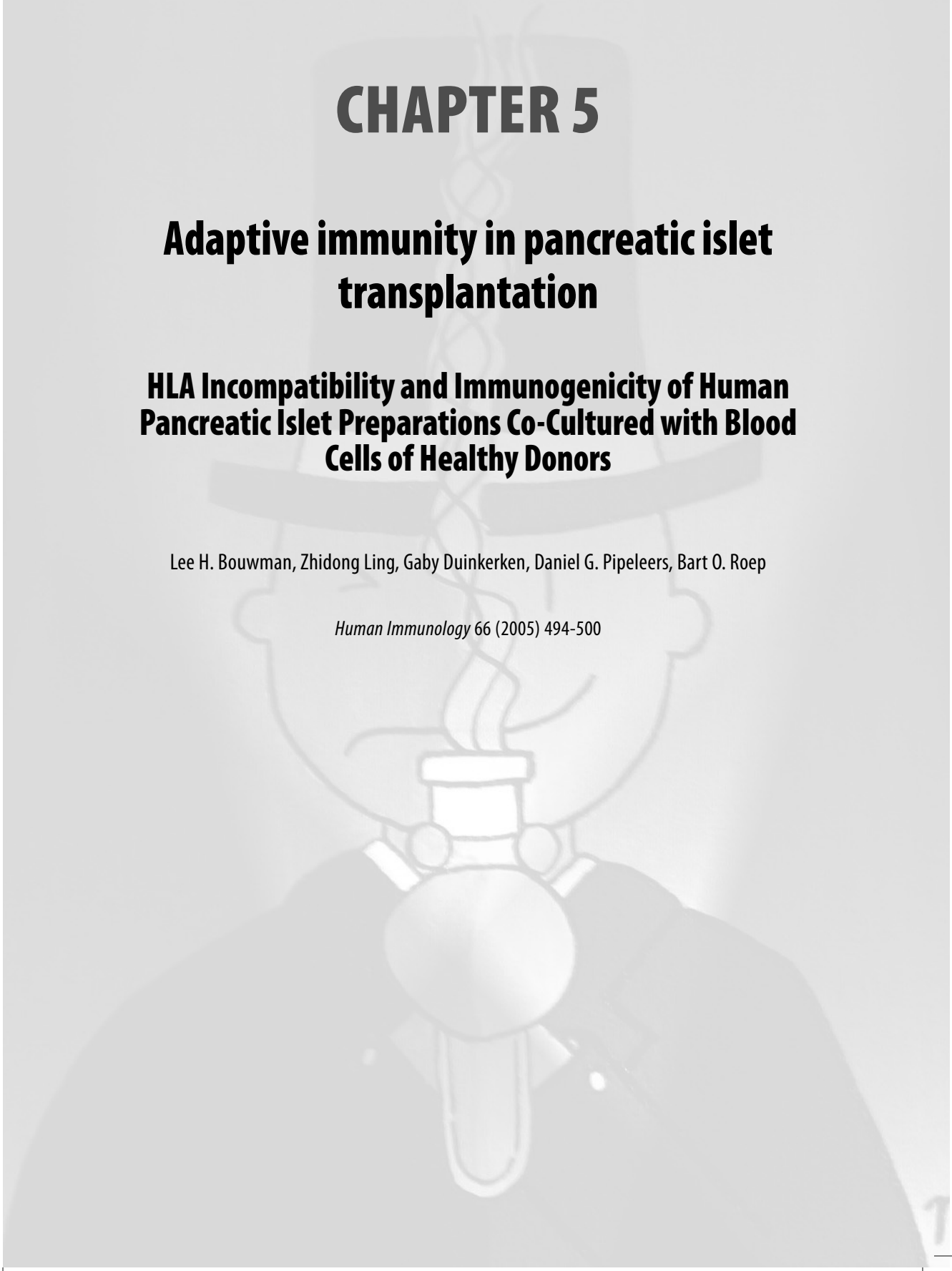

ABSTRACT

Type 1 Diabetes Mellitus (T1D) is a T-cell mediated autoimmune disease characterized by the destruction of beta cells in the pancreas. An attractive novel therapy for type 1 diabetes is pancreatic islet transplantation, provided that recurrent islet autoimmunity and allograft rejection can be prevented.

We analysed the response of peripheral blood mononuclear cells (PBMC) from healthy blood donors to human islet-cell preparations with a composition similar to that of islet grafts used in clinical transplantation trials. It was examined whether the degree of MHC incompatibility between PBMC and donor islet cells is related to the degree of proliferative T-cell responses during co-culture of HLA-matched and mismatched PBMC with human islet cell-preparations [i.e. mixed islet lymphocyte reaction (MILR)].

Prominent T-cell responses were observed in the vast majority of cases of double HLA class II-mismatches. Intermediate T-cell responsiveness was observed in single HLA class II mismatches, whereas HLA-matches did not induce a T-cell response.

Our results identify the potential immunogenicity of islet preparations transplanted between HLA-DR incompatible subjects regardless of an autoimmune background of the recipient.

INTRODUCTION

Since the major histocompatibility complex (MHC) molecule plays an essential role in the activation of T cell responses, the genes encoding these molecules have been implicated in susceptibility to serve T cell mediated autoimmune diseases. Several HLA alleles have been shown to be major genetic risk factors in development of type 1 diabetes. Vulnerability for type 1 diabetes is genetically dominated by the HLA gene region accounting for 42% of the familial inheritance of type 1 diabetes[1].

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[2]. It has long been acknowledged that T-cells play a crucial role in the immunopathogenesis of type 1 diabetes, the hallmark of autoimmune diabetes being lymphocytic invasion of pancreatic islets[3-10].

A potential therapy for diabetes is transplantation of insulin-producing beta-cells of isolated pancreatic islets, provided that recurrence of T-cell autoreactivity against islet determinants, as well as induction of alloimmunity to donor antigens, is prevented[11-13]. This attractive and recently successful therapy for T1D is overshadowed by the need for permanent immune suppression. Without the administration of these non-specific and potentially harmful immunosuppressive drugs, graft failure seems inevitable. Islet transplantation is thus limited to diabetic patients already receiving immune suppression for a previous organ transplant, or to patients with severe hypoglycemia unawareness or uncontrollable hyperglycemia. The introduction of a new glucocorticoid free immunosuppressive regime, the so-called Edmonton protocol, has improved the outcome of islet transplantation considerably[14]. This protocol includes sirolimus, tacrolimus and dacluzimab. All these immunosuppressive drugs share the same basic quality that they all inhibit T-cell stimulation and proliferation, identifying once again T-cells as key-players in this rejection process.

Prediction and prevention of ongoing beta-cell destruction after islet transplantation, resulting in long-term graft survival is of utmost importance. In order to be able to optimise the current islet transplantation, it is essential to study the reaction of T-cells to islets.

