Recombinant mannan-binding lectin (MBL) for therapy

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Abstract
Mannan-binding lectin (MBL) is a plasma protein involved in the innate immune response. It binds to a number of micro-organisms and promotes killing of these through complement activation either directly or through opsonization. Clinical evidence indicates that in a variety of situations genetically determined low MBL levels are associated with increased susceptibility to infections. Infusions of plasma-derived MBL into MBL-deficient individuals was found to be safe in preliminary trials, but we considered that sufficient production and product safety could only be achieved through synthesis of recombinant MBL. A transfected human cell line produces MBL showing the same biological activity as plasma-derived MBL, and an essentially identical profile on MS. The production has been scaled up and clinical trials will start this year.

Introduction
Mannan-binding lectin (MBL) is a plasma protein produced by hepatocytes [1]. It is an oligomer of structural subunits each composed of three O-glycosylated polypeptide chains of identical amino acid sequence (see [2] for a recent review). Each subunit thus presents three carbohydrate-recognition domains (‘CRDs’), which show affinity for terminal, non-reducing sugars presenting horizontal 3- and 4-hydroxyl groups (e.g. mannose, glucose, N-acetylgalcosamine and N-acetylmannosamine, but not galactose). The affinity for monosaccharides is low (10⁻³ to 10⁻⁴ M) but high-avidity binding (10⁻⁸ to 10⁻¹⁵ M) to pathogen-associated molecular patterns is ensured through multiple binding sites. Many micro-organisms, including bacteria, fungi and viruses have thus been reported to bind MBL. Importantly even weak binding, undetectable by flow cytometry, can mediate killing [3]. MBL does not bind to normal tissues, but binding to cancer cells and apoptotic cells has been observed.

MBL circulates in complexes with MBL-associated serine proteases, MASP-1, -2 and -3, and a small, non-enzyme peptide, MAp19 or sMAP [4]. The MASPs have a domain structure identical to that of C1r and C1s of the classical complement pathway C1 complex, and are involved in the activation of downstream complement components. When MBL binds to ligands, MASP-2 is activated and then, like C1s, cleaves C4 and C2 to form the C3 convertase, C4bC2b. This initiates the MBL pathway of complement activation. MASP-1 activates C3, albeit at low efficiency, but may still be subserving a biological activator role [5]. No complement-associated activity has been reported for MASP-3, and the function of MAp19 also remains unknown.

MBL deficiency
The MBL pathway is deficient in a significant proportion of the population due to low levels of MBL [6]. The levels of MBL are determined by genetic polymorphism in the promotor region as well as in the part of exon 1 that encodes the first part of the collagenous sequence of the peptide. The frequency of the different allotypes varies between different populations; the allotypes termed B, C and D represent mutant allotypes of the wild type, termed A.

It is often mistakenly stated that the exon 1 mutant allotypes are the most frequent causes of low levels of MBL. Indeed, most papers on associations of ‘low MBL’ with various diseases neglect the fact that (at least among Caucasians) the promoter allotype X, with a gene frequency of 0.20, is more common than the most frequent structural allotype in this population, the B allotype (gene frequency 0.14) [7].

Importantly, even among individuals with identical allotypes the concentration of MBL in plasma may vary considerably (more than 5-fold) in samples taken from normal individuals in good health [7]. Very little variation is seen in samples withdrawn over 1 year [8], but elevated MBL concentrations are observed during infections or after major operations with increases of up to 3-fold [9]. Possibly this relatively small (compared with 1000-fold steady-state inter-individual differences) acute phase response has prompted investigators to base their investigations solely on allotype analyses – and, sadly to say, in most cases they are only encompassing the structural allotypes. It should be stressed that there is no substitute for measuring the actual level of MBL in plasma. The reliance on structural allotypes will, everything else being equal, be expected to result in finding a

Key words: complement, innate immunity, mannan-binding lectin (MBL), recombinant mannan-binding lectin, therapy.

Abbreviations used: MBL, mannan-binding lectin; pMBL, plasma-derived MBL; rMBL, recombinant MBL; MASP, MBL-associated serine protease; MALDI-MS, matrix-assisted laser desorption-ionization MS.

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Figure 1 | (A) Comparison of the size distribution of the post-translationally modified MBL polypeptide chains of pMBL and rMBL by MALDI-MS and (B) the effects of long-term cold storage

(A) pMBL was purified from pooled plasma by affinity chromatography on mannan-derivatized agarose and rMBL was purified from culture supernatant. The molecular mass in Da corresponding to each peak is given. (B) rMBL was also analysed after storage for 12 months at 4°C and at −20°C.

lower than the true significance of the MBL association with whatever is being investigated.

An entirely different complication appears to be the finding of significant amounts of MBL in structural mutant allelotype homozygous individuals [10]. MBL in such individuals appears to be of lower molecular size than the MBL present in subjects homozygous for wild-type MBL alleles [11]. This MBL does not bind significantly to mannan, and does not activate complement. Similarly, by sandwich assay using a series of new monoclonal anti-MBL antibodies, we have recently found apparent MBL levels of up to 0.5 µg/ml in homozygous B allelotype individuals. Quantifications based on reduced SDS/PAGE Western blots indicate that this may be somewhat overestimated.

