The Ancient Complement System: Role in Physiology and Defense

Saskia Kuipers

This thesis was prepared at the Eijkman-Winkler Center for Microbiology, Infectious Diseases and Inflammation, University Medical Center, Utrecht, and in the Department of Microbiology, Public Health Laboratory Twente Achterhoek, Enschede, the Netherlands.

ISBN 90 015899 5
Cover design Titia Kuipers: From ascidian to man
Printed by Febodruk BV, Enschede

The publication of this thesis was financially supported by Department of Microbiology, Public Health Laboratory Twente Achterhoek, Enschede, the Netherlands.

The Ancient Complement System: Role in Physiology and Defense

(met een samenvatting in het Nederlands)

Een wetenschappelijke proeve op het gebied van de Medische Wetenschap

Proefschrift
ter verkrijging van de graad van doctor aan de Universiteit van Utrecht
op gezag van Rector Magnificus
involge het besluit van het College voor Promoties
in het openbaar te verdedigen
op donderdag 25 September 2003
om 12:45 uur
door

Saskia Kuipers

Geboren op 21 mei 1964 te Arnhem

Promotiecommissie

Promotor:	Prof. dr. H. van Dijk
Co-promotor:	Dr. C.M. Verduin
Overige leden:	Prof. dr. M.R. Daha Prof. dr. J.L.L. Kimpen Prof. dr. F.P. Nijkamp Prof. dr. J.E. Veldman Prof. dr. B.J.M. Zegers

'Our first task is to construct the physical reality in which we live. The second task is the creation of ourselves – of that very self that lives within this outer reality. Both tasks require equal attention. Keeping the balance between them is a very sacred and demanding art.'

Olga Kharitidi, 1996

Voor mijn ouders
Voor Joris

CONTENTS

Chapter 1

General introduction

Chapter 1

INTRODUCTION:
THE COMPLEMENT SYSTEM IN HISTORICAL PERSPECTIVE

Abbreviations

AP	: alternative complement pathway
C1-INH	: C1 esterase inhibitor
CP	: classical complement pathway
CR1	: complement receptor 1 (CD35)
CRP	: C-reactive protein
DAF	: decay-accelerating factor (CD55)
Ig	: immunoglobulin
LP	: lectin complement pathway
MBL	: mannose-binding lectin
MCP	: membrane cofactor protein (CD46)
MHC	: major histocompatibility complex
RCA	: regulators of complement activation
SLE	: systemic lupus erythomatosus

Classification of species or phyla in evolution

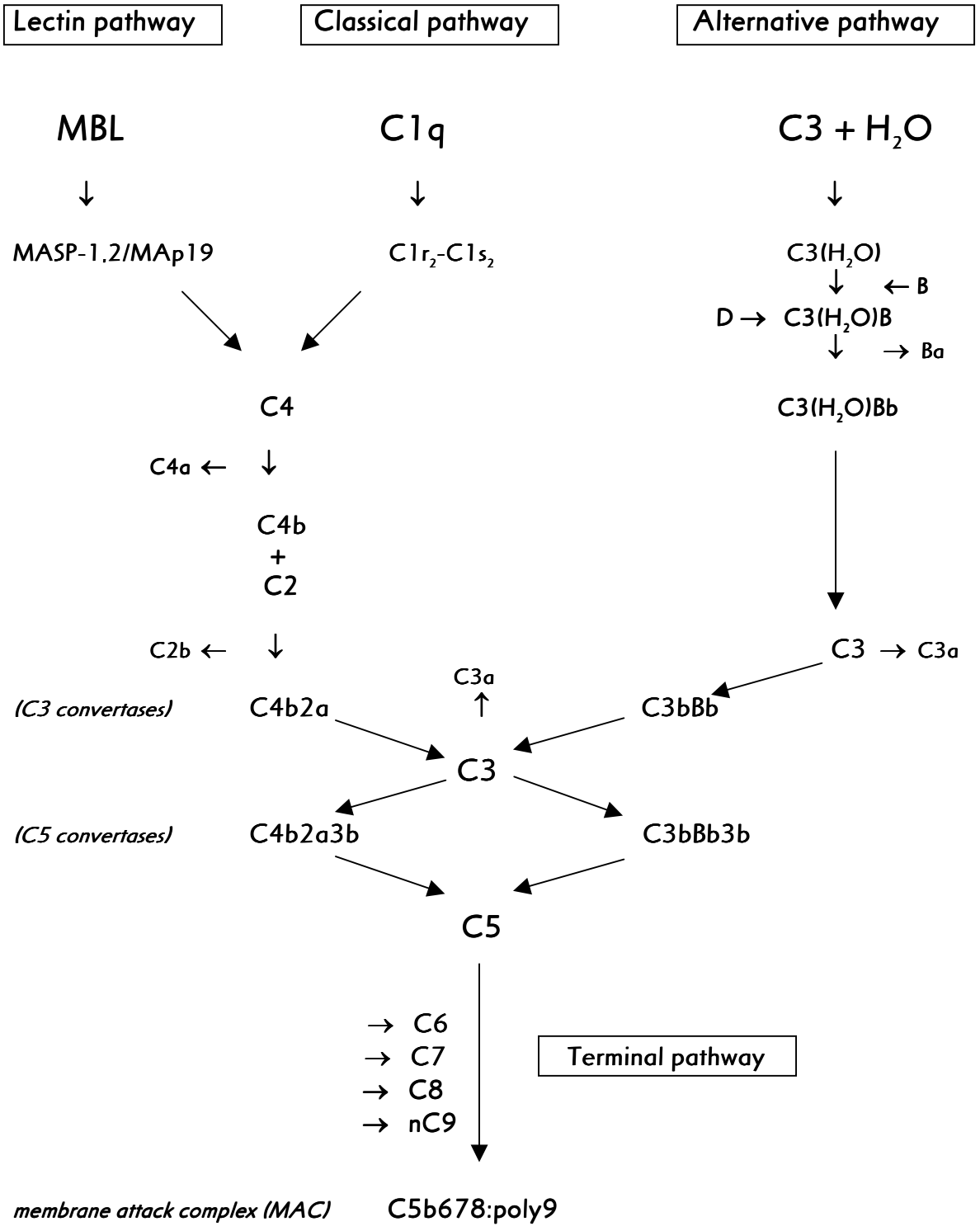
Agnatha	: jawless vertebrates like hagfish and lamprey
Ascidian:	: belongs to the subphylum urochordata
Chordata:	: phylum comprising urochordata, cephalochordata and vertebrata
Cyclostome	: e.g. lamprey
Deuterostome	: comprises two major phyla, the chordata (including mammals) and the echinodermata
Echinodermata	: (ekhinos = sea urchin, derma = skin) phylum including sea urchins, sea stars, and seacucumbers
Invertebrates	: echinoderms, and protochordates like *Clavelina picta* and the ascidian *Halocynthia roretzi*
Teleost fish	: bony fish like trout, sand bass, and puffer fish
Tunicate	: belongs to the phylum of the urochordata
Urochordata	: subphylum
Vertebrates	: classified in jawless (Agnatha) or jawed species

THE COMPLEMENT SYSTEM

1.1 Overview of the complement system (Fig. 1)

The immune system in human and other mammals is composed of two major subsystems (Table 1): the innate immune system and the adaptive immune system. The adaptive immune system, which is organized around B- and T lymphocytes, is very flexible in its adaptation to molecular changes in varying pathogens. However, reactions to unknown foreign contacts are slow, since it takes three to seven days before clonal selection and expansion of lymphocytes ensures a specific immune response. On the other hand, the innate immune system, which can be defined as all immune defenses that lack immunological memory, is quick to respond (in minutes to hours), and has evolved from ongoing associations between host and pathogen [1,2,3,4]. Nowadays, the innate immune response is understood to be essential to the function of the adaptive immune response [5].

Figure 1. An overview of the complement system: the three complement activation pathways of the complement system and the effector pathway (terminal pathway).



The number of C9 molecules in a MAC can vary between 1 and 18.

Table 1. A simplified version of human immunity [1,2].

Innate immunity	**Cellular**	**Phagocytes:** monocytes, macrophages, neutrophils **Dendritic cells** **Natural killer cells**
	Humoral (soluble)	**Complement:** - MBL or lectin complement activation pathway (LP) - Alternative complement activation pathway (AP) - Classical complement activation pathway (CP) **Acute phase proteins** **Cytokines** e.g. interferon **Chemokines**
Adaptive immunity	**Cellular** **Humoral**	**T lymphocytes, B lymphocytes** **Antibodies** (secreted by B lymphocytes or memory cells)

The complement system, focus of this thesis, is an important system in innate immunity and consists of over thirty different proteins. Synthesis of complement proteins occurs mainly in the liver, and in monocytes and macrophages, phagocytic cells that are part of innate immunity. Activation of the complement system results in a triggered enzyme cascade, analogous to cascades in coagulation, fibrinolysis, and the kinin pathway [1].

The complement system is involved in the innate recognition and elimination of antigen-antibody complexes and foreign microbes or molecules from the body. Clearance is mostly mediated by the mononuclear phagocytic system, consisting of monocytes and macrophages in liver, spleen, lymph nodes, or in the inflamed tissue itself, e.g. the lungs. In primates, erythrocytes have the function of transporting immune complexes to the liver or spleen for immune clearance [6,7]. Since human erythrocytes carry complement receptor 1 (CR1) molecules on their surface, immune complexes adhere to the CR1 molecules with the aid of C4b and C3b, by a process called 'immune adherence' or 'the red cell adhesion phenomenon' [8,9,10]. In mice, platelets carry CR1 receptors, which makes these cells mediators of immune adherence [11].

Although complement was first described by the end of the 19th century, it was only in the 1960s that separation techniques became available which allowed the purification and the functional characterization of the components involved in the antibody-dependent classical pathway, and the antibody-independent alternative pathway. The antibody-independent lectin pathway was recognized only a decade ago.

1.2 Complement activation pathways and C3 conversion

The key position in the complement system is held by C3. This complement component is the most abundant, being present in normal plasma at levels up to 1.3 mg/ml [12]. The activation of C3 into C3b and C3a is mediated by so-called C3-convertases, which are the products of the three different complement activation pathways. In turn, C3b exposes a highly reactive thioester group that then reacts with foreign materials, e.g. immune complexes or carbohydrates.

The complement activation pathways include, in order of descending evolutionary age, the lectin pathway (LP), the 'alternative' pathway (AP) and a third pathway,

inappropriately named the 'classical' pathway (CP), though this pathway is phylogenetically the youngest. These activation pathways either lead to coating of microbes, immune complexes, and/or cells with C3b (opsonization) or to the initiation of 'terminal pathway' activation, which involves a series of events resulting in cellular membrane destabilization by pore formation. Thus, killing of e.g. Gram-negative bacteria and virus-infected cells is achieved.

1.2.1 The lectin pathway of complement activation

The lectin pathway (LP) was only discovered in 1989 [13]. Activation of the lectin pathway starts with the recognition and binding of foreign bodies by a serum lectin called mannose-binding lectin (MBL). MBL is a high-molecular-weight protein, present in blood plasma in minute amounts (about 1.7 µg per ml). See Figure 2. MBL belongs to the family of collagenous lectins (collectins), together with human CL-L1 [14], CL-P1 [15], lung surfactant proteins A and D, and the bovine proteins conglutinin [16], collectin-43 [17], and collectin-46 [18].

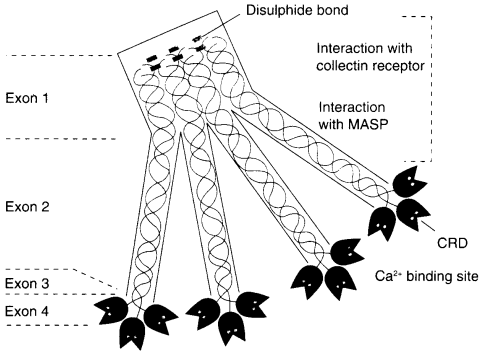


Figure 2. A side view of the tetrameric form of human mannose-binding lectin (MBL), based on four identical subunits. Each MBL head contains three C-type lectin domains that are carbohydrate recognition sites (CRD). MASPs interact with the collagenous regions. The four exons of the human MBL gene encode for the protein regions shown. MASP: MBL-associated serine protease [25].

Upon binding to foreign bodies, MBL undergoes a conformational change leading to the coordinate activation of at least two MBL-associated proteins (MASP-1, and MASP-2) and the generation of the active form of MASP-2, the LP-dependent C4/C2 convertase [19]. MASP-1 is capable of direct C3 and C2 cleavage [20,21], but MAp19 lacks proteinase activity and its function is unknown [22,23]. The role of a recently discovered fourth MBL-associated serine proteinase, MASP-3, an alternative splicing product of the *MASP1/3* gene, probably involves downregulation MASP-2 activity as shown by *in-vitro* experiments [24]. C4/C2 convertase essentially generates three products from its substrates C2 and C4. These are C4bC2a and the split products C4a and C2b. Substrate-bound C4bC2a is a C3 convertase, allowing the conversion of C3 into C3a and C3b, which is the key reaction leading to terminal complement pathway initiation.

MBL mediates the recognition of foreign particles by its three identical sugar-binding domains with specificity for mannose, N-acetylglucosamine, and fucose [25]. The cell wall of fungi is relatively rich in mannan (polymerized mannose), while peptidoglycan of Gram-positive bacteria contains large amounts of N-acetylglucosamine, making these microorganisms ideal substrates for the lectin pathway [26,27]. IgA, a heavily mannosylated immunoglobulin and important mediator in mucosal immunity, activates

the lectin pathway, and is thought to act synergistically with MBL in defense [28]. One can also speculate on the MBL-activating properties of IgD. A cellular receptor for MBL has only been recognized recently, i.e. CR1 (complement receptor 1, CD35, C3b/C4b receptor) that is present on the surface of erythrocytes and inflammatory cells [29].

Table 3. *Initiators of the three complement activation pathways.*

complement activation pathways	initiators of complement activation pathways
lectin complement pathway	microbes with exposed mannose, fucose, GlcNAc*
	lipoproteins**
	apoptotic cells #
alternative complement pathway	bacteria, viruses, fungi, tumor cells
classical complement pathway	certain viruses and Gram-negative bacteria
	immune complexes
	apoptotic cells
	ligand-bound CRP ##

*GlcNAc: N-acetyl-D-glucosamine. ** This thesis, chapter 7. # Ogden et al [30] and Raaschou-Jensen [31]. ## CRP: C-reactive protein.

1.2.2 The alternative pathway (AP) of complement activation

The alternative pathway was first described in 1956 by Pillemer et al. [32]. However, it took more than 15 years until the pathway became generally accepted by complementologists [33]. Nowadays, we know that the main function of the antibody-independent alternative pathway is to increase the number of C3b and inactivated C3b (C3bi) molecules on the substrates of complement activation. Thereby, the recognition and clearance of foreign bodies and immune complexes is accomplished. C3b deposition on the activating surface leads to an explosive instigation of the 'amplification loop', which is kept in check by the cell-bound complement inhibitors DAF (CD55) and MCP (CD46), and the soluble serum factors H and I. Activating surfaces include bacteria (LPS, teichoic acid), fungi, parasites, viruses, virally infected cells, and tumor cells like lymphoblastoid cells [12].

Once formed, C3-convertase(s) will be amplified by amplification loop. This may be achieved in the following way: a C3 convertase will activate C3 into C3a and C3b, the very last of which will be covalently bound to the surface of activation (e.g. a microorganism). Next, C3b will bind AP component factor B which, in turn, will be activated by AP component factor D, also termed 'adipsin' [12]. The AP-dependent C3-convertase C3bBb is then formed, as well as split product Ba, which is a leukotaxin. Properdin, the first identified AP component, stabilizes C3bBb, thereby greatly increasing its half-life, and promoting the generation of AP-dependent C5 convertases (C3bBbC3b). Down-regulation of the amplification loop is effectuated through factor I, which uses soluble factor H as a cofactor or cell-surface proteins MCP and CR1.

1.2.3 The classical pathway of complement activation

Phylogenetically considered the youngest complement activation route, the classical pathway (CP) depends on IgG and/or IgM antibodies that are present in higher life forms

(mammals), but also cartilagenous and bony fish. The CP is very similar to the lectin pathway and may even have been derived from it, since CP component C1, consisting of C1q, C1r and C1s, is phenotypically and functionally related to the MBL/MASP-1/MASP-2 complex [34]. Moreover, the LP and CP share components C4 and C2. Experimental evidence showed that the first CP component C1q can bind Gram-negative bacteria with exposed lipid A, and exists in association with proenzymic $C1r_2s_2$. The C3b receptor (CR1), present on erythrocytes and leukocytes, can act as a receptor for both C1q [35] and MBL [29], which again stresses the functional relationship between C1 and MBL.

Table 3. *Physiologic activities ascribed to components of the human complement system, adapted from [1].*

ACTIVITY	COMPLEMENT PROTEINS INVOLVED
Host defense	
opsonization	covalently bound C3 and C4 fragments
chemotaxis and leukocyte activation	C4a, C3a, C5a (anaphylatoxins); anaphylatoxin receptors on leukocytes
lysis of bacteria, cells, particles	MAC (C5b-9)
Clearance of particles	
immune complexes	C1q; covalently bound C3 and C4 fragments
apoptotic cells	C1q; covalently bound C3 and C4 fragments; MBL[#]
lipoproteins *	MBL; covalently bound C3 and C4 fragments
ds-DNA, ss-DNA **	MBL ? **
Interface innate and adaptive immunity	
augmentation of antibody repsonses	C3b and C4b bound to IC and Ag; C3 receptors on B cells and APCs
enhancement of immunological memory	C3b and C4b bound to IC and Ag; C3 receptors on follicular dendritic cells

[#] Ogden et al, 2001; Raaschou-Jensen, 2001. * This thesis, chapter 7. ** This thesis, chapter 7. IC: immune complexes. Ag : antigen. APC: antigen-presenting cell.

1.2.4 The terminal complement pathway

Starting point of the terminal complement pathway is the formation of C5 convertases (C4bC2aC3b and/or C3bBbC3b) which mediate the conversion of C5 into C5b and C5a, a potent chemoattractant. The former complexes with C6 and C7, the resultant of which is a soluble complex with affinity for membranous bilayers. Upon insertion into e.g. Gram-negative bacteria, C8 will bind to the complex, thereby forming a new membrane-bound enzyme (C5b-8) with membrane-bound C9 polymerase activity. Under the influence of C5b-8, some 13 C9 molecules become polymerized, resulting in a functional cylindric pore through the membrane. Depending on the presence of bacterial capsules and/or the total number of C9 molecules, target cells either resist membrane attack or are lysed through osmotic imbalance.

1.3 Regulators and modulators of the complement system

Unwanted activation of the complement cascade is prevented by both cell-bound and fluid-phase regulators and modulators. Soluble complement inhibitors include the following proteins:

C1 esterase inhibitor (C1-INH): an inhibitor of the classical complement pathway through equimolar binding to C1r and C1s, leading to the dissociation of the C1 complex [12]. In a similar way, C1-INH binds to MASP-2 and inhibits MASP-2-associated C4-cleaving activity, thereby inhibiting the lectin complement pathway too [36]. The protein is present in human serum at 0.15 to 0.35 mg/ml. It belongs to the serpin (serine proteinase inhibitor) superfamily that is present in plants, invertebrates and vertebrates, functioning in complement, coagulation, fibrinolysis, contact activation, and transport [12].

C3b inactivator (factor I): acts in conjunction with factor H in the conversion of C3b into C3bi. This intermediate can still act as an opsonin, but not as an active enzyme in the AP [12].

α_2-Macroglobulin (α_2M): belongs to the C3/C4/C5 thioester protein family and functions as a natural regulator of proteinases. Also, α_2M may prevent LP activation through direct binding, or it can interfere with MASP-induced C4 cleavage [37,38]. However, this finding has not been confirmed by others, including ourselves [39-41].

Factor H and factor H-like molecules: act at the level of factor B binding to target-bound C3b, thereby preventing the formation of AP-dependent C3 convertases. Binding of factor H also regulates the LP through binding of C3b [42]. C-reactive protein (CRP) may inhibit factor H by binding which, in turn, controls the LP, but the significance of this finding is not clear [43]. Factor H belongs to the family of regulators of C3 and C4 activation and degradation, a family of proteins displaying repeating elements called complement control proteins (CCP's), and whose genes are located in a cluster on chromosome 1.

Cell-bound complement regulators include complement receptor 1 (CR1), which has a factor-H-like co-enzyme function versus factor I, DAF and MCP, together called the three membrane-bound regulators of complement activation (RCA) proteins [44]. In man, CR1 is a transmembrane glycoprotein present on all types of blood cells except thrombocytes. In the circulation, 90 to 95 % of CR1 is expressed by erythrocytes, on the surface of which CR1 acts as a carrier protein [6,9,10] for bound immune complexes towards liver and spleen for transfer to macrophages [45]. The ligands for CR1 are C1q [35,46], MBL [29], C4b, C3b, and C3bi [12]. CR1 deactivates convertases by decay acceleration, and has cofactor activity for factor I-mediated cleavage of C3b and C4b.

1.4 The evolution of the complement system

1.4.1 Findings in nature

Phylogenetic studies have shown that the terminal and the classical pathways are relatively young (350-400 million years) when compared with the alternative and lectin pathways (over 700 million years old; Fig.3) [47]. The evolutionary studies have revealed that the three complement activation pathways and the terminal pathway are present in cartilaginous fishes (e.g. sharks) and higher vertebrates, while the classical and terminal pathways are lacking in the most primitive existent vertebrates like the lamprey (a jawless fish; Agnatha)[47-49].

The adaptive immune response, which must have appeared about 450 million years ago [50], is found in cartilaginous fish (sharks), in bony fish like salmon, and in the higher life forms, but is inefficient or absent in jawless fish and invertebrates [51]. In contrast, the complement system of cold-blooded vertebrates shows more diversity and greater activity than in higher life forms [47]. For example, the alternative pathway in fish is up to ten times as active as compared to higher vertebrates, which may offer a kind of compensation for the lack of adaptive immunity [50,52]. The AP component factor D has been found only in bony fish and higher evolved organisms. The only known member of the RCA (regulators of complement activation) family found in lower vertebrates, like teleost fish, are factor H and sand bass proteins SBp1 and SBCRP-1 [53]. The terminal complement components C5b, C6, C7, C8a, C8b, and C9 homologues are present in bony fish and higher life forms, but the most ancient fish lack a functional terminal pathway.

Which complement homologues have been identified in the lower part of the evolutionary tree? Evolutionary homologues of the key protein C3 have been identified in sea urchins and tunicates, lower phyla sharing characteristics with vertebrates [54]. In the invertebrate tunicate *Styela plicata*, a calcium-dependent lectin has been isolated. Also, two other complement components, MASP and factor B, have been identified in invertebrates like deuterostomes, e.g. sea urchins and sea stars [55], confirming a simple complement system with less components and fewer pathways in the lower phyla. In ascidians, no C2 or C4 is found, but C3 molecules with opsonic activity are present, as are MASP homologues, although no functional study has been done to demonstrate C3-cleaving activity of these homologues, yet.

Until recently, the alternative pathway was considered to be more primitive and hence older than the lectin pathway in evolutionary terms. However, certain important findings question this view [48,49,56]. For example, factor D has been found only in bony and cartilagenous fish and in higher species, but not in the jawless fish (Agnatha) and other lower life forms. Even more convincing is the evidence that lectins and MASPs are present not only in vertebrates but also in invertebrates. In deuterostomes, e.g. sea urchins and sea stars, C3 homologues, MASP and factor B chave been identified [55,57]. Moreover, in the ascidian *Halocynthia roretzi*, beside C3, two MASPs have been found, with close similarity to MASP-1 [58]. In the evolutionary tree, ascidians belong to the urochordata with a phylogenetical position between vertebrates and true invertebrates. Finally, MBL-like proteins have been found in invertebrates like the

protochordate *Clavelina picta* [59], the tunicate *Styela plicata* [54], and in insects like the cockroach *Blaberus discoidalis* [60].

In conclusion, these finding provide evidence that the lectin-dependent complement pathway was present prior to the emergence of vertebrates in evolution [51,58].

1.4.2. Protein families

The evolution of the complement system coincided with the appearance of the adaptive immune system. This was concluded from the discovery of recombinase activating genes (RAG) in jawed vertebrates, resulting in the ability to carry out gene arrangements and to generate duplicated genes available for altered function [55]. The finding of similarities in protein sequence and function, as well as the close organization of some complement genes, have led to the theory that, in higher vertebrates, the complement cascade evolved from a few primordial genes through gene duplication [61].

Similarities in protein sequence and function are found in the α_2M/C4/C3/C5-thioester protein family. The only thioester protein found in invertebrates like the horseshoe crab *Limulus polyphemus* is α_2-macroglobulin [62], lending support to the idea that α_2-macroglobulin predates the other members of the family. However, the genes expressing the different thioester proteins are not found on one particular chromosome, but on different chromosomes.

A second protein family is formed by the RCA family. Found in bony fish, this protein family consists of CR1, CR1-like proteins, CR2, factor H, factor H-like proteins, DAF, and MCP. Later in evolution, however, the RCA family probably evolved into two different groups based on varying homology of protein sequences [53]. Sponges already contained molecules with CCP repeats that are highly homologous to the RCA family [63], providing evidence for the existence of precursors earlier in evolution. However, the evolution of this family of molecules remains to be elucidated.

The serine proteinases involved in the complement system consist of C1r, C1s, C2, factor B, factor D, factor I and the MASPs. As a third example of protein families, the constituents of the C1r/C1s/MASP-1/MASP-2 family are found to be phenotypically and functionally related [41]. MASP-1 is more similar to C1r than to C1s. Based on amino-acid sequence, two groups of serine proteases can be identified in the complement system [64]. The first group is the AGY-type (encoding an active serine site encoded by AGY), found solely in vertebrates, and which includes C1r, C1s, and MASP-2, each protein being encoded by a single exon. The second group is called the TCN-type, encoding a histidine loop also found in digestive enzymes and coagulation factors. The TCN-type group comprises MASP-1, factors B, D, and I, and C2 [65]. These finding suggest that the TCN-type is the evolutionary prototype of the MASP/C1r/C1s family. Probably, the classical pathway, being very similar to the lectin pathway, has evolved from the more ancient MBL/MASPs pathway at the evolutionary stage between cyclostomes and cartilaginous fish [65].

Ancient complement components of the major histocompatibility complex (MHC) class III molecules are C4A, C4B, C2, and factor B, but the question if the MHC is, or has been, present in life-forms lower than jawed fish remains unanswered. Gene

duplications that gave rise to C4/C3/C5 and factor B/C2 occurred only in vertebrates, but not in the invertebrate lineage. Again, this provides evidence for the theory that the lectin pathway is older than the alternative pathway [56,58].

1.5 The ancient complement system

The afore-mentioned information suggests that in ancient times (before the jawed fish evolved) some kind of primordial complement system must have existed, consisting of the following components (Fig. 3):

1. **C3**, the presence of with indicates at least a primitive complement system; ascidian C3 is opsonic, i.e. it enhances the phagocytosis of particles by coelomocytes or ascidian blood cells,

2. the **lectin-MASP** pathway, as the first self and non-self discriminatory system leading to deposition of C3b on target particles,

3. the **alternative pathway**, functioning as a C3b-amplification system; this pathway harbors **factor B** (Bf); Bf derived from sea urchins and ascidians possess additional short consensus repeat (SCR) domains and low-density lipoprotein receptor (LDLr) domains of which the function is unknown [58].

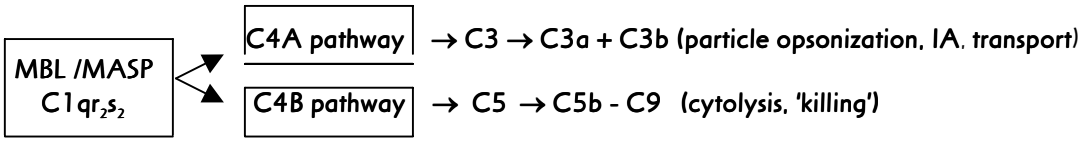
4. a possible equivalent of the 'immune adherence' phenomenon, which, in primates, involves the **C3b-receptor** (CR1 = CD35) on erythrocytes, similar to the thrombocyte compartment in rodents. Human erythrocytes carry between 500 and 1200 CR1 molecules on their surface [12,66], which are able to bind C3b-coated particles. These particles are then transported from their site of formation or production to target organs like the spleen, the liver, or any 'macrophage-rich' predator organ that may take care of the elimination of foreign particles in earlier life forms. The presence of CR1 in the lower phyla is unknown, and the evolution of CR1 is still unsolved (personal communication, 2002, Dr. F. Beurskens, Dr. Lloyd Klickstein, Brigham Women's Hospital, Boston, USA). However, CR3 and CR4 have been identified in lower phyla. Targets of C3b binding in lower phyla may consist of thrombocytes or more primitive hemocytes.

1.6 Can complement deficiencies clarify complement function?

Complete C4 deficiency is extremely rare but invariably leads to autoimmunity (SLE, and renal immune complex disease), and increased susceptibility to bacterial infections. Partial C4A or C4B deficiencies in the Caucasian population have a combined frequency of 31.6%. However, deficiency of C4B, which is more hemolytically active, has only incidentally been described to be associated with Gram-negative infections , while the body of literature on partial C4A deficiency points towards an increased risk of developing immune complex disease like SLE. Target-bound C4b of the A isotype is a better ligand for CR1 than C4b of the B isotype [67].

Figure 3. *Evolution of the complement system as published by Sunyer et al [47].* Reproduced with permission from the publisher.

Figure 4. *Proposed pathway for opsonization and immune adherence (IA).*



Over 12% of the Dutch population has low MBL serum levels (< 0.42 mg/L), as is shown is chapter 3, which is in line with MBL deficiency figures in other Caucasian populations. The prevalence of MBL deficiency in sub-Saharan Africa is much higher, over 30%, with a high C allele frequency of 0.24 [68]. One could speculate that MBL deficiency has or has had a relative evolutionary advantage, e.g. because it makes the MBL-deficient subject more resistant towards certain lethal infectious disease with e.g. mycobacteria and leishmania parasites [69-71]. In the northern European population, MBL deficiency is associated with severe atherosclerosis [72,73], but in Africa, where the prevalence of MBL deficiency amounts to 30%, overall incidence of atherosclerosis is low and a possible association with MBL has not been studied [74].

If MBL protects against severe atherosclerosis, which mechanism is involved and what does that tell us about the physiological function of MBL? Does MBL resemble surfactant protein D (SP-D) in its function as a mediator in lipid homeostasis [75]? One might speculate that MBL removes lipid particles involved in atherosclerosis from the bloodstream, which may be in line with findings that MBL can act as a scavenger molecule participating in clearance of dying host cells [30,31]. One may also hypothesize that lipid particles activate MBL, which are then removed from the circulation via opsonization and degradation in a target organ like the liver. In plants, mannose- and N-acetylglucosamine-specific lectins may be involved in the transport and/or accumulation of storage compounds [76]. Another interesting fact is that some plant lectins are potent antilipolytic agents [77].

In conclusion, complement deficiencies may help unravel the physiological functions of the individual complement components or of the complement activation pathways as a whole.

1.7 Introduction to this thesis

The thesis comprises the next chapters and addresses the following research questions:

Chapter 1. General introduction

> This first chapter introduces the subject and the players in the field of the body defense against infectious and chronic disease(s), and puts the complement system in historical perspective.