In rat studies, it has been shown that T-cells obtained prior to islet transplantation can react against islet allografts in a mixed islet-leukocyte culture [15]. This reactivity was similar to that of T-cells isolated after grafting. We have previously demonstrated a marked increase in T-cell alloreactivity in immune suppressed type 1 diabetes patients rapidly upon implantation of human islet allografts from multiple donors[11]. This rise was exclusive to patients that acutely rejected the islet allograft, and absent

in cases of successful restoration of beta-cell function. The contribution of HLA class II to immunogenicity of islets was further underscored by our recent observation that islet autoantigens are processed and presented by vascular endothelial cells expressing MHC class II leading to activation of autoreactive T-cells [16], implying that MHC class II could be important in human islet graft-failure by autoreactivity[17]. Little is known about the extent that human islet preparations could be target of alloreactivity in relation with the degree of HLA mismatching with human healthy blood donors.

To evaluate the potential immunogenicity of human islet preparations under immunocompetent conditions, we investigated the ability of peripheral blood mononuclear cells (PBMC), isolated from immunologically uncompromised healthy blood donors, to react human islet preparations in relation with the degree of HLA-DR incompatibility. Mixed islets lymphocyte cultures [18,19] were performed with a panel of HLA-DR matched and mismatched healthy blood donors. For comparison, mixed lymphocyte reactions (MLR) using PBMC of blood donors with PBMC of the islet donor, were carried out and analysed in relationship to the pattern of reactivity found in the MILR.

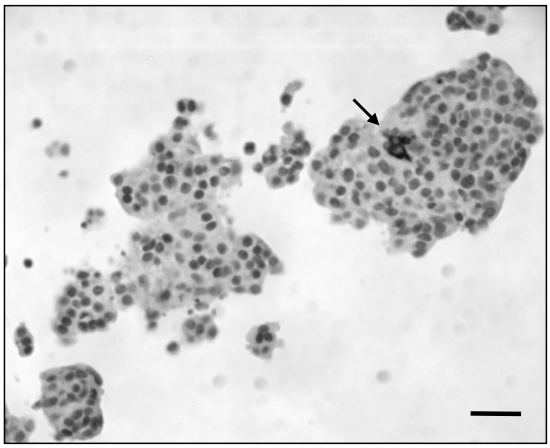

Figure 1: Representative image of human pancreatic cell preparations immunostained for HLA-DR. Positive cells (arrow) were present at a low percentage (<1%) and occurred usually as single units. The scale bar represents 50µm.

MATERIAL AND METHODS

Human islet isolation and culture

Human pancreata, were obtained from organ donors (eleven preparations from 10 donors, age 2-60 years) at European hospitals affiliated with ß-Cell Transplant, a European Concerted Action on islet cell transplantation in diabetes. Islets were

prepared in the Central Unit of this multicenter program (Medical Campus, Vrije Universiteit Brussel) [12]. Detailed description of methods of isolation, phenotypic and functional characterization of the islet preparations is available elsewhere [12]. This study was conducted with cultured Islet cell preparations that were comparable to those incorporated in grafts that are implanted in patients. They are composed of endocrine and non-endocrine duct cells with, in average, 48 percent insulin-positive cells (Table 1). Their content in MHC-II expressing cells is consistently lower than 1 percent (Figure 1). The available beta cell mass was too low for inclusion in grafts so that the preparations became available for approved research projects if they fulfilled the legal and ethical criteria that were set for such use. The isolated islet preparations were cultured in Ham's F10 medium[12] for 1-5 days before transfer to Leiden, where they were processed immediately for immunological studies.

Table 1: Characterization of human islet preparations.

Donors	Age	Sex	HLA-DR	Culture Time (days)	Cellular composition (% total)				
					Exocrine	Non-granulated	Endocrine	Dead	Insulin positive
1	17	M	DR4 DR6(13)	5	0	40	58	2	58
2	22	M	DR2(15) DR6(13)	1	15	31	50	4	42
3	40	F	DR2(16) DR3(17)	5	0	58	32	10	28
4	20	M	DR4	8	0	46	50	4	42
5	12	M	DR8 DR12	5	0	50	42	8	44
6	2	M	DR2 DR4	4	0	28	62	10	57
7	52	M	DR6 DR13	3	2	28	64	6	49
8	42	F	DR1	6	0	12	82	6	61
9	48	F	DR2 DR6	3	0	8	76	16	63
10a	60	M	DR4 DR6	4	0	31	66	3	54
10b	60	M	DR4 DR6	4	0	66	30	4	30

Peripheral Blood mononuclear cells (PBMC) isolation

PBMC were obtained from healthy blood donors visiting the blood bank in Leiden, the Netherlands by Ficoll separation after obtaining informed consent.

HLA-DR typing

The HLA-DR genotypes of both blood- and pancreas-donors were determined by conventional genotyping in the vast majority of cases as described in detail [20]. Incidentally, pancreata were HLA typed by serology only. Therefore, all HLA genotypes were translated to serotype to allow comparison.

Mixed islet lymphocyte reaction (MILR)

Islet preparations in a concentration range (10.000 to 150.000 endocrine cells per well) were co-cultured with 150.000 PBMC per well, in micro well tissue plates (Griener) for 5 days at 37°C, 5% carbon dioxide in air. Islet preparations and PMBC were HLA-DR matched or mismatched. RPMI culture medium was used containing 10% fetal calf serum and 100 units/ml penicillin and 100 µg/ml streptomycin. Samples were incubated in triplo. On day 5 [H^3]-Thymidine was added to each well for 16 hours in an end concentration of 1µCi/ml. The MILR response was measured by incorporation of [H^3]-Thymidine in proliferating lymphocytes using a liquid scintillation counter (LKB Wallac, Croydon UK). Dose-response curves differed between islet preparations, and optimal responses were determined individually. For standardization and comparison between islet preparations, the MILR was considered positive when the signal exceeded the average signal of stimulatory cells alone plus three times the standard deviation.

Furthermore, in two cases a double mismatched MILR was performed, in the presence and absence of monoclonal antibodies directed against HLA-DR (B8.11.2), HLA-DQ (SPV-L3) and HLA class 1 (W6.32). The human islet preparations were incubated in the presence of 10 µg/ml of monoclonal antibodies directed against HLA-DR (B8.11.2), HLA-DQ (SPV-L3) and HLA class 1 (W6.32).

Mixed lymphocyte reaction (MLR)

In the MLR, PBMC of both blood donors and islet donors were used. γ-irradiated PBMC (3000 R) of the islet donor were incubated with blood donor PMBC in a 1:1 ratio, 200.000 cells per well (Griener). Samples were incubated in triplicate for 5 days at 37°C, 5% carbon dioxide in air. On day 5 [H^3]-Thymidine was added to each well for 16 hours in an end concentration of 1 µCi/ml. The MLR response was measured by incorporation of [H^3]-Thymidine in proliferating lymphocytes. The MLR responses were considered positive when the signal exceeded the average signal of stimulatory cells alone plus 3 times the standard deviation.