Most fortunately, our original monoclonal anti-MBL antibody, 131-1 [12], selectively determines the levels of functional MBL. This is illustrated by the close correlation ($r = 0.95$) between C4-cleaving activity and MBL levels as determined by a sandwich immunoassay using 131-1 [13].

Pleasingly, there is also a close correlation ($r = 0.97$) between the lectin activity determined by binding to mannan and the sandwich assay [14].

Defining MBL deficiency

In early reports MBL deficiency was defined as levels below the sensitivity of the assay. With the ELISAs employed the assay sensitivity was likely to be about 1 ng/ml in the tested sample, i.e. serum diluted 20–100-fold. We developed a more sensitive assay based on detection by time-resolved fluorometry with a sensitivity of below 50 pg/ml, i.e. 1 ng of MBL/ml of undiluted plasma or serum. With this assay we have not encountered a plasma sample with less than 3 ng of MBL/ml among the more than 10 000 individual
samples tested. This is important since one potential obstacle for therapeutic use of MBL could be the induction of anti-MBL antibodies. If, as we believe, there are no completely MBL-deficient individuals, then antibody responses are unlikely.

For a rational definition of MBL deficiency one needs to know how much is required for adequate biological function. Such information can only be obtained by investigating patients. Results from investigations on adult leukaemia patients undergoing chemotherapy suggest that less than 500 ng of MBL/ml significantly increases the risk of the patient contracting serious infections (pneumonia, bacteremia; \( P < 0.0001 \)) [15]. A similar study on leukaemic children showed increases in duration of febrile episodes in patients with less than 1 \( \mu \)g of MBL/ml [16]. The risk-determining threshold appears to be lower (100 ng/ml) in an entirely different group of patients, women suffering from recurrent spontaneous abortions [17,18]. The percentage of individuals with levels below 500 ng of MBL/ml of plasma is about 40% among Caucasians, while about 12% have levels below 100 ng/ml [7].

**Reconstitution therapy**

The ability of MBL–MASP complexes to activate complement suggests that the binding of MBL to microorganisms may be of biological significance. Indeed, MBL enhances the phagocytosis of bacteria and yeast, and opsonin deficiency was in 1989 linked to MBL deficiency [19]. The opsonin deficiency was discovered in children with severe recurrent diseases, including diarrhoea, leading to death, and relief was obtained with infusion of plasma with opsonin activity [20]. Subsequently, a large number of reports have substantiated a relationship between low MBL levels and increased susceptibility to infections [21]. Early in the 1990s we speculated on the possibility of producing sufficient quantities of MBL from donor plasma to enable therapeutic intervention. We developed a small-scale procedure for purification from Cohn fraction II/III. This was scaled up at Statens Serum Institut, Copenhagen, Denmark, for the purification of plasma-derived MBL (pMBL) for the first clinical trials [22]. No adverse reactions could be observed, including no anti-MBL response after several infusions spaced over 2 years in an adult MBL-deficient volunteer. Support for larger-scale trials was sought from the European Union, but was turned down on the grounds that planning for therapeutics based on human plasma was ethically unacceptable. This, among other considerations, not least the unknown risk of inadvertent transmission of infectious agents, and the not unlikely possibility of limited production capacity, prompted us to seek the development of procedures for the production of recombinant MBL (rMBL).

**rMBL**

We decided to opt for human cell lines as producers to optimize the likelihood of faithfully reproducing the post-translational modifications of MBL. A human endothelial kidney cell line was transiently transfected and cultured in protein-free medium. A range of MBL oligomers were secreted, but a simple fractionation on mannose-derivatized beads allowed purification of oligomers with a distribution comparable with that of pMBL [23]. The production of pharmaceuticals is not possible within the Danish University system. It is therefore necessary to turn to private industry. Thus NatImmune A/S was established. NatImmune decided to establish its own development laboratories 2 years ago, and a certified cell bank of transfected cells constitutively producing rMBL has since been established. Process development work performed at NatImmune has determined conditions for cell-culture production in protein-free medium, and a purification process, including mandatory virus inactivation and host-cell DNA degradation and host-cell protein-removal steps necessary for a modern manufacturing process, was developed. This production process has been transferred to a contract producer where manufacturing is currently taking place.

The rMBL was compared with pMBL by MS and by its biological activity, and these analyses were also used...
Biological activity of MBL evaluated as C4 activation mediated by the MBL–MASP complex

(A) pMBL (dashed line) and rMBL were mixed with an equal volume of MBL-deficient serum and incubated for 10 min at 37 °C before diluting in barbital-buffered saline containing 5 mM CaCl2 and 5 µg/ml purified C4. The dilutions were added to microwells coated with mannan and incubated at 