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

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CHAPTER 4

MBL and liver transplantation

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

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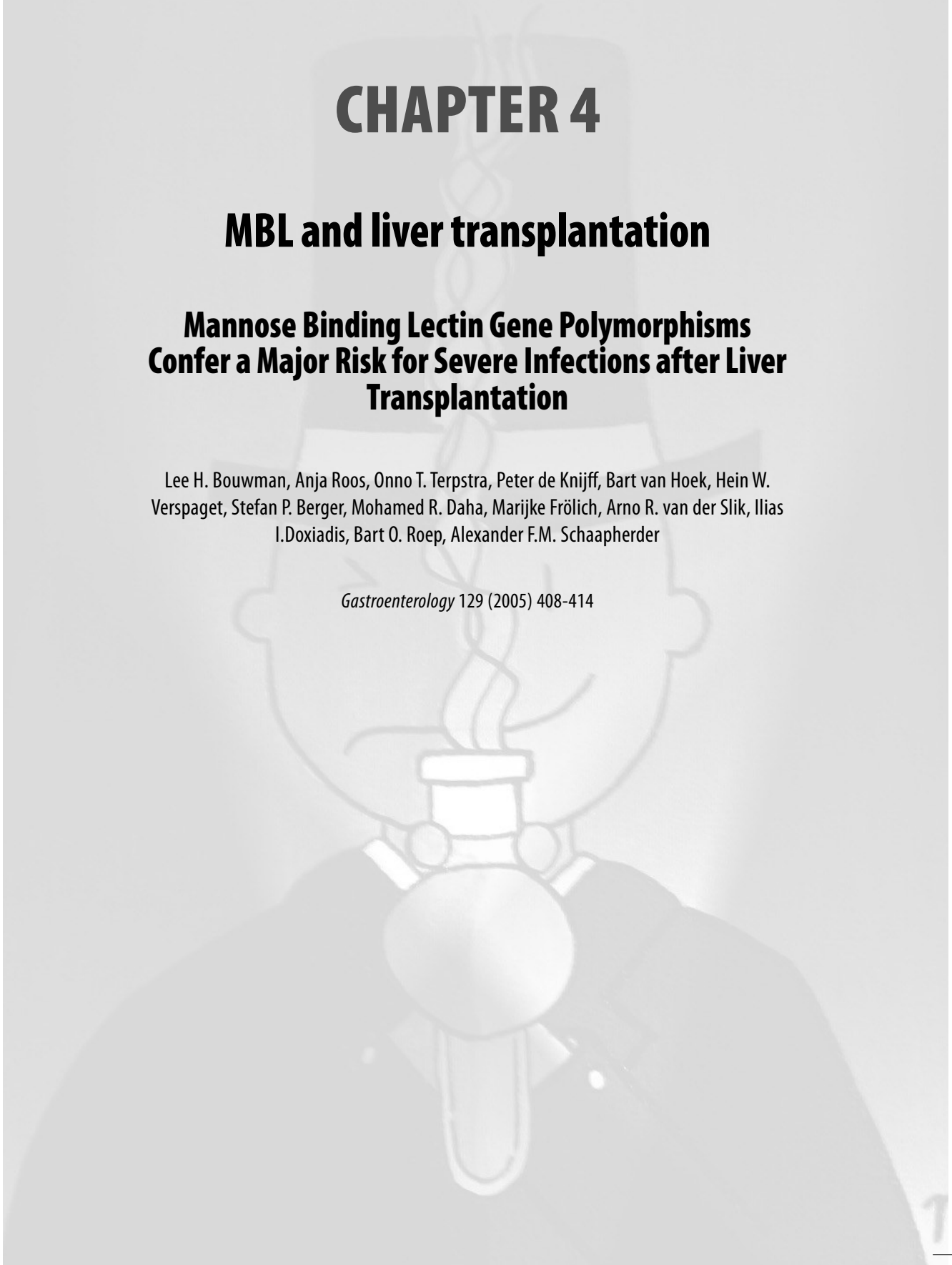

ABSTRACT

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

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

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

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

INTRODUCTION

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

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

Exon 1 of the *mbl-2* gene, which is located at chromosome 10, contains three known single nucleotide polymorphisms (SNPs) at codons 52 (Arg→Cys), referred to as allele 'D', codon 54 (Gly→Asp, allele 'B') and codon 57 (Gly→Glu, allele 'C') (3). These SNPs are associated with low serum concentrations, disturbed polymerization and impaired function of MBL (2, 4). Dependent on ethnicity, the allele frequency of variant alleles B, C and D, commonly referred to as O-alleles, may be above 40% (wildtype = A/A). In addition to the three SNPs in exon 1, there are several other polymorphic sites located in the MBL promoter region, including SNPs located at positions -550 (H/L variant), and -221 (X/Y variant), both G to C nucleotide substitutions. The common allele A of exon 1 is associated with the following haplotypes: HYA, LYA and LXA, exhibiting respectively high, intermediate and low promoter activity and serum MBL levels. The structural alleles carry the following haplotypes: LYB, LYC and HXD (5, 6).