Statistical analysis

Statistical analysis for group comparison was performed using Chi-square analyses with two-sided Fisher exact tests. Statistical significance was achieved if the *P* value was less than 0.05. Frequencies of both positive and negative MILR and MLR were compared with the chi-square test or the Fisher's exact probability test.

RESULTS

Comparison between MILR and HLA-DR haplotype

Mixed islet lymphocyte reactions (MILR) were performed with islet and PBMC preparations that were HLA-DR matched, single mismatched and doubled mismatched (Table 2). T cell responses were exclusively seen in HLA mismatch combinations (p=0.0004). In the group of double HLA-DR mismatches, 12/13 resulted in potent T cell proliferative responsiveness (double mismatched vs. matched: p=0.0003). The one combination without reactivity involved the islet preparation with the highest percent endocrine cells (82%). Intermediate T cell responses were observed in 6/9 tested combinations with a single HLA-DR mismatch (single mismatch vs. matched: p=0.017), whereas none of the 6 HLA-DR matched combinations induced a T cell response. In rare cases, sufficient numbers of islets were available to allow HLA blocking studies. The observed T cell proliferative responses were to a large extent suppressed when the mixed islet lymphocyte reactions were performed in presence of a monoclonal antibody directed against HLA-DR, whereas no inhibition was seen with monoclonal antibodies directed against HLA-DQ and HLA class 1 (data not shown).

Table 2: MILR in relation with HLA-DR matching. Indicated are positive or negative proliferative responses of HLA-matched, single or double mismatched PBMC of healthy donors against human islet-preparations, tested in the MILR ("nr." refers to the donor number indicated in Table 1).

MILR	**HLA-DR mismatches**		
	2	**1**	**0**
POS	DR3,3 x DR4,6 (nr.1)* DR3,3 x DR2,6 (nr.2) DR4,10 x DR 2,3(nr.3) DR1,8 x DR4,4(nr.4) DR6,13 x DR2,4(nr.6) DR3,3 x DR6,13(nr.7) DR2,6 x DR 1,1(nr.8) DR1,7 x DR2,6 (nr. 9) DR 15,15 x DR 4,6 (nr.10a) DR 1,1 x DR 4,6 (nr.10a) DR 16,11 x DR 4,6 (nr.10b) DR 3,7 x DR 4,6 (nr.10b)	DR6,6 x DR4,6(nr.1)* DR 2,6(14) x DR 2,6(13)(nr.2) DR2,2 x DR2,3(nr.3) DR1,8 x DR8,12(nr.5) DR2,6 x DR6,13(nr.7) DR1,8 x DR1,1(nr.8)	
NEG	DR 3,7 x 1,1(nr.8)	DR3,3 x DR2,3(nr.3) DR2,6 x DR2,4(nr.6) DR1,6 x DR 1,1(nr.8)	DR2,6 x DR2,6(nr.2) DR2,3 x DR2,3(nr.3) DR6,13 x DR6,13(nr.7) DR1,1 x DR1,1(nr.8) DR 4,6 x DR 4,6 (nr.10a) DR 4,6 x DR 4,6 (nr.10b)

Mismatch vs. Match:	p = 0,0004
Double mismatch vs. Match:	p = 0,0003
Single mismatch vs. Match:	p = 0,017

* No MLR done

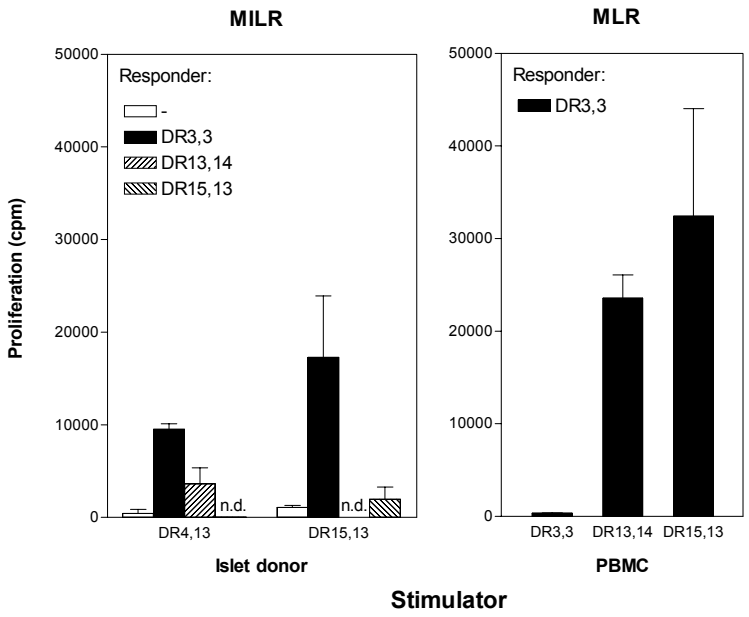

Figure 2: Representative comparison between MILR and MLR. Raw counts per minute (cpm) are indicated to illustrate background responses of islet preparations, stimulator or responder PBMC alone as well as the amplitude of the proliferative response to . (n.d.: not done)

Comparison between MILR and MLR

The lymphocyte reactivity to allogeneic islet cell preparations (MILR) was compared to that measured against lymphocytes (MLR) that were isolated from the islet cell donor (table 2). The MLR tests were positive for 15/16 cases where a positive MILR had been measured; the one MLR negative/ MILR positive case related to the only child (2y) that was tested as islet donor (Table 3). In the group of negative MILR tests, 3/10 exhibited a positive MLR. Overall, a significant relation was seen between recognition of islet donor PBMC and islet preparations by healthy donor derived PBMC ($p=0.0013$; Table 3). When the analysis was restricted to the 12 to 60 year old donor group the correlation between MILR and MLR was even stronger ($p=0.0003$).

Table 3: The correlation between the presence (POS) and absence (NEG) of proliferative T-cell responses in co-cultures of PBMC isolated from healthy blood donors with human islet preparation. Responsiveness was significantly concordant between MILR and MLR (p=0.0013).

MILR	MLR	
	POS	NEG
POS	DR 3,3 x DR 2,6	DR 6,13 x DR 2,4
	DR 4,10 x DR 2,3	
	DR 1,8 x DR 4,4	
	DR 3,3 x DR 6,13	
	DR 2,6 x DR 1,1	
	DR 1,7 x DR 2,6	
	DR 15,15 x DR 4,6	
	DR 1,1 x DR 4,6	
	DR 16,11 x DR 4,6	
	DR 3,7 x DR 4,6	
	DR 2,6(13) x DR 2,6(14)	
	DR 2,2 x DR 2,3	
	DR 1,8 x DR 8,12	
	DR 2,6 x DR 6,13	
	DR 1,8 x DR 1,1	
	DR 3,7 x DR 1,1	DR 2,6 x DR 2,4
NEG	DR 3,3 x DR 2,3	DR 2,6 x DR 2,6
	DR 1,6 x DR 1,1	DR 2,3 x DR 2,3
		DR 6,13 x DR 6,13
		DR 1,1 x DR 1,1
		DR 4,6 x DR 4,6
		DR 4,6 x DR 4,6

DISCUSSION

Our study demonstrates that cultured human islet cell preparations with a composition similar to that used in our clinical trial, have a strong immunogenic potential dependent of the degree of their HLA incompatibility with responder leukocytes. Importantly, matched islet preparation persistently led to absence of alloreactivity *in vitro*. This may bear implications for clinical islet cell transplantation. Human islet transplantation has been considered to be a promising therapy for T1D patients for the last 31 years[21,22]. A major obstacle that remains to be tackled in allograft islet transplantation, is the rejection process resulting in graft failure. The current knowledge of the immunogenesis of the rejection process is at best incomplete. This might be due to the fact that graft tissue is inaccessible after transplantation, making clinical and histological studies very difficult and demanding. Hence, most studies addressing the specific allogeneic rejection response are murine based and cannot be extrapolated directly to the human situation. Clinical trials studying the rejection process rely upon the presence of C-peptide, indicating beta cell function. The immunological status of the islet-transplantation however remains concealed[23].

Multiple donor islet grafts further cloud the different roles of involved actors in this immunological play. In the present study we selectively studied the degree of alloreactivity in PBMC of immunocompetent healthy blood donors against allogeneic islet preparation with different degrees of HLA incompatibility and composition.

HLA has long been recognized to be of clinical importance in solid organ transplantation as one of the primary causes of Th1/HLA class II antigen-rejection. However the importance of HLA in islet transplantation and rejection has been disputed. One study showed low or even absent T-cell response directed against pancreatic islets *in vitro* [23]. The authors hypothesized that low T-cell proliferative responses could be a direct result of the inhibitory effects of exocrine enzymes upon lymphocytes, utilizing the role of class II MHC. However, we have demonstrated that alloreactivity in type 1 diabetes patients transplanted with human islet allografts strongly correlated with acute rejection and loss of graft function *in vivo* [11]. Moreover, it has been suggested that HLA mismatching could provide better allograft survival of transplanted pancreatic islets in autoimmune diabetes, as was shown in mice. It has also been argued that avoidance of MHC class II sharing between donor and recipient, could avoid the recurrence of autoimmunity and subsequently the destruction of beta cells[24]. However, there is little evidence from clinical trials on islet transplantation that islet allografts benefit from mismatching with the recipient. Moreover, our present data clearly demonstrate that HLA matching significantly reduces the risk for alloreactivity, while induction of alloreactivity appears to be the most pronounced cause of graft failure[11]. We appreciate that in an era of donor shortage and multiple donors being required to obtain a sufficient islet load for transplantation, donor selection based upon HLA is inconceivable. Therefore, tailored immune suppresion is of utmost importance in order to optimize the outcome of islet transplantation.

In order to address the involvement of HLA in human islet transplantation, we studied the response of peripheral blood mononuclear cells (PBMC) from healthy blood donors to human islet-preparations. Healthy donors were chosen, rather than diabetic patients, to evaluate the primary T-cell response and exclude recurrent islet autoimmunity. Contrary to a previous report [23], a T-cell proliferative reaction could be invoked upon stimulation of healthy donor T-cells with double HLA-mismatched islet preparations in all but one mixed islet lymphocyte reactions. In contrast, none of MILR performed with HLA-DR matched T-cells and islet preparations displayed any T-cell responsiveness. Partial HLA-DR mismatching could invoke an intermediate T-cell response in the majority of cases, which accorded with the MLR.

As previously mentioned, there was only one exception where a positive MLR did not correlate with a positive MILR despite a complete HLA class II disparity. This occurred in the case of an islet preparation from a 2 year-old pancreas donor, suggesting that donor age may be a factor related to immunogenicity.

In conclusion, our data clearly demonstrate the immunogenic nature of islet preparations of clinical norm, even in healthy donors lacking auto-reactive islet T-cells. The immunogenicity is dependent largely on the degree of HLA matching with responder leukocytes. HLA-DR matching is accompanied by lack of immune reactivity against the islet preparation. Our findings underscore the difficulty of islet allotransplantation, but provide leads to reduce the extent of alloreactivity in clinical islet transplantation.

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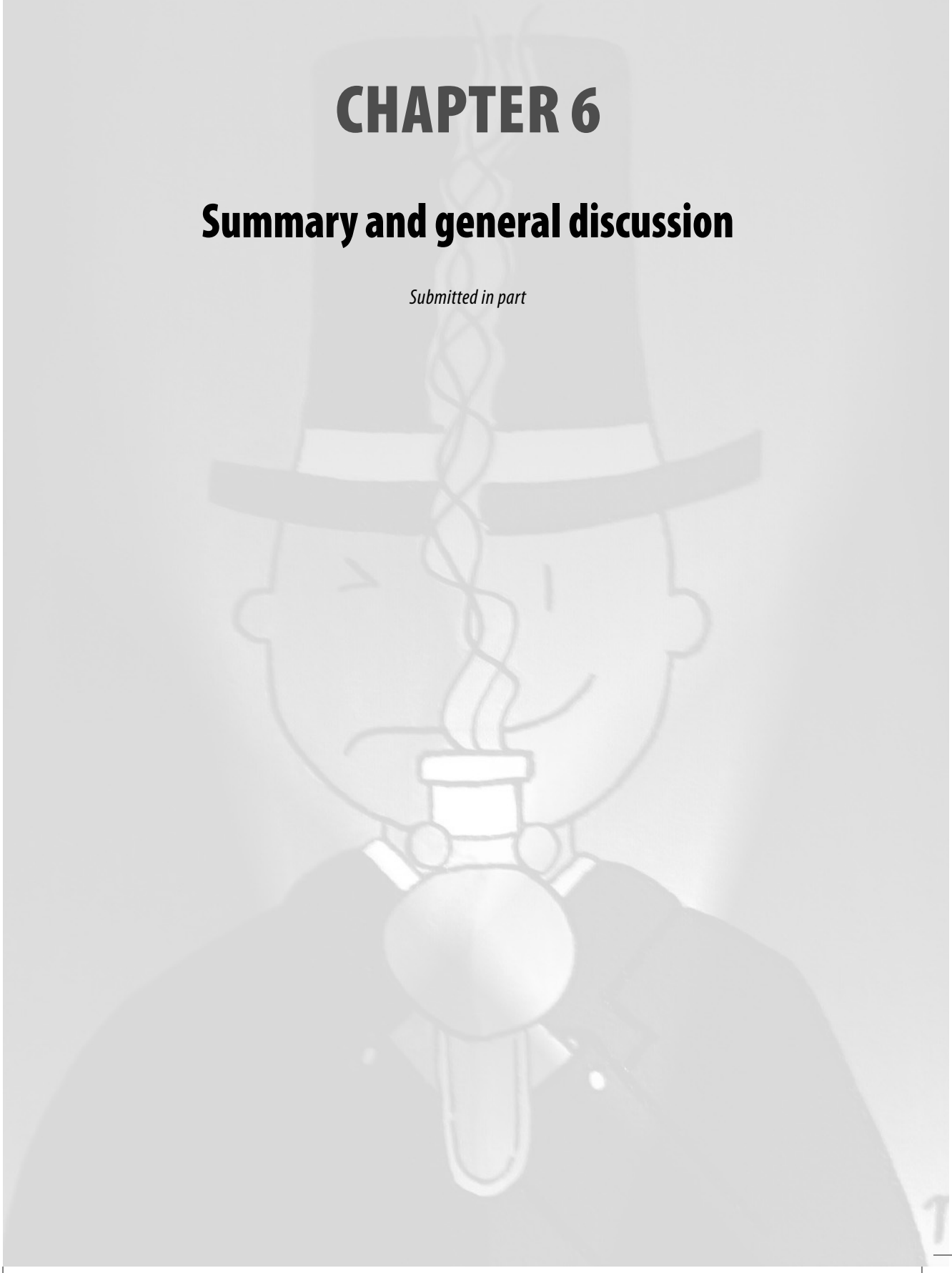
CHAPTER 6

Summary and general discussion

Submitted in part



SUMMARY AND GENERAL DISCUSSION

The aim of the thesis was to obtain insight in the immunological aspects of transplantation and diabetes, with the emphasis on mannose binding lectin (MBL) as a crucial part of the innate immune system. The results of this thesis underscore the Jekyll-and-Hyde character of MBL as it is shown that MBL deficiency is detrimental in liver transplantation patients wheras it could be beneficial in type 1 diabetes. In order to fully appreciate the genetic, functional and serological impact of MBL under discrete pathological conditions, novel assays were required and designed. As opposed to the vast majority of groups studying MBL in relation to disease, presently it was opted to expand the focus of the current research by tallying not only MBL genetics and MBL serum levels but also MBL function. By doing so, a discrepancy was identified between MBL serum levels and functional MBL activity under specific circumstances, underlining the necessity to study all dimensions of MBL when correlating the lectin pathway (LP) to disease. Furthermore, being capable to study MBL in transplantation and diabetes resulted in ability to link specific parts of innate and adaptive immunity.

Mannose binding lectin, as a key player of natural immunity, is capable of binding common carbohydrate structures of a diversity of microorganisms (including bacteria, viruses and fungi) and facilitates elimination of these pathogens (1). In the general population there is a wide variety in MBL serum concentrations as well as functional MBL activity (2). This great diversity in concentration and function has been shown to be determined by single nucleotide polymorphisms in exon 1 of the *mbl2* gene and its promoter(3; 4). Currently, it has well been acknowledged that an impaired MBL function significantly increases the risk for infections in individuals who largely depend on their innate immune system for anti-microbial defense, like immune-compromised patients and young children (5-9). It is therefore intriguing that the frequency of variant alleles, resulting in low MBL production, is above 40% depending on ethnicity. This percentage is even higher when promoter SNPs are taken into account (3). The high frequency of variant genotypes resulting in low levels of high molecular MBL weight and decreased MBL function indicate that in particular cases impaired MBL function and low serum concentrations may be beneficial. This has been suggested to be the case for mycobacterial infections, inflammatory bowel disease like ulcerative colitis, and transplantation (1; 10; 11).

Molecular and immunological characteristics of MBL

Chapter 2 is build up of three parts and shows the close interaction between the different complement pathways. The first part of this chapter discusses the functional characterization of the lectin pathway in human serum. Here, a novel functional as-

say is described that enables quantification of autologous complement activation via the LP in full human serum up to the formation of the membrane attack complex. This assay offers novel possibilities for both patient diagnostics and research.

The second part of this chapter is continuation of the observation shown in the previous section, that normal human serum contains IgG, IgA and IgM antibodies against mannan. The high frequency of MBL deficiency suggests that MBL-mediated innate immunity can be compensated by alternative defense strategies. To examine this hypothesis, complement activation by MBL-binding ligands was studied. The results show that the prototypic MBL ligand mannan can induce complement activation via both the LP and the classical pathway (CP). Furthermore, antibody binding to mannan restored complement activation in MBL-deficient serum in a C1q-dependent manner. Cooperation between the CP and the LP was also observed for complement activation by the protein 60 from *Listeria monocytogenes*. It is concluded that antibody-mediated CP activation can compensate for impaired target opsonisation via the LP in MBL-deficient individuals.

The last part of this chapter describes binding of MBL to polymeric serum IgA as a novel mechanism for activation of the lectin pathway. Furthermore, it is shown that MBL binding to IgA results in complement activation and propose that this leads to a synergistic action of MBL and IgA in antimicrobial defense, especially in the mucosal immune system.

It is concluded that the LP of the complement system as a part of the innate immunity is interwoven with the adaptive immune system, a characteristic that previously was only allocated to the CP within the complement system.

MBL and type I diabetes

Type 1 (insulin-dependent) diabetes mellitus (T1D) is an autoimmune disease characterized by the specific destruction of beta cells in the pancreas. The role of the adaptive immune system in the autoimmune process leading to type 1 diabetes is well established (12). Presently the interest for the innate immune system in the immunopathogenesis of T1D is mounting (13-15). Having previously shown that MBL is able to meticulously link the adaptive and innate immune system, it is speculated that MBL could be involved in the pathogenesis of type 1 diabetes. It could be hypothesized that low MBL serum levels could result in an impaired clearance and inactivation of pathogens responsible for beta cell destruction. Contrarily, insulitis resulting in tissue damage could activate the lectin pathway via MBL, facilitating additional beta cell damage and a more fulminant insulitis. **Chapter 3** describes MBL in relationship to type 1 diabetes at clinical presentation. In this study new-onset juvenile type 1 diabetic patients are investigated in comparison with their non-diabetic siblings and healthy unrelated control subjects. The polymorphisms of MBL exon

1 and promoter were determined and serum MBL concentration and MBL-complex activity were measured. Initially, the genetic MBL constitution did not account for disease predisposition, since the genetic epidemiology did not show pronounced differences between patient and unrelated control subjects. However, genetic stratification based upon MBL polymorphisms in both the coding exon 1 and promoter region, proved to be essential to reveal T1D associated differential MBL functionality. 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. 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. It is concluded 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. The study described in this chapter is the first to show an association between MBL and the onset of T1D. Although MBL is a major recognition molecule of the lectin pathway, it should be appreciated that various other molecules are involved, including MBL-associated serine protease and fluid phase complement inhibitors like C1 esterase inhibitor. In order to be able to fully comprehend the involvement of the lectin pathway in the onset of T1D, further extension of research is required and should include all relevant players. However, with our present knowledge it can be hypothesized that high MBL levels and MBL activity could facilitate an immune response by inflicting or maintaining damage during the insulitis phase of T1D. In accordance with the danger model of the immune system (16), the presence of damage is essential for an (auto)immune response

MBL and liver transplantation

Infection is the primary cause of death after liver transplantation. As transplant patients require immune suppressive drugs in order to insure graft survival, they rely to a great extent on their innate immunity to counteract infections. Studying MBL as a major component of the innate immune system, the effect of MBL gene polymorphisms on the susceptibility to infection after liver transplantation was evaluated in **chapter 4**. Furthermore, although it is generally assumed that MBL is hepatically produced, it was sought to establish the role of the liver in production of serum MBL. Patients undergoing orthotopic liver transplantation were investigated. MBL promoter and exon 1 polymorphisms were determined in both patients and in liver donors. MBL serum concentration was monitored shortly before and during one year after transplantation. MBL deficiency was evaluated in association to the occurrence of clinically significant infections during this period. This study shows that

transplantation of MBL-wildtype recipients with donor livers carrying MBL-variant alleles resulted in a rapid and pronounced decrease of serum MBL levels. This serum conversion was associated with the disappearance of high molecular weight MBL. No indication for extrahepatic production of serum MBL could be obtained. The presence of MBL variant alleles in the MBL gene of the donor liver, but not in the recipient, was associated with a strongly increased incidence of clinically significant infections following transplantation in a gene-dose-dependent way. It was concluded that serum MBL is produced by the liver under strong genetic control, and established that the liver is the primary producer of serum MBL. Following liver transplantation, the MBL genotype of the donor liver is a major risk determinant for life-threatening infections.

The ability to unambiguously identify a group of patients severely prone to infection post transplantation is of significant clinical value. In an era of donor shortage, donor selection based upon MBL genotype is inconceivable. However, this study suggests that patients receiving an 'MBL-variant' liver could benefit from MBL replacement therapy similar to that presently being studied in phase I/II and III studies (17; 18). Furthermore, prophylactic approaches including intensified clinical follow-up, preemptive antimicrobal therapy, and prolonged selective digestive decontamination could be considered dependent on the MBL genotype of the liver donor. Moreover, administering intravenous immunoglobulins (IVIg) should be considered. By administering a diverse repertoire of immunoglobulins that possess the same wide spectrum of antibacterial, antiviral and antifungal specificities as MBL, IVIg could compensate MBL deficiency via the CP. As clearly shown in chapter 2, complement activation by prototypic MBL ligands can be achieved in MBL-deficient serum via the antibody-mediated activation of the classical pathway, thus compensating MBL deficiency.

Having shown the direct implications of MBL deficiency in liver transplantation, randomized clinical intervention studies should be set up in transplant patients receiving an MBL variant liver in order to diminish the increased susceptibility to life-threatening infections.

Adaptive immunity in pancreatic islet transplantation

As shown and discussed in chapter 3, type 1 diabetes is an autoimmune disease in which both adaptive and innate immune processes are involved, ultimately leading to beta cell destruction. An attractive novel therapy for type 1 diabetes is pancreatic islet transplantation. In order to successfully apply islet transplantation as a treatment for diabetes, two distinct tribulations have to be resolved. Primary, the recurrence of islet autoimmunity, a process theoretically equivalent to the pathogeneses of T1D (chapter 3), should be prevented. It is conceivable that MBL is also involved

in this process as implied in chapter 3, however this topic remains subject to future investigation. Secondly, to assure pancreatic islet graft survival, it is fundamental to prevent alloimmunity resulting in allograft rejection. In order to optimise the current islet transplantation, it is essential to study the reaction of T-cells to islets. **Chapter 5** describes the effect of HLA incompatibility and immunogenicity of human pancreatic islet preparations and peripheral blood mononuclear cells (PBMC) of healthy blood donors. The aim of this study was to examine whether the degree of MHC incompatibility between PBMC and donor islet cells is related to the degree of proliferative T-cell responses. The experiments included co-culturing of PBMC with human islet-cell preparations with a composition similar to that of islet grafts used in clinical transplantation trials [i.e. mixed islet lymphocyte reaction (MILR)]. PBMCs isolated from immunologically uncompromised healthy blood donors were used to exclusively study alloreactivity independently of recurrence of autoreactivity. Prominent T-cell responses were observed in the vast majority of cases of complete HLA class II-mismatches. Intermediate T-cell responsiveness was observed in single HLA class II mismatches, whereas HLA-matches islet preparations did not induce a T-cell response.

These results identify the potential immunogenicity of islet preparations transplanted between HLA-DR incompatible subjects regardless of an autoimmune background of the recipient. These findings underscore the difficulty of islet allotransplantation, but provide leads to reduce the extent of alloreactivity in clinical islet transplantation.

CONCLUDING REMARKS

The interplay between innate and adaptive immunity is complex and multifaceted as chapters 2 and 3 of this thesis show. The beneficial effect of high MBL serum levels in liver transplant patients stand in vast contrast to the potential harmful effect of elevated serum MBL levels in diabetic patients emphasizing the pluripotency of mannose binding lectin. This paradox is reflected in chapters 3 and 4. Since the first report of the clinical implication of MBL deficiency somewhat four decades ago (19), our knowledge of the lectin pathway has expanded tremendously. Strikingly however, clinical implementation of our current knowledge occurs tediously lethargic. Presently, MBL replacement therapy is being studied in phase I/II and III studies (17; 18). However several other therapeutic interventions can be put forward to compensate for MBL deficiency in immunocompromised patients, including intensified clinical follow-up and preemptive antimicrobal therapy. Intravenous and subcutaneous

immunoglobulin administration might also be an alternative therapy to counterweigh a malfunctioning lectin pathway (20; 21).

Finally, pancreatic islet transplantation as a potential therapy for diabetes currently remains an immunological pitfall. Major hurdles include the occurrence of alloimmunity and the recurrence of autoimmunity. Further complicating immunological disentanglement is the fact that both innate and adaptive immunity appear to be involved. Presently islet graft rejection can only be counteracted by pharmacological immune suppression. The substitution of insulin replacement therapy by immune suppressive drugs is a debatable issue, which should be evaluated conscientiously by physicians and patients, taking both short and long term perils into account. Currently, diabetic patients appear to be stuck between a rock and a hard place. Therefore studies aimed to identify immunological aspects of islet transplantation are of eminent importance in order to optimalize immune suppressive therapy and to reduce morbidity and mortality.

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NEDERLANDSE SAMENVATTING

De term immuniteit is afgeleid van het Latijnse woord *immunitas*, dat refereert aan de vrijstelling die Romeinse senatoren hadden van civiele verplichtingen, zoals bijvoorbeeld dienstplicht en wettelijk vastgelegde vervolging. Sinds het Romeinse tijdperk heeft de term immuniteit meer de betekenis gekregen van het resistent zijn tegen infecties. Het immuunsysteem is een complexe verzameling van cellen en moleculen, die het lichaam laat functioneren in een habitat vol ziekteverwekkers (pathogenen), zoals bacteriën en virussen.

Het immuunsysteem kan worden ingedeeld in natuurlijke en specifieke immuniteit. Deze indeling is gebaseerd op het vermogen van het specifieke deel van het immuunsysteem om bij herhaalde infecties een versterkte immuunrespons te genereren. Natuurlijke immuniteit is het deel van het immuunsysteem dat reeds bij de geboorte aanwezig is en continu is qua intensiteit bij herhaalde infecties.

Er zijn situaties waarin een te hard werkend immuunsysteem nadelig kan zijn. Twee voorbeelden waarin excessieve immuunrespons negatieve gevolgen heeft zijn auto-immuunziekten (bijvoorbeeld type 1 diabetes) en transplantatie. Het onvermogen om lichaamseigen cellen en eiwitten te onderscheiden van pathogene eiwitten en micro-organismen kan resulteren in auto-immuunziekten, welke leiden tot weefsel- en/of orgaanschade. Na een transplantatie kan het immuunsysteem het getransplanteerde orgaan als pathogeen herkennen en een immuunrespons initiëren, hetgeen kan leiden tot zowel acute- als chronische afstoting.

Het doel van dit proefschrift is om inzicht te verkrijgen in immunologische aspecten van transplantatie en diabetes. De nadruk ligt op Mannose Binding Lectin (MBL) als cruciaal onderdeel van de natuurlijke immuniteit.

Hoofdstuk 1 beschrijft de verschillende moleculaire en immunologische eigenschappen van Mannose Binding Lectin als onderdeel van het complementsysteem.

Het complementsysteem bestaat uit drie verschillende routes: de klassieke route, de alternatieve route en de lectine route. Hoewel alle drie routes van het complement systeem op verschillende wijzen worden geactiveerd, resulteren ze alle in aktivatie van het complement systeem. De vijf biologische functies van het complement systeem zijn 1) complement-gemedieerde cytolysis (celdood); 2) opsonisatie van lichaamsvreemde organismen; 3) activatie van ontsteking; 4) klaren van lichaamseigen- en lichaamsvreemde débris; 5) versterken van de specifieke immuunrespons (figuur 1, hoofdstuk 1).

De lectine route wordt geactiveerd wanneer MBL bindt aan koolhydraatstructuren welke aanwezig zijn op een groot aantal micro-organismen. In de algemene populatie bestaat er een grote variatie aan MBL-concentratie in het bloed. Dit concentra-

tieverschil is erfelijke bepaald door verschillende variante genen die leiden tot een lage MBL-productie en gestoorde MBL-functie.

Een deficiënte lectine route door een gestoorde MBL-functie dan wel een te lage MBL-concentratie, resulteert in vatbaarheid voor infectie. Een overdreven lectine route gemedieerde complementactivatie zou kunnen leiden tot weefsel- en orgaanschade. Deze bipolaire verhouding wordt in hoofdstuk 1 besproken aan de hand van een overzicht van verschillende ziekten, geassocieerd met een hoge dan wel lage MBL concentratie.

Moleculaire en immunologische karakteristieken van MBL

In **hoofdstuk 2** worden in drie delen functionele karakteristieken van MBL beschreven. Het eerste deel van dit hoofdstuk beschrijft de functionele karakterisatie van de lectine route in normaal humaan bloed. Een nieuwe techniek wordt beschreven waarmee complementactivatie via de lectine route kan worden gemeten. Het tweede deel beschrijft de relatie tussen MBL-genotype en MBL-functionaliteit. Verder wordt er in dit deel ingegaan op de observatie, dat humaan bloed antilichamen bevat die gericht zijn tegen mannan, een koolhydraat dat typisch door MBL wordt gebonden. De hoge frequentie aan variante MBL genen in de populatie zou een aanwijzing kunnen zijn, dat er een alternatief immuun mechanisme is, dat compenseert voor MBL deficiëntie. Om deze hypothese te staven werd complement activatie bestudeerd door middel van moleculen welke typisch worden gebonden door MBL (MBL-liganden). Hieruit bleek dat het prototypische MBL-ligand, mannan, complementactivatie kan bewerkstelligen via zowel de lectine route als de klassieke route. Verder kan ook het eiwit "protein 60" van de bacterie *Listeria monocytogenes*, zowel via de lectine route als via de klassieke route het complementsysteem activeren. Er kan geconcludeerd worden dat, tot op zekere hoogte, de klassieke route van het complement systeem kan compenseren voor een deficiëntie van de lectine route in MBL-deficiënte individuen.

Het laatste deel van dit hoofdstuk beschrijft de binding van MBL aan polymeer serum IgA als nieuw mechanisme van complement activatie via de lectine route. Deze vorm van complement activatie zou kunnen leiden tot een synergistisch effect op de antimicrobiële werking van MBL en IgA, met name in het mucosale immuunsysteem.

MBL en type I diabetes

Type 1 (insuline afhankelijke) diabetes mellitus (T1D) is een auto-immuunziekte, die gekarakteriseerd wordt door de specifieke destructie van insuline producerende bètacellen in het pancreas (alvleesklier). De rol van het specifieke immuunsysteem in het complexe auto-immuun dat resulteert in T1D is alom geaccepteerd. Er zijn

echter steeds meer aanwijzingen, dat ook de natuurlijke immuniteit betrokken is bij het ontstaan van T1D. **Hoofdstuk 3** beschrijft MBL in relatie tot type 1 diabetes. In deze studie werden juveniele type 1 diabetische patiënten bij de klinische presentatie van hun ziekte bestudeerd. Patiënten werden vergeleken met hun gezonde (niet-diabetische) broers of zussen ("familie controles") en met gezonde bloeddonoren. Zowel MBL-bloedconcentratie als MBL-activiteit werden bepaald, alsook het MBL-genotype. Genetisch werden er geen verschillen aangetoond tussen de patiënten en de gezonde bloeddonoren. Echter, nadat zowel patiënten als hun familiecontroles ingedeeld waren aan de hand van hun genetische MBL achtergrond, bleken diabetische patiënten zowel een hogere MBL-bloedconcentratie te hebben als een hogere MBL-activiteit. Verder bleek de MBL-activiteit meer verhoogd te zijn dan verklaard kon worden aan de hand van MBL-concentratiestijging. Geconcludeerd wordt dat MBL-concentratie en activiteit verhoogd is bij T1D patiënten bij klinische presentatie, hetgeen een mogelijke rol voor MBL weglegt in de pathogenese (wijze waarop een ziekte ontstaat) van type 1 diabetes.

MBL en levertransplantatie

Infectie is de primaire doodsoorzaak na levertransplantatie. Om geen afstotingsreactie tegen het getransplanteerde orgaan te ontwikkelen, moeten transplantatiepatiënten geneesmiddelen innemen die het immuunsysteem onderdrukken. Omdat deze geneesmiddelen de specifieke immuniteit onderdrukken, vallen de patiënten voor een groot deel terug op hun natuurlijke immuniteit om infecties te voorkomen. In **hoofdstuk 4** wordt het effect beschreven van MBL-deficiënte genotypen op de vatbaarheid van infecties na levertransplantatie. Verder wordt getoetst of de lever de voornaamste producent is van MBL, zoals wordt aangenomen. Negenenveertig levertransplantatiepatiënten werden bestudeerd. MBL-concentratie in het bloed werd kort voor transplantatie en gedurende 1 jaar na transplantatie gemeten. Van zowel de donorlever als van de patiënt werd het volledig MBL-genotype bepaald. Gedurende een periode van 1 jaar na transplantatie werden levensbedreigende infecties gecorreleerd aan het MBL-genotype van de ontvanger en aan het MBL-genotype van de donorlever. De in dit hoofdstuk beschreven studie laat zien dat na transplantatie, de donorlever de MBL-serumconcentratie bepaalt, onafhankelijk van het genotype van de ontvanger. Verder toont deze studie dat patiënten die een MBL-deficiënte donorlever ontvangen een bijna viermaal hoger risico hebben op levensbedreigende infecties in het eerste jaar na transplantatie.

Specifieke immuniteit bij transplantatie van eilandjes van Langerhans

Zoals reeds besproken in hoofdstuk 3 is type 1 diabetes een auto-immuunziekte waarbij zowel specifieke- als natuurlijke immuniteit betrokken zijn, die uiteindelijk

resulteert in destructie van insuline-producerende bètacellen in het pancreas. Een nieuwe en hoopgevende therapie voor T1D is het transplanteren van bètacellen in eilandjes van Langerhans. Echter, om een dergelijke transplantatie succesvol te laten zijn moeten twee grote obstakels worden overwonnen. Ten eerste moet de terugkeer van het auto-immuunproces, leidend tot bètaceldestructie, worden voorkómen (hoofdstuk 3). Ten tweede moet worden voorkómen dat er afstoting plaats vindt van de getransplanteerde eilandjes. Hoofdrolspelers in de specifieke immuniteit en in afstootreacties zijn de T-cellen van het immuunsysteem. Om een goede overleving van de getransplanteerde eilandjes te krijgen is het van groot belang de reactie van T-cellen te bestuderen, om zo gericht geneesmiddelen te kunnen ontwikkelen om de afstootreactie tegen te kunnen gaan.

De werking van T-cellen berust op het herkennen van HLA-moleculen. Dit zijn moleculen die op (bijna) alle cellen van het lichaam aanwezig zijn en aangeven of een cel lichaamseigen, dan wel lichaamsvreemd is. In **Hoofdstuk 5** wordt het onderzoek beschreven waarin de immuunreactie van T-cellen wordt onderzocht aan de hand van het verschil van HLA-moleculen tussen T-cellen en eilandjes. Geconcludeerd wordt dat reeds bij geringe verschillen van HLA tussen T-cellen van de ontvanger en donoreilandjes een T-cel immuunrespons waarneembaar is. Deze immuunrespons treedt niet op wanneer T-cellen van de ontvanger HLA-identiek zijn aan de donoreilandjes.

In de klinische praktijk is implementatie van basaal wetenschappelijke onderzoeksresultaten een zeer moeizame onderneming. In dit proefschrift is getracht de consequenties van basaal wetenschappelijke bevindingen voor de kliniek in kaart te brengen. De toekomst moet leren of deze vertaalslag van fundamentele wetenschap naar klinische praktijk waardevol is gebleken.

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CURRICULUM VITAE

The author of this thesis was born on January 18th 1974 in Belfast, United Kingdom. With his parents he moved to The Netherlands where he followed his secondary school at the Prins Willem van Oranje MAVO in Krimpen aan den IJssel and the Comenius College in Capelle aan den IJssel. After having graduated from the atheneum in 1993 he joined the Royal Dutch Navy and attended the Royal Dutch Naval Academy in Den Helder. Having successfully passed the practical Royal Dutch Marine Corps officers training in 1996, he began to study Biomedical Science at the University of Leiden in that same year. In 2001 the author received his masters degree having completed his graduation project at the Nephrology department of the Leiden University Medical Center. His graduation project included complement system research and was supervised by Professor M.R. Daha and Dr. A. Roos. For this work he received the "Dick Held" junior research prize of the Royal Dutch Medical Association. In 1998 the author was admitted to medical school at the University of Leiden. In 2000 he received his medical masters degree (*cum laude*) and in 2002 he qualified as a Medical Practitioner (*cum laude*). Before embarking on his research project that evolved in this thesis, the author worked as a surgical senior house officer in the Accident and Emergency department of the Leiden University Medical Center. Currently he is a surgical resident at that same hospital (Head: Professor J.H. van Bockel). Furthermore, the author is presently a member of the editorial board of the *World Journal of Gastoenterology*.

LIST OF ABBREVIATIONS

ANOVA	analysis of variance
AP	alternative pathway
BSA	bovine serum albumin
CDR	C-terminal carbohydrate-recognition domain
CP	classical pathway
CSI	clinically significant infections
ELISA	enzyme linked immunosorbent assay
GVB	gelatin veronal buffer
I/R	ischemia/reperfusion
LP	lectin pathway
mAb	monoclonal antibody
MAC	membrane attack complex
MASP	MBL-associated serine protease
MBL	mannose-binding lectin
MILR	mixed islet/lymphocyte reaction
MLR	mixed lymphocyte reaction
MP	MBL pathway
OLA	oligonucleotide ligation assay
OLT	orthotopic liver transplantation
p60	protein 60 of *Listeria monocytogenes*
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
R	correlation coefficient
RF	rheumatoid factor
SLE	systemic lupus erythematosus
SNP	single nucleotide polymorphism
SNPs	single nucleotide polymorphisms
T1D	type 1 Diabetes Mellitus