Chapter 2. A case of familial meningococcal disease due to deficiency in mannose-binding lectin (MBL)

(*The Proceedings of the 2nd Eijkman Symposium on Tropical Diseases: From Molecule to Bedside. Marzuki S, Verhoef, J, Snippe H, eds; Kluwer/Plenum 2003*)

This overview explains how patient-related findings open new horizons, as this was the case with meningococcosis and the lectin complement activation pathway.

Chapter 3. Prevalence of abnormal serum MBL levels in the Dutch population

In this study, we investigated, by use of a competitive ELISA, the prevalence of MBL deficiency in the normal Dutch population. For this study, we used a representative panel of 419 randomly chosen serum samples from donors to the blood bank, and from young and elderly orthopedic patients visiting the out-patient department. The ages of these serum donors ranged from 3 to 85 years.

Chapter 4. A functional microtiter assay for MBL in human serum

(*Journal of Immunological Methods 2002; **268:** 149-157*)

In this chapter, we describe the details of a functional MBL assay devised to estimate functional MBL levels (including MASP activity) in large panels of human sera. The assay devised is based on the principle of yeast (*Saccharomyces cerevisiae*)-induced activation of MBL and the subsequent bystander hemolysis of chicken erythrocytes used as 'innocent bystander cells' in the assay.

Chapter 5. Differential microorganism-induced MBL activation

(*FEMS Immunology and Medical Microbiology 2003; **36:** 33-39*)

This study describes the use of the hemolytic MBL assay, as described in chapter 4, to compare the strength of MBL activation by different bacteria and yeasts. Unexpectedly, in the case of two *Neisseria meningitidis* type B and C strains, less than one colony-forming unit (CFU) was still able to generate hemolysis. This seemingly homeopathic result could be explained by spontaneous meningococcal 'blebs' formation, and subsequent MBL activation by these particles. Clinically, this result may play an important role in invasive meningococcal disease. *Mycobacterium bovis* BCG was another potent activator. Gram-negative bacilli like *Pseudomonas aeruginosa* and *Legionella pneumophila* could activate MBL, but only in higher microbial concentrations.

Chapter 6. Deficiency of MBL is a novel risk factor for legionellosis

Legionella pneumophila is an intracellular bacterium that can survive and replicate in monocytes and macrophages. Does MBL have a protective role in legionellosis or does it augment susceptibility to this serious infection? Functional MBL titers were studied in sera from 124 proven and probable cases of legionellosis from the Bovenkarspel outbreak in the Netherlands in 1999, as well

as in sera from 60 individuals who had been exposed to the *Legionella pneumophila* type 1 outbreak in without developing clinical disease. Results were compared to those of 389 controls from the Dutch population.

Chapter 7. The 'immune adherence 'phenomenon: a versatile imune transport and elimination system?

The 'immune adherence' phenomenon, which describes the adherence of opsonized (C3b-laden) particles to erythrocytes via the C3b receptor (CR1), thus enabling transport of particles to the fixed elimination organs, is discussed in this chapter. Particles known to be opsonized are immune complexes and microbes, but additional experiments using the functional MBL assay show that lipoproteins are also capable of activating the complement system. Reduced opsonization not only leads to reduced clearance of infectious particles and immune complexes, thus enhancing infectious susceptibility and autoimmune disease, but it may also play a role the clearance of other particles including the primary lipoproteins chylomicrons and very-low density lipoprotein (VLDL) from the circulation.

Chapter 8. A genetic link between systemic lupus erythematosus (SLE) and thyroid autoimmunity?

In this study, we present a closer look at a family around a 13-year old girl with the first signs of the non-organ-specific autoimmune disease systemic lupus erythematosus (SLE). In her serum, extremely low levels of C4 were detected. In collaboration with our American colleague Dr. Chack-Yung Yu from Columbus (Ohio, USA), we studied the C4A allotypes in the affected subject and and found out that the quantity of C4A in her serum was zero (C4AQ0), although she had inherited a single C4B gene from each parent. In five family members on the maternal side, thyroid autoimmune disease had been diagnosed. Partial C4A deficiency was found in these individuals as well. This chapter offers insight into the complex discussion in the field of autoimmune diseases linked to deficiency of complement factor C4A.

Chapter 9. General discussion.

A summary of findings is given, including suggestions for further research.

REFERENCES

1. **Walport, M.J**. Complement. First of two parts. N Eng J Med 2001; **344:** 1058-1066.
2. **Delves, P.J., Roitt, I.M**. The immune system. First of two parts. N Engl J Med 2000; **343:** 37-49.
3. **Medzhitov, R., C. Janeway**. Innate immunity. N Eng J Med 2000; **343:** 338-344.
4. **Courtney Smith, L., K. Azumi, M. Nonaka**. Complement systems in invertebrates. The ancient alternative and lectin pathways. Immunopharmacol 1999; **42:** 107-120.
5. **Fearon, D.T., R.M. Lockley**. The instructive role of innate immunity in the acquired immune response. Science 1996; **272**:50-3.
6. **Nelson, R.A**. The immune-adherence phenomenon. Science 1953; **118:** 733-737.
7. **Cornacoff, J.B., L.A. Hebert, W.L. Smead, M.E. van Aman**. Primate erythrocyte-immune complex-clearing mechanism. J Clin Invest 1983; **71:** 236-247.
8. **Davis, L.J., H.C. Brown**. The adhesion phenomenon, a specific serological reaction occurring in trypanosomiasis. Trans Royal Soc Trop Med 1927; **21:** 113.
9. **Siegel, I., T.L. Liu, N Gleicher.** The red-cell immune system. Lancet 1981; **2:** 556-559.
10. **Nelson, D.S**. Immune-adherence. Adv Immunol 1963; **3:** 131.
11. **Miller, G.W., A.D. Steinberg, I. Green, V. Nussenzweig**. Complement-dependent alterations in the handling of immune complexes by NWZ/W mice. J Immunol 1975; **114:** 1166-1170.
12. **Law, S.K.A., K.B.M. Reid**. Complement in focus, 2nd edition. 1995. Oxford University Press. Oxford, GB.
13. **Kuhlman, M., Joiner, K., Ezekowitz, R.A.B**. The human mannose-binding protein functions as an opsonin. J Exp Med 1989; 169: 1733-1745.
14. **Ohtani, K., Y. Suzuki, S. Eda, T. Kawai, T. Kase, T. Shimada, H. Keshi, Y. Sakai, A. Fukuoh, T. Sakamoto, N. Wakamiya**. Molecular cloning of a novel human collectin from liver (CL-L1). J. Biol. Chem. 1999; 13681-13689.
15. **Ohtani, K., Y. Suzuki, S. Eda, T. Kawai, T. Kase, H. Keshi, Y. Sakai, A. Fukuoh, T. Sakamoto, H. Itabe, T. Suzutani, M. Ogasawara, I. Yoshida, N. Wakamiya.** The membrane-type collectin CL-P1 is a scavenger receptor on vascular endothelial cells. J Biol Chem 2001; **276:** 44222-44228.
16. **Holmskov, U.L**. Collectins and collectin receptors in innate immunity. APMIS 2000; Suppl **100:** 1-59.
17. **Hansen, S., D. Holm, L. Vitved, C. Bendixen, K.B.M. Reid, K. Skjoedt, U. Holmskov**. Abstract I-6. Proceedings of the Vth International Workshop on C1, the first component of the complement system, and collectins. 2001.
18. **Hansen, S., M. Dahl, C. Stover, N. Lynch, S. Thiel, J.C. Jensenius, W. Schwaeble**. Abstract III-3. Proceedings of the Vth International Workshop on C1, the first component of the complement system, and collectins. 2001.
19. **Thiel, S., T. Vorup-Jensen, C.M. Stover, W. Schwaeble, S.B. Laursen, K. Poulsen, A.C. Willis, P. Eggleton, S. Hansen, U. Holmskov, et al**. A second serine protease associated with mannan-binding lection that activates complement. Nature 1997; 386: 506-510.
20. **Matsushita, M., T. Fujita**. Cleavage of the third component of complement (C3) by mannose-binding protein-associated serine protease (MASP) with subsequent complement activation. Immunobiol 1995; **194**: 443-448.
21. **Matsushita, M, S. Thiel, J.C. Jensenius, I.Terai, T. Fujita**. Proteolytic activities of two types of mannose-binding lectin-associated serine protease. J Immunol 2000; **165:** 2637-2642.
22. **Stover, C.M., S. Thiel, M. Thelen, N.J., Lynch, T. Vorup-Jensen, J.C. Jensenius, W Schwaeble**. Two constituents of the initiation complex of the mannan-binding lectin activation pathway of complement are encoded by a single structural gene. J Immunol 1999; **162**:3481-3490.
23. **Takahashi, M., Y. Endo, T. Fujita, M. Matsushita**. A trncated form of mannose-binding lectin-associated serine protease (MASP)-2 expressed by alternative polyadenylation is a component of the lectin complement pathway. Int Immunol 1999; **11:** 859-863.

24. **Dahl, M.R., S. Thiel, M. Matshushita, T. Fujita, A.C. Willis, T. Christensen, T.Vorup-Jensen, J.C. Jensenius**. *MASP-3 and its association with distinct complexes of the mannan-binding lectin complement activation pathway. Immun 2001;* **15**:127-135.
25. **Turner, M.W**. *Mannose-binding lectin: the pluripotent molecule of the innate immune system. Immunol Today 1996;* **17:** 532-540.
26. **Emmerik, L.C. van, Kuijper, E.J., Fijen, C.A.P., Dankert, J., Thiel, S**. *Binding of mannan-binding protein to various bacterial pathogens of meningitis. Clin Exp Immunol 1994;* **97:** 411-416.
27. **Neth, O., D.L. Jack, A.W. Dodds, H. Holzel, N.J. Klein, M.W. Turner**. *Mannose-binding lectin binds to a range of clinically relevant microorganisms and promotes complement deposition. Infect Immun 2000;* **68:** 688-693.
28. **Roos, A., L.H. Bouwman, D.J. van Gijlswijk-Janssen, M.C. Faber-Krol, G.L. Stahl, M.R. Daha**. *Human IgA activates the complement system via the mannan-binding lectin pathway. J Immunol 2001;* **167:** 2861-2868.
29. **Ghiran, I., S.F. Barbashov, L.B. Klickstein, S.W. Tas, J.C. Jensenius, A. Nicholsen-Weller**. *Complement receptor 1/CD35 is a receptor for mannan-binding lectin. J Exp Med 2000;* **192**:1797-1807.
30. **Ogden, C.A., deCathelineau, A., Hoffmann, P.R., Bratton, D., Ghebrehiwet, B., Fadok, V.A., Henson, P.M**. *C1q and mannose-binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. J Exp Med 2001;* **194:** 781-795.
31. **Raaschou-Jensen, N., H.O. Madsen, L.P. Ryder, C. Koch, P. Garred**. *Mannose-binding lectin tags cells undergoing apoptosis. Abstract IV-9. Proceedings of the Vth International Workshop on C1, the first component of the complement system, and collectins. 2001.*
32. **Pillemer, L., L. Blum, I.H. Lepow, O.A. Ross, E.W. Todd, A.C Wardlaw**. *Science 1954;* **120:** 279-284.
33. **Lepow, I.H, F.S Rosen**. *Pathways to the complement system. N Engl J Med 1972;* **286:** 942-943.
34. **Vorup-Jensen, T., Petersen, S.V., Hansen, A.G., Poulsen, K., Swaeble, W., Sim, R.B., Reid, .B.M., Davis, S.J., Thiel, S., Jensenius, J.C**. *Distinct pathways of mannan-binding lectin (MBL) and C1-complex autoactivation revealed by reconstitution of MBL with recombinant MBL-associated serine protease-2. J Immunol 2000;* **165:** 2093-2100.
35. **Klickstein, L.B., S. Barbashov, T. Liu, R.M. Jack, A. Nicholson-Weller**. *Complement receptor type 1 (CR1, CD35) is a receptor for C1q. Immun 1997;* **7:** 345-355.
36. **Matsushita, M., T. Fujita**. *Inhibition of mannose-binding protein-associated serine protease (MASP) by C1 inhibitor. Mol Microbiol 1996;* **33(S1):** 44.
37. **Terai, I., K. Kobayashi, M. Matsushita, T. Fujita, K. Matsuno**. α_2-Macroglobulin binds to and inhibits mannose-binding protein-associated serine protease. Int Immunol 1995; **7**:1579-1584.
38. **Storgaard, P., D. Holm, E. Nielsen, E. Skriver, O. Andersen, S.E. Svehag**. *Mannan-binding protein forms complexes with* α_2*-macroglobulin. A proposed model for the interaction. Scand J Immunol 1995;* **42:** 373-380.
39. **Armstrong, P.B., J.P. Quigley**. α_2-Macroglobulin: an evolutionary conserved arm of the innate immune system. Dev Comp Immunol 1999; **23**: 375-390.
40. **Petersen, S.V., S. Thiel, L. Jensen, T. Vorup-Jensen, C. Koch, J.C. Jensenius**. *Control of the classical and the MBL pathway of complement activation. Mol Immunol 2000;* **37:** 803-811.
41. **Wong, N.K.H., M. Kojima, J. Dobó, G. Ambrus, R.B. Sim**. *Activities of the MBL-associated serine proteases (MASPs) and their regulation by natural inhibitors. Mol Immunol 1999;* **36:** 853-861.
42. **Suankratay, C., C. Mold, Y. Zhang, T.F. Lint, H Gewurz**. *Mechanism of complement-dependent hemolysis via the lectin pathway: role of the complement regulatory proteins. Clin Exp Immunol 1999;* **117:** 442-448.
43. **Suankratay, C., Mold, C., Zhang, Y., Potemba, L.A., Lint, T.F., Gewurz, H**. *Complement regulation in innate immunity and the acute-phase response: inhibition of mannan-binding lectin-initiated complement cytolysis by C-reactive protein. Clin Exp Immunol 1998;* **113:** 353-359.

44. **Hourcade, D., M.K. Liszewski, M. Krych-Goldberg, J.P Atkinson**. Functional domains, structural variations and pathogen interactions of MCP, DAF and CR1. Immunopharmacol 2000; **49:** 103-116.
45. **Hebert, L.A**. The clearance of immune complexes from the circulation of man and other primates. Am J Kidney Dis 1991; **17:** 353-361.
46. **Tas, S.W., L.B. Klickstein, S.F. Barbashov, A. Nicholson-Weller**. C1q and C4b bind simultaneously to CR1 and additively support erythrocyte adhesion. J Immunol 1999; **163:** 5056-5063.
47. **Sunyer, J.O., I.K. Zarkadis, J.D. Lambris**. Complement diversity: a mechanism for generating immune diversity? Immunol Today 1998; **19:** 519-523.
48. **Nonaka, M., T. Fujii, T. Kaidoh, S. Natsuume-Sahai, M. Nonaka, N. Yamaguchi, M. Takahashi**. Purification of a lamprey component protein homologous to the third component of mammalian complement system. J Immunol 1984; **133:** 3242-3249.
49. **Nonaka, M**. Evolution of the complement system. Curr Opin Immunol 2001; **13:** 69-73.
50. **Sunyer, J.O., L. Tort**. Natural hemolytic and bactericidal activities of sea bream (*Sparus aurata*) serum are affected by the alternative complement pathway. Vet Immunol Immunopathol 1995; **45:** 333-345.
51. **Zarkadis, I.K., D. Mastellos, J.D. Lambris**. Phylogenetic aspects of the complement system. Dev Comp Immunol 2001; **25:** 745-762.
52. **Koppenheffer, T.L**. Serum complement component of ectothermic vertebrates. Dev Comp Immunol 1987; **11:** 279-286.
53. **Krushkal, J., O. Bat, I. Gigli**. Evolutionary relationships among proteins encoded by the regulator of complement activation gene cluster. Mol Biol Evol 2000; 17: 1718-1730
54. **Nair, S.V., S. Pearce, P.L. Green, D. Mahajan, R.A. Newton, D.A. Raftos**. A collectin-like protein from tunicates. Comp Biochem Physiol 2000;**125:** 279-289.
55. **Courtney Smith, L., L.A. Clow, D.P. Terwilliger**. The ancestral complement system in sea urchins. Immunol Rev 2001; **180:** 16-34.
56. **Dodds, A.W**. Which came first, the classical/lectin pathway or the alternative pathway of complement? Abstract I-3. Proceedings of the Vth International Workshop on C1, the first component of the complement system, and collectins. 2001.
57. **Al-Sharif, W.Z., J.O. Sunyer, J.D.Lambris, L. Courtney Smith**. Sea urchin coelomocytes specifically express a homologue of the complement component C3. J Immunol 1998; **160:** 2983-2997.
58. **Nonaka, M., K Azumi**. Opsonic complement system of the solitary ascidian, *Halocynthia roretzi*. Dev Comp Immunol 1999; **23:** 421-427.
59. **Vasta, G.R., M. Quesenberry, H.Ahmed, N. O'Leary**. C-type lectins and galectins mediate innate and adaptive immune functions: their roles in the complement activation pathway. Dev Comp Immunol 1999; **23:** 401-420.
60. **Wilson, R., C.Chen, N.A. Ratcliffe**. Innate immunity in insects: the role of multiple, endogenous serum lectins in the recognition of foreign invaders in the cockroach, *Blaberus dicoidalis*. J Immunol 1999; **162:** 1590-1596.
61. **Bentley, D.R**. Structural superfamilies of the complement system. Exp Clin Immunogenet 1988; **5:** 69-80.
62. **Quigley, J.P., P.B Armstrong**. An endopeptidase inhibitor found in *Limulus* plasma: an ancient form of α_2-macroglobulin. Ann NY Acad Sci 1983; **421:** 119-24.
63. **Pahler, S., B. Blumbach, I. Muller, W.E. Muller,**. Putative multi-adhesive protein from the marine sponge *Geodia cydonium*: cloning of the cDNA encoding a fibronectin-, an SRCR-, and a complement control protein module. J Exp Zool 1998; **282:** 332-43.
64. **Endo, Y., M. Takahashi, M. Nakao, H. Saiga, H. Sekine, M. Matsushita, et al**. Two lineages of mannose-binding lectin-associated serine protease (MASP) in vertebrates. J Immunol 1998; **161**:4924-30.
65. **Matsushita, M., Y. Endo, M. Nonaka, T. Fujita.** Complement-related serine proteases in tunicates and vertebrates. Curr Opin Immunol 1998; **10:** 29-35.

66. **Wilson, J.G., E.E. Murphy, W.W. Wong, L.B. Klickstein, J.H. Weis, D.T. Fearon**. Identification of a restriction fragment length polymorphism by a CR1 and cDNA that correlates with the number of CR1 on erythrocytes. J Exp Med 1986; **164:** 50-9.
67. **Reilly, B.D., Mold, C**. Quantitative analysis of C4Ab and C4Bb binding to the C3b/C4b receptor (CR1, CD35). Clin Exp Immunol 1997; **110:** 310-316.
68. **Madsen, H.O., M.L. Satz, B. Hogh, A. Svejgaard, P. Garred**. Different molecular events result in low protein levels of mannan-binding lectin in populations from southeast Africa and South America. J Immunol 1998; **161:** 3169-75.
69. **Garred, P., M. Harboe, T. Oettinger, C. Koch, A. Svejgaard.** Dual role of mannan-binding protein in infections: another case of heterosis? Eur J Immunogenet 1994; **21:** 125-31.
70. **Garred, P., C. Richter, A.B. Andersen, H.O. Madsen, I. Mtoni, A. Svejgaard, J. Shao.** Mannan-binding lectin in the sub-Saharan HIV and tuberculosis epidemics. Scan J Immunol 1997; **46:** 204-208.
71. **De Miranda Santos, I.K.F., C.H.N. Costa, H. Krieger, M.F. Feitosa, D. Zurakowski, B. Fardin, R.B.B. Gomes, D.L. Weiner, D.A. Harn, R.A.B. Ezekowitz, and J.E. Epstein.** Mannan-binding lectin enhances susceptibility to visceral leishmaniasis. Infect Immun 2001; **69:** 5212-5215.
72. **Madsen, H.O., V. Videm, A. Svejgaard, J.L. Svennevig, P. Garred.** Association of mannose-binding lectin deficiency with severe atherosclerosis. Lancet 1998; **352**: 959-560.
73. **Hegele, R.A., M.R. Ban, C.M. Anderson, J.D. Spence**. Infection- susceptibility alleles of mannose-binding lectin are associated with increased carotid plaque area. J Invest Med 2000; **48**: 198-202.
74. **Muna, W.F**. Cardiovascular disorders in Africa. World Health Stat Q 1993; **46:** 125-133.
75. **Borregaard, N., P. Elsbach, T. Ganz, P. Garred, A. Svejgaard**. Innate immunity:from plants to humans. Immunol Today 2000; **21:** 68-70.
76. **Van Damme, E.J.M., W.J. Peumans, A. Pusztai, S. Batdocz**. Handbook of plant lectins:poperties and biomedical applications. John Wiley & Sons, Chichester, GB, 1997.
77. **Ng, T.B., W.W. Li, Yeung, H.W**. Int J Biochem 1989; **21:** 149-155.

Chapter 2

A case of familial meningococcal disease due to deficiency in mannose-binding lectin (MBL)

S. Kuipers[1], P.C. Aerts[1], O.J.J. Cluysenaer[2], A.K.M. Bartelink[2], R.A.B. Ezekowitz[3], W.A.Bax[2], M.M.M. Salimans[4], and H. van Dijk[1.4]

[1] Eijkman-Winkler Center for Microbiology, Infectious Diseases and Inflammation, University Medical Center, Utrecht, the Netherlands, [2] Department of Internal Medicine, Meander Medical Center, Amersfoort, [3] Department of Pediatrics, Harvard Medical School, Boston, MA, USA, [4] Department of Medical Microbiology and Immunology, Meander Medical Center, Amersfoort,the Netherlands

Adapted from: The Proceedings of the 2nd Eijkman Symposium on Tropical Diseases: From Molecule to Bedside. Marzuki S, J. Verhoef, H. Snippe (Eds); Kluwer/Plenum 2003

ABSTRACT

A case of familial meningococcal meningitis is described, which involved a 18-year old boy, his mother, and his grandfather, all three suffering from meningococcal disease in young adolescence (17-19 y), albeit with one or two generations in between. By studying the genetic variants of MBL, we found out that the three family members described carried the B/D variant *mbl* genes instead of the homozygous *mbl* A/A trait. Serum MBL levels within the family varied from below 0.15 μg per ml (for the B/D variant) up to 7.0 μg per ml, with 1.67 μg per ml as mean serum level of a control population (n= 419). In the normal population, incidental cases with serum levels of up to 13 μg-equivalents per ml were also observed, which occurred predominantly in elderly people and which are most likely to be explained by the acute-phase reactant behavior of MBL protein.

In the autumn of 1997 in the Netherlands, there was a small epidemic of type-C meningococcal disease that, more or less, overlapped the 'bible belt'. The disease was most active in and around a village called Nijkerkerveen, which was also the village of birth of our male index patient. The boy's meningitis was well diagnosed and responded to treatment with penicillin, resulting in uneventful recovery. Laboratory investigations on his serum showed normal immunoglobulin levels, while complement levels (CH50, AP50, and C1q, C3, C4A and C4B serum levels) were in the normal range as well, which suggests an undisturbed humoral defense including the hitherto known complement pathways, the classical and alternative complement pathways [1]. So far, however, the rationale behind the familial behavior of the disease remained obscure.

Two years later, the European Immunology Meeting was held in Amsterdam, where the last author of this paper happened to be one of the chairmen at the session on complement. At that occasion, connections between susceptibility to meningococcal disease and carriership of mannose-binding lectin (MBL) variant alleles were reported. These reports were the reason to reopen the familial meningitis case, now with the sole purpose to identify putative MBL variant alleles and matching decreased serum MBL levels. To this end, we developed a competitive ELISA making use of reagents kindly donated by coauthor Dr. R.A.B. Ezekowitz [1]. With this ELISA, MBL protein levels were found in the index family ranging from < 0.15 up to 7 μg MBL per ml, with a mean value of 1.67 μg/ml.

After this event, we decided to develop a functional microtiter assay for MBL, based on the principle of yeast-induced bystander lysis of chicken erythrocytes [2]. In this assay, live cells of *Saccharomyces cerevisiae* are used as activators of MBL, which give rise to C5b6-formation, that – together with terminal complement components C7, C8, and C9 – is able to lyse innocent bystander cells. For this purpose, we use chicken erythrocytes, but trypsin-treated guinea pig erythrocytes may be used as well. In this assay, serum deficient in MBL is used as reagent serum (R-MBL), for which we regularly employ serum of the mother of the index patient. Characteristics of the assay are a minimum detection level of 0.20 μg-equivalents MBL per ml, a linear relationship with

MBL mass ($r = 0.869$; $p < 0.0001$), and inhibition of hemolysis by C1-inhibitor (C1-INH), but not by α_2-macroglobulin, and by the sugars mannose, N-acetylglucosamine, and fucose, but not by galactose.

With the newly developed functional MBL assay, we were able to test a great many serum samples (over 5000), including those from young children with recurrent pneumococcal otitis media, young children with malignancies, a large family with recurrent furunculosis, and serum samples from individual cases with recurrent infections, frequent miscarriages, and atherosclerosis.

Take-home message

Ever since we met our case of familial meningococcal disease in the Netherlands, nothing remained the same:

We learned that the complement system does not only have two activation pathways, the classical complement activation pathway and the alternative complement activation pathway, but also a third one: the MBL-dependent 'lectin complement activation pathway', besides the terminal pathway, which is a short-cut between the three real activation pathways and one of the effector mechanisms of the complement system: the formation of membrane-attack complexes (MAC). The latter can kill certain gram-negative microorganisms like *Neisseria meningitidis*. Moreover, phylogenetic studies by colleagues in the field have taught us that the lectin pathway is by far one of the most ancient pathways of the complement system, dating from at least 700 million years. The classical and terminal pathways, however, are much younger, originating from 450 million years ago. Scientists in our field are now involved in studies which should tell us whether the lectin or the alternative pathway is the most ancient complement activation pathway. If the latter will be true, the lectin pathway may function as the initiator of the alternative complement pathway.

Since MBL is such an ancient molecule, there are also ill-functioning variant alleles, based on single-base substitutions that predestinate for serious (chronic) disease, including infections, autoimmunity, and atherosclerosis. However, variant alleles may also be protective, which holds especially true for the African (C-type) variant that protects from mycobacterial diseases like tuberculosis and leprosy. Tubercle bacilli are 'encapsulated' by lipoarabinomannan (LAM), which is recognized by intact but not by variant MBLs, the latter of which do not allow activation of the lectin pathway. We presume that the leprosy bacterium carries a similar, capsule-like structure, which also helps to activate the lectin pathway resulting in opsonization by and 'hiding' of the phagocytosed bacteria in mononuclear phagocytes; the phenomenon involved in the chronicity of mycobacterial disease. The protective effect of variant MBLs, encoded for by a defective gene, may then be explained by the fact that they are unable to activate the MBL-associated serine proteinases (MASPs, which are molecules very much related to the C1q-associated C1r and C1s), preventing lectin pathway activation and thereby chronic disease with specific intracellular pathogens.

With help of our functional assay, we studied the prevalence of MBL deficiency in different populations. The incidence of MBL deficiency in the Dutch population was

established to be 13%, mostly due to the B and D variants, and LX promoter polymorphism. The prevalence of MBL deficiency in African countries is, however, about 35%, which underlines again the importance of variant alleles in the resistance to mycobacterial disease.

Since MBL is independent of antibodies, one may speculate that the lectin pathway is the innate-immunity counterpart or even the predator of antibodies, as it is able to recognize sugar moieties on all type of microorganisms. Again with help of our functional assay, we tested the ability of different microorganisms to activate the lectin pathway [3]. The MBL-activating ability was expressed in number of microbes giving rise to a certain degree of activation (Z-value [number of active sites per target cell] = 0.6). The data obtained showed that again meningococci were the most powerful activators of the human lectin pathway, with less than one colony-forming unit giving rise to optimum lectin-pathway activation.

In conclusion, MBL is a very versatile protein that deserves very thorough study in order to elucidate its exact role in the defense from different pathogens and diseases.

REFERENCES

1. **Willem A. Bax, Onno J.J. Cluysenaer, Anton K.M. Bartelink, Piet C. Aerts, R. Alan B. Ezekowitz, Hans van Dijk.** Association of familial deficiency of mannose-binding lectin and meningococcal disease. Lancet 1999; **354:** 1094-1095.
2. **Saskia Kuipers, Piet C. Aerts, Anders G. Sjöholm, Theo Harmsen, Hans van Dijk.** A hemolytic assay for the estimation of mannose-binding lectin levels in human serum. J Immunol Methods 2002; **268:**149-157.
3. **Saskia Kuipers, Piet C. Aerts, Hans van Dijk.** Differential microorganism-induced mannose-binding lectin activation. FEMS Immunology Med Microbiol 2003; **36**: 33-39.

Chapter 3

Prevalence of abnormal serum mannose-binding lectin (MBL) levels in the Dutch population

S. Kuipers[1], M.M.M. Salimans[2], T.Harmsen[1], R.J. Kraaijenhage[3], J.A.J. Faber[4], K. Takahashi[5], H. van Dijk[1, 2]

[1] Eijkman-Winkler Center for Microbiology, Infectious Diseases and Inflammation, University Medical Center, Utrecht, the Netherlands, [2] Department of Medical Microbiology and Immunology, Meander Medical Center, Amersfoort, the Netherlands, [3] Department of Clinical Chemistry, Meander Medical Center, Amersfoort, the Netherlands, [4] Center for Biostatistics, Utrecht, the Netherlands, [5] Department of Pediatrics, Harvard Medical School, Boston, MA,USA

(submitted for publication)

ABSTRACT

Mannose-binding lectin (MBL) is an ancient serum protein involved in the initiation of complement activation via the so-called lectin pathway (LCP). Since MBL activation is antibody-independent, the LCP is considered part of the non-specific (innate) immune system. Probably due to its antiquity, the *mbl* gene and/or its promoter region may contain several point mutations, with diminished function(s) of the protein in consequence of decreased oligomerization. In all age groups, defective genes are associated with an increased susceptibility of the carrier to infection. In MBL-deficient patients with cystic fibrosis, or those receiving chemotherapy or stem cell transplantation, infectious complications are more prevalent. From literature it is known that MBL deficiency is associated with severe atherosclerosis too. Therefore, for defined patient groups, MBL substitution therapy may well become common practice in the very near future. The purpose of the present study was to define normal and decreased MBL serum levels, and to establish the prevalence of MBL deficiency in a randomly chosen cross-section of the multicultural Dutch population (n = 419; age 3-85 y). MBL deficiency was tested by a competitive, enzyme-linked immunosorbent assay making use of a commercially available peroxidase-labeled monoclonal anti-MBL antibody. In our study, individuals could be distinguished with decreased MBL levels (0.20-0.42 mg/L) in 9.1% of the study population, and ultra-low antigenic MBL levels (< 0.20 mg/L) in 3.1%, which occurred most frequently below the age of 60. From the age of 60 onwards, 7.8% of the donors presented with enhanced serum MBL levels. No significant correlation was established between C-reactive protein (CRP) and MBL levels, which makes it highly unlikely that high MBL levels are due to an acute phase response.

INTRODUCTION

Mannose-binding lectin (MBL) is the leading protein in the lectin pathway of complement activation (LCP). The protein belongs to the family of C1q-related collectins, proteins consisting of trimeric subunits with a collagenous tail and globular sugar-binding domains [1]. MBL has affinity for mannose, N-acetylglucosamine, and fucose moieties on Gram-negative and Gram-positive bacteria [2-4], yeasts [3-5], parasites [6,7], and viruses [8,9].

The antibody-independent LCP is considered to be part of the innate immune system, able to respond to foreign invaders within minutes to hours. The main function of the LCP is the coating of microorganisms with C3b and/or C3bi, which makes these microbes vulnerable to phagocytosis and killing by polymorph nuclear cells and phagocytes of the mononuclear lineage [10].

Deficiencies of MBL are associated with all kinds of - mostly infectious - diseases. These include respiratory tract infections in early childhood [11], meningococcal disease [12,13], invasive pneumococcal disease [14], and severe infections complicating cystic fibrosis (CF) [15], chemotherapy [16,17] and stem cell transplantation [18]. Intriguingly,

MBL deficiency is also related to severe atherosclerosis, but the background of this linkage has not been satisfactorily explained yet [19]. Purified-MBL therapy has been given to young MBL-deficient patients [20], and it is highly probable that in the near future CF and cancer patients will be screened for MBL deficiency in order to reduce infectious complications during treatment [21].

The prevalence of MBL deficiency has been estimated in different study populations, both by antigenic and genetic means. Genetic studies show decreased MBL serum levels in the subjects with variant alleles *B*, *C*, and/or *D* as compared to the homozygous wild-type *A* gene, and in promoter polymorphisms *H/L*, *X/Y*, and *P/Q*. So far, the largest population studied for serum MBL levels included over a thousand normal Japanese individuals ranging in age from 3 to 100 years [22]. Similar, but smaller studies have been performed in different populations around the world [23-29]. So far, the prevalence of MBL deficiency in the Netherlands was unknown.

The purpose of the present paper was to study the distribution of MBL serum levels in the Dutch multicultural population. To this end, a highly sensitive, competitive ELISA was used [12]. The genetic background of low MBL serum levels was studied by testing a selection of serum samples showing low and ultralow MBL levels for *mbl* gene mutations and promoter polymorphisms using an ultrasensensitive PCR applicable to serum instead of isolated DNA. By comparing MBL values with CRP levels, the putative impact of the acute phase response on serum MBL levels was studied.

MATERIALS AND METHODS

Sources and preparation of human sera. Human sera were collected from 419 individuals. Sera were from healthy regular donors to a blood bank (age 18-65 years; n = 257), patients to an general pediatric outpatient department (age: 3-18 years, n = 93; first visit), and pre-operative patients to an orthopedic outpatient department (age 65-85 years; n = 69; first visit). The male to female sex ratio was 1.2: 1. The medical history of these individuals, e.g. the occurrence of MBL deficiency-associated diseases, was unknown. Informed consent was obtained from all individuals. All sera were stored at –70°C until further use. Two reference sera with MBL levels of 0.396 µg/ml and 1.25 µg/ml were a kind donation by Dr. P. Garred (Tissue Typing Laboratory, Copenhagen, Denmark).

MBL and anti-MBL antibodies. Purified MBL was a kind gift from Dr. Alan B. Ezekowitz, Department of Pediatrics, Massachusetts General Hospital, Boston, MA, USA. Peroxidase-labeled anti-MBL antibodies were commercially obtained (anti-MBL monoclonal antibody clone 131-1, Statens Serum Institute, Copenhagen, Denmark).

Determination of MBL serum levels. Serum MBL levels were estimated by a protein-specific competitive ELISA, which was executed as follows: high-binding ELISA plates (Costar, Corning, NY, USA) were coated with 1.5 mg MBL per ml. The coated wells were blocked with 2% protein hydrolysate (kindly donated by Dr. Glas, Friesland Dairy Foods, Leeuwarden, the Netherlands). Sera to be tested were serially ($10^{-0.5}$) diluted.

Subsequently, mixtures were prepared consisting of 1/12,500-diluted horseradish-peroxidase-labeled anti-MBL monoclonal antibody and serially diluted serum. Mixtures were allowed to incubate for one hour at room temperature. After each incubation step, the wells were thoroughly washed. Tetramethylbenzidine dissolved in acetic acid (pH = 5.5) was used as the chromogenic substrate. Following substrate conversion, the reaction was stopped by 0.5 M H_2SO_4. Optical densities were read at 450 nm. The lower detection limit of the assay was established as 0.026 µg/ml. Serial dilutions of the reference sera (MBL levels of 0.396 and 1.25 µg/ml) were used as intermediate- and normal-level MBL references for the competitive ELISA in every run.

Genotyping of MBL gene and promoter variants in sera with low MBL levels. The MBL genotypes of 29 out of 51 sera with decreased levels (≤ 0.42 µg/ml) were determined. DNA could be isolated from these serum samples according to the manufacturer's instructions using an ultrasensitive, commercially available DNA isolation kit (QIAamp DNA Blood Mini Kit, Qiagen, Hilden, Germany). Genomic PCRs were performed in 50-µl volumes containing specific primers for the alleles in the exon 1 region (*B*, *C*, and *D* variants, and wild-type *A*) and primers for the promoter region types *H* and *L*, *X* and *Y* of the *mbl* gene, as described previously by Madsen et al. [30]. Briefly, DNA was amplified by an initial general polymerase chain reaction, followed by site-directed mutagenesis (SDM) PCR for the wild-type *A*, and the variant alleles types *B*, *C*, and *D*. Restriction-enzyme digestion of the PCR products was performed in order to distinguish between variant alleles. Promoter polymorphism types *L*, *X*, and *Y*, as well as the *cis/trans* locations of the promoter variants *H/L* and *X/Y* relative to the structural variants were determined as described by Madsen et al. [30]. For the detection of promoter polymorphism type H, primer 5'-TTA-CCC-AGG-CAA-GCC-TGT-G-3' was used in 40 cycles of 30 sec at 94°C, 60 sec at 68 °C, and 60 sec at 72°C.

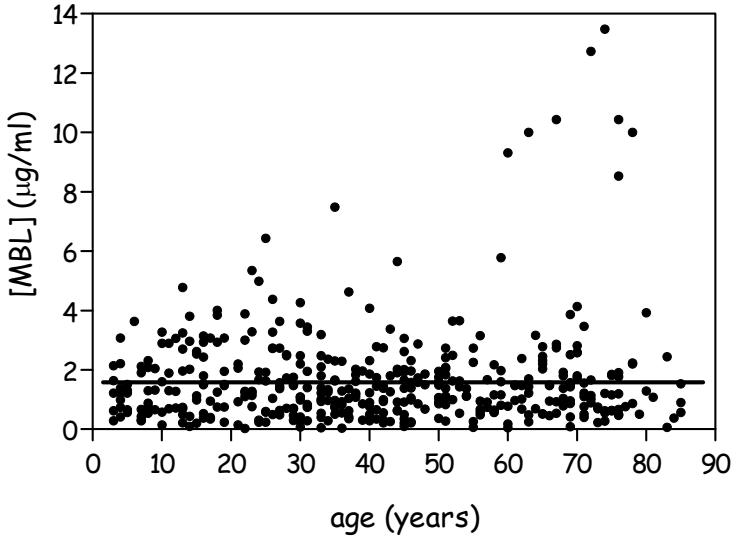
Determination of the correlation between CRP and MBL in a subpopulation. In 40 randomly chosen sera, MBL values (in µg/ml) were determined and compared with CRP levels in the same sample. CRP (in mg/L) was determined by analysis on a BNII nephelometer (Dade Behring, Darmstadt, Germany).

Statistical analysis. Mean MBL levels in the study population were determined by calculating the arithmetic and geometric means of all samples. The median was defined as the MBL levels of the 210th sample in the MBL-level-ranked study population. Age-related reference centiles were constructed according to Altman [31]. Briefly, after modeling the mean as a function of age, the age-specific standard deviation was estimated by regressing the absolute residuals on age, with the assumption that the data were, or could be transformed to be, normal at each age using the properties of the half normal distribution. The Pearson correlation coefficient *r* was used to correlate MBL and CRP serum levels.

RESULTS

The frequency of reduced serum MBL levels in the Dutch population was investigated using a competitive ELISA using recombinant MBL and monoclonal anti-MBL antibody as coating and developing agents, respectively. Sera tested were from a representative cross-section through the Dutch population with donors in the age range from 3 to 85 years (n = 419; average age: 40.8 years).

Figure 1. A scatter plot of MBL levels in serum from Dutch subjects (n = 419) as measured by competitive ELISA. The age (years) is given on the x-axis, while serum MBL levels (in µg/ml) are shown on the y-axis. A line representing the arithmetic mean (MBL level: 1.67 µg/ml) is superimposed in the figure.



Individual serum MBL levels ranged from 0.026 to 13.5 µg/ml. The geometric mean of the whole study population amounted to 1.15 µg/ml, while the median and arithmetic means were 1.27 and 1.67 µg/ml, respectively. Upon exclusion of values below 0.42 µg/ml, representing MBL-deficient sera, the median and arithmetic means were 1.45 and 1.88 µg/ml, respectively. A scatter plot of MBL levels, on which the arithmetic mean was superimposed, is shown in Fig. 1. When age-related reference centiles were constructed using absolute residuals [31] (Fig. 2), no significant correlation was found between log MBL levels and age.

Fig. 3 shows the frequency distribution of logarithmically ranked MBL serum levels. The overall pattern of MBL levels did not meet the criteria of a normal distribution, but rather that of a distribution with maxima at 0.07, 0.32, and 1.78 µg MBL/ml. The first group consisted of undetectable MBL serum levels (< 0.20 µg/ml), the second group of diminished MBL levels (between 0.20 and 0.42 µg/ml), and a third group of normal MBL levels (> 0.42 µg/ml).

Figure 2. *MBL serum levels in 419 subjects in the age of 3 to 85 years: age-related reference centiles using absolute residuals, according to the method of Altman. No significant positive or negative correlation could be established between log MBL levels and age.*

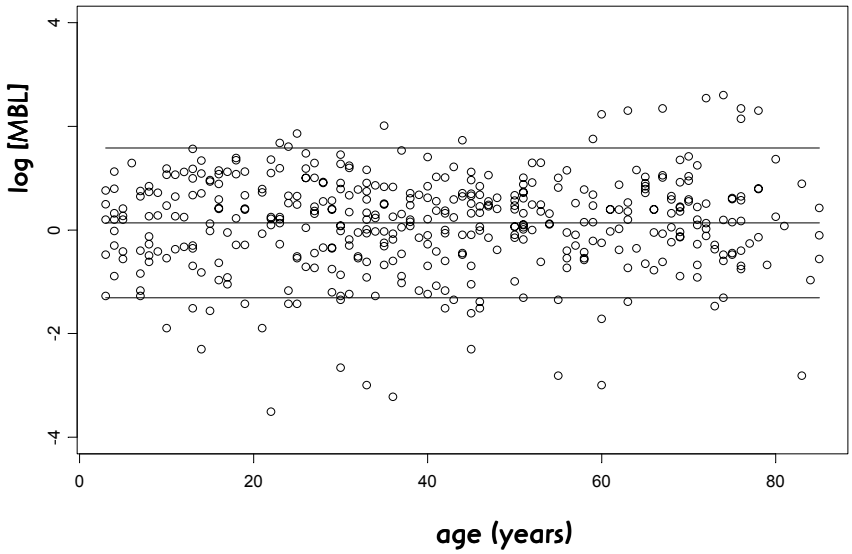
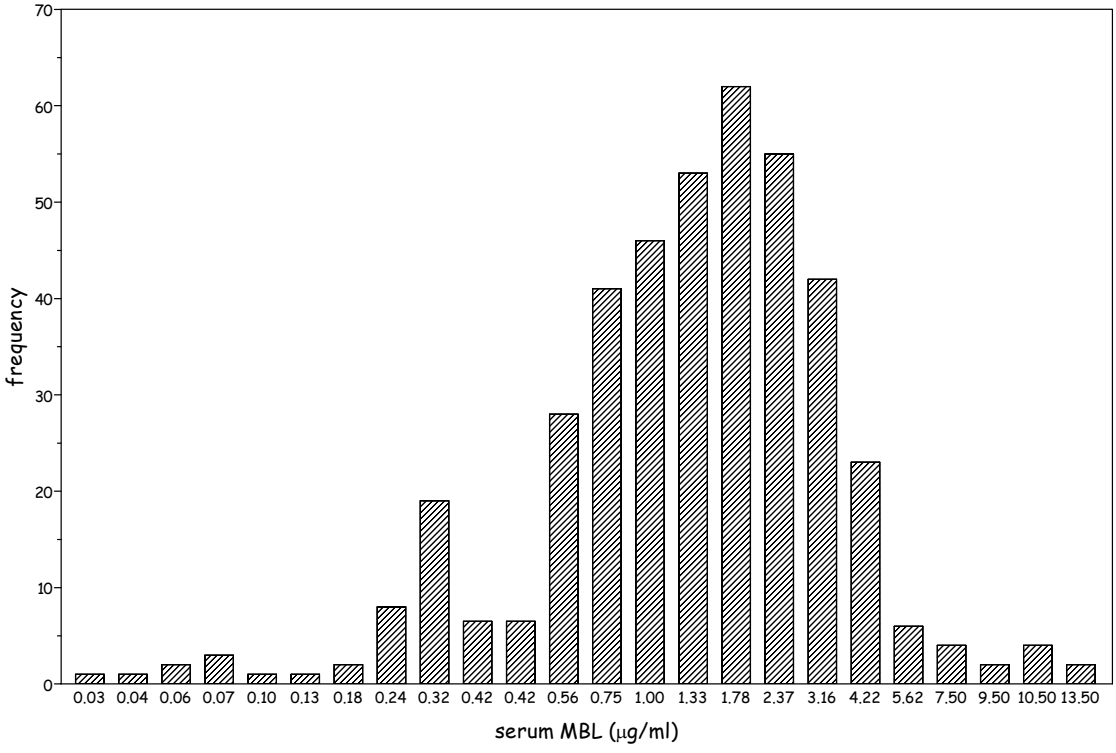


Figure 3. *Frequency distribution of MBL in human sera (n = 419). MBL values are given on a logarithmic scale on the abscissa. On the y-axis, the number of individual serum samples is shown. Serum MBL levels could be divided into 3 distinct subgroups: MBL levels below 0.20 µg/ml, MBL levels between 0.20 and 0.42 µg/ml, and MBL levels higher than 0.42 µg/ml.*



Fifty-one individuals (12.2 %) showed serum MBL levels below 0.42 µg/ml, of which thirteen individuals showed MBL levels below 0.20 µg/ml (3.1% of population). Clearly enhanced MBL levels were found in eight donors in the age range of 60 to 85 years (2% of population; 7.8% of donors > 60 years; MBL > 7.5 µg/ml). In order to exclude that high MBL levels were infection-related, a comparison was made between MBL and CRP levels in 40 randomly selected sera (MBL range between 0.166 and 7.256 µg/ml). A weak negative correlation (r = - 0.11) was found between serum MBL and CRP levels (Fig. 4).

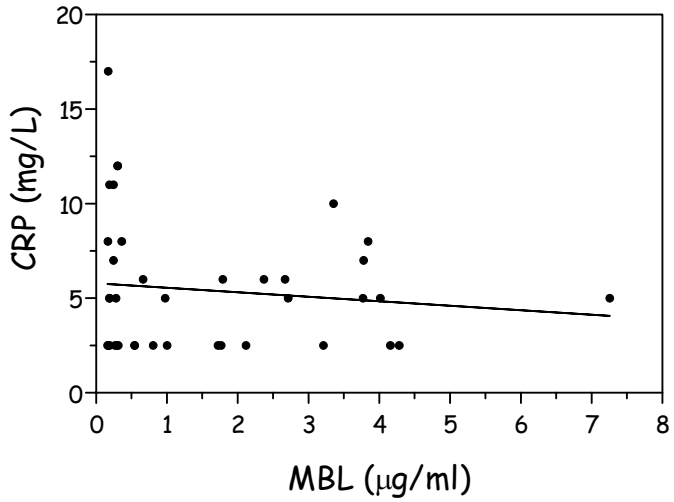


Figure 4. *Scatter plot showing a weak, inverse correlation (r = - 0.11) between MBL and CRP levels in serum. The correlation was not significant. CRP is a well-known acute phase reactant, whereas MBL shows a weak response in the acute phase.*

Sera showing decreased MBL levels (< 0.42 µg/ml) were investigated for codon mutations of the *mbl* gene and for promoter polymorphisms *H/L* and *X/Y* (Table 1). In this subpopulation (n = 29), 11 individuals bore the wild-type *A/A* genotype combined with promoter polymorphisms of mainly the *LX* type (8 out of 11). The heterozygous wild-type genotypes *A/B* and *A/D* were present in ten and five out of 29 subjects, respectively.

The homozygous variant allele *B/B* genotype with *LY* promoter polymorphism was established in 2 out of 29 individuals. No codon 57 mutation (type C) was observed in our MBL-deficient subpopulation. *LXA/HYA* (24%), and *LXA/LYB* (21%) genotypes were found throughout the range between 0.026 and 0.381 µg/ml, while the *LXA/DHY* genotype was found in the MBL range of 0.246 and 0.381 µg/ml. The *X* allele was found only in combination with *L*, the *LY* haplotype only with the *B* variant, while the *D* variant was found only in combination with *HY*, which is in line with linkage disequilibrium data published by Madsen et al. [25].

Table 1. Low-level MBL sera were investigated for the presence of codon variants type *B*, *C*, and *D*, according to increasing MBL levels.

MBL serum level (µg/ml)	MBL genotype
0.026	*LXA / HYA*
0.039	*LXA / LXA*
0.054	*LYB / LYB*
0.055	*LYB / LYB*
0.060	*LXA / HYA*
0.070	*LYA / LYB*
0.104	*LXA / LYA*
0.181	*HYA / HYA*
0.216	*LXA / LYB*
0.217	*LXA / LYB*
0.229	*HYA / LYB*
0.237	*LXA / LYB*
0.245	*HYA / HYD*
0.246	*LYA / HYD*
0.254	*LXA/ HYD*
0.258	*LXA / HYA*
0.272	*LYB / LYB*
0.275	*LYB / HYD*
0.278	*LXA / LYB*
0.290	*LXA / LYB*
0.296	*LXA / HYA*
0.299	*LXA / LYB*
0.311	*LYA / HYA*
0.312	*LXA / HYA*
0.343	*LXA / HYA*
0.349	*LYA / HYD*
0.358	*HYA / HYA*
0.366	*HYA / LYB*
0.381	*LXA / HYD*

DISCUSSION

This study aimed at investigating serum MBL levels in a representative cross-section of the Dutch population in which, so far, a large-scale study had not been conducted. In literature, MBL deficiency has been associated with increased susceptibility to several infectious and non-infectious diseases [11-19]. Measuring MBL levels in defined patient populations is becoming increasingly interesting, since MBL substitution therapy is currently under development (personal communication; Garred, 2002).

In our study, antigenic MBL serum levels could be divided into three subgroups, including a low-level group (< 0.20 µg/ml), an intermediate group with MBL levels ranging from 0.20 to 0.42 µg/ml, and an MBL-sufficient group (MBL > 0.42 µg/ml). In the distribution profile no age-dependency was found, even when values were transformed into residuals according to Altman [31]. Age variation in the serum MBL concentration, as described in Finnish children younger than 12 years [32], was not

confirmed by our study. However, our results indicated that maximum MBL levels per age category tended to increase with age. Very high MBL levels (> 7.5 µg/ml) were limited to people over the age of sixty.

Our findings are at variance with data from the Japanese population, which showed declining values with advancing age, the highest values being found in the age group of 3 to 9 years [22]. Our data are comparable to values in a group of 123 Danish blood donors, in which the median MBL serum level was 1.23 µg/ml, and 13% non-*A/A* genotypes MBL were found [29]. A striking finding was the decreasing number of donors with very low MBL levels (< 0.20 µg/ml) with increasing age. This is in agreement with the observation that MBL-deficient individuals are more prone to infection and to serious inflammatory diseases like atherosclerosis.

Besides as complement component, MBL has been described as a mild acute-phase reactant, doubling or tripling MBL serum levels [33,34]. In a second Finnish study, serum MBL levels in MBL-deficient patients with bacterial infection remained undetectable, while mean MBL levels in patients with documented bacterial infections were significantly higher than in healthy controls (9.88 versus 4.48 µg/ml) [32]. In the latter study, C-reactive protein (CRP) showed a significant though weak negative correlation with MBL. However, in a recent study in HIV-positive patients, no correlation was found between CRP and MBL [35]. By comparing CRP levels to MBL levels in our study population, we established a weak but non-significant correlation between the two variables, thereby excluding acute bacterial infection in this group (Fig. 4).

The genetic basis for MBL deficiency codon mutations is found in exon 1 of the *mbl* gene on chromosome 10. Single-base substitutions, found at codons 52, 54, and 57, are indicated as variant alleles *D*, *B*, and *C*, respectively. MBL of wild-type structural gene homozygotes include MBL dimers, trimers, tetramers and oligomers. MBL oligomers, which are particularly effective in complement activation [36], are absent or diminished in individuals with codon mutations. Point mutations also lead to distortion of the collagen helix of the protein [37].

Changes in the dimorphic loci in the promoter region of the MBL gene may also influence MBL levels, though to a much lesser degree than the codon variants [10,38,39]. Promoter polymorphisms are called *H* (high) versus *L* (low), *X* versus *Y* (*Y* leading to higher MBL levels than *X*), and *P* versus *Q*. Since *P* promoter polymorphism is part of both high-expressing and low-expressing haplotypes, studying *P/Q* polymorphism as such does not provide additional information [25]. Only the *LX* haplotype down-regulates MBL as efficiently as a structural variant. In a very recent paper, other promoter polymorphisms have been described [28], and it may well be that more minor aberrations will be uncovered in the near future.

In our study, human serum was the only source available for MBL genotyping. Normally, upon centrifugation, a minute amount of genetic material is released from nucleated cells, enabling amplification for genotyping. In some serum samples, very low amounts of DNA were detected, necessitating a second PCR with the same primer sets on the PCR product from the first run.

Genotyping was performed in a subpopulation of subjects with low MBL levels (n = 29; Table 1). The heterozygous, wild-type genotypes *A/B* and *A/D* were present in 10 and 5 out of 29 subjects, respectively, while one individual showed a *B/D* genotype. The homozygous variant allele *B/B* genotype could be established in 2 out of 29 individuals with low MBL levels. *LYB* haplotypes were found in 13 out of 29 low-level sera. Type *B* mutant is the most frequently found gene mutation in the Caucasian population (0.11-0.16), while type *D* mutant occurs at less than 0.05 all over the world [10]. No codon 57 mutation (type *C*) was observed in our MBL-deficient subpopulation, indicating that no one of African decent had participated in this randomly chosen study population. In 11 individuals, the wild-type *A/A* genotype was found combined with promoter polymorphisms of mainly the *LX* type (8 out of 11), compared to the *LY* and *HY* type. In Caucasoids, the *LXA* and *LYA* haplotypes are less frequent (24 and 23% respectively) than the high MBL-producing *HYA* haplotypes (33%).

Our results show that over 12% of the present Dutch population has decreased serum MBL levels. This percentage is in the same order of magnitude as described in Denmark and the United Kingdom. In a recent study with a different study population (n = 121), we showed that functional MBL levels in serum correlated significantly to ELISA levels (r = 0.869) [40], suggesting that MBL function is diminished in individuals with variant alleles or promoter polymorphisms.

In conclusion, we were able to establish normal MBL levels and to quantify decreased MBL levels as a measure of deficiency. In our study, over 12% of the Dutch population was typed MBL-deficient. Defining MBL levels in patients is becoming more and more important since decreased MBL serum levels, which cause increased susceptibility to infection and to inflammatory diseases like atherosclerosis, may be treatable in the very near future.

REFERENCES

1. **Holmskov, U., C.J Jensenius**. Structure and function of collectins: humoral C-type lectins with collagenous regions. Behring Mitt 1993; **93:** 224-235.
2. **Emmerik, L.C. van, E.J. Kuijper, C.A.P. Fijen, J. Dankert, S. Thiel**. Binding of mannan-binding protein to various bacterial pathogens of meningitis. Clin Exp Immunol 1994; **97:** 411-416.
3. **Neth, O., D.L. Jack, A.W. Dodds, H. Holzel, N.J. Klein, M.W. Turner**. Mannose-binding lectin binds to a range of clinically relevant microorganisms and promotes complement deposition. Infect Immun 2000; **68:** 688-693.
4. **Kuipers, S., P.C. Aerts, H. van Dijk**. Differential microorganism-induced mannose-binding lectin activation. FEMS Immunol and Medical Microbiol 2003; **36:** 33-39.
5. **Bull, F.G., N.R. Turner**. A serum mannan-binding protein and candidiasis. Sabouraudia 1984; **22:** 347-350.
6. **Klabunde, J., A.C. Uhleman, A.E. Tebo, J. Kimmel, R.T. Schwarz, P.G. Kremsner, F.J. Kun**. Recognition of *Plasmodium falciparum* proteins by mannan-binding lectin, a component of the human innate immune system. Parasitol Res 2002; **88:** 113-117.
7. **Green, P.J., T. Feizi, M.S. Stoll, S. Thiel, A. Prescott, M.J. McConville**. Recognition of the major cell surface glycoconjugate of *Leishmania* parasites by the human mannan-binding protein. Mol Biochem Parasitol 1994; **66:** 319-328.

8. **Hartshorn, K.L., K. Sastry, M.R. White, E.M. Anders, M. Super, R.A. Ezekowitz, A.I. Tauber**. Human mannose-binding protein functions as an opsonin for influenza A viruses. J Clin Invest 1993; **91:** 1414-1420.
9. **Haurum, J.S., S. Thiel, I.M. Jones, P.B. Fischer, S.B. Laursen, J.C. Jensenius**. Complement activation upon binding of mannan-binding protein to HIV envelope glycoproteins. AIDS 1993; **7:** 1307-1313.
10. **Turner, M.W**. Mannose-binding lectin (MBL) in health and disease. Immunobiol 1998; **119:** 327-339.
11. **Koch, C., M. Melbye, P. Sorensen, P. Homoe, H.O. Madsen, K. Molbak, C.H. Hansen,** insufficiency in early childhood. JAMA 2001; **285:** 316-328.
12. **Bax, W.A., O.J.J. Cluysenaer, A.K.M. Bartelink, P.C. Aerts, R.A.B. Ezekowitz, H. van Dijk**. Association of familial deficiency of mannose-binding lectin and meningococcal disease. Lancet 1999; **354:** 1094-1095.
13. **Hibberd, M.L., M. Sumiya, J.A. Summerfield, R. Booy, M. Levin, and the Meningococcal Research Group**. Association of variants of the gene for mannose-binding lectin with susceptibility to meningococcal disease. Lancet 1999; **353:** 1049-1053.
14. **Roy, S., Knox, K., Segal, S., Griffiths, D., Moore, C.E., Welsh, K.I., Smarason, A., Day, N.P., McPheat, W.L., Crook, D.W., Hill, AVS, and the Oxford Pneumococcal Surveillance Group**. MBL genotype and risk of invasive pneumococcal disease: a case-control study. Lancet 2002; **359:** 1569-1573.
15. **Garred, P., T. Pressler, H.O. Madsen, B. Fredriksen, A. Svejgaard, N. Hoiby, M. Schwartz, C. Koch**. Association of mannose-binding lectin heterogeneity with severity of lung disease and survival in cystic fibrosis. J Clin Invest 1999; **104:** 431-437.
16. **Peterslund, N.A., C. Koch, J.C. Jensenius, S. Thiel**. Association between deficiency of mannose-binding lectin and severe infections after chemotherapy. Lancet 2001; **358:** 637-638.
17. **Neth, O., I. Hann, M.W. Turner, N.J. Klein**. Deficiency of mannose-binding lectin and burden of infection in children with malignancy: a prospective study. Lancet 2001; **358:** 614-618.
18. **Mullighan, C.G., S. Heatley, K. Doherty, F. Szabo, A. Grigg, T.P. Hughes, A.P. Schwarer, J. Szer, B.D. Tait, L. Bik To, P.D. Bardy**. Mannose-binding lectin gene polymorphisms are associated with major infection following allogeneic hemopoietic stem cell transplantation. Blood 2002; **99:** 3524-3529.
19. **Madsen, H.O., V. Videm, A. Svejgaard, J.L. Svennevig, P. Garred**. Association of mannose-binding lectin deficiency with severe atherosclerosis. Lancet 1998; **352:** 959-960.
20. **Valdimarsson, H., M. Stefansson, T. Vikingsdottir, G.J. Arason, C. Koch, S. Thiel, J.C. Jensenius**. Reconstitution of opsonizing activity by infusion of mannan-binding lectin (MBL) to MBL-deficient humans. Scand J Immunol 1998 ; **48 :** 116-123.
21. **Ezekowitz, R.A.B**. Mannose-binding lectin in the prediction of susceptibility to infection. Lancet 2001; **358:** 598-599.
22. **Terai, I., K. Kobayashi, T. Fujita, K. Hagiwara**. Human serum mannose binding protein (MBP): development of an enzyme-linked immunosorbent assay (ELISA) and determination of levels in serum from 1085 normal Japanese and in some body fluids. Biochem. Med Metabol Biol 1993; **50:** 111-119.
23. **Turner, M.W., L. Dinan, S. Heatley, D.L. Jack, B. Boettcher, S. Lester, J. McCluskey, D. Roberton**. Restricted polymorphism of the mannose-binding lectin gene of indigenous Australians. Hum Mol Genet 2000; **9:** 1481-1486.
24. **Crosdale, D.J., W.E. Ollier, W. Thomson, P.A. Dyer, J.C. Jensenius, R.W. Johnson, K.V. Poulton**. Mannose binding lectin (MBL) genotype distributions with relation to serum levels in UK Caucasoids. Eur J Immunogenet 2000; **27:** 111-117.
25. **Madsen, H.O., M.L. Satz, B. Hogh, A. Svejgaard, P. Garred**. Different molecular events result in low protein levels of mannan-binding lectin in populations from southeast Africa and South America. J Immunol 1998; **161:** 3169-3175.

26. **Aittoniemi, J., A. Miettinen, P. Laippala, E. Isolauri, J. Viikari, T. Ruuska, E. Soppi**. Age-dependent variation in the serum concentration of mannan-binding protein. Acta Pediatr 1996; **85:** 906-909.
27. **Lipscombe, R.J., Y.L. Lau, R.J. Levinsky, M. Sumiya, J.A. Summerfield, M.W. Turner**. Identical point mutation leading to low levels of mannose binding protein and poor C3b mediated opsonisation in Chinese and Caucasian populations. Immunol Lett 1992; **32:** 253-258.
28. **Jüliger, S., P.G. Kremsner, M.P. Alpers, J.C. Reeder, J.F.J. Kun**. Restricted polymorphisms of the mannose-binding lectin gene in a population of Papua New Guinea. Mutat Research 2002; **505:** 87-91.
29. **Garred, P., S. Thiel, H.O. Madsen, L.P. Ryder, J.C. Jensenius, A. Svejgaard**. Gene frequency and partial protein characterization of an allelic variant of mannan binding protein associated with low serum concentrations. Clin Exp Immunol 1992; **90:** 17-21.
30. **Madsen, H.O., P. Garred, S. Thiel, J.A. Kuttzhals, L.U. Lamm, L.P. Ryder, A. Svejgaard**. Interplay between promotor and structural gene variants control basal serum of mannan-binding protein. J Immunol 1995; **155:** 3013-3020.
31. **Altman, D.G**. Construction of age-related reference centiles using absolute residuals. Stat Med 1993; **12:** 917-924.
32. **Aittoniemi, J., E. Rintala, A. Miettinen, E. Soppi**. Serum mannan-binding lectin (MBL) in patients with infection: clinical and laboratory correlates. APMIS 1997; **105:** 617-622.
33. **Ezekowitz, R.A.B., L.E. Day, G.A. Herman**. A human mannose-binding protein is an acute-phase reactant that shares homology with other vertebrate lectins. J Exp Med 1988; **167:** 1034-1046.
34. **Thiel, S., U. Holmskov, L. Hviid, S.B. Laursen, J.C. Jensenius**. The concentration of the C-type lectin, mannan-binding protein, in human plasma increases during an acute phase response. Clin Exp Immunol 1992; **90:** 31-35.
35. **Heggelund, L., T.E. Mollnes, T. Ueland, B. Christophersen, P. Aukrust, S.S. Froland**. Mannose-binding lectin in HIV infection: relation to disease progression and highly active antiretroviral therapy. J Acquir Immune Defic Syndr 2003; **32:** 354-361.
36. **Yokota, Y., T. Arai, T. Kawasaki**. Oligomeric structures required for complement activation of serum mannan-binding proteins. J Biochem 1995; **117:** 414-419.
38. **Bella, J., M. Eaton, B. Brodsky, H.M. Berman**. Crystal and molecular structure of a collagen-like peptide at 1.9 A resolution. Science 1994; **226:** 75-81.
39. **Summerfield, J.A., S. Ryder, M. Sumiya, M. Thursz, A. Gorchein, M.A. Monteil, M.W. Turner**. Mannose binding protein gene mutations associated with unusual and severe infections in adults. Lancet 1995; **345:** 886-889.
40. **Kuipers, S., P.C. Aerts, A.G. Sjöholm, T. Harmsen, H. van Dijk**. A hemolytic assay for estimation of functional mannose-binding lectin levels in human serum. J Immunol Meth 2002; **268:** 149-157.

Chapter 4

A hemolytic assay for the estimation of functional mannose-binding lectin (MBL) levels in human serum

S. Kuipers[1], P.C. Aerts[1,2], A.G. Sjöholm[3], T. Harmsen[1], and H. van Dijk[1]

[1] Eijkman-Winkler Center for Microbiology, Infectious Diseases and Inflammation, University Medical Center, Utrecht, the Netherlands, [2] Department of Pediatric Infectious Diseases, Wilhelmina Children's Hospital, Utrecht, the Netherlands, [3] Institute of Laboratory Medicine, Section for Microbiology, Immunology, and Glycobiology, Lund University, Lund, Sweden

Journal of Immunological Methods 2002; 268: 149-157

Abbreviations

AP50	: hemolytic alternative pathway activity
CH50	: hemolytic overall complement activity
ChE	: chicken erythrocytes
C1-INH	: C1 esterase inhibitor
EDTA	: ethylene diamine tetraacetic acid
EGTA	: ethylene glycol bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ELISA	: enzyme-linked immunosorbent assay
HPS	: human pooled serum
MAp19	: MBL-associated protein of 19 kDa
MASP	: MBL-associated serine proteinase
MBL	: mannose-binding lectin
MoAb	: monoclonal antibody
PMN	: polymorphonuclear granulocyte
VBS	: veronal-buffered saline

ABSTRACT

A simple assay was developed to estimate functional mannose-binding lectin (MBL) levels in serum, based on the principle of yeast-induced bystander lysis of chicken erythrocytes. The assay is sensitive to inhibition by EGTA (which allows alternative pathway activation), EDTA, mannose, N-acetylglucosamine and C1 esterase inhibitor (C1-INH), whereas it was not inhibited by galactose. A high-titer human anti-mannan antibody-containing serum with 0.06 µg MBL per ml gave a functional signal corresponding to 0.12 µg-equivalents MBL per ml, indicating that anti-mannan antibodies are poorly hemolytic in the assay. The assay is well suited for the large-scale testing of patient samples for a functional MBL activity.

INTRODUCTION

Mannose-binding lectin (MBL) is a high-molecular-weight protein present in blood plasma at low concentrations (1.7 µg/ml). Together with the human proteins CL-L1 [1], CL-P1 [2], and lung surfactant proteins A and D, and the bovine proteins conglutinin, collectin-43 [3] and collectin-46 [4], the protein belongs to the family of C1q-related 'collagenous lectins' (collectins). MBL is the first component of the lectin pathway (LP) of complement activation and binds microorganisms and foreign particles via specific sugar residues (mannose, N-acetylglucosamine, and fucose) by its three identical lectin moieties, thereby acting as an opsonin [5]. When foreign bodies are bound by MBL, three MBL-associated proteins (MASP-1, MASP-2, and MAp19) [6-9] become coordinately activated, resulting in the generation of the active form of MASP-2, the LP-dependent C4/C2 convertase. The role of a recently discovered, fourth MBL-associated protein (MASP-3) probably involves downregulation of MASP-2 as shown by in-vitro experiments [10]. Substrate-bound C4bC2a is a C3 convertase, which allows the conversion of C3 into C3a and C3b, the key reaction leading to terminal-pathway initiation. Besides indirect C3 activation, the lectin pathway may also be involved in direct C3 activation [11]: MASP-1 can cleave C3 independently [12]. An ex-vivo

model has shown that MBL decreases pro-inflammatory cytokine production in meningococcal disease [13].

MBL deficiencies are quite common in man. In fact, low-level haplotypes can occur in up to 30% of the human population [14], and are associated with all kinds of infectious and infection-related diseases. MBL treatment of individuals with recurrent infections secondary to severe MBL deficiency has proven useful in certain cases [15], which strongly suggests that there is a future for MBL substitution therapy. The genetic basis of MBL deficiency is found in variant alleles and promotor polymorphisms. All *mbl* gene codon mutations are found in exon 1 on chromosome 10. Single-base substitutions, found at codons 52, 54, and 57, are indicated as the variant alleles D, B, and C, respectively. These variant alleles lower the serum MBL levels [15]. Changes in the promoter region of the MBL gene, called H (high), L (low), X, and Y, also have a profound impact on MBL serum levels, with LX leading to low MBL levels, LY to intermediate levels and HY to high levels. The P promotor polymorphism is found in both high- and low-expressing haplotypes; therefore an isolated search for the P/Q promotor polymorphism is probably not useful [16,17].

Over the past twelve years, several enzyme immunoassays have been developed to estimate antigenic MBL levels in serum. One common disadvantage of these antigenic assays, however, is that they fail to measure functional MBL, i.e. the joint activity of the MBL-MASPs complex. Recently, functional MBL assays either based on the lysis of mannan-coated erythrocytes [18] or mannan-induced C4b-deposition were developed [19]. In the present paper, we describe a new hemolytic test for the estimation of functional MBL in serum and compare the data obtained with those from a competitive MBL ELISA presented earlier [20]. In short, the yeast *Saccharomyces cerevisiae* was used as an MBL activator in a dilution series of human serum, to which MBL-deficient serum was added as a source of all complement components except MBL. Chicken erythrocytes were used as the target for hemolysis. The influence of classical pathway activation by anti-mannan IgG antibodies on the outcome of the assay was studied using an MBL-deficient serum with high anti-mannan antibody levels. MBL-specific oligosaccharides mannose and N-acetyl-D-glucosamine, but not galactose, substantially reduced hemolysis, which is in line with the idea that the former two sugars compete with yeast cells for MBL binding. The MBL regulatory function of C1 esterase inhibitor (C1-INH), which regulates the lectin pathway by binding to MASP-1 and MASP-2 in an equimolar manner [21] could be confirmed using our assay.

MATERIALS AND METHODS

Chemicals. D(+)-mannose (Mr 180.2) was obtained from Sigma, Zwijndrecht, the Netherlands (catalogue number M-4625), N-acetyl-D-glucosamine (GlcNAc; Mr 221.2) from Serva, Heidelberg, Germany (catalogue number 10290), D(+)-galactose (Mr 180.2) from Sigma (catalogue number G -0750), and C1-inhibitor from Calbiochem-Novabiochem Corp., La Jolla, CA, USA (1 mg/ml; catalogue number 204883).

Buffers. VBS^{++}: Veronal (5 mM)-buffered saline (VBS), pH 7.4, containing 0.15 mM Ca^{2+} and 0.5 mM Mg^{2+}. EGTA-VB: VBS containing 8 mM EGTA and 2.5 mM Mg^{2+}. EDTA-VB: VBS containing 10 mM EDTA.

Human serum. Blood collected from 20 healthy coworkers at our laboratory and from 121 donors to a blood bank was allowed to clot for 30 min, and was subsequently centrifuged at 1,500x g for 10 min. Individual samples were stored at -70°C until further use. The sera from the laboratory workers were subsequently pooled (human pooled serum, HPS), and stored similarly. Serum from a subject known to have a very low MBL level [20] was used as the MBL-reagent serum in the functional assay. Serum with a high IgG anti-mannan antibody titer (IgG 8.7 mg/l, IgM 5.6 mg/l) from an MBL-deficient individual (antigenic MBL level of 0.06 µg/ml) was used to study the influence of anti-mannan antibodies on very low MBL levels. Informed consent was obtained from all donors.

Baker's yeast. *Saccharomyces cerevisiae* cells were cultured on Sabouraud agar plates (Merck, Darmstadt, Germany) for 48 h at 37°C before being used as lectin-pathway activators. Yeast particles were enumerated microscopically by the Thoma slide counting chamber method (W. Schreck, Hofheim, Germany).

Functional assay for mannose-binding lectin. The functional MBL assay, based on the principle of MBL-dependent bystander hemolysis, was executed in U-well microtiter plates (Greiner, Frickenhausen, Germany). First, sera to be tested were serially diluted in the test plate in order to obtain 50-µl samples in a dilution series of $10^{-0.5}$ (1/10 to 1/3,162), starting with a serum dilution of 10% in VBS^{++}. Then, a 10-ml test mixture in VBS^{++} was prepared per microtiter plate. Such a mixture contained a standardized amount of freshly cultured baker's yeast *Saccharomyces cerevisiae* (3.0×10^7 cells), 150 µl reagent serum as a source of all complement components except MBL, and 10^9 chicken erythrocytes (ChE) as the target cells of bystander hemolysis. Direct hemolysis of the erythrocytes induced by yeast cells was excluded by incubating the erythrocytes and yeast cells together just with reagent serum. Human pooled serum (MBL titer: 1.67 µg/ml as determined by ELISA [20] was used as the MBL reference sample in the assay.

A total of 100 µl of test mixture was added to each test well containing serum dilutions, and the microtiter plate was put on a water-based incubator operating at 37°C [22]. After incubation, the erythrocytes were spun down and 50-µl samples of each supernatant were transferred to a flat-bottom plate with 200-µl samples of water in each well. Hemoglobin release was measured in an ELISA reader operating at a wavelength of 405 nm. Percentages of hemolysis were calculated using controls for 100% (water-lysed) and 0% hemolysis (buffer control). The percentages were then transformed to the number of active sites per cell according to the equation published by Borsos and Rapp [23]:

$$Z \text{ (number of active sites per cell)} = -\ln (1 - \text{fraction erythrocytes lysed})$$

Titers were read at $Z = 0.200$. Functional MBL titers were defined as titers in µg equivalents MBL per ml, relative to the known reference. Sera were also tested in EGTA-VB to study alternative pathway activation and in EDTA that inhibits the three complement activation pathways. For this and all other results presented, a minimum of

three replicate assays was performed. The inter- and intra-assay variations (n >10) were determined as well.

Inhibition of MBL activation in the functional assay by competing carbohydrates. This was done by preincubating test serum samples with mannose, N-acetyl-D-glucosamine (GlcNAc), or galactose at 37˚C for 30 min, starting with a concentration of 2.5 mg carbohydrate per well. A checkerboard titration was done in the following manner: HPS was diluted in all the horizontal rows of the microtiter plate, and dilutions (1/3) of the carbohydrates were tested (833, 278, 92.6, 30.9, 10.3, and 3.4 µg/well), in the vertical rows.

The effect of C1-inhibitor in the functional assay. C1 esterase inhibitor (C1-INH) with a concentration of 1 mg/ml was added to the functional assay. The highest concentration tested was 350 µg/ml, which is the physiological concentration of C1-INH. Final concentrations of 175 and 87.5 µg/ml C1-INH were also tested. Per well, 350 µg C1-INH or a dilution thereof was mixed with serial dilutions of human pooled serum. The mixture was allowed to incubate at 37˚C for 30 min before the addition of yeast cells, MBL-deficient serum and chicken erythrocytes. Putative direct hemolysis by C1-INH was ruled out by adding C1-INH to erythrocytes and MBL-deficient serum only.

Anti-MBL monoclonal antibody (MoAb) and purified human MBL. The commercially available anti-MBL IgG1 antibody clone 131-1 (1 mg/ml; lot 01044PA01) was obtained from the Statens Serum Institute in Copenhagen, Denmark. Human recombinant MBL, which was a kind gift from Dr. R.A.B. Ezekowitz (Boston, MA, USA), was more than 95 % pure.

MBL-specific competitive enzyme-linked immunoassay (ELISA). MBL levels in human sera were estimated using a protein-specific competitive ELISA. This was executed as follows: ELISA plates were first coated with 1.5 µg/ml recMBL in phosphate-buffered saline (PBS), and blocked with 4% skim milk in PBS. Then, mixtures were prepared consisting of 1/5,000-diluted anti-MBL monoclonal antibody in PBS and equal volumes of serial $10^{-0.5}$ dilutions of test samples, which were allowed to incubate for 1 h at room temperature. After incubation, the amounts of bound antibody were tested by 1/6,000-diluted peroxidase-labeled anti-mouse IgG antibody in PBS (Nordic, Tilburg, the Netherlands). Tetramethylbenzidine was used as the chromogenic substrate. After each incubation step, the wells were thoroughly washed. Two sera with MBL levels of 0.396 µg/ml and 1.248 µg/ml (a kind donation of Dr. P. Garred, Copenhagen, Denmark) were used as intermediate- and high-level MBL references, respectively.

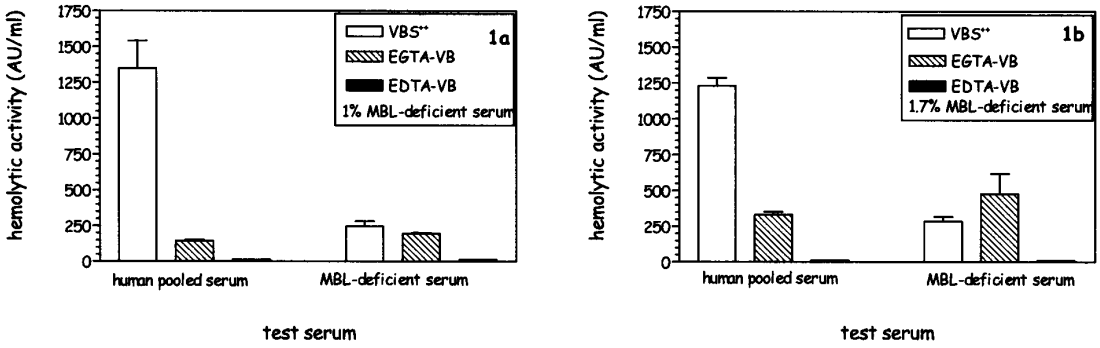
Human anti-mannan antibodies. A serum sample from an MBL-deficient subject (ELISA titer: 0.06 µg/ml), with a high anti-mannan antibody titer (IgG: 8.7 mg/l; IgM 5.6 mg/l; A.G.S.), was tested for functional MBL in order to study the influence of classical pathway activation on the outcome of the assay.

Statistical methods. The Pearson correlation coefficient was calculated and used to analyze data generated by both the functional MBL test and the competitive MBL ELISA that was applied to the sera of the 121 blood donors. Inter- and intra-assay coefficients of variation were estimated from 10 measurements of HPS using the equation: $(s : x) \times 100\%$, where s is the sample standard deviation and x the sample mean.

RESULTS

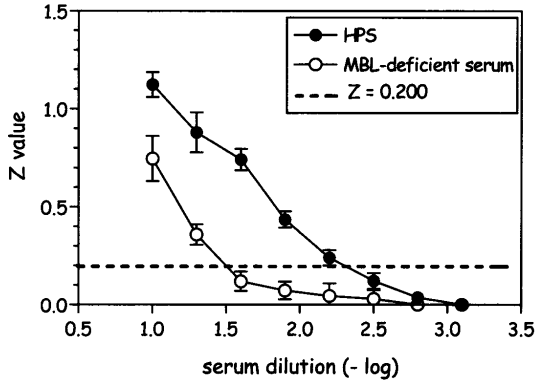
The known MBL-deficient donor serum was tested in the functional assay against the human serum pool, which had an established antigenic MBL level of 1.67 µg/ml. The level of antigenic MBL in our reagent serum was 0.206 µg/ml. Several reagent serum concentrations were tested initially in the functional assay, but a final concentration of 1% reagent serum proved to yield the best results with the least contribution (equal to an MBL concentration of 0.02 µg per ml) of the alternative pathway, measured with EGTA-VB instead of VBS^{++} (Fig. 1). Based on the MBL-deficient serum from the regular donor, a Z value of 0.200 proved to best estimate functional MBL titers (Fig. 2). The intra-assay variation was 5.6% and the inter-assay variation was 5.2%.

Fig. 1. The effects of 1% (1a) and 1.7% MBL-deficient serum (1b), as the reagent in the functional MBL assay, on the apparent functional activities of human pooled serum (HPS), expressed in arbitrary units



(AU) per ml. Note that the alternative pathway activity of 1.7% MBL-deficient serum (measured in EGTA-VB) exceeds the apparent MBL activity (measured in VBS^{++}). Vertical bars indicate standard errors of the mean.

Fig. 2. Functional MBL levels are read at a Z value of 0.2.



Sera from 121 donors to a blood bank were tested with both a competitive ELISA and the functional assay. A highly significant ($P < 0.0001$) positive correlation ($r = 0.869$) was found between the MBL ELISA results and the functional MBL levels, as shown in Figure 3. The intercept of the line is at about 0.2 µg/ml equivalent and not at zero.

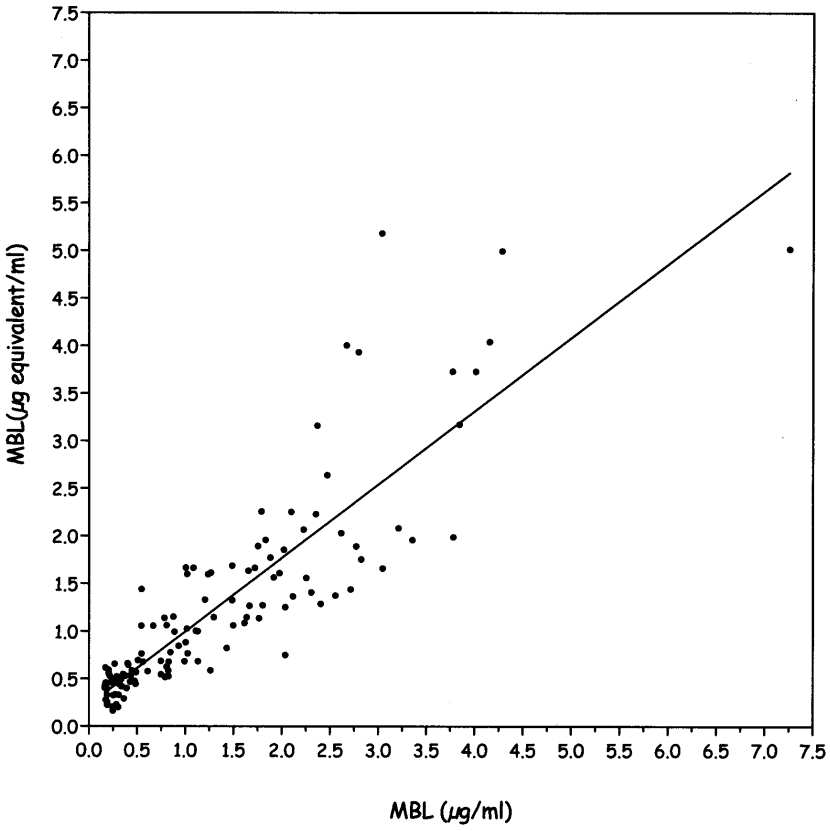


Fig. 3. Comparison of functional MBL levels obtained with the functional assay (shown on the y-axis), with data obtained with a competitive ELISA (shown on the x-axis). Sera tested were from donors to a blood bank (n=121).

The MBL-deficient serum with high anti-mannan antibody titers scored 0.06 µg MBL/ml when tested with ELISA, and 0.12 µg-equivalent MBL/ml when tested with our functional assay. We also studied the inhibition of lectin pathway activation by the carbohydrates GlcNAc, D(+)mannose, and D(+)galactose using our functional assay. GlcNAc (Fig. 4a) and mannose (not shown here) fully blocked MBL-mediated hemolysis, while galactose did not (Fig. 4b).

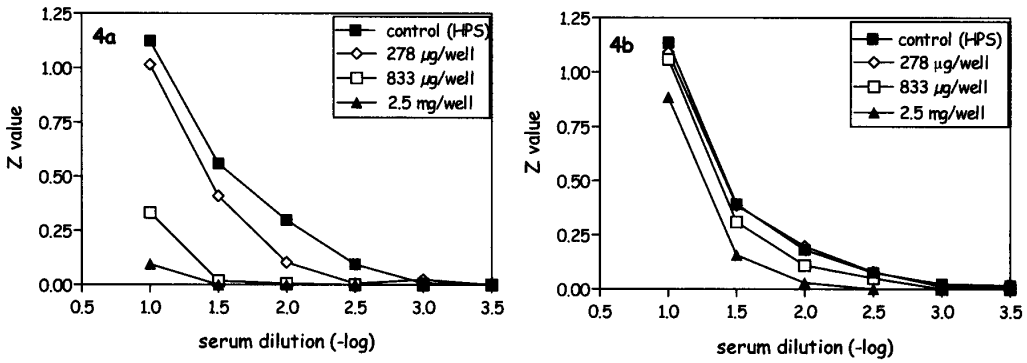


Fig. 4. Saccharide-mediated inhibition of MBL activity by the saccharide N-acetyl-D-glucosamine (GlcNAc) (4a), but not by galactose (4b).

When the MASP-1-and MASP-2-binding C1-inhibitor was added to the MBL functional assay, hemolysis was blocked in a concentration-dependent way (Fig. 5).

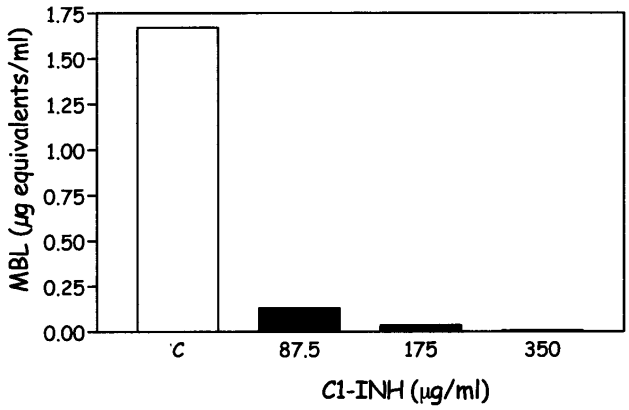


Fig. 5. The influence of C1-esterase inhibitor (C1-INH) on MBL serum levels in the functional assay. C1-INH is a known inhibitor of complement activation. Human pooled serum (MBL: 1.67 µg/ml) without C1- INH is used as a control (C).

DISCUSSION

This paper describes a new hemolytic assay for estimating the functional activity of mannose-binding lectin (MBL), the leading component of the lectin complement pathway. Baker's yeast cells were chosen as MBL-activating particles for this assay, because their cell wall is rich in mannan and because *Saccharomyces cerevisiae* was the first reported microorganism to be associated with MBL [24]. Before MBL was recognized as a complement component, defective yeast opsonization had already been described in 25% of children with frequent unexplained infections [25]. This defective yeast opsonization had also been noted in the sera from children with an increased incidence of infection or atopy [26]. Defective yeast opsonization was functionally measured using opsonized baker's yeast as the substrate for PMN-dependent phagocytosis [25, 27], by studying neutrophil iodination response [28] and by measuring chemoluminescence emitted by PMNs upon incubation with yeast cells opsonized with normal or deficient sera [27]. Tests involving PMN function, however, suffer from the disadvantage that their results may differ from PMN donor to donor due to the many Fc-receptor polymorphisms that occur in the population.

Although another assay has been described for the measurement of MBL-initiated hemolysis based on the lysis of mannan-coated erythrocytes [18], the test we describe here seems to be less complex and less laborious, while the accuracy is acceptable (about 5% inter-and intraday variability). The test we present here appears to be very useful for measuring functional MBL, not only in the normal serum-level range, but also in case of deficiencies. Other, less sensitive assays, e.g. those based on nephelometry, often lack linearity at the low-level range. A strange, but reproducible, observation was that the functional assay does not detect MBL levels below 0.20 µg MBL equivalent per ml. This intrinsic property of the functional assay is not easy to explain, unless we suppose that the chains encoded by variant MBL alleles have some residual functional activity.

As far as the MBL activator in this functional assay is concerned, all yeasts are efficient activators of the alternative complement pathway, but the advantage of *S. cerevisiae* is that it lacks pathogenicity. With regard to a possible contribution to the eventual MBL titer of the alternative pathway constituents in the MBL-reagent serum, we found that this is not more than 5%, at least when using 1% MBL-deficient serum, which we consider to be negligible.

The apparently low MBL activity found in MBL-deficient serum with high anti-mannan immunoglobulin levels indicate that classical pathway activation by anti-mannan antibodies is not likely to contribute to major false-positive MBL results. This is in line with findings by Super et al. [29] who did not observe any correlation between the levels of IgG1, IgG3, or IgM anti-mannan antibodies and level of C4 or C3bi binding. This allowed us to conclude that, in an experimental system using low serum concentrations, MBL cleaves complement component C4 in an antibody-independent manner. In samples with low C4 serum levels, which theoretically is possible in homozygous C4A or C4B deficiency, MBL activation still leads to hemolysis in the assay due to MASP-1 induced C3 cleavage. The latter 'bypass mechanism' will not be found in a C4-binding assay as described previously [30].

The two carbohydrates with equatorial 3- and 4-OH groups (mannose and GlcNAc) inhibited MBL-dependent bystander lysis in a dose-dependent manner. In contrast, galactose, which does not fulfill these spatial demands, showed less inhibition of functional MBL, thereby confirming earlier findings by Holmskov et al. [31]. These researchers demonstrated the saccharide selectivity of collectins using a solid-phase assay, in which human MBL was best inhibited by GlcNAc, less by mannose, and not at all by galactose.

C1 esterase inhibitor (C1-INH), which is a known inhibitor of complement activation via the classical pathway subcomponents C1r and C1s, has been recognized as an inhibitor of the lectin pathway through binding to MASP-1 and MASP-2 [32]. In our functional assay, profound inhibition was seen at levels of 350 and 175 µg per ml, which confirms the finding by Matsushita et al. (2000) that C1-INH is a potent inhibitor of MBL-MASPs in a hemolytic system. Results obtained with the putative lectin pathway inhibitors α_2-macroglobulin [33-35] and aprotinin were inconclusive and therefore not described in this paper.

Recently, using this functional MBL assay, we tested more than 750 serum samples from young children suffering from unexplained, repeated episodes of otitis media due to pneumococcal infection. Our results showed a very high incidence of MBL deficiency in these children as measured by the functional assay. The lower cut-off value used (0.2 µg MBL equivalents per ml) was based on an epidemiological study in the Dutch population (submitted for publication; very low serum MBL: < 0.2 µg MBL equivalents/ml; low serum MBL: 0.2 – 0.42 µg MBL equivalents/ml). The incidence of functional MBL deficiency ranged from 24.6 to 35.3 percent, depending on the age group tested, versus 4.9 percent in the control group (unpublished results). Based on these and the results mentioned above, this novel functional MBL assay could be very useful for large-scale testing in the medical immunology laboratory.

ACKNOWLEDGEMENTS

The authors want to thank Mr. Henny de Kruijff, Van Miert's Poultry Slaughterhouse, Breukelen, the Netherlands, for providing the chicken erythrocytes.

REFERENCES

1. **Ohtani, K., Y. Suzuki, S. Eda, T. Kawai, T. Kase, H. Keshi, Y. Sakai, A. Fukuoh, T. Sakamoto, H. Itabe, T. Suzutani, M. Ogasawara, I. Yoshida, N. Wakamiya**. The membrane-type collectin CL-P1 is a scavenger receptor on vascular endothelial cells. J Biol Chem 2001; **276:** 44222-44228.
2. **Ohtani, K., Y. Suzuki, S. Eda, T. Kawai, T. Kase, T. Shimada, H. Keshi, Y. Sakai, A. Fukuoh, T. Sakamoto, N. Wakamiya**. Molecular cloning of a novel human collectin from liver (CL-L1). J Biol Chem 1999; **274:** 13681-13689.
3. **Holmskov, U**.2000. Collectins and collectin receptors in innate immunity. APMIS 100, 1-59.
4. **Hansen, S., D. Holm, L. Vitved, C. Bendixen, K.B.M. Reid, K. Skjoedt, U. Holmskov**. CL-46, a novel collectin highly expressed in bovine thymus. 2001. Abstract in: Proceedings of the Vth International Workshop On C1, the first component of complement, and Collectins. P. 5.
5. **Turner, M.W**. Mannose-binding lectin: the pluripotent molecule of the innate immune system. Immunol Today 1996; 17: 532-540.
6. **Matsushita, M., T. Fujita**. Activation of the classical complement pathway by mannose-binding protein in association with a novel C1s-like serine protease. J Exp Med 1992; **176:** 1497-1502.
7. **Thiel, S., T.Vorup-Jensen, C.M. Stover, W.J. Schwaeble, S.B. Laursen, K. Poulsen, A.C. Willis, P. Eggleton, S. Hansen, U. Holmskov, et al.** A second serine protease associated with mannan-binding lectin that activates complement. Nature 1997; **386:** 506-510.
8. **Stover, C.M., S. Thiel, M. Thelen, N.J. Lynch, T. Vorup-Jensen, J.C. Jensenius, W.J. Schwaeble**. Two constituents of the initiation complex of the mannan-binding lectin activation pathway of complement are encoded by a single structural gene. J Immunol 1999; **162:** 3481-3490.
9. **Thiel, S., S.V. Petersen, T. Vorup-Jensen, M. Matsushita, T. Fujita, C.M. Stover, W.J. Schwaeble, J.C. Jensenius**. Interaction of C1q and mannan-binding lectin (MBL) with C1r, C1s, MBL-associated serine proteases 1 and 2, and the MBL-associated protein Map19. J Immunol 2000; **165:** 878-887.
10. **Dahl, M.R., S. Thiel, M. Matsushita, T. Fujita, A.C. Willis, T. Christensen, T. Vorup-Jensen, J.C. Jensenius**. MASP-3 and its association with distinct complexes of the mannan-binding lectin complement activation pathway. Immun 2001; **15**: 127- 135.
11. **Kuhlman, M., K. Joiner, R.A.B. Ezekowitz**. The human mannose-binding protein functions as an opsonin. J Exp Med 1989;**169:** 1733-1745.
12. **Matsushita, M., and Fujita, T**. Cleavage of the third component of complement (C3) by mannose-binding protein-associated serine protease (MASP) with subsequent complement activation. Immunobiol 1995; **194:** 443-448.
13. **Jack, D.L., R.C. Read, A.J. Tenner, M. Frosch, M.W.Turner, N.J. Klein**. 2001. Mannose-binding lectin regulates the inflammatory response of human professional phagocytes to *Neisseria meningitidis* serogroup Br J Infect Dis 2001; **184:**1152-1162.
14. **Ezekowitz, R.A.B**. Mannose-binding lectin in prediction of susceptibility to infection. Lancet 2001; **358:** 598- 599.
15. **Valdimarsson, H., M. Stefansson, T. Vikingsdottir, G.J. Arason, C. Koch, S. Thiel, J.C. Jensenius**. Reconstitution of opsonizing activity by infusion of mannan-binding lectin (MBL) to MBL-deficient humans. Scand J Immunol 1998; 48: 116-123.
16. **Madsen, H.O., P. Garred, S. Thiel, J.A.L. Kurtzhals, L.U. Lamm, L.P. Ryder, A. Svejgaard**. Interplay between promotor and structural variants control basal serum level of mannan-binding protein. J Immunol 1995;**155:** 3013-3020.

17. **Madsen, H.O., M.L. Satz, B. Hogh, A. Svejgaard, P. Garred**. *Different molecular events result in low protein levels of mannose-binding lectin in populations from Southeast Africa and South America.* J Immunol 1998; **161:** 3169-3175.
18. **Suankratay, C., X-H. Zhang, Y. Zhang, T.F. Lint, H. Gewurz**. *Requirement for the alternative pathway as well as C4 and C2 in complement-dependent hemolysis via the lectin pathway.* J Immunol 1998; **160:** 3006-3013.
19. **Petersen, S.V., S. Thiel, L. Jensen, R. Steffensen, J.C. Jensenius**. *An assay for the mannan-binding lectin of complement activation.* J Immunol Methods 2001; **257:** 107-116.
20. **Bax, W.A.., O.J.J. Cluysenaer, A.K.M. Bartelink, P.C. Aerts, R.A.B. Ezekowitz, H. van Dijk**. *Association of familial deficiency of mannose-binding lectin and meningococcal disease.* Lancet 1999; **354:** 1094.
21. **Matsushita, M., S. Thiel, J.C. Jensenius, I. Terai, T. Fujita**. *Proteolytic activities of two types of mannose-binding lectin-associated serine protease.* J Immunol 2000; **165:** 2637-2642.
22. **Klerx, J.P.A.M., C.J. Beukelman, H. van Dijk, J.M.N. Willers**. *Microassay for colorimetric estimation of complement activity in guinea pig, human and mouse serum.* J Immunol Methods 1983; **63:** 215-220.
23. **Borsos, T., H.J. Rapp.** *Chromatographic separation of the first component of complement and its assay on molecular basis.* J Immunol 1963; **91:** 851.
24. **Bull, F.G., N.J. Turner**. *A serum mannan-binding protein and candidiasis.* Sabouraudia 1984; **22:** 347-350.
25. **Soothill, J.F., B.A. Harvey**. *Defective opsonization. A common immunity deficiency.* Arch Dis Child 1976; **51:** 91-99.
26. **Richardson, V.F., V.F. Larcher, J.F. Price**. *A common congenital immunodeficiency predisposing to infection and atopy in infancy.* Arch Dis Child 1983; **58:** 799-802.
27. **Turner, M.W**. *Evaluation of C3b/C3bi opsonization and chemoluminescence with selected yeasts and bacteria using sera of different opsonic potential.* Immunol. 1986; **58:** 111-115.
28. **Roberton, D.M., N.K. Dhanjal, R.J. Levinsky, J.F. Mowbray, M.W. Turner**. *Polymorphonuclear neutrophil iodination response as an estimate of defective opsonization.* Clin Exp Immunol 1981; 43: 208-214.
29. **Super, M., R.J. Levinsky, M.W. Turner**. *The level of mannan-binding protein regulates the binding of complement-derived opsonins to mannan and zymozan at low serum concentrations.* Clin Exp Immunol 1990; **79:** 144-150.
30. **Petersen, S.V., S. Thiel, L. Jensen, T. Vorup-Jensen, C. Koch, J.C. Jensenius.** *Control of the classical and the MBL pathway of complement activation.* Mol Immunol. 2000; **37:** 803-811.
31. **Holmskov, U., R. Malhotra, R.B. Sim, J.C. Jensenius**. *Collectins: collagenous C-type lectins of the innate immune defense system.* Immunol Today 1994; **15:** 67-74.
32. **Wong, N.K.H., M. Kojima, J. Dobó, G. Ambrus, R.B. Sim**. *Activities of the MBL-associated serine proteases (MASPs) and their regulation by natural inhibitors.* Mol Immunol 1999; **36:** 853-861.
33. **Storgaard, P., E.H. Nielsen, E. Skriver, O. Andersen, S-E. Svehag**. *Mannan-binding protein forms complexes with α_2-macroglobulin. A proposed model for interaction.* Scand J Immunol 1995;**42:** 373-380.
34. **Terai, I., K. Kobayashi, M. Matsushita, T. Fujita, K. Matsuno**. α_2-macroglobulin binds to and inhibits mannose-binding protein-associated serine protease. Int Immunol 1995; **7:** 1579-1584.
35. **Armstrong, P.B., J.P Quigley**. α_2-macroglobulin: an evolutionary conserved arm of the innate immune system. Dev Comp Immunol 1999; **23:** 375-390.

Chapter 5

Differential microorganism-induced mannose-binding lectin (MBL) activation

Saskia Kuipers[1,2], Piet C. Aerts[2,3], and Hans van Dijk[1]

[1] Eijkman-Winkler Center for Microbiology, Infectious Diseases and Inflammation, University Medical Center Utrecht (UMCU), the Netherlands, [2] Department of Microbiology, Regional Public Health Laboratory Twente Achterhoek, Enschede, the Netherlands, [3] Department of Pediatric Infectious Diseases, Wilhelmina Children's Hospital, UMCU, Utrecht, the Netherlands

FEMS Immunology and Medical Microbiology 2003; 36: 33-39.

ABSTRACT

Mannose-binding lectin (MBL) is a serum complement factor playing a dominant role in first-line defense. When MBL binds to specific sugar moieties on microorganisms, the lectin complement pathway (LCP) is activated. Changes in the *mbl* gene and promotor may result in MBL with less activity, predisposing the individual to recurrent infections. Using a functional MBL assay, we investigated at what concentration different microbes activated MBL. Less than 1 CFU of *Neisseria meningitidis* group B and C still activated MBL, which may be ascribed to filterable blebs. *Nocardia farcinica* and *Legionella pneumophila* activated MBL well, which raises new questions about host susceptibility. In contrast to other research, *Pseudomonas aeruginosa* activated the LCP potently.

INTRODUCTION

Mannose-binding lectin (MBL) is an ancient complement protein discovered only a few decades ago [1,2]. Being the leading protein of the lectin complement pathway (LCP), it belongs to the family of calcium-dependent collagenous lectins (collectins) [3]. This C1q-like molecule has three identical sugar-binding domains with specificity for mannose, N-acetyl-D-glucosamine (GlcNAc), and fucose, but not for antibodies. When one or more of these sugars are present on a microbial surface, binding and subsequent activation of MBL will take place, which indirectly leads to C3b and C3bi deposition on the microbial surface as well.

The LCP comprises MBL and its associated serine proteases, and complement components C4, C2, and C3. The latter three components are shared with the phylogenetically younger classical complement pathway, which becomes activated when microorganisms bind immunoglobulins. MBL activation results in direct microbial opsonization and/or microbial lysis via membrane attack complexes [4], a property that makes the lectin complement pathway part of the innate immune system.

Certain microorganisms appear to be ideal substrates for MBL binding and subsequent lectin pathway activation. E.g, the fungal cell wall is relatively rich in mannan (polymerized mannose) [5], the mycobacterial cell wall rich in lipoarabinomannan (LAM) [6], and GlcNAc constitutes an important building stone of peptidoglycan of bacterial cell walls [7]. Bacterial capsules also contain a variety of polysaccharides including GlcNAc, mannose, fucose, and derivatives thereof, which suggests that certain capsular types may activate the lectin pathway as well. In fact, MBL binding to various bacterial, fungal, viral, and parasitic agents has been demonstrated by means of flow cytometry [8,9], radio-immunoassay [10,11], ELISA [11,12,13], or electron microscopy [14]. MBL binding has been tested functionally using a C4b-deposition assay [15,16]. However, discrepancies in MBL binding and activation by microbes may be found, depending on the method used.

Recently, we devised a novel, hemolytic assay for the estimation of functional MBL, based on the phenomena of *Saccharomyces cerevisiae*-induced MBL activation and subsequent bystander lysis of chicken erythrocytes [17]. MBL was the rate-limiting factor in that assay, making the test highly specific for MBL. In the present study, we used the same assay to compare MBL activation by different microorganisms.

MATERIALS AND METHODS

Human pooled serum. Blood collected from healthy workers of our laboratory was allowed to clot for 60 min before centrifugation (1,500 x g) at room temperature for 10 min. Sera were then pooled (human pooled serum; HPS), and stored in100-µl aliquots at -70°C until further use.

MBL-deficient serum. Serum from an MBL-deficient subject was used as MBL-reagent serum. This serum was also stored in 100 µl aliquots at -70°C.

Microbial strains. The microbial strains tested in the functional MBL assay comprised a selection of defined strains, including those from the American Type Culture Collection (ATCC) and the British National Culture Type Collection (NCTC). The Gram-positive bacterial strains tested included: *Staphylococcus aureus* Cowan I, *Micrococcus lysodeikticus*, *Enterococcus faecalis* ATCC 29212, unencapsulated *Streptococcus pneumoniae* strain Rx1 (kindly provided by Dr. Larry McDaniel, Jackson, MI, USA), and group-A beta-hemolytic *Streptococcus* strain NY-5, which is a strong exotoxin SPE-A1 producer [18].

The Gram-negative bacterial strains included were: *Legionella pneumophila* NCTC 11233, *Helicobacter pylori* (patient isolate), *Escherichia coli* ATCC 25922, *Salmonella typhimurium* strains M206 and the M206 Re mutant (with LPS lacking the O-antigenic side chain, composed only of keto-deoxyoctonic acid and lipid A) [19], and *Yersinia enterocolitica* serotype O:3. Four *Neisseria gonorrhoeae* strains differing in piliation and opacity protein (F62, N300 (piliated, Opa-negative), N303 (piliated, Opa_{50}), and N392 (piliated, Opa_{30})) [20,21] were a kind gift from Dr. M. Dehio, Tübingen, Germany), and *Neisseria meningitidis* group B and C (patient isolates) were also included in our assay for MBL activation.

Non-fermenting Gram-negative strains tested were: *Burkholderia cepacia* ATCC 25417, *Pseudomonas aeruginosa* ATCC 27853, *P. aeruginosa* ATCC 10145, and a mucoid *P. aeruginosa* isolate cultured directly from the sputum of a cystic fibrosis patient.

The actinomycete *Nocardia farcinia* was a clinical isolate. The National Institute for the Environment and Public Health (RIVM, Bilthoven, the Netherlands) provided the *Mycobacterium bovis* BCG strain. *Candida albicans* ATCC 14053 was obtained commercially. Baker's yeast (*Saccharomyces cerevisiae*) was used as the reference organism at a concentration of 6×10^6 yeast cells ml^{-1}.

Microbial cultures. Strains were cultured according to standard procedures [22]. *Legionella pneumophila* was cultured on BCYE agar (Oxoid, Basingstoke, UK), gonococci on GC agar (Becton Dickinson, Cockeysville, MD, USA), both meningococcal strains on Columbia blood agar (Oxoid), *Helicobacter pylori* on Belo Horizonte medium (Oxoid), *Burkholderia cepacia* on *Burkholderia cepacia* agar (Oxoid), the yeasts *Candida albicans* and *Saccharomyces cerevisiae* on Sabouraud agar (Merck, Darmstadt, Germany), and *Mycobacterium bovis* BCG in Dubos liquid medium (Difco, Le Pont du Claix, France) supplemented with glycerin (Merck) and bovine albumin (Sigma, Zwijndrecht, the Netherlands). All other bacterial strains were cultured on Columbia blood agar.

For use in the functional assay, the microorganisms were suspended in veronal-buffered saline with 0.15 mM Ca^{2+} and 0.5 mM Mg^{2+} (VBS^{++}), until an absorbance value of 1.0 at 660 nm was reached. The microbial suspensions were serially diluted (1/2 up to

1/6,4 x 10^7). In order to study activation by subcellular products of meningococci, meningococcal suspensions were filtered through a 0.22 µm syringe filter (Gelman Sciences, Ann Arbor, MI, USA). Bacterial concentrations at OD 1.0 varied from 1.5 x 10^8 colony-forming units (CFU) per ml for *Neisseria species* to 1.1 x 10^9 for *B. cepacia*. Microorganisms were enumerated microscopically by the Thoma slide counting chamber method (W. Schreck, Hofheim, Germany).

Functional microtiter assay for MBL activation by microorganisms. The functional MBL test used to study MBL activation by microorganisms was derived from a recently developed assay based on *Saccharomyces cerevisiae*-induced bystander C5b6-mediated hemolysis of chicken erythrocytes (ChE) [17]. In that assay, serum to be tested for functional MBL was serially diluted ($10^{-0.5}$). A fixed amount of MBL-activating baker's yeast cells (3.0 x 10^5 cells/well), MBL-deficient serum (1.5 µl/well) providing all complement components except MBL, and target cells (1x 10^7 ChE/well) was added. The conditions were chosen in such a way that MBL was the rate-limiting factor in that assay.

In the present assay, MBL-activating *S. cerevisiae* was substituted by other microorganisms. The different microbial concentrations were added in a 50-µl volume per well. A checkerboard titration was done as follows: HPS (as the source of MBL) was serially ($10^{-0.5}$) diluted, starting with a dilution of 10% HPS in VBS^{++} per well (final concentration 3.3% HPS) in the horizontal lines of the microtiter plate, while the serially diluted microorganisms were added to the vertical rows. Per microtiter plate, a 5-ml mixture was made containing 150 µl MBL-deficient serum and 10^9 ChE in VBS^{++}. A 50-µl sample was taken from this mixture and added to each test well. Then, the plate was incubated at 37°C for 1 h, and spun for 10 min in a microtiter centrifuge. Thereafter, 50-µl samples of each supernatant were transferred to a flat-bottom plate containing 200 µl water per well. Hemoglobin release was measured in an ELISA reader operating at a wavelength of 405 nm.

Percentages of hemolysis were calculated using controls for 100% (water-lysed) and 0% (buffer incubated) hemolysis. The percentages were then transformed according to the equation published by Borsos and Rapp [23]:

$$Z = -\ln\ (1 - \text{fraction of erythrocytes lysed})$$

in which Z is the mean number of active sites per chicken erythrocyte. Human pooled serum (HPS) in EGTA-VB (8 mM ethylene glycol bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid with 2.5 mM Mg^{2+}) was used to test alternative pathway activation by the various microbes. Direct hemolysis of the erythrocytes by microbial products was excluded by incubating microorganisms and erythrocytes together with MBL-deficient serum only. Microbial MBL activation was related to the MBL activity of 3 x 10^5 *S. cerevisiae* cells/well in a 50-µl volume, added to a HPS dilution series in the same microtiter plate as the microbe tested. The microbial concentration giving rise to a Z value of 0.6 (calculated by: $Z_{\text{microorganism}} / Z_{\textit{S. cerevisiae}} = 0.6$) was used as a means to rank the microorganisms based on their MBL-activating capacities. All experiments were repeated at least twice.

Specificity of microbial MBL activation. To test the specificity of microbe-induced MBL activation, experiments were also executed in the presence of serial (1/3) dilutions

of either D(+)-mannose (Sigma) or N-acetyl-D-glucosamine (Serva, Heidelberg, Germany). The highest saccharide concentration tested was 2.5 mg per well. MBL-containing human pooled serum (20%; 25 µl) samples were preincubated with the saccharide (25 µl) at 37°C for 30 min. After incubation of the saccharides with HPS, microbial cells were added. Sugar-inhibitable hemolysis was considered MBL-mediated.

RESULTS

A functional MBL assay was used to compare MBL activation by different microorganisms. In the test, microbial numbers as well as the MBL concentration was varied. Curves obtained were mostly straight parallel lines, showing less hemolysis with decreasing numbers of bacteria. Incidentally, optimum curves were obtained. By determining the intersection of the straight lines with the horizontal line Z = 0.6, the number of microorganisms activating MBL in the assay could be compared (Table 1).

Table 1. Ranking of microorganisms according to their MBL-activating capacities. Z value stands for the amount of hemolysis in the functional assay, showing the active sites per erythrocyte. CFU: colony-forming units.

Microbial strain	Concentration at Z = 0.6 (CFU)
Neisseria meningitidis group B	1
Neisseria meningitidis group C	1
Nocardia farcinica	5
Neisseria gonorrhoeae F62	50
Neisseria gonorrhoeae N300	50
Neisseria gonorrhoeae N303	50
Neisseria gonorrhoeae N392	50
Salmonella typhimurium M206	75
Salmonella typhimurium M206 Re mutant	300
Mycobacterium bovis BCG	400
Saccharomyces cerevisiae	1350
Pseudomonas aeruginosa ATCC 27853	5000
Pseudomonas aeruginosa ATCC 10145	5000
Pseudomonas aeruginosa mucoid (CF)	5000
Candida albicans ATCC 14053	3×10^5
Legionella pneumophila NCTC 11233	5×10^6
Yersinia enterocolitica	3×10^7
Micrococcus lysodeikticus	3×10^7
Staphylococcus aureus Cowan I	3×10^8
Helicobacter pylori	3×10^8
Escherichia coli ATCC 29522	3×10^8
Enterococcus faecalis	no activation at all

In order of decreasing MBL-activating abilities, the following microbes were ranked: *Neisseria meningitidis* groups B and C, *Nocardia farcinica*, the four gonococcal strains, (showing equal MBL-activating capacities irrespective of pili or opacity protein), *Salmonella typhimurium*, the Re mutant of *S. typhimurium* (lacking the O-antigenic side chain, theoretically having less MBL-activating sites), *Mycobacterium bo*vis BCG, mucoid and non-mucoid *Pseudomonas aeruginosa* (showing no difference on outcome between mucoid and nonmucoid strains), *Candida albicans*, *Legionella pneumophila*, *Yersinia enterocolitica*, *Micrococcus lysodeikticus*, *Staphylococcus aureus* Cowan I, *Escherichia coli*, and *Helicobacter pylori*. The *Enterococcus faecalis* strain did not activate MBL at all.

The competitive, inhibitory effect of MBL-specific saccharides on lectin pathway activation is shown for the reference organism *S. cerevisiae* and for the unencapsulated Gram-positive bacterium *Micrococcus lysodeikticus. S. cerevisiae*-induced MBL activation could be counteracted by adding to the sera to be tested 2.5 mg/well or 1/3 dilutions of this suspension of D(+)-mannose, the carbohydrate substrate with the greatest affinity for MBL (Fig. 1a). N-acetyl-D-glucosamine competed with *M. lysodeikticus* for MBL binding in the same manner, as is shown in Fig. 1b. MBL-activation by all other microbes tested could be inhibited by the saccharides too.

The finding that *Neisseria meningitidis* groups B and C still caused MBL-mediated hemolysis despite low bacterial concentrations demanded additional experimental information. After passing the meningococcal suspensions through a microbial filter, no differences were seen between the amount of MBL-induced hemolysis caused by the unfiltered suspension and the filtered one (not shown).

Some microbial strains produced hemolysins that lysed the chicken erythrocytes. Therefore, these strains could not be evaluated in the functional MBL assay. This was true for group A β-hemolytic streptococcus strain NY-5, *Burkholderia cepacia* ATCC 25417, and the heat-killed, unencapsulated *Streptococcus pneumoniae* strains Rx1.

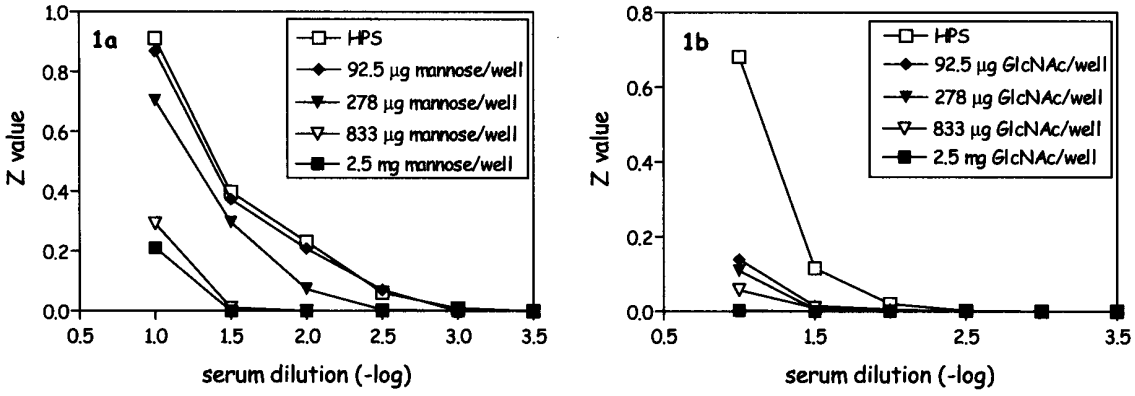


Fig. 1. Competition of carbohydrates and bacteria for MBL binding. (1a) Mannose inhibition of *Saccharomyces cerevisiae*-induced MBL activation. (1b) N-acetyl-D-glucosamine (GlcNAc) inhibition of *Micrococcus lysodeikticus*-induced MBL activation.

DISCUSSION

Many experiments regarding complement activation by different microorganisms were carried out either before the definite recognition of the alternative pathway (around 1980) [24], or when the lectin pathway was yet to be discovered (before 1989). This necessitates reevaluation of the capacities of different microorganisms to activate the three complement pathways. The ancient lectin pathway, activation of which was the subject of this study, leads to antibody-independent microbial coating with C3b, and ingestion in cells of the monocyte phagocyte lineage or, in case of e.g. *Neisseriae*, in terminal complement pathway (TCC)-mediated microbial lysis. As multiple Fc-receptor polymorphisms of polymorph nuclear (PMN) leukocytes severely complicate opsonic studies, the focus of this paper is restricted to functional activation of MBL by microorganisms.

For the purpose stated above, we modified a recently developed, functional MBL assay, based on the principle of yeast-induced, C5b-6-mediated bystander hemolysis of chicken erythrocytes [17]. The hemolytic assay, specific for MBL by using MBL-deficient reagent serum, combines *Saccharomyces cerevisiae*-induced MBL pathway activation with bystander hemolysis of chicken erythrocytes. The assay is easier to perform than complement fixation assays, and very reproducible. As shown previously, the influence of classical complement pathway activation is minimized in hemolytic assays when using diluted serum of less than 5 percent [25] . Competition of MBL with carbohydrates is a measure of the remaining complement activity induced by the HPS used (Fig. 1). The influence of antibodies on classical pathway activation in this MBL assay was studied with *S. cerevisiae*, using serum from an MBL-deficient subject; the effect of this IgG- and IgM-containing serum was negligible.

By substitution of baker's yeast, the MBL-activating capacity of microbes other could be evaluated. The most striking finding was that, depending on the microbe tested, either straight lines or optimum curves were obtained (X-axis: number of microorganisms; Y-axis: Z value, or number of active sites per cell). The 'optimum curve' phenomenon can be explained by microbe-induced depletion of early complement components C2 and C4 upon testing higher microbial concentrations, resulting in less hemolysis than expected.

Several of our results differ dramatically from those found by others for several microorganisms tested. For example, very weak or absent MBL binding by *Pseudomonas aeruginosa* was found by other researchers [15,26], and functional MBL activation by *P. aeruginosa* strains (n = 7) could not be detected in a functional C4-binding assay [15]. *P. aeruginosa* belongs to the non-fermenting group of Gram-negative bacteria involved in severe pulmonary infections in cystic fibrosis (CF) patients, resulting in a shortened life-span in those patients carrying variant MBL alleles [27]. MBL activation as shown in our assay provides a better explanation for the severe infections in MBL-deficient CF patients.

Extremely low concentrations (≤ 1 CFU) of group B and C meningococci still activated MBL to a Z value of 0.6 in the functional assay. A new finding is that, even after filtration of the bacterial suspensions, unambiguous MBL activation was found, pointing towards a soluble MBL-activating factor. A candidate factor may be the 'blebs', vesicles formed by parts of the meningococcal outer membrane. Blebs, consisting partly of LPS, are secreted

in serum and cerebrospinal fluid within seconds *in vitro* as well as *in vivo* [28]. Although previous experiments showed that MBL can bind to sialylated meningococcal LPS [29,30], and to opacity and porin B proteins (M.M. Estabrook, D.J. Jack, N.J. Klein, and G.A. Garvis, Abstr. 5th Internat. Workshop on C1, and collectins, abstr.II-3, 2001), our findings show the importance of a soluble, filterable factor in meningococcal pathology, which raises new questions, and may explain the increased susceptibility of MBL-deficient persons to meningococcal disease [31].

Gonococcal pili and opacity protein variations do not contribute to differences in the lectin pathway-activating capacity. Also, the ability of gonococci to activate MBL functionally is less pronounced when compared to meningococci.

S. cerevisiae, the first reported microorganism associated with MBL [1], is a yeast of low pathogenicity. Its potent MBL-activating capacity can be related to the high polysaccharide content of the cell wall, which consists mainly polymers of N-acetylglucosamine (chitin), glucose (β-glucan), and mannose (mannan) [5]. The cell wall of *C. albicans* resembles that of *S. cerevisiae*, but the two yeast species differ in pathogenicity [5]. Surprisingly, more *Candida albicans* cells were needed to achieve the same amount of hemolysis when compared to *S. cerevisiae*. Whereas clinical disease with *S. cerevisiae* is rare, *Candida species* cause mucosal and systemic disease in both normal and immunocompromized patients. Immunity to *Candida* infections is complement-related [32], but also dependent on the quantity and quality of peripheral phagocytes and on T-cell function.

Since MBL binding leads to opsonization and internalization in monocytes and monocyte-derived macrophages, it can be theorized that normal serum MBL levels predispose to more severe disease with intracellular pathogens, which survive and replicate in monocytes and macrophages. This theory has been confirmed in patient studies for tuberculosis in HIV-negative individuals [33,34,35] and for Ethiopeans suffering from leprosy, which is caused by *Mycobacterium leprae* [36]. Also, a direct correlation has been found between high MBL levels and the development of invasive (visceral) leishmaniasis, which is caused by the intracellular protozoa *Leishmania chagasi* [37]. As expected, very low concentrations of the intracellular microorganism *Mycobacterium bovis* BCG activated MBL in our assay. This can be explained by vivid MBL binding to the mycobacterial cell wall components peptidoglycan and lipoarabinomannan (LAM). The role of MBL in e.g. *M. avium-intracellulare* infections in HIV-patients, demands further research.

The intracellular pathogen *Legionella pneumophila* activates the lectin pathway, which may represent a quick means of transport of the bacterium into pulmonary macrophages after inhalation by the host. In the macrophages, bacterial replication takes place within 2-4 hours [38], which may result in severe pneumonia. MBL deficiency may protect against severe legionellosis, which is currently under investigation.

Our study confirms the strong MBL-activating ability of the intracellular *Salmonella species*. The decreased MBL-activating capacity in the functional assay of the Re mutant can be explained by the shorter LPS side chain, composed only of lipid A and keto-desoxy-octonic acid. Other authors confirmed that MBL binds avidly to *Salmonella typhimurium* core LPS structures, and that this type of binding is abrogated by the addition of O-antigen [39].

Until now, no study has looked at the role of innate complement proteins and the pathogen *Nocardia farcinica*, which causes pulmonary infections beside brain abscesses in patients with dysfunctional cellular immunity. One would expect that nocardial infections would be more common, knowing that many people carry variant MBL alleles. However, this microorganism grows slowly and is usually killed by macrophages and T-lymphocytes [40].

The pathogen *Yersinia enterocolitica* activates MBL in our assay, a finding demanding further research. After ingestion of meat and refrigerated food, this pathogen causes gastroenteritis and, rarely, reactive arthritis. The role of MBL in *Helicobacter pylori*-associated gastritis, gastric ulceration and lymphoma (MALT-type B-cell) also remains to be elucidated. Recent research showed that *H. pylori* activated the classical pathway in the absence of specific antibodies, at least via LPS [41], but specific lectin pathway was not studied. However, as *H. pylori* activated MBL in the functional assay, it is more likely that the lectin pathway is the major complement pathway involved in opsonization of the bacterium.

Testing the MBL-activating capacities of microorganisms in fixed circumstances may not be representative for what happens in the host, and it is more likely that our functional assay approaches the more complex *in vivo* situation. Therefore, ranking of microorganisms in terms of MBL activation can give a different impression from the ranking of microbes in terms of MBL-binding.

Several bacteria show α- or β-hemolytic activity when cultured on sheep blood agar, and this quality makes these strains unsuitable for testing in the functional MBL assay using chicken erythrocytes. For instance, *Streptococcus pyogenes* contains powerful hemolysins. The same holds true for *Burkholderia cepacia*, and the unencapsulated *Streptococcus pneumoniae* strain.

In conclusion, our functional MBL assay can be used to evaluate MBL binding and activation by most microbes, providing new insight in the double role of MBL in genetic susceptibility to microbial diseases. The present study shows that extremely low concentrations of meningococci lead to profound MBL activation, as do filterable meningococcal products. Contradictory to other *in vitro* studies, *Pseudomonas aeruginosa* does activate MBL, providing experimental evidence for the clinical outcomes in MBL-deficient CF patients. New insight was gained in the activation of MBL by *Legionella pneumophila*, *Nocardia farcinica* and *Helicobacter pylori*, raising exiting new questions as to the role of this ancient protein in (intracellular) infection.

REFERENCES

1. **Bull, F.G., M.W. Turner**. A serum mannan-binding protein and candidiasis. Sabouraudia 1984; **22:** 347-350.
2. **Kawasaki, N, T. Kawasaki, I. Yamashina**. Isolation and characterization of a mannan-binding protein from human serum. J Biochem 1983; **94:** 937-947.
3. **Holmskov, U**. Collectins and collectin receptors in innate immunity. APMIS 2000; **100:** 1-59.
4. **Turner, M.W**. The lectin route of complement activation. Res Immunol 1996; **147:** 110-115.
5. **LaJean Chaffin, W., J.L. Lõpez-Ribot, M. Casanova, D. Gozalbo, J.P. Martïnez**. Cell wall and secreted proteins of *Candida albicans*: identification, function, and expression. Microbiol Molec Biol Rev 1998; **62:** 130-180.

6. **Chatterjee, D., K. Lowell, B. Rivoire, M. McNeil, P.J. Brennan**. Lipoarabinomannan of *Mycobacterium tuberculosis*. J Biol Chem 1992; **267:** 6234-6239.
7. **Heijenoort, J. van**. Formation of the glycan chains in the synthesis of bacterial peptidoglycan. Glycobiol 2001; **11:** 25R-36R.
8. **Neth, O., D.L. Jack, A.W. Dodds, H. Holzel, N.J. Klein, and M.W. Turner**. Mannose-binding lectin binds to a range of clinically relevant microorganisms and promotes complement deposition. Infect Immun 2000; **68:** 688-693.
9. **Townsend, R., R.C. Read, M.W. Turner, N.J. Klein, D.L. Jack**. Differential recognition of obligate anaerobic bacteria by human mannose-binding lectin. Clin Exp Immunol 2001; **124**: 223-228.
10. **Emmerik, L.C. van, E.J. Kuijper, C.A.P. Fijen, J. Dankert, S. Thiel**. Binding of mannan-binding protein to various bacterial pathogens of meningitis. Clin Exp Immunol 1994; **97:** 411-416.
11. **Polotsky, V.Y., J.T. Belisle, K. Mikusova, R.A.B. Ezekowitz, K.A. Joiner**. Interaction of human mannose-binding protein with *Mycobacterium avium*. J Infect Dis 1997 ; **175 :** 1159-1168.
12. **Peters, C., M. Kawakami, M. Kaul, T. Ilg, P. Overath, T. Aebischer**. Secreted proteophosphoglycan of *Leishmania mexicana* amastigotes activates complement by triggering the mannan binding lectin pathway. Eur J Immunol 1997; **27 :** 2666-2672.
13. **Saifuddin, M., M.L. Hart, H. Gewurz, Y. Zhang, G.T. Spear**. Interaction of mannose-binding lectin with primary isoaltes of human immunodeficiency virus type 1. J Gen Virol 2000; **81:** 949-955.
14. **Klabunde, J., J. Berger, J.C. Jensenius, M-Q Klinkert, U.E. Zelck, P.G. Kremsner, J.F.J. Kun**. *Schistosoma mansoni*: adhesion of mannan-binding lectin to surface glycoproteins of cercariae and adult worms. Exp Parasitol 2000; **95:** 231-239.
15. **Davies, J., O. Neth, E. Alton, N. Klein, M.W. Turner**. Differential binding of mannose-binding lectin to respiratory pathogens in cystic fibrosis. Lancet 2000; 355: 1885-1886.
16. **Haurum, J.S., S. Thiel, I.M. Jones, P.B. Fischer, S.B. Laursen, J.C. Jensenius**. Complement activation upon binding of mannan-binding protein to HIV envelope glycoproteins. AIDS 1993; **7:** 1307-1313.
17. **Kuipers, S., P.C. Aerts, A.G. Sjöholm, T. Harmsen, H. van Dijk**. A hemolytic assay for estimation of functional mannose-binding lectin levels in human serum. J Immunol Meth 2002; **268:** 149-157.
18. **Mascini, E.M., M.A.J. Hazenberg, E.A.E. Verhage, S.E. Holm, J. Verhoef, and H. van Dijk**. A new procedure for the purification of streptococcal pyrogenic exotoxin A from *Streptococcus pyogenes* supernatant. Clin Diagn Lab Immunol 1996 ; **3:** 779-781.
19. **Bloembergen, P**. PhD thesis. 1987. University of Utrecht, the Netherlands. Experimental auto-immunity in mice.
20. **Kupsch, E-M., B. Knepper, T. Kuroki, I. Heuer, T.F. Meyer**. Variable opacity (Opa) outer membrane proteins account for the cell tropisms displayed by *Neisseria gonorrhoeae* for human leukocytes and epithelial cells. EMBO J 1993; **12:** 641-650.
21. **Scheuerpflug, I., T. Rudel, R. Ryll, J. Pandit, T.M. Meyer**. Roles of PilC and PilE proteins in pilus-mediated adherence of *Neisseria gonorrhoeae* and *Neisseria meningitidis* to human erythrocytes and endothelial and epithelial cells. Infect Immun 1999; 67: 834-843.
22. **Murray, P.R., E.J. Baron, M.A. Pfaller, F.C. Tenover, R.H. Yolken**. 7th ed. Manual of Clinical Microbiology. 1999. ASM Press, Washington D.C., USA.
23. **Borsos, T., H.J. Rapp**. Chromatographic separation of the first component of complement and its assay on molecular basis. J Immunol 1963; **91:** 851-858.
24. **Lepow, I.H**. Louis Pillimer, properdin and scientific controversy. J Immunol 1980; **125:** 471-478.
25. **Super, M., R.J. Levinsky, M.W. Turner**. The level of mannan-binding protein regulates the binding of complement-derived opsonins to mannan and zymozan at low serum concentrations. Clin Exp Immunol 1990; **79:** 144-150.
26. **Jack, D.L., N.J. Klein, M.W. Turner**. Mannose-binding lectin:targeting the microbial world for complement attack and opsonophagocytosis. Immunol Rev 2001; **180:** 86-99.
27. **Garred, P., T. Pressler, H.O. Madsen, B. Frederiksen, A. Svejgaard, N. Høiby, M. Schwartz, C. Koch**. Association of mannose-binding lectin gene heterogeneity with severity of lung disease and survival in cystic fibrosis. J Clin Invest 1999; **104:** 431-437.

28. **Devoe, I.W., J.E. Gilchrist**. Release of endotoxin in the form of cell wall blebs during in vitro growth of *Neisseria meningitidis*. J Exp Med 1973; **138:** 1156-1167.
29. **Jack, D.L., A.W. Dodds, N. Anwar, C.A. Ison, A. Law, M. Frosch, M.W. Turner, N.J. Klein**. Activation of complement by mannose-binding lectin on isogenic mutants of *Neisseria meningitidis* serogroup B J Immunol 1998; **160:** 1346-1353.
30. **Jack, D.L., G.A. Jarvis, C.L. Booth, M.W. Turner, N.J. Klein**. Mannose-binding lectin accelerates complement activation and increases serum killing of *Neisseria meningitidis* serogroup C J Infect Dis 2001; **184:** 836-845.
31. **Bax, W.A., O.J.J. Cluysenaer, A.K.M. Bartelink, P.C. Aerts, R.A.B. Ezekowitz, and H. van Dijk**. Association of familial deficiency of mannose-binding lectin and meningococcal disease. Lancet 1999; **354:** 1094-1095.
32. **Kozel, T.R., L.C. Weinhold, D.M. Lupan**. Distinct characteristics of initiation of the classical and alternative complement pathways by *Candida albicans*. Infect Immun 1996; **64:** 3360-3368.
33. **Garred, P., H.O. Madsen, J.A. Kurtzhals, L.U. Lamm, S.Thiel, A.S. Hey, A. Svejgaard**. Diallelic polymorphism may explain variations of the blood concentration of mannan-binding protein in Eskimos, but not in black Africans. Eur J Immunogenet 1992; **19:** 403-412.
34. **Garred, P., C. Richter, A.B. Andersen, H.O. Madsen, I. Mtoni, A. Svejgaard, J. Shao**. Mannan-binding lectin in the sub-Saharan HIV and tuberculosis epidemics. Scand J Immunol 1997; **46:** 204-208.
35. **Turner, M.W., L. Dinan, S. Heatley, D.L. Jack, B. Boettcher, S. Lester, J. McCluskey, D. Roberton**. Restricted polymorphism of the mannose-binding lectin gene of indigenous Australians. Hum Mol Genet 2000; **9:** 1481-1486.
36. **Garred, P., M. Harboe, T. Oettinger, C. Koch, A. Svejgaard**. Dual role of mannan-binding protein in infections: another case of heterosis? Eur J Immunogenet 1994; **21:** 125-131.
37. **De Miranda Santos, I.K.F., C.H.N. Costa, H. Krieger, M.F. Feitosa, D. Zurakowski, B. Fardin, R.B.B. Gomes, D.L. Weiner, D.A. Harn, R.A.B. Ezekowitz, J.E. Epstein**. Mannan-binding lectin enhances susceptibility to visceral leishmaniasis. Infect Immun 2001; **69:** 5212-5215.
38. **Horwitz, M.A**. The legionaires' disease bacterium (*Legionella pneumophila*) inhibits phagosome-lysosome fusion in human monocytes. J Exp Med 1983;**158:** 2108-2126.
39. **Devyatyarova-Johnson, M., I.H. Rees, B.D. Robertson, M.W. Turner, N.J. Klein, D.L. Jack**. The lipopolysaccharide structures of *Salmonella enterica* serovar typhimurium and *Neisseria gonorrhoeae* determine the attachment of human mannose-binding lectin to intact organisms. Infect Immun 2000; **68:** 3894-3899.
40. **Beaman, B.L., L. Beaman**. *Nocardia species*: host-parasite relationships. Clin Microbiol Rev 1994; **7:** 213-264.
41. **Berstad, A.E., K. Hogason, G. Bukholm, A.P. Moran, P. Brandtzaeg**. Complement activation directly induced by *Helicobacter pylori*. Gastroenterol 2001;**120:** 1108-1116.

Chapter 6

Deficiency of mannose-binding lectin (MBL) is a novel risk factor for *Legionella pneumophila* pneumonia

S. Kuipers[1, 2], E.P.F. IJzerman[3], K.D. Lettinga[4] and H. van Dijk[2, 5]

[1]Department of Microbiology, Public Health Laboratory Twente Achterhoek, Enschede, [2]Eijkman-Winkler Center for Microbiology, Infectious Diseases, and Inflammation, University Medical Center, Utrecht, [3]Regional Laboratory for Public Health, Haarlem, [4]Department of Internal Medicine, Academic Medical Center, Amsterdam, [5] Department of Medical Microbiology and Immunology, Meander Medical Center, Amersfoort

(submitted for publication)

ABSTRACT

Deficiency of mannose-binding lectin (MBL), one of the most important proteins of innate immunity, alters susceptibility to infection. Using a hemolytic assay, we investigated functional MBL serum levels in a cohort of pneumonia patients from a legionellosis outbreak in the Netherlands in 1999 (n = 124), and compared these to 447 controls. A significantly higher incidence of MBL deficiency (serum MBL<0.20 µg equivalents/ml; X=96.7; P<0.001) was found in the patients, whereas MBL deficiency in 59 asymptomatic, exposed individuals was comparable to the controls. We conclude that MBL deficiency is a novel risk factor for *Legionella pneumophila* pneumonia.

Legionella pneumophila is an intracellular gram-negative bacterium capable of surviving and replicating in cells of the human mononuclear phagocytic system. Infection may lead to an acute selflimiting flu-like illness called Pontiac fever, or to a severe pneumonia that has a calculated case fatality rate of 11-18 %. In 1999 in Bovenkarspel, the Netherlands, a large outbreak of pneumonia caused by *L. pneumophila* type 1 occurred at a well-attended flower show. The outbreak included 188 patients, of who 21 died. The source of the outbreak could be traced to a whirlpool in one of the exhibition halls. [1]

Together with complement components C1 and C3b, mannose-binding lectin (MBL) belongs to the three initiators of complement activation, in case of MBL via the so-called lectin pathway (LP). LP activation begins when MBL binds to mannose, fucose or N-acetylglucosamine moieties on microbes, thus leading to microbial opsonization, intracellular uptake and killing in monocytes and macrophages. [2] Three structural gene mutations determine serum MBL levels, further modulated by several promoter polymorphisms. [2,3] A calculated 32% of cases of disease with the gram-negative bacterium *Neisseria meningitidis* are associated with one or two MBL gene variants, with decreased serum MBL levels. [4] It is unknown whether MBL deficiency enhances susceptibility to infection with the gram-negative bacterium *Legionella pneumophila.* In this study, we investigated functional MBL levels in patients with *L. pneumophila* pneumonia in order to find out if there is a relation between severe clinical disease and deficiency of MBL.

All patients included in this study were visitors to the West-Frisian Flora Show (WF), which was held in February 1999 in Bovenkarspel. Case definitions were formulated by the Outbreak Management Team. A *L. pneumophila* 'case' was defined as a visitor to the WF with a clinically and radiographically confirmed pneumonia, and either a positive *L. pneumophila* type 1 culture or a positive urinary *L. pneumophila* type 1 antigen assay or a serologically confirmed rise in *L. pneumophila* antibody titer, without evidence of another microbial cause. A 'probable case' was defined as a visitor to the WF with a clinically and radiographically confirmed pneumonia, who did not meet the laboratory criteria for a confirmed case but in whom no other infectious agent could be identified. [1]

The legionellosis group (n = 124) consisted of 92 cases and 32 probable cases. Their ages ranged from 20 to 86 years, with an arithmetic mean of 64.1 years (SD 10.4). The male to female ratio was 1.5 : 1. The control group consisted of 378 donors to the blood bank in Utrecht (age: 19 to 67 years), and 69 preoperative visitors to an orthopedic

outpatient department (age: 67 to 85 years). The mean age was 45.0 years (SD 17.2), and the male to female ratio 1.7 : 1. A third group consisted of 59 asymptomatic WF workers with positive *L. pneumophila* serology. Ages ranged from 15 to 76 years, with a mean of 45.7 years (SD 14.3), and a male to female ratio of 0.9 : 1.

Serum samples (stored at – 80 °C) from the Bovenkarspel legionellosis patients were tested them for functional MBL activity as described, [5] and the results were compared with the control group and the group of asymptomatic WF workers. In our laboratory, decreased functional MBL levels have been defined as levels below 0.42 µg per ml, and MBL deficiency as levels below 0.20 µg/ml. [5]

Functional MBL titers in 640 samples tested ranged from < 0.13 to 6.04 µg equivalents per ml. Statistical analysis was done using a 3×2 χ^2 test. As shown in the table, a significantly higher incidence of MBL deficiency (MBL < 0.20 µg equivalents per ml) was found in the patients as compared to the population controls (X [3x2 table] = 96.7; $P < 0.001$). One WF worker (1.7 %) was found to be MBL deficient, while normal and decreased MBL levels were found in 79.7 % and 18.6 % of sera, respectively (table).

Table. Functional serum mannose-binding lectin (MBL) levels in 124 cases of proven and probable legionellosis related to the Bovenkarspel outbreak in 1999. A group of 378 donors to a blood bank and 69 preoperative visitors to an orthopedic outpatient department were used as controls. A group of 59 asymptomatic workers with positive *L. pneumophila* serology was also included.

Individuals	Functional MBL levels in µg eq/ml		
	Deficient (< 0.20)	**Low level** (0.20-0.42)	**Normal** (> 0.42)
Patients	37[a] (29.8%)	20 (16.2%)	67 (54%)
Controls	13 (2.9%)	45 (10.1%)	389 (87%)
Exposed workers	1 (1.7%)	11 (18.6%)	47 (79.7%)

[a] A significantly higher incidence of MBL deficiency was found in patients with *Legionella pneumophila* type 1 pneumonia when compared to the controls (X [3x2 table] = 96.7; $P << 0.001$).

In the Netherlands, 13 % of the population shows decreased functional MBL levels.[5] Severe MBL deficiency has been found in 1.7 % to 3.1 % of the population, varying according to the population tested. Our results, both of the population controls and the WF workers, are in line with genotypic and phenotypic findings in the Caucasian population. [2,3] MBL is evenly distributed among sexes, and in adults no age dependency is found. Although a weak acute phase reactant, in MBL-deficient individuals MBL levels cannot increase substantially even in the event of an infection, as was confirmed in our patient group.

Until now, the role of the MBL in the pathogenesis of legionellosis has not been described. Several non-genetic risk factors for legionellosis are known, including smoking, age, corticosteroid use, and chronic obstructive pulmonary disease. However, since there is no evidence that MBL phenotypes and genotypes influence the above named risk factors in our study, it was unnecessary to match patients and controls for additional risk

factors in the assessment of functional MBL levels in serum. Our hypothesis that MBL deficiency increases susceptibility to infection with *Legionella pneumophila* could be confirmed, although pathophysiological mechanisms that can explain our results remain to be elucidated.

In conclusion, in our case control study we found that extremely low MBL levels in serum are strongly associated with development of severe disease with *Legionella pneumophila*. This finding adds *L. pneumophila* to the list of microorganisms posing a threat to those individuals with MBL deficiency.

REFERENCES

1. **Den Boer, J.W., E.P.F. Yzerman, J. Schellekens, K.D. Lettinga, H. Boshuizen, J.E. van Steenbergen, A. Bosman, S. van den Hof, H.A. van Vliet, M.F. Peeters, R.J. van Ketel, P. Speelman, J.L. Kool, M.A. Conyn-van Spaendonck**. A large outbreak of Legionnaires' disease at a flower show, the Netherlands, 1999. Emerg Infect Dis 2002; **8:** 37-43.
2. **Turner, M.W**. The lectin route of complement activation. Res Immunol 1996; **147:** 110-15.
3. **Mead, R, D. Jack, M. Pembrey, L. Tyfield, M.W. Turner, and the ALSPAC study team**. Mannose-binding lectin alleles in a prospectively recruited UK population. Lancet 1997; **349:** 1669-70.
4. **Hibberd, M.L., M. Sumiya, J.A. Summerfield, R. Booy, M. Levin, and the Meningococcal Research Group**. Association of variants of the gene for mannose-binding lectin with susceptibility to meningococcal disease. Lancet 1999; **353:** 1049-53.
5. **Kuipers, S., P.C. Aerts, A.G. Sjöholm, T. Harmsen, H. van Dijk**. A hemolytic assay for the estimation of functional mannose-binding lectin levels in human serum. J Immunol Meth 2002; **268:** 149-57.

Chapter 7

Erythrocyte-mediated transport and elimination

Saskia Kuipers[1,2], Theo Harmsen[1], and Hans van Dijk[1]

[1] Eijkman-Winkler Center for Microbiology, Infectious Diseases, and Inflammation, University Medical Center, Utrecht, the Netherlands, [2] Department of Microbiology, Public Health Laboratory Twente Achterhoek, Enschede, the Netherlands

(submitted for publication)

It is almost beyond doubt that the main function of erythrocytes is to exchange and transport gases like oxygen and carbon dioxide to and from the lungs, respectively. In a similar manner, erythrocytes are able to transport toxic carbon monoxide, which shows that erythrocyte transport is not only used for beneficial purposes. In this discussion paper we will show that the above statements definitely are not the only truth about erythrocytic transport, and that at least part of erythrocyte function is devoted to the transport and/or elimination of particles, including bacteria, (malaria) parasites, and lipoproteins. In the fast-growing field of immunology, it is beginning to be understood that erythrocytes are able to carry immune complexes to the liver, spleen and lungs [1]. This whole process seems to be governed by a single receptor (CR1 = C3b(i) receptor) that is present on primate erythrocytes but lacking on erythrocytes of lower mammals. However, there is substantial evidence that in lower mammals like e.g. rodents, platelets rather than erythrocytes fulfill the CR1 carrier role as described in primates.

EXAMPLES OF ERYTHROCYTE IMMUNOLOGY

Example 1: Erythrocytes do not only bind gases. They are also able to bind and transport drugs like α-methyldopa and certain antimetabolites [2]. A teleological explanation for these phenomena could be that the binding of drugs to erythrocytes prevents the vessel wall from adverse reactions with the drug, which may protect the vessel wall from possible effects of type II hypersensitivity reactions (drug fever), or from drug-induced, direct toxicity.

Example 2: Erythrocytes are also able to transport intact bacteria through the blood stream, probably to protect the vessel wall from unwanted inflammatory effects that may ultimately lead to premature vascular disease and, in the long run, even to atherosclerosis, or to target live bacteria to professional elimination sites including the spleen, liver and/or lungs. This erythrocyte-mediated bacterial transport and elimination mechanism has been described first by RA Nelson in 1953 who introduced the term 'immune adherence' (Fig. 1) for this unique bacterial-erythrocyte interplay [3,4].

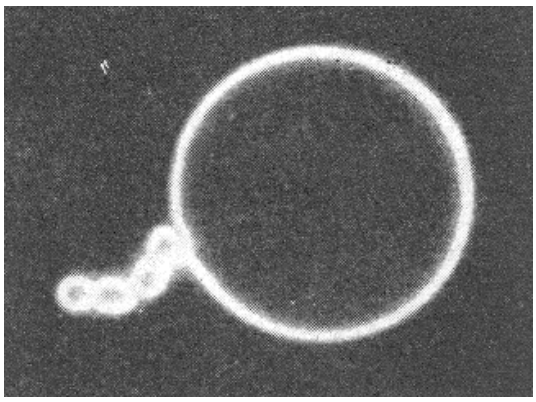


Figure 1. Darkfield microscopy of *Streptococcus pneumoniae* immune adherence to an erythrocyte (R.A. Nelson, 1953).

Later, as already pointed out, this mechanism appeared to be mediated by a limited number (400 to 700) of erythrocyte-bound CR1 receptors on the erythrocyte. Although these numbers seem negligible, the large amounts of erythrocytes in circulation

ensure that this erythrocyte-mediated, immune elimination system is a very smart and effective one [4-7]. However, again thinking in teleological terms, the question remains which complement pathway opsonizes bacteria, making them ligands for CR1. It is known that the lectin and alternative complement activation pathways are, in evolution, the most ancient. Our first guess would be the lectin complement pathway, activation of which is mediated by mannose, N-acetylglucosamine, and fucose on gram-positive and gram-negative bacteria, yeast, mycobacteria, viruses, and parasites. This option seems logical, since the alternative complement pathway could easily amplify the signal initiated by the lectin pathway, while the alternative complement pathway is not a strong activation pathway per se. Later in evolution, the classical pathway could be the second best option, but only for people with a broad specter of specific antibodies or with an intact MBL pathway. However, in case of holes in their antibody repertoire and/or a diminished MBL function, individuals will probably need substitution therapy with parenteral IgG or MBL to sustain their failing immunity.

Example 3: In the present proposal we would like to discuss how we would solve the question by which complement activation pathway the 'immune adherence' phenomenon is triggered. To answer this question, we propose to apply our three in-house, functional microtiter complement assays to measure *in-vitro* activation via the classical, the alternative, and the mannose-binding lectin pathway (shortly: the lectin pathway) [8,9]. To this end, we intend to use sera from different donors with deficiencies in MBL (e.g. the *B/D* variant), C1q, factor B, factor D, and IgG_2.

Example 4: In this short proposal, we also intend to highlight two recent findings related to the 'immune adherence' phenomenon and to quite similar erythrocyte-related phenomena, including the binding of gut-derived lipoproteins (chylomicrons) and very-low-density lipoproteins (VLDL) to circulating erythrocytes. From these *ex-vivo* experiments, we learned that the erythrocytes do not only bind chylomicrons and VLDL in the circulation, but that they also transport and target the lipoproteins to sites of elimination, including the liver (the organ of the lipoprotein synthesis), the spleen and the lungs, in a manner similar to microorganisms (Fig. 2).

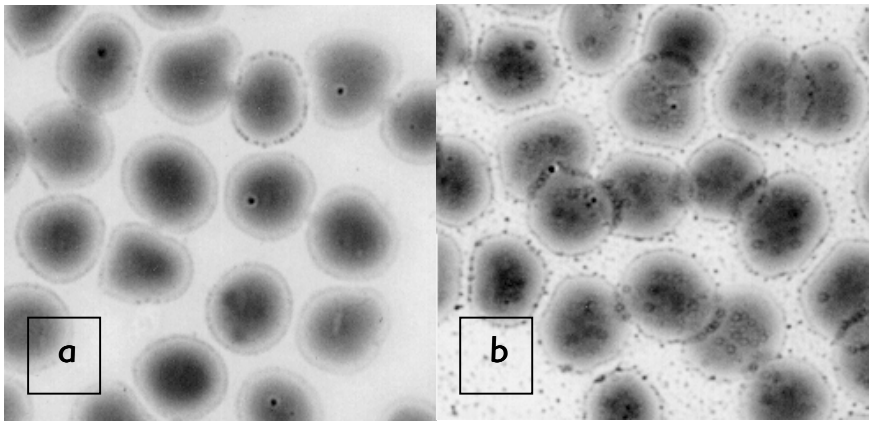
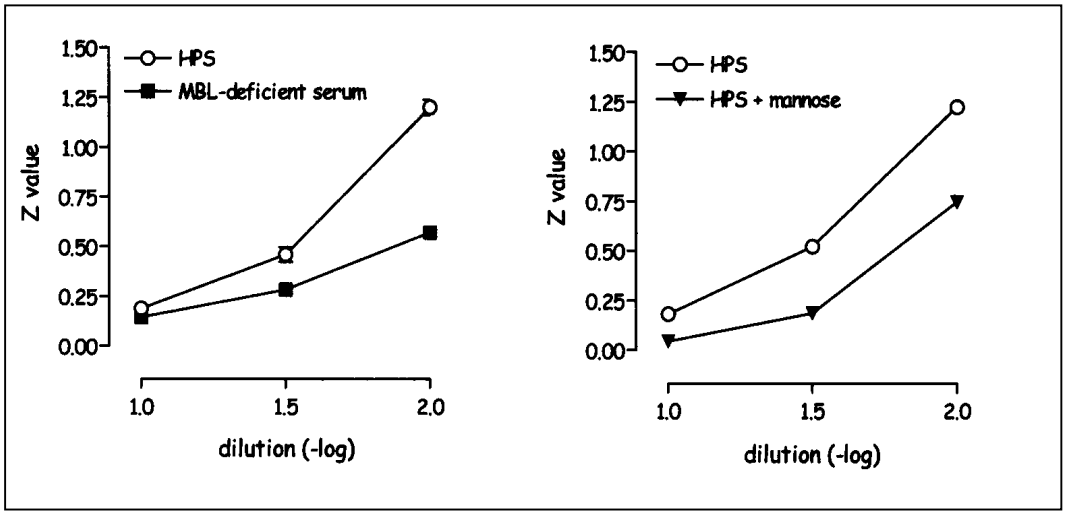


Figure 2. Smears of erythrocytes obtained from a healthy MBL-sufficient individual 3 hours after a 12-hours fast (2a) and 3 h after an oral fat load (2b). Lipid particles were stained with Sudan black.

This finding strongly suggests that chylomicrons and VLDL both activate the complement system, and that these particles are opsonized and erythrocyte-bound. The question then arises via which pathway complement activation is achieved. So far, two pieces of evidence exist suggesting that this new phenomenon may be mediated by the lectin pathway (Fig. 3) [10]; first of all because the lipoproteins mentioned are heavily glycosylated with the sugars mannose, N-acetyglucosamine, and fucose [11], which all three happen to be strong binding sites for mannose-binding lectin and, secondly, that according to literature deficiency of mannose-binding lectin, which occurs quite frequently in our population (more than 12%; recent results), is associated with severe atherosclerosis [12].

Figure 3. Lectin-dependent complement activation using a hemolytic assay as published by Kuipers et al. [9] using chylomicrons instead of *Saccharomyces cerevisiae* as activators of mannose-binding lectin (MBL). The figure on the left shows lipoprotein-induced MBL activation in human pooled serum (HPS) with MBL levels of 1.67 µg per ml versus MBL-deficient serum with an MBL level of 0.20 µg per ml. The right figure shows that lipoprotein-induced MBL activation can be inhibited by the addition of the saccharide mannose (2.5 mg/well).



Example 4: Colleagues working in the field of DNA vaccinology are often confronted with problems of diminished efficacy of their preparations [13], in our view due to early elimination that is likely to interfere with effectivity. We suggest here that also the poor efficacy of DNA vaccines may be a phenomenon that is mediated by MBL, which is most likely activated by the pentose-moieties in their constructs.

Purpose of the proposal: Purpose of our proposal is to confirm the importance of the lectin complement pathway in the protective, erythrocyte-mediated transport and elimination of chylomicrons, VLDL, DNA (constructs), and bacteria.

REFERENCES

1. **Nairn, R., M. Helbert**. 2002. In: Immunology for Medical Students, p 162. Mosby International Ltd., London, UK.
2. **Schrijvers, D., M. Highly, E. de Bruyn, A.T. van Oosterom, J.B. Vermorken**. Role of red blood cells in pharmacokinetics of chemotherapeutic agents. Anticancer Drugs 1999;**10:** 147-53.
3. **Nelson, R.A**. The immune-adherence phenomenon. Science 1953;**118:** 733-737.
4. **Nelson, D.S**.1965. Immune adherence. In : Complement, Ciba Foundation Symposium, p 222-241. Ed. Wolstenholme, G.E.W. and Knight, J. J. & A. Churchill, Ltd., London, UK.
5. **Fearon, D.T**. Identification of the membrane glycoprotein that is the C3b receptor of the erythrocyte, polymorphonuclear leukocyte, B lymphocyte and monocyte. J Exp Med 1980;**152:** 20-30.
6. **Siegel, I., T.L. Liu, N. Gleicher**. The red-cell immune system. Lancet 1981; **2:** 556-559.
7. **Cornacoff, J.B., L.A. Hebert, W.L. Smead, M.E.van Aman**. Primate erythrocyte-immune complex clearing mechanism. J Clin Invest 1983; **2:** 236-247.
8. **Klerx, J.P.A.M. C.J. Beukelman, H. van Dijk, J.M.N. Willers.** Microassay for colorimetric estaimation of complement activity in guinea pig, human and mouse serum. J Immunol Meth 1983; **63:** 2150-220.
9. **Kuipers, S., P.C. Aerts, A.G. Sjöholm, T. Harmsen, H. van Dijk**. A hemolytic assay for estimation of functional mannose-binding lectin levels in human serum. J Immunol Meth 2002; **268:** 149-157.
10. **Kuipers, S., M. Castro Cabezas, T. Harmsen, B. Dekker, H. van Dijk**. The mannose-binding lectin-severe atherosclerosis paradox solved? Int. Immunopharmacol 2002; **2:** 1363.
11. **Sasak, W.V., J.S. Lown, K.A. Colburn**. Human small-intestinal apolipoprotein B-48 oligosaccharide chains. Biochem J 1991; **274:** 159-165.
12. **Madsen, H.O., V. Videm, A. Svejgaard, J.L. Svennevig, P. Garred**. Association of mannose-binding lectin deficiency with severe atherosclerosis. Lancet 1998; **352:** 959-960.
13. **Plank, C., K. Mechtler, F.R. Szoka Jr, E. Wagner**. Activation of the complement system by synthetic DNA complexes: a potential barrier for intravenous gene delivery. Human Gene Therapy 1996; **7:** 1437-1446.

Chapter 8

A genetic link between systemic lupus erythomatosus and thyroid autoimmune disease?

S. Kuipers[1], E.K. Chung[2], L. Meijer[3], P.H.G. Hogeman[3], R.J. Kraaijenhage[4], C.Yung Yu[2], H. van Dijk[1,5]

[1] Eijkman-Winkler Center for Microbiology, Infectious Diseases, and Inflammation, University Medical Center, Utrecht, the Netherlands, [2] Children's Research Institute, the Ohio State University, Columbus, Ohio, USA, [3] Department of Pediatrics, Meander Medical Center, Amersfoort, the Netherlands, [4] Department of Clinical Chemistry, Meander Medical Center, Amersfoort, the Netherlands, [5] Department of Medical Microbiology and Immunology, Meander Medical Center, Amersfoort, the Netherlands

(submitted for publication)

Abbreviations:

AITD	: autoimmune thyroid disease
RCCX	: genetic module coding for C4, steroid 21-hydroxylase CYP21, Ser/Thr protein kinase RP, and tenascin.
SLE	: systemic lupus erythematosus

ABSTRACT

Systemic lupus erythematosus (SLE) is an autoimmune disease affecting vessels and organs by precipitating immune complexes. Homo- and heterozygous deficiency of complement component C4A is the most common risk factor of human SLE from most races. We describe a female patient in her early teens with, as yet, partial SLE. Both parents, and 8 out of 9 uncles and aunts on the maternal side were partially C4A deficient. Surprisingly, in four of the relatives on the maternal side autoimmune thyroid disease had been diagnosed. The association between SLE and thyroid autoimmunity in patients and their families has been an ongoing subject of investigation. This paper focuses on C4 deficiency in this multicase family with autoimmune disease as described above.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a non-organ-specific autoimmune disease associated with deficiencies in the early components of the complement system such as mannose-binding lectin (MBL), C1, C2 and C4 [1-8]. Partial deficiency of the two isotypes of C4, C4A or C4B, is the most common inherited immune deficiency in humans. Together, C4A and C4B determine total serum C4 levels [9]. Serum C4 plays an important role in both the lectin and the classical pathway of complement activation, which both lead to coating of immune complexes and (microbial) particles with opsonic complement component C3b. Although C4A and C4B are highly related, their action is more or less polarized, C4B being more hemolytically and bactericidally active and engaged in the activation pathways, while C4A is more involved in immune complex clearance [10].

In almost 35% of individuals from all over the world, the presence of C4A*Q0 or C4B*Q0 alleles has been demonstrated [6]. Deficiencies of MBL and C4A, but not of C4B, are linked to systemic autoimmunity. Complete deficiencies of both C4A and C4B, though rarely found, increases susceptibility to infections and, being an independent risk factor for SLE, almost invariably leads to SLE or lupus-like disease [3,11]. Identification of C4A and C4B allotypes are allowed by gross differences in electric charges of the variants, resulting in different electrophoretic migration patterns. In plasma from the patient's parents the C4 haplotype A3B1 was found, which is the most frequent haplotype among Caucasians (frequency 0.466) [12]. Over 19 C4A and 22 C4B allotypes are recognized besides the null alleles C4A*Q0 and C4B*Q0.

Autoimmunity can cluster within families, thereby supporting the presence of genetic susceptibility. For example, 27% of SLE patients have a positive family history for autoimmune disease [13]. We describe a girl who was diagnosed with partial SLE in her

early teens. Her family history included other cases of SLE as well as thyroid autoimmunity. C4 protein allotyping was performed besides the study of C4 polymorphisms, C4 gene dosage, and RCCX modules in all members of this multicase family with autoimmune diseases.

CASE HISTORY

A 13-year old girl was seen by her general practitioner (GP) with the following complaints: fatigue, paleness, headache, fever, sore throat, upper respiratory tract infection and cervical lymphadenopathy. The symptoms disappeared after a week. However, two months later, the same symptoms recurred. The GP diagnosed anemia and started oral iron substitution therapy. The pediatrician was consulted for the hemolysis.

On examination, the pediatrician saw a pale-looking girl, with no other physical signs besides lymphadenopathy. Blood tests revealed a hypochromic, macrocytic anemia (hemoglobin 5.2 mmol/l) with a positive direct Coombs' test (anti-IgG: 3+, anti-C3: 2+), which is characteristic for an immune-mediated hemolytic anemia. Autoantibody testing revealed positive antinuclear and anti-ds-DNA antibodies, combined with low hemolytic (CH50 33%, AP50 72%) and immunochemic complement values (C1q 52%, C3 0.39 g/l and C4 0.03 g/l). Immunofluorescence studies performed on a skin biopsy showed granular depositions positive for IgG (++), IgA (+/++), IgM (+++), and C3c (++) along the basal membrane, in a few papillae, and to a lesser degree in small subepidermal vessels (IgG: +/-, IgA: +/-, IgM: +/++, C3c: +). Viral infections were ruled out. Findings matched with an immune complex disease accompanied by a mild vasculitis. The results were consistent with autoimmune hemolytic anemia, probably based on an early, yet incomplete SLE with, instead of four, three positive criteria of the American College of Rheumatology [14]. The family history revealed autoimmune thyroid disease (AITD) in four individuals on the maternal side, and systemic lupus erythomatosus (SLE) in the deceased aunt on the paternal side, who had also presented with SLE in early childhood.

During follow-up at the outpatient clinic, the index patient presented with the same symptoms as before. Aggravated hemolysis (hemoglobin 4.8 mmol/l) necessitated treatment with prednisone 60 mg daily. After two weeks, the hemoglobin level raised to normal. Subsequently, the prednisone dosage was tapered over four months. Ever since, the hemolytic complement levels have been normal, while serum C3 and especially C4 levels have remained subnormal.

MATERIALS AND METHODS

Human blood samples. Peripheral blood samples were taken from the SLE patient, her father and mother, and the 9 aunts and uncles on the maternal side after informed consent had been obtained.

Complement component C3 and C4 serum levels. These were determined on a BN II nephelometer (Dade Behring, Marburg, Germany) according to standard methods.

Anti-thyroid antibodies. Anti-thyroid peroxidase (TPO) antibodies and anti-thyroglobulin (TG) were estimated in serum using a BN II nephelometer as well (Dade Behring, Marburg, Germany) according to standard methods.

Complement component C4 phenotype analysis and the relative quantities of C4A and C4B allotypes. Peripheral blood plasma from EDTA samples from the patient and her parents was used for C4 allotypic polymorphism testing following standard procedures [27]. In short, the plasma was treated with neuraminidase (Sigma, St Louis, MO, USA) and carboxypeptidase B (Sigma), and then resolved by high-voltage agarose gel electrophoresis (HVAGE) based on gross electric charge differences of plasma proteins. C4 was detected by immunofixation with goat anti-human C4 serum (Incstar, Stillwater, MN), which was followed by blotting and staining with SimplyBlue Safestain (Invitrogen, Carlsbad, CA, USA). The relative intensities of the stain correspond with the relative number of *C4A* and *C4B* alleles.

Isolation of genomic DNA and Southern blot analysis. Genomic DNA, to be used for restriction fragment length polymorphism (RFLP) analysis, was isolated from peripheral blood cells in EDTA samples using the Pure-gene DNA isolation kit from Gentra Systems (Minneapolis, MN, USA).

C4 genotyping and determination of C4A and C4B gene dosage. A two-step strategy was employed to determine the number of C4A and C4B genes present in a diploid genome (i.e. gene dosage) from each family member. First, primers ***b***, ***c***, and ***d*** were used for module-specific PCR to decipher the total number of C4 genes [28]. Second, primers Y24IN and E29.3 were used for amplification of the C4d region (1.3 kb in size), which was later subjected to LSP-*Psh*AI RFLP analysis for the elucidation of the relative number of C4A and C4B genes [27]. The sequence for primer ***b*** was, 5′ GCT CAA GCT GTG AGG AGA ACT 3′; ***c***, 5′ TAT CAC AGG CTC TGG CCC CA 3′; ***d***, 5′ TTC GTG GTC CAG TAC AGG GA 3′; Y24IN, 5′ CAG AAG GGT GAG TGT CAC CTG AG 3′; and E29.3, 5′ TTG GGT ACT GCG GAA TCC CC 3′. The PCR conditions were as follows: 1 cycle at 94°C for 3 min; 35 cycles at 94°C for 45 sec, 62°C for 45 sec, 72°C for 1 min; and 1 cycle at 72°C for 10 min. 20% of the amplified products from the module-specific PCR reaction were electrophoresed in 1% agarose gels. A digital image of the gel was taken and the intensity of each band was quantitatively analyzed by Nucleotech-GelExpert, version 4.0. The amplified products from the C4d region were purified and subjected to the LSP method using 0.2 μM [γ-^{32}P] ATP-labeled primer E29.3, as described in [28]. LSP products were restriction digested with *Psh*AI overnight. DNA fragments were resolved by electrophoresis using 1.2% agarose gels. The gel was subsequently processed by a Southern blot technique, subjected to autoradiography, and the radioactivity of each band was registered by phosphorimaging and quantified by Molecular Dynamics ImageQuant Software version 5.0, as described in [28]. Details of the RCCX modules and C4A and C4B gene dosages were demonstrated by TaqI RFLP, showing the presence and dosage of RP1-C4 long (7.0 kb), RP1-C4 short (6.4 kb), RP2-C4 long (6.0 kb) and RP2-C4 short (5.4 kb), and by PshAI-PvuII RFLP elucidating the relative dosage of C4A (1.7 kb) and C4B (2.2 kb). Furthermore, pulsed field gel electrophoresis (PFGE) of PmeI-digested genomic DNA was employed to show the RCCX length variants in haplotypes unambiguously [28].

HLA typing. In order to be able to confirm the MHC haplotype situation between the patient and her parents, molecular human leukocyte antigen (HLA)-ABC (class I) and HLA-DRB1 (class II) typing was performed. In short, leukocytes were isolated from blood

drawn from these individuals, and genomic DNA was extracted. Sequence based HLA typing was performed according to standard procedures [29].

RESULTS

Allotyping experiments using blood from the patient and her parents revealed the total absence of C4A in the patient's blood when compared to her parents (Fig. 1). Plasma originating from the parents clearly stained for C4A3, whilst their daughter's plasma showed total absence of C4A3. In plasma of both parents vivid C4B1 staining was found. The relative intensity of the C4B1 stain in the paternal plasma already shows the presence of more C4B alleles when compared to the maternal plasma or that of the patient (Fig. 1).

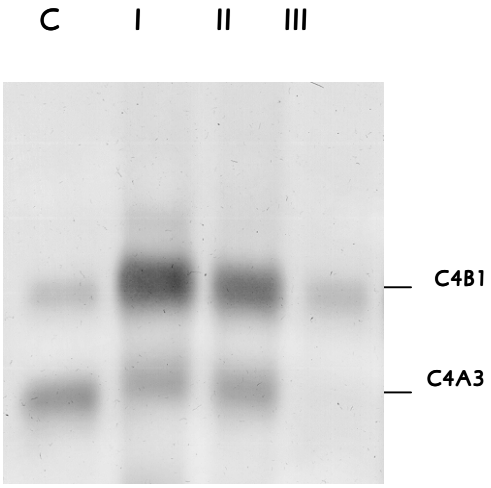
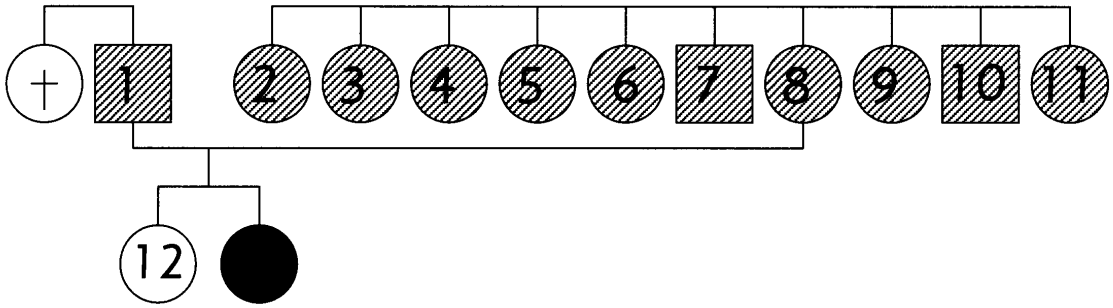


Figure 1. *Plasma samples from the positive control (C), father (I), mother (II) and the patient (III) were subjected to agarose gel electrophoresis and immunofixation with antiserum to C4 (C4 allotyping), showing deficiency of C4A in the patient. Note the strong staining of C4B1 in the father's serum that, after genotyping, could be ascribed to the presence of six C4B alleles.*

A pedigree of this family is shown in Fig 2. Serum C4 levels and the number of C4A and C4B genes present in the diploid genome of the patient and each family member (i.e. gene dosage) are shown in Table 1.In short, the patient (13) has homozygous C4A deficiency while the C4B gene dosage is normal with 2 copies of the C4B gene in the diploid genome (monomodular RCCX (M): one C4 gene per chromosome). On the contrary, the patient's sister (12) has 5 C4 genes, comprising two C4A genes and three C4B genes divided between one bimodular RCCX (B: each copy of chromosome 6 with two C4 genes) and one trimodular RCCX (T: each copy of chromosome 6 with three C4 genes). The patient's mother (8) has 3 C4 genes in one monomodular and one bimodular RCCX (B/M). From the family segregation analysis, it may be inferred that the patient's father (1) has one trimodular RCCX, which was transmitted to the patient's sister (12), and one monomodular RCCX, which was transmitted to the patient (black circle).

Figure 2. A genealogical tree showing the homozygous C4A-deficient girl with incomplete SLE (black), her non-C4A-deficient sister (12), her parents and family members on the maternal side with heterozygous C4A deficiency (1-11). The subjects 2, 3, 4, and 7 suffer from autoimmune thyroid disease (AITD).



However, the results of homozygous bimodular RCCX haplotypes and those of trimodular/monomodular haplotypes are indistinguishable by module-specific PCR. Therefore, PmeI-PFGE and TaqI RFLP analyses were employed to definitively demonstrate the presence of trimodular-LSS (*L*ong-*S*hort-*S*hort) and monomodular-S (*S*hort) RCCX structures in the patient's father (Fig. 3).

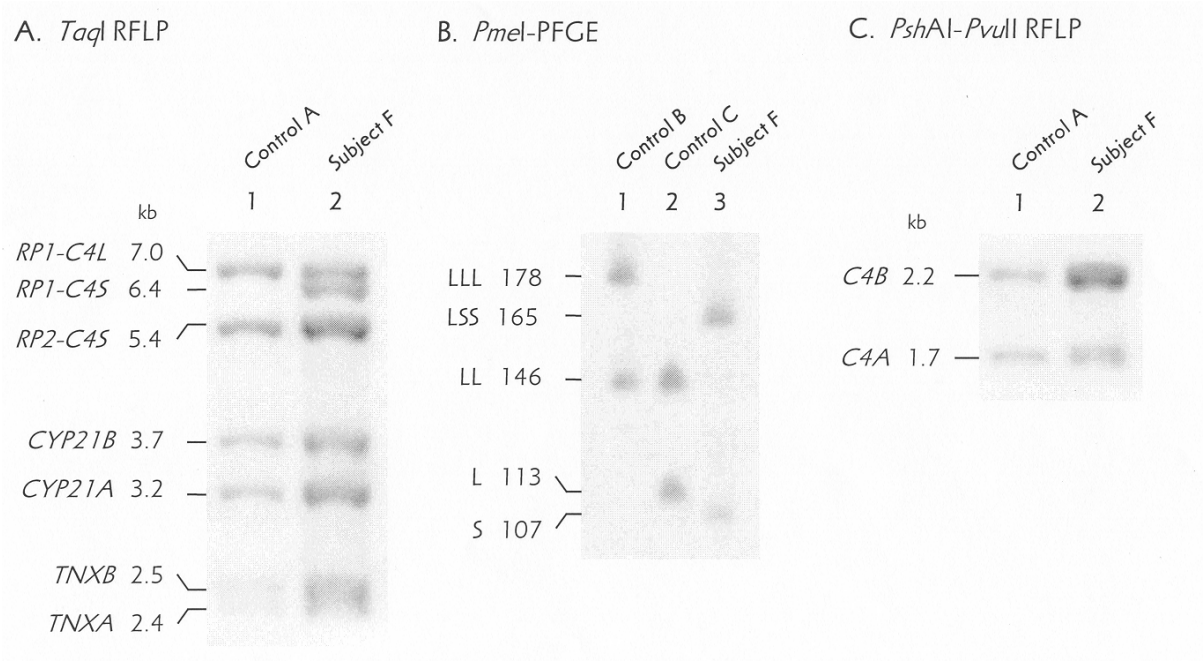


Figure 3. An example of RCCX determination in blood samples from patient's father (F). A trimodular RCCX structure with one long and two short C4 genes on one haploid genome and a monomodular RCCX with a single short C4 gene on the other haploid genome (C4A-C4B-C4B / C4B).

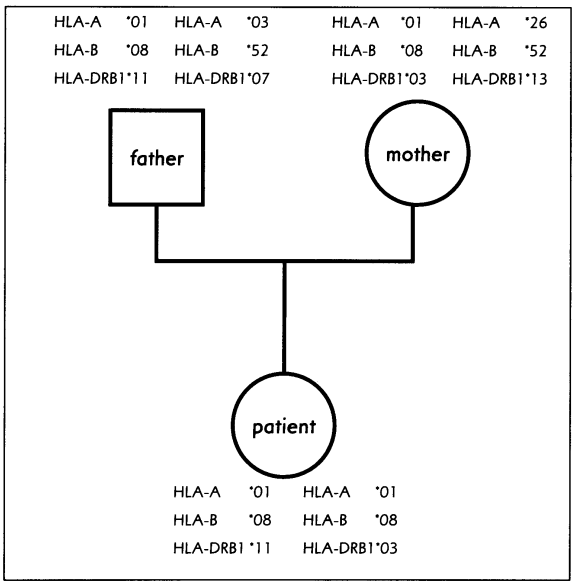
Table 1. *The index patient (no 13) and her family members: serum C4 levels, total C4 gene dosage, number of C4A and C4B genes, RCCX modules, antithyroid antibody status, and autoimmune diseases as currently diagnosed in this family are included in this table.*

No	Sex	Age (years)	Total C4 gene #	*C4A* #	*C4B* #	Module *	Serum C4 (g/l)	Anti-TG (IU/ml)	Anti-TPO (IU/ml)	Autoimmune disease
0	F	died	n.a.				n.a.	n.a.	n.a.	SLE
1	M	47	4	1	3	M / T	0.28	< 172	< 20	
2	F	54	3	1	2	M / B	0.22	< 172	139	AITD
3	F	52	3	1	2	M / B	0.21	< 172	121	AITD
4	F	51	3	1	2	M / B	0.23	< 172	340	AITD
5	F	49	3	1	2	M / B	0.18	< 172	< 20	
6	F	49	3	1	2	M / B	0.19	< 172	< 20	
7	M	48	3	1	2	M / B	0.23	< 172	< 20	AITD
8	F	46	3	1	2	M / B	0.13	< 172	173	
9	F	45	3	1	2	M / B	0.12	1119	624	
10	M	43	3	1	2	M / B	0.19	< 172	< 20	
11	F	40	3	1	2	M / B	0.16	< 172	57	
12	F	16	5	2	3	B / T	0.21	904	< 20	
13	F	13	2	0	2	M / M	0.03			SLE / AIHA

*M: monomodular RCCX, each copy of chromosome 6 contains one C4 gene; B: bimodular RCCX, each copy of chromosome 6 has two C4 genes; T: trimodular RCCX; each copy of chromosome 6 has three C4 genes. Complement factor C4 levels were measured by nephelometry. Anti-TG: antithyroglobulin antibodies. Anti-TPO: antithyroid peroxidase antibodies. AIHA: auto-immune hemolytic anemia. AITD: auto-immune thyroid disease. SLE: systemic lupus erythematosus. N.a.: not available.

HLA typing of class I and class II alleles (HLA-A, B, C and HLA-DRB1, respectively) in the patient and her parents further revealed that the paternal monomodular-S RCCX structure of the patient belonged to the HLA-A1 B8 DRB1*11 haplotype, whereas the maternal monomodular-S RCCX structure belonged to the HLA-A1 B8 DRB1*03 haplotype (Fig. 4).

Figure 4. HLA typing of the patient and her parents.



Anti-thyroid peroxidase antibodies (anti-TPO) and anti-thyroglobulin antibodies (anti-TG) were measured in sera of all family members. The results are shown in Table 1. Clinical thyroid autoimmunity was present in four family members (subjects 2, 3, 4, and 7). Three of the four family members with thyroid autoimmunity showed increased anti-TPO. All four individuals suffering from clinical thyroid disease were heterozygous C4A deficient.

DISCUSSION

Although the increased prevalence of autoimmune thyroid diseases (AITD) in systemic lupus erythematosus (SLE) patients and their families has been reported by several researchers over the years, with a reported prevalence of AITD in SLE up to 27%, the supposedly combined genetic factor(s) leading to these immune complex diseases still remain to be elucidated [15,16]. SLE patients have a 5.7 times higher chance of developing thyroid autoimmunity [16]; other researchers have found a marked, though age-dependent increase in both thyroid disease and antithyroid antibodies in SLE patients themselves [17]. In the case of SLE, C4A*Q0 alleles are part of the multifactorial etiology also including other components of the lectin and classical pathways of complement activation, Fc receptor polymorphisms, lymphokines, apoptotic factors, and HLA-DR2 and -DR3, the serological definitions currently termed DRB1*15 or DRB1*16, and DRB1*03, respectively [8,18]. In thyroid autoimmunity, the only genetic factors known to have a role in etiology are HLA-DR3 (or DRB1*03) molecules and the *CTLA-4* gene [19]. Although the mother of the SLE patient was DRB1*03 positive, the father was not, even though SLE was prevalent in his family. For this reason we did not pursue this line of genetic research. As an indication of necessity for more research, genome-wide screening of SLE patients has been proposed to unravel more

factors involved in the pathogenesis of systemic and thyroid autoimmunity [15]. Since the prevalence of autoantibodies is known to be raised in relatives of multicase SLE families like the family described above, the primary goal of this study was to compare data with genetic aberrations of the C4 gene and to test thyroid autoantibody levels in this multicase SLE family.

The extremely low serum levels of complement component C4 in the above described 13-year old patient pointed in the direction of C4A deficiency, since the main function of the C4A isotype is the mediation of immune complex clearance from the bloodstream whereas the C4B isotype is hemolytic and bactericidal. These differences may be explained by the fast binding of C4B to hydroxyl groups and the slower binding of C4A to amine groups [20]. Only deficiency of C4A, but not of C4B, predisposes to systemic autoimmunity. Analysis of deficiencies of the early complement components showed that, after allotyping and genetic experiments, this patient's partial SLE was based on an incidental homozygous C4A deficiency combined with a normal C4B gene dosage.

Identification of C4A and C4B allotypes are allowed by gross differences in electric charges of the variants, resulting in different electrophoretic migration patterns. In plasma from the patient's parents the C4 haplotype A3B1 was found, which is the most frequent haplotype among Caucasians (frequency: 0.466) [12]. Over 19 C4A and 22 C4B allotypes are recognized besides the null alleles C4A*Q0 and C4B*Q0.

C4A and C4B are encoded by genes located in the class III region of the major histocompatibility complex (MHC) on chromosome 6. The central MHC is a tightly packed and partially overlapping set of genes on chromosome 6, not only encoding complement factor C4, but also in the ancient complement proteins C2 and factor B [20]. The C4 gene is a constituent of the genetic module termed RCCX, coding not only for C4, but also for steroid 21-hydroxylase CYP21, nuclear Ser/Thr protein kinase RP, and the extracellular matrix protein tenascin TNX [9]. In a single *C4* haplotype, single loci exist for *RP*, *CYP21* and *TNX*, but up to four *C4* genes may be found in a haploid genome, and each C4 locus may encode C4A and/or C4B [9,21]. The total C4 level in serum is determined both by the relative number of C4A and C4B genes present in the genome and by the presence or absence of disease leading to C4 consumption in vivo [9].

In the index patient, Southern blot analysis was suggestive for complete C4A deficiency (absence of C4A protein). Subsequently, using newly established PCR genotyping techniques applied to plasma of all family members, the total number of C4 genes, RCCX modules present on chromosome 6, and the ratio between C4A and C4B were determined in blood samples from the patient, her sister and parents, and from nine sisters and brothers of the mother. Both parents, and the nine family members from the maternal side, had only one C4A gene in the diploid genome. As an example, only 55% of Caucasians have the two-locus structure with C4A-C4B configurations in the MHC, the remainder has unequal numbers of those genes [8].

Partial C4A or C4B deficiencies in the Caucasian population have a combined frequency of 31.6% [8], confirming earlier findings that non-expression of 1, 2, or 3 C4 alleles is found in 35%, 9% and 1%, respectively [3]. We could show that our young SLE patient inherited the HLA-A1 B8 DR3 haplotype with a single *C4B* gene from her mother, and the HLA- A1 B8 DR11 with one *C4B* gene from her father.

Autoimmune thyroid disease (AITD) was diagnosed in one brother and three sisters of the patient's mother. Heterozygous C4A-deficiency was found in these individuals, indicating a possible genetic link between SLE and AITD based on C4A deficiency. Serum antibodies against thyroid peroxidase, the enzyme that iodinates thyroglobulin, are associated significantly with early stages of AITD [22], and have been associated with C4AQ0 (null alleles) in literature [23]. Anti-thyroglobulin antibodies seem to appear later in thyroid disease and are usually found in sera from patients with Hashimoto's disease.

Although thyroid cells are relatively resistant to complement attack, expression of CD59 is upregulated in AITD. However, how all other molecules and proteins upregulated in AITD and the complement system interact and connect is not entirely clear [18]. Why only thyroid cells from individuals suffering from AITD express MHC class II molecules, in particular HLA-DR, is unknown.

Plasma C4 is synthesized mainly in the liver, although local C4 production may be found in organs like the kidneys, heart, synovium, as well as in the skin. Significant levels of C4 are synthesized in endocrine organs like adrenals and the thyroid gland [24]. Whether deficiency in thyroid C4 production plays a role in AITD is unknown. How then may partial C4A deficiency result in thyroid autoimmune disease? It is very likely that decreased local C4 production in the thyroid leads to lessened local immunoclearance of, for example, thyrogastric autoantobodies, which have been associated with a null allele at the C4A locus [23]. Another line of thought may be followed when realizing that in totally C4A-deficient patients the organs affected are those that normally synthesize C4.

As C4 is necessary for the opsonization of immune complexes, allowing 'immune adherence' of immune complexes (ICs) to C3b-receptors (CR1, CD35) on erythrocytes, thus ensuring IC transport to the organs of elimination [25,26] one may speculate on the pathological consequences of reduced immune complex clearance in both organ-specific and non-organ-specific disease. C4 is also involved in chemotaxis of inflammatory cells and phagocytosis of ICs, apoptotic particles and, probably, DNA. However, it must be kept in mind that the effect of a single genetic factor must be interpreted within the shared genetic background in families. Clearly, more studies are needed to elucidate other mechanisms involved.

In summary, we have demonstrated early onset (partial) SLE in a complete C4A-deficient Caucasian child, and thyroid autoimmunity in several heterozygous C4A-deficient family members. More studies in multicase SLE families may uncover the genetic etiologies involved in organ-specific and non-organ-specific autoimmune disease.

REFERENCES

1. **Lau, Y.L., C.S. Lau, S.Y. Chan, J. Karlberg, M.W. Turner**. Mannose-binding protein in Chinese with systemic lupus erythematosus. Arthr Rheum 1996; **39:** 706-708.
2. **Fielder, A.H.L., M.J. Walport, J.R. Batchelor, R.I. Rynes, C.M. Black, I.A. Dodi, G.R. Hughes**. Family study of the major histocompatibility complex in patients with systemic lupus erythematosus: importance of null alleles of C4A and C4B in determining disease susceptibility. Br J Med 1983; **286:** 425-428.
3. **Hauptmann, G., G. Tappeiner, J. Schifferli**. Inherited deficiency of the fourth component of human complement. Immunodeficiency Rev 1988; **1:** 3-22.

4. **Hartung, K., M.P. Baur, R. Coldewey, M. Fricke, J.R. Kalden, H.J. Lakomek, H.H. Peter, D. Schendel, P.M. Schneider, S.A. Seuchter, et al.**, Major histocompatibility complex haplotypes and complement C4 alleles in systemic lupus erythematosus. J Clin Invest 1992; **90:** 1346-1351.
5. **Moser, K.L., B.R. Neas, J.R. Salmon, H. Yu, C. Gray-McGuire, N. Asundi, G.R. Bruner, J. Fox, J. Kelly, S. Henshall, D. Bacino, M. Dietz, R. Hogue, et al**. Genome scan of human systemic lupus erythematosus: evidence for linkage on chromosome 1q in African-American pedigrees. Proc Natl Acad Sci USA 1998; **95:**1469-1474.
6. **Atkinson, J.P., P.M. Schneider**. Genetic susceptibility and class III complement genes. In: **Lahita RG** (Eds.) Systemic Lupus Erythematosus. 3rd ed. San Diego: Academic Press, 1999, pp 91-104.
7. **Sullivan, K.E., N.A. Kim, D. Goldman, Petri M**.A. C4A deficiency due to a 2 bp insertion is increased in patients with systemic lupus erythematosus. J Rheumatol1999; **26:** 2144-2147.
8. **Barilla-LaBarca, M-L., J.P. Atkinson**. Rheumatic syndromes associated with complement deficiency. Curr Opin Rheumatol 2003; **15:** 55-60.
9. **Blanchong, C.A., B. Zhou, K.L. Rupert, E.K. Chung, K.N. Jones, J.F. Sotos, W.B. Zipf, R.M. Rennebohm, C.Y. Yu**. Deficiencies of human complement component C4A and C4B and heterozygosity in length variants of *RP-C4-CYP21-TNX* (RCCX) modules in Caucasians: the load of RCCX genetic diversity on major histocompatibility complex-associated disease. J Exp Med 2000; **191:** 2183-2196.
10. **Law, S.K.A., K.B.M. Reid**. (Eds.) Complement in focus. 2nd Ed. Oxford University Press, 1995.
11. **Rupert, K.L., J.M. Moulds, Y. Yang, F.C. Arnett, R.W. Warren, J.D. Reveile, B.L. Myones, C.A. Blanchong, C. Yung Yu**. The molecular basis of complete complement C4A and C4B deficiencies in a systemic lupus erythomatosus patient with homozygous *C4A* and *C4B* mutant genes. J Immunol 2002; **169:** 1570-1578.
12. **Awdeh, Z.L., C.A. Alper**. Inherited structural polymorphism of the fourth component of human complement. Proc Natl Acad Sci USA 1980; **77:** 3576-3580.
13. **Pistiner, W., D.J. Wallace, S. Nessim, A.L. Metzger, J.R. Kleinberg**. Lupus erythematosus in the1980s: a survey of 570 patients. Semin Arthritis Rheum 1991; **21**: 55-64.
14. **Hochberg, M.C**. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum1997; **40:** 1725.
15. **Corporaal, S., M. Bijl, C.G.M. Kallenberg**. Familial occurrence of autoimmune diseases and autoantibodies in a Caucasian population of patients with systemic lupus erythematosus. Clin Rheumatol 2002; **21**:108-113.
16. **Pyne, D., D.A. Isenberg**. Autoimmune thyroid disease in systemic lupus erythomatosus. Ann Rheum Dis 2002; **61:** 70-72.
17. **Vianna, J.L., Haga, H.J., Asherton, R.A., Swana, G., Hughes, G.R.V.** A prospective evaluation of antithyroid antibody prevalence in 100 patients with systemic lupus erythematosus. J Rheumatol 1991; **18:**1193-1195.
18. **Christiansen, F.T., W.J. Zhang, M. Griffiths, S.A. Mallal, R.L. Dawkins**. Major histocompatibility complex (MHC) complement deficiency, ancestral haplotypes and systemic lupus erythematosus (SLE): C4 deficiency explains some but not all of the influence of the MHC. J Rheumatol 1991; **18**:1350-1358.
19. **Weetman, A.P**. Autoimmune thyroid disease: propagation and progression. Eur J Endocinol 2003; **148:** 1-9.
20. **Beurskens, F.J.M., H. van Dijk**. Does complement component C4A protect from autoimmune disease? Immunol Today 1997; **18:** 199.
21. **Yu, C.Y., Z. Yang, C.A. Blanchong, W. Miller**. The human and mouse MHC class III region: a parade of 21 genes at the centromeric region. Immunol Today 2000; **21:** 320-328.
22. **Hollowell, J.G., N.W. Staehling, W.D. Flanders WD, W.H. Hannon, E.W. Gunter, C.A. Spencer, L.E. Braverman**. Serum TSH, T(4), and thyroid antibodies in the United States population (1988 to 1994): National Health and Nutrition Examination Survey (NHANES III). J Clin Endocrinol Metab 2002; **2:** 489-499.
23. **McCann, V.J., J. McClusky, H. Kelly, P.H. Kay, P.J. Zilko, F.T. Christiansen, R.L. Dawkins**. Thyroid autoimmunity and MHC associated alleles at the C4 locus in patients with type 1 (insulin-dependent) diabetes. Diabetologia 1984; **27:** 124-125.
24. **Colten, H.R., G. Garnier**. Regulation of complement protein gene expression. In: Volanakis, J.E., Frank, M.M. (Eds). The human complement system in health and disease. Marcel Dekker, New York, pp 217-240.
25. **Nelson, R.A**. The immune-adherence phenomenon. Science 1953; **118:** 733-737.
26. **Cornacoff, J.B., L.A. Hebert, W.L. Smead, M.E. van Aman**. Primate erythrocyte-immune complex clearing mechanism. J Clin Invest 1983; **2:** 236-247.
27. **Sim, E., S.J. Cross**. Phenotyping of human complement component C4, a class-III HLA antigen. Biochem J 1986; **239:** 763-767.

28. **Chung, E.K., Y. Yang, K.L. Rupert, K.N. Jones, R.M. Rennebohm, C.A. Blanchong, C.Y. Yu**. Determining the one, two, three, or four long and short loci of human complement *C4* in a major histocompatibility complex haplotype encoding C4A or C4B proteins. Am J Hum Genet 2002; **71:** 810-822.
29. **Schreuder, G.M.Th., C.K. Hurley, S.G. Marsh, M. Lau, M. Maiers, C. Kollman, H. Noreen**. The HLA dictionary 2001: a summary of HLA-A, -B, -C, -DRB1/3/4/5, -DQB1 alleles and their association with serologically defined HLA-A, -B, -C, DR and –DQ antigens. Hum Immunol 2001; **62:** 826-849.

Chapter 9

General discussion

The Ancient Complement System: Role in Microbial Clearance, Lipid Transport and Prevention of Autoimmunity

This study concentrates on the complement system, which is part of the nonspecific or 'innate' immune system. The complement system may be considered a primordial enzyme system that was, before enhancing cytolysis or 'killing', primarily involved in the enhancement of cellular transportation of 'opsonized' or C3b-laden particles through the circulation towards eliminatory organs in the human body.

In humans and other primates, the adherence of these opsonized particles to the C3b-receptor (CR1) on cells is called 'immune adherence'. Although erythrocytes do not carry the largest number of CR1s on their surface, their receptors are clustered in such a way that erythrocyte 'immune adherence' is more efficient than on e.g. neutrophils. Moreover, about 95% of CR1s in the circulation are found on erythrocytes, making the role of erythrocytes in transport and elimination of microbes, immune complexes and tumor cells indispensable.

Opsonization of immune complexes and transport thereof to the spleen and liver is a well-recognized function of the human complement system, as is microbial opsonization. Thus, immune complex precipitation disease in vessels (e.g. SLE) and organs (e.g. thyroid autoimmunity) is prevented. Microbial opsonization facilitates their adherence to erythrocytes and, subsequently, microbial transport to one of the fixed organs of phagocytosis where intracellular killing of most of the microbes can take place. However, certain (myco)bacteria and parasites have adapted and evolved in such a way that intracellular survival and even replication is ensured.

Our studies concerned the investigation of the prevalence of MBL deficiency in the Dutch population (chapter 3), and the development of a hemolytic MBL assay, which may be of great value for the further analysis of MBL and/or MBL-associated protein deficiency (chapter 4). By adapting the functional MBL assay, microorganism-induced MBL activation could be investigated, leading to a new way of looking at MBL-activating potencies of microbes, including their filterable products, e.g. presumably 'blebs' which are products of meningococci (chapter 5). Furthermore, *Legionella pneumophila* was found capable of activating the MBL pathway, although this bacterium proved to be a weak *in-vitro* MBL activator. However, this raised the question as to what the role of MBL in legionellosis might be. In 1999 in the Netherlands, an outbreak of legionellosis occurred at the well-attended Flower Show in Bovenkarspel. By comparing the MBL status of sera from that patient cohort versus age-matched controls from the Dutch population, we could conclude that MBL deficiency is a risk factor for pneumonia with *Legionella pneumophila.* The prevalence of MBL deficiency (< 0.20 μg per ml) in a group of asymptomatic, but exposed workers was similar to the controls (Chapter 6).

Complement factor C4 is shared by both the lectin and classical complement pathways. Like all complement proteins, it is synthesized mainly in the liver. However, the thyroid and the adrenals – being endocrine organs – also produce complement C4

transcripts but, as yet, the reason for these organs to synthesize C4 is unknown. Possibly, the link between (auto)immunity and the endocrine system can be sought at the level of the diseased organ, as has been described in chapter 8. The great genetic diversity of human *C4A* and *C4B* genes leads to both quantitative and qualitative variations of the polymorphic C4A and C4B proteins, possibly as an evolutionary answer to selection pressure against a great many microorganisms.

In this thesis, the possibility is considered that other targets of opsonization by the complement system are, or can be:

(a) Lipoproteins, of which at least apolipoprotein B is covered with the MBL-binding carbohydrates mannose, N-acetylglucosamine and fucose, which activate the complement system via the lectin pathway, as is shown in chapter 7. Normally, after an abundant meal, fat is absorbed though the small intestine, where exogenous lipids are taken up and incorporated into lipoproteins. These are secreted as chylomicrons into the lymph. After entering the systemic circulation, a modulation takes place, mainly involving triglyceride hydrolysis by the enzyme lipoprotein lipase (LPL). The remaining particles are called 'remnants'. In the normal situation, most of these pro-atherogenic particles are transported to the liver where they are cleared from the circulation by a receptor-mediated mechanism. How the transport to the liver takes place is, at present, not fully understood. Lipoproteins activate MBL, which is supposedly followed by lipid particle opsonization and subsequent adherence of lipoproteins, chylomicrons, or remnants to erythrocytes to be, presumably, transported to and cleared by the liver. Although more proof is certainly needed, *ex-vivo* erythrocytes do carry more fat particles on their surface after lipid intake as compared to fasting erythrocytes. Moreover, MBL deficiency leads to severe atherosclerosis. It seems that the lectin pathway is necessary for clearance of lipids, although proving this hypothesis will need various longstanding experiments.

(b) DNA, which is known to activate an as yet unknown complement pathway and which, hypothetically, may have been the target of the primitive immune system via MBL binding to pentose sugars; these experiments still need to be done.

By now, it is clear that the lectin and alternative pathways of complement activation are the most ancient ones when compared to the classical and terminal complement pathways. Possibly, the two pathways constitute two intimately linked parts of a single activation pathway in the primitive, jawless deuterosomes, whereas the antibody-dependent, so-called 'classical' pathway of complement activation must have evolved about 450 million years ago, when antibodies developed. Thinking in evolutionary terms, one may consider that the ancient complement system - as it was - expanded its original function in response to microbes, simply because infectious diseases are a heavily selective principle that may kill animals (including humans) before sexual maturity. This is why the original function of the ancient complement system may have been maintained, while the complement system itself drifted away towards a defense mechanism and evolved by the inclusion of foreign components and by gene duplication, e.g. in the C3/C4/C5/α_2M family. Other evolutionary examples are the MBL-derived C1-complex

that has capacity to bind IgM and IgG antibodies, its inhibitor C1-INH (C1 esterase inhibitor) that actually deserves the more correct name of MBL-INH, and the perforin-related terminal complement pathway consisting of the components C5b, C6, C7, C8, and C9. MBL was a target for the newly evolved IgA.

According to our hypothesis, the lectin pathway may have initially been created in evolution as a mediator of clearance of so-called 'naked' DNA and/or as an opsonizing step in the clearance of lipid particles from the circulation. Moreover, it was also kindly adapted by the immune system to function as a kind of matrix for evolutionary development of the extensive complement system as currently described in the immunological textbooks.

During thousands of years, point mutations have arisen in MBL, possibly under the influence of radioactive events and UV light during evolution. These deviations from the normal *mbl* gene and promoter gave rise to lowered MBL serum levels. The mutations have been positively selected in over 11% of the northern European population and in more than 30% of the African population, the latter most probably under the influence of infectious diseases like tuberculosis, leprosy, and visceral leishmaniasis, as examples of severe infection with intracellular pathogens for which MBL deficiency may be protective.

MBL seems to provide a selective advantage for infection with certain intracellular pathogens like mycobacteria and *Leishmania*. Our finding that MBL deficiency increases susceptibility for disease with *Legionella pneumophila*, also an intracellular microorganism, does not fit that idea. Scanty reports in mice and man suggest that MBL modulates inflammation by triggering proinflammatory cytokine release. The contradictory results raise new ideas for research, e.g. susceptibility of individuals with normal serum MBL levels for the intracellular bacterium *Salmonella typhi*, and the subsequent need for vaccination against typhoid fever in travelers. Moreover, one may also argue about vaccination against meningococcal disease in subjects with normal serum MBL levels, and speculate on complement studies in the 'meningitis belt' in Africa that could elucidate a connection between MBL deficiency and the occurrence of outbreaks of meningococcal meningitis.

The positive selection of defective MBL alleles in the population is an important cause of MBL deficiency-associated hypertriglyceridemia and subsequent atherosclerosis. A similar genetic selection may have occurred with the C4A gene. Homozygous C4A deficiency leads to autoimmune diseases like systemic lupus erythematosus (SLE), while heterozygous C4A, which is also linked with SLE, is associated with autoimmune thyroiditis as well. C4A null alleles (C4A*Q0) have not only been associated with an increase in circulating immune complexes, but also with premature peripheral atherosclerosis. These data indirectly support our hypothesis of the necessity of early complement components that are shared by the lectin and the classical pathway, in the elimination of lipid particles from the circulation.

Taken together, our findings and hypotheses may lead to an exciting new line of research that can, eventually, lead to the rewriting of immunological and physiological textbooks.

In conclusion, the complement system does not only play an important role in the field of immunology and (medical) microbiology but it may very well be, as is shown in this thesis, one of the key players in the fields of endocrinology and lipidology. The more we understand and are able to prove that physiological systems are entwined, both in nature, and in man as part of nature, the bigger the notion will be that joining forces between medical doctors in various fields and researchers in biology will exponentially increase our knowledge and understanding of this intriguing aspect of the world.

NEDERLANDSE SAMENVATTING EN DISCUSSIE

Dit proefschrift heeft als onderwerp het zogenoemde **Complement Systeem**, een uitgebreid en – evolutionair gezien – zeer oud afweersysteem tegen wezensvreemde micro-organismen. Het complement systeem bestaat uit meer dan 30 eiwitten, en is in staat om heel snel te reageren op ongewenste indringers van buitenaf, dat wil zeggen binnen seconden tot dagen. Dit is in tegenstelling tot de afweerstoffen, die pas na één of meerdere weken worden gevormd.

De centrale rol in dit systeem wordt gespeeld door het eiwit **C3** dat via meerdere activatie routes kan worden aangezwengeld (zie **hoofdstuk 1**, figuur op blz. 12), waarna omzetting van C3 naar C3a en C3b volgt. Het aldus ontstane C3b kan zich hechten aan oppervlakten van ongewenste micro-organismen, immuuncomplexen en cellen, een proces dat 'opsonisatie' wordt genoemd. Opsonisatie is het 'hapklaar maken' van deeltjes voor verdere verwerking in speciale opruimcellen ('eetcellen' of macrofagen) in milt, lever en longen. Zo worden met C3b-beladen ('geopsoniseerde') microben via de C3b-receptor gebonden aan rode bloedcellen en zo door de bloedbaan getransporteerd in de richting van de lever en milt. In de vaste, fagocyterende cellen van bijvoorbeeld de milt vindt intracellulaire afdoding plaats van de meeste soorten microben.

Het complement systeem kan op drie manieren worden geactiveerd (zie blz. 12). De drie complement activatie routes heten:

- de **lectine route**, met mannose-bindend lectine (**MBL**) als hoofdspeler. MBL bindt gemakkelijk aan de suikers mannose, N-acetylglucosamine en fucose. Deze suikers kunnen voorkomen aan de buitenkant van bacteriën, virussen, parasieten en schimmels. Als de binding tot stand is gebracht, dan wordt de lectine route geactiveerd.
- de **alternatieve route**, welke wordt aangezwengeld door oppervlakten van microben en tumorcellen.
- de **'klassieke' route**, die antilichamen nodig heeft om te kunnen worden geactiveerd

Er bestaat ook een vierde complement route, de zogeheten **terminale complement route**, een effector route waarvan het eindprodukt ('membrane attack complex') in staat is om te doden door gaatjes te maken in cellen en microben (bijv. de meningokok).

In **hoofdstuk 2** wordt uitgelegd wat de basis heeft gevormd voor dit proefschrift: een kleine epidemie van hersenvliesontsteking door groep C meningokokken in Nijkerkerveen en een verhoogde risico bij een getroffen familie, waarbij een duidelijk verband werd vastgesteld tussen doorgemaakte meningokokkenziekte en zeer lage MBL spiegels in het bloed.

Om vast te kunnen stellen hoe verlaagde MBL spiegels in bloedserum zijn verdeeld over de algehele Nederlandse bevolking, is een steekproef verricht bij een groep van

ruim 400 gezonde Nederlanders in de leeftijd tussen 3 en 85 jaar. Dit onderzoek staat beschreven in **hoofdstuk 3**. Wij vonden in deze onderzoeksgroep een verminderde hoeveelheid MBL bij 12.2% van onze onderzoeksgroep, en bijna onmeetbare MBL spiegels bij 3.1%. Verlaagde MBL spiegels zijn te wijten aan veranderingen in het *mbl* gen en in de promoter, waarbij er 7 verschillende genetische combinaties bekend zijn die tot op zekere hoogte de MBL bloedspiegel bepalen. Omdat MBL een oud eiwit is, zijn er waarschijnlijk in de loop van de duizenden jaren puntmutaties ontstaan in het genetisch materiaal, mogelijk ten gevolge van radioactiviteit en UV-straling. Deze mutaties zijn terug te vinden bij meer dan 11% van de Noord-Europese bevolking, en bij meer dan 30% van de bevolking van Afrika. Dat in Afrika zo'n hoog percentage afwijkend MBL wordt gevonden, is mogelijk te wijten aan de evolutionaire invloed van infectieziekten als tuberculose, lepra, en kala azar (viscerale leishmaniasis), ziekten veroorzaakt door langzaam groeiende, intracellulaire verwekkers waarbij een tekort aan MBL juist lijkt te beschermen.

De twee functionele testen die in de klinische praktijk worden gebruikt om tekorten in het complement systeem op te sporen zijn gericht op de klassieke en alternatieve complement activatie routes. Deze testen zijn niet geschikt om een tekort aan MBL te detecteren. In **hoofdstuk 4** wordt een door ons ontwikkelde functionele MBL test beschreven; deze kan van grote betekenis zijn voor de verdere analyse van deficiëntie van MBL en/of de hulpeiwitten, de zogeheten MASPs (MBL-associated serine proteases). De ingrediënten zijn eenvoudig: testserum, bakkersgist als MBL-activator, MBL-deficiënt serum als leverancier van alle complement factoren behalve MBL, en rode bloedcellen van kippen die fungeren als willekeurig slachtoffer van het 'membrane attack complex', waardoor de rode bloedcellen uiteen vallen. De relatieve mate van roodkleuring in het testsysteem is dan een maat voor MBL activiteit.

Door de bakkersgist uit de functionele MBL test te vervangen door (verschillende concentraties van) andere micro-organismen, kon worden onderzocht in welke mate de onderzochte micro-organismen MBL activatie teweeg brengen, zoals beschreven in **hoofdstuk 5**. Op deze manier kan het belang van de lectine route bij de afweer tegen verschillende bacteriën en gisten worden gemeten. Ook concludeerden we dat filtreerbare bacteriële producten van meningokokken (mogelijkerwijs stukjes buitenmembraan) een grote rol spelen bij complement activatie via MBL. Met behulp van de functionele test werd de MBL-activerende eigenschap van *Legionella pneumophila* onderzocht, en alhoewel deze bacterie een zwakke MBL activator bleek, werd de nieuwsgierigheid gewekt naar de rol van MBL bij *Legionella* infectie.

De onderzoeksvraag "welke rol speelt MBL bij *Legionella* infectie?" is nader uitgewerkt in **hoofdstuk 6**. In 1999 vond in Bovenkarspel, waar de drukbezochte West-Friese Flora werd gehouden, een *Legionella* epidemie plaats. Door MBL te meten in patiëntensera versus controlesera uit de Nederlandse bevolking, konden wij concluderen dat een ernstig tekort aan MBL een risicofactor vormt voor longontsteking met *Legionella*

pneumophila. De prevalentie van MBL tekort in een groep asymptomatische, doch bewezen besmette werkenden bij de Flora was vergelijkbaar met de controlegroep, die qua leeftijd overeen kwam.

Als men resultaten uit hoofdstuk 2 en 5 combineert met de uitkomsten van hoofdstuk 6, kan men concluderen dat MBL-deficiënte personen een toegenomen kans hebben op infecties met sterke MBL activatoren die ook relatief snel groeien in het laboratorium. Uit deze conclusie kunnen ideeën voor verder onderzoek ontspruiten, zoals bijvoorbeeld naar vatbaarheid voor tyfus (*Salmonella typhi*) bij reizigers en de daarmee samenhangende noodzaak voor vaccinatie. Als men in dezelfde trant door redeneert, dan kunnen er vraagtekens worden gezet bij de meningokokken-vaccinatie voor personen met een normale MBL serum spiegel, en kan men speculeren over uitkomsten van complement studies in de 'meningitis belt' in Afrika.

In **hoofdstuk 7** laten we zien dat lipoproteïnen in staat zijn om MBL te activeren. Het is bekend dat apolipoproteïne B, een belangrijk suikerig vet van de chylomicronen, aan de buitenkant is bezaaid met mannose, N-acetylglucosamine, en fucose, alle drie sterk MBL-bindende suikers. Normaliter wordt, na een overvloedige maaltijd, vet geadsorbeerd door de dunne darm, waar de vetten worden opgenomen en ingebouwd in lipoproteïnen. Deze worden uitgescheiden als chylomicronen (vetbolletjes) naar de lymfe waarna, als de bloedbaan is bereikt, een aanpassing plaatsvindt. Dit betreft vooral afbraak via hydrolyse van triglyceriden door het enzym lipoproteïne lipase (LPL). De resterende deeltjes worden 'remnants' genoemd. Theoretisch gezien zou er 'opsonisatie' van vetpartikels kunnen plaatsvinden, gevolgd door aanhechting van lipoproteïnen, chylomicronen, of 'remnants' aan rode bloedcellen om daarna te worden getransporteerd naar de lever, waar ze worden onttrokken aan de circulatie via een receptor gemedieerd mechanisme. Hoe dit transport plaats vindt is onvoldoende bekend. Wat verder bekend is, is dat een tekort aan MBL leidt tot ernstige atherosclerose.

Opsonisatie van immuuncomplexen is een bekende functie van het complement systeem. Na binding aan rode bloedcellen via CR1 volgt transport van deze complexen naar de milt en lever, Op deze manier wordt het neerslaan van immuuncomplexen in de bloedvaten en organen voorkomen. Als immuuncomplexen wel neerslaan in de bloedvaten dan kan dat leiden tot systemische lupus erythematosus (SLE). In **hoofdstuk 8** worden leden van een familie besproken die aan SLE of schildklier-autoimmuniteit lijden. Er is bij deze familie gekeken naar een samenhang tussen C4A deficiëntie en klinische ziekte. Zoals bij bijna alle complement eiwitten het geval is, wordt C4 vooral in de lever gesynthetiseerd. Hoewel ook de schildklier en bijnieren – ook endocriene (hormoonproducerende) organen – complement C4 transcripten produceren is het tot nu toe onduidelijk waarom deze organen C4 aanmaken. Het is mogelijk dat er een verband tussen (auto)immuniteit en het endocriene systeem gevonden kan worden op het niveau van het zieke orgaan zelf. De grote genetische diversiteit in *C4A* and *C4B* genen leidt tot een grote kwantitatieve en kwalitatieve variatie in C4A en C4B eiwitten,

waarschijnlijk een evolutionair antwoord op de selectiedruk veroorzaakt door de verscheidenheid aan micro-organismen.

In dit proefschrift wordt beschreven dat er naast micro-organismen, tumorcellen en immuuncomplexen, mogelijkerwijs andersoortige partikels zijn die complement activeren, zoals lipoproteïnen en DNA (nucleïnezuur). Hoewel (nog) niet door ons bewezen, is het goed theoretisch mogelijk dat DNA via de lectine route het complement systeem activeert. Op theoretische gronden mag men aannemen dat via de pentose suikers binding van DNA aan MBL zou kunnen plaatsvinden. Zogeheten 'naakt' DNA zou het eerste doelwit van het primitieve afweersysteem kunnen zijn geweest. Volgens onze hypothese is de lectine route vooraleerst ontstaan als een mediator van de klaring van DNA en/of als een opsoniserende stap in de klaring van vetdeeltjes uit de bloedbaan. Hoe dan ook, het primitieve complement systeem werd een soort matrix voor de evolutionaire ontwikkeling van het uitgebreide complement systeem zoals op dit moment beschreven.

Door de uitselectie van afwijkende MBL allelen (een van de genen die op een bepaalde plaats in het chromosoom kan voorkomen) in de bevolking zou een belangrijke oorzaak kunnen zijn voor de MBL-deficiëntie-geassocieerde hypertriglyceridemie en atherosclerose. Een vergelijkbare selectie zou plaatsgevonden kunnen hebben bij het C4A gen. Homozygote C4A deficiëntie (het ontbreken van genen op beide chromosomen) leidt tot auto-immuunziekten als 'systemic lupus erythematosus' (SLE) terwijl heterozygote C4A deficiëntie (met ongelijke allelen), welke ook een link heeft met SLE, tevens is geassocieerd met auto-immuun schildklierontsteking. Het ontbreken van (een deel van) de C4A genen is niet alleen geassocieerd met een toename van circulerende immuuncomplexen in de bloedbaan, maar ook met vroegtijdige perifere atherosclerose. Deze data ondersteunen op een indirecte manier onze hypothese dat complementfactoren uit zowel de lectine als de klassieke route een rol spelen bij de eliminatie van vetdeeltjes uit de circulatie.

Dit alles in overweging nemende, zouden onze bevindingen en hypothesen kunnen leiden tot een spannende, nieuwe onderzoekslijn die eventueel zou kunnen leiden tot het herschrijven van immunologische en fysiologische boeken.

Kortom, het complement systeem speelt niet alleen een belangrijke rol in de immunologie en (medische) microbiologie maar, zoals dat wordt beschreven in dit proefschrift, het zou ook een belangrijke speler kunnen zijn in het veld van de endocrinologie en lipidologie. Hoe meer we begrijpen en kunnen bewijzen dat fysiologische systemen onderling verbonden zijn, zowel in de natuur als in de mens die daarvan deel uitmaakt, hoe groter het besef dat door het samenwerken van artsen in verschillende specialismen en onderzoekers in de biologie onze kennis en ons begrip van dit boeiende aspect van de wereld exponentieel zal groeien.

DANKWOORD

Hans, jij hebt mij de kans gegeven om met plezier onderzoek te doen, en ik prijs mijzelf gelukkig een mentor te kennen als jij. Het gemak waarmee je samenhang ontdekt tussen verschillende fysiologische systemen is heel inspirerend geweest. Jouw veelzijdigheid heeft er ook toe geleid dat we niet alleen onderzoeksmogelijkheden, maar ook de rest van 's levens thema's niet onbesproken voorbij hebben laten gaan. En bumperen is ook een vak! Pieternel, je hebt jaren je man aan mij uitgeleend op doordeweekse avonden zonder ook maar één keer te mopperen, hulde!
Prof. Verhoef, hoofd van het Eijkman-Winkler Centrum, wil ik graag bedanken voor de kans die ik kreeg om de promotie af te maken. Zonder nulaanstelling was het er zeker niet van gekomen.
Cees, jij was degene die in het begin van mijn opleiding met mij meedacht om mijn wens tot het doen van onderzoek in vervulling te doen gaan, zowel onder de lunch en tijdens het fietsen naar huis. Het spreekt voor zich dat een arts-microbioloog die ook gepromoveerd is op 'Complement' een waardige copromotor zal zijn!
Theo, ELISA-tovenaar, muizenverslinder en strijder tegen onrecht, je hebt mij jaren geleden je steun toegezegd en dat ook altijd laten merken. Ik ben jou veel verschuldigd.
Piet, mijn inwijding in de geheimen van de eiwitzuivering en complement testen heb ik aan jou te danken. En dat terwijl dat niet eens echt je taak was! Het zegt veel over je bereidheid anderen te helpen. Bertie, altijd een rots in de branding als er proeven gedaan moesten worden; als er weer eens kippenbloed gehaald moest worden was jij er als de kippen bij. Elisabeth, EB, als groentjes zijn we samen het onderzoek in gerold; wat hebben die plakeiwitten ons te grazen gehad! Niettemin zijn er monoklonalen naar je vernoemd. Alle studenten die in de loop van de jaren zich in ons onderwerp hebben verdiept: dank voor de inzet. Mireille, Dr. "het komt wel goed", we zijn samen in het "Diak" begonnen in 1995, jij met je onderzoek en ik met mijn opleiding. In oktober a.s. zijn we allebei gepromoveerd arts-microbioloog. Dat we nog veel samen mogen kokkerellen en duiken (en Spaans leren)! Ellen, het sprak vanzelf dat je mijn paranimf zou zijn, want jij was natuurlijk een fantastisch voorbeeld voor mij, ook al had ik niet jouw snelheid van schrijven. Alle avond-en weekendwerkers in het Eijkman-Winkler Centrum: dank voor alle gezelligheid.
Ron Hendrix, als directe collega en directeur van het Laboratorium Microbiologie Twente Achterhoek vond jij dat ik, net als jij, de kans moest krijgen om het promotie onderzoek af te maken, en je was ook nooit te beroerd om een dienst over te nemen. Ik hoop dat je, nu ik weer meer aanwezig ben (ook in de geest), zelf weer meer zal gaan pipetteren en schrijven. En anders schrijf ik toch gewoon jouw resultaten op! Flip Rothbarth, zowel als directe collega in Twente en als collega-uit-de-Randstad heb je mij altijd gesteund en moed ingepraat. Waar ik mij ook bevond in den lande, je wist mij altijd te vinden. De 'Vrijdagen' in Utrecht waren alleen mogelijk door steun van alle collega microbiologen: Wim, Eveline, en later Bert.
Petra, al zijn de foto's niet gelukt, je bereidwilligheid om in te springen verdient alle lof. Richard, het priegelwerk van het enige kleurenplaatje in het boekje komt op jouw naam te staan; je bent niet voor niets de fotograaf van het Enschedese lab.
Ouders, na gedane arbeid komt nu meer tijd van leven, maar ambitie zit –net als reislust– misschien toch in de genen. Titia, natuurlijk moesten jouw tekentalenten op de voorkant.

Liesbeth, gelukkig kan de lay-out jouw goedkeuring dragen. Marijke, nu wordt ik toch een echte dokter. Dianne, fantastisch dat je mij vaak 's ochtends belde om me weer eens een hart onder de riem te steken. Suzanne F., je bleef belangstellend zelfs al heb ik als afschrikwekkend voorbeeld gediend. Alie, gelukkig boden de ritjes tussen Twente en Utrecht ook wel eens de gelegenheid om in Deventer te pauzeren. Je commentaar op eerdere versies van de samenvatting was zeer treffend. Frank en Mary, wat is het alweer lang geleden dat we in Maine de discussie bespraken.
Veel dank ben ik ook verschuldigd aan mijn Peugeot 405, die mij tienduizenden kilometers veilig heen en weer reed tussen Twente en Utrecht alvorens total loss te geraken na alweer een vermoeiende rit. Je kreeg een waardige opvolger in de vorm van een lekker verende Citroën.
Mijn allerliefste liefste Incapriester Joris, jouw geduld met mij en al die fratsen die ik weer ging uithalen in de avonden en weekenden, en je onvoorwaardelijke liefde en steun zijn randvoorwaarden geweest voor het slagen van dit promotieproject. Gelukkig dat jij ook nog onderzoek deed naar de tenten, slaapzakken, en kookspullen die onontbeerlijk zijn voor geslaagde uitwaaivakanties in de Alpen, Altai, Andes, en de Sinaï. Ik hoop dat we samen nog veel onderzoek gaan doen naar de verbazingwekkende natuur en cultuur in deze wereld.
Allen die hebben mogen meegenieten van mijn gesjees en gesjouw met koffers en zware boodschappentassen vol literatuur, van mijn slaapgebrek, tijdgebrek en gejaagdheid, en allen die ik niet met name heb genoemd: het kan alleen maar nog beter worden!

CURRICULUM VITAE

De schrijver van dit proefschrift werd op 21 mei 1964 geboren als tweede van een tweeling in het toenmalige Diakonessenziekenhuis in Arnhem. Na bezoek aan de kleuterschool 'de Narcis' liep zij door naar de lagere school 'De Sterrenberg'. Eind 1971 vertrok het gehele gezin Kuipers, bestaande uit de ouders met vier dochters, vanuit Arnhem naar India, waar zij zich vestigden in de plaats Poona (thans Pune genaamd). Op dat moment brak – letterlijk – een oorlog uit. Na een half jaar privē onderwijs mocht het leergierige meisje per auto met chauffeur naar de meisjesschool "St. Mary's", alwaar onder de krachtige leiding van Anglicaanse nonnen werd kennisgemaakt met het Britse schoolsysteem, inclusief prijzenstelsel. Eenmaal terug in Nederland doorliep zij VWO-B aan het Christelijk Atheneum te Arnhem. Vanaf 1982 studeerde zij Geneeskunde aan de Universiteit Utrecht. In 1987 keerde ze terug naar India voor een stage in een lepracentrum in Tamil Nadu. In 1989 werden een aantal co-assistentschappen gelopen in Suriname en Curaçao, waarna eind 1990 het artsexamen met goed gevolg werd afgelegd.

In 1991 was zij ruim 100 uur per week werkzaam in Frimley, Surrey, Engeland, als assistent Chirurgie bij lever- en pancreasspecialist Mr. I. Paterson. Daarna volgde gedurende een jaar een assistentschap op de Intensive Care van ziekenhuis 'de Weezenlanden' in Zwolle onder leiding van thoraxanesthioloog B. Mooi. In januari 1993 werd het Amerikaanse artsdiploma behaald (FMGEMS). Om brood mēt kaas te kunnen verorberen werd daarna de functie vervuld van Clinical Research Associate bij MIRAI in Amsterdam.

De loopbaan in de Microbiologie begon in februari 1994 met een AGNIO-schap Medische Microbiologie bij het SSDZ (Stichting Samenwerking Delftse Ziekenhuizen) en Reinier de Graaf Gasthuis te Delft. Dit was ook de plaats waar zij haar geliefde Joris F. van Eekhout ontmoette. Van 1995 tot 1999 was zij in opleiding tot arts-microbioloog in het Academisch Ziekenhuis Utrecht bij Prof.dr. J. Verhoef, en in het Diakonessenhuis Utrecht bij Dr. R. Diepersloot. Begin 1997 begon zij aan de voorbereiding van dit proefschrift onder leiding van Prof.dr. H. van Dijk.

Sinds 1 maart 1999 is zij als arts-microbioloog verbonden aan het Laboratorium Microbiologie Twente Achterhoek te Enschede. Van daaruit is zij werkzaam als consulent klinische microbiologie en infectieziekten, en is tevens staflid van het Streekziekenhuis Midden-Twente te Hengelo.