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

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

PATIENTS AND METHODS

Patients

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

MBL genotyping

DNA from all 49 liver donors and from 25 recipients was routinely isolated from blood or tissue samples. MBL SNPs at codon 52, codon 54 and codon 57 of the *mbl2* gene were typed by pyrosequencing (P. de Knijff and A. Roos, submitted). The MBL genotype of carriers of one or two variant allele(s) (B, C, or D alleles) was designated as A/O and O/O, respectively, whereas the MBL genotype of only wildtype allele carriers at all three positions were designated as A/A. For analysis, carriers of A/O and O/O MBL genotypes were considered as one group (MBL-variant).

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

72°C. For visualization, the amplification products were run on a 1.5% (w/v) agarose MP gel (Boehringer Mannheim) prestained with ethidium bromide.

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

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

MBL concentration

MBL serum concentrations were measured blinded in all serum samples by sandwich ELISA essentially as previously described with some modifications (4). Briefly, plates were coated with mAb 3E7 (anti-MBL mAb kindly provided by Dr. T. Fujita, Fukushima, Japan) at 5 µg/ml. Sera were diluted in PBS containing 0.05% Tween-20 and 1% BSA. MBL was detected using dig-conjugated mAb 3E7, followed by HRP-conjugated sheep anti-dig antibodies (Boehringer).

Western blotting

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

Cholinesterase concentration

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

Clinical data

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

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

Statistical analysis

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

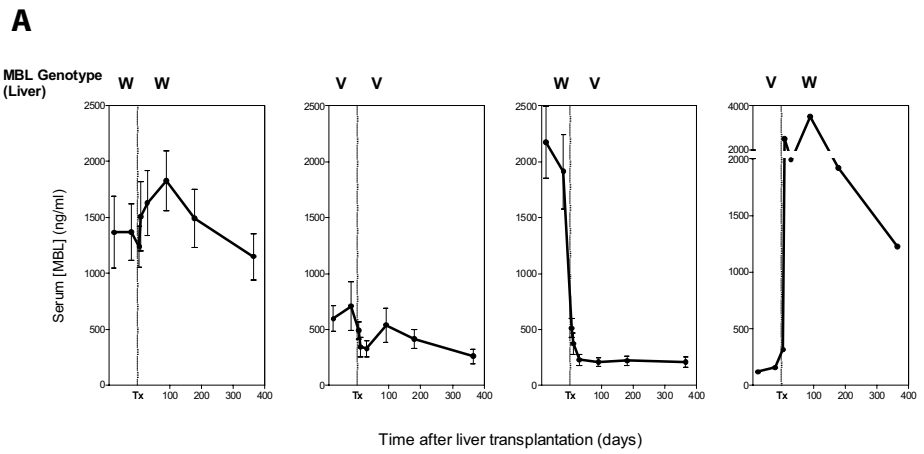

Figure 1: Hepatic production of serum MBL.

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

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

RESULTS

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

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

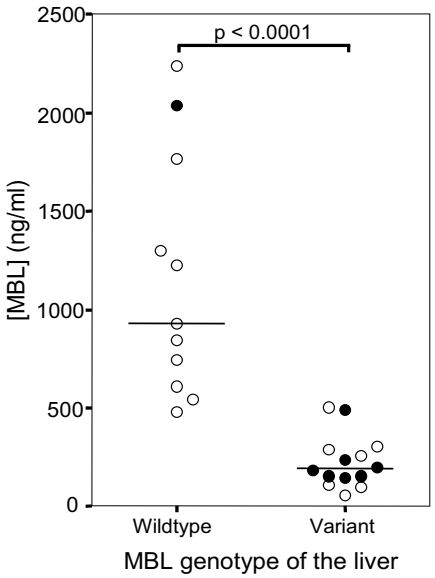

Figure 2: Donor MBL genotype determines MBL serum concentration after transplantation.
MBL serum concentration at 1 year following transplantation in patients stratified according to the MBL genotype of the donor liver. Patients with clinically significant infections (CSI) are represented by black dots.

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

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

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

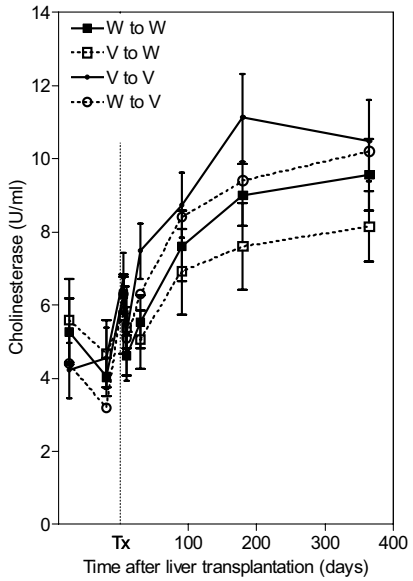

Figure 3: Cholinesterase as a marker for liver function.
Cholinesterase serum concentration in liver transplant recipients stratified according to the MBL genotype of the liver [W: MBL wildtype (A/A); V: MBL-variant (A/0 or 0/0)]. Error bars indicate the standard error of the mean. (W to W: Recipients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

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

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

DISCUSSION

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

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

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

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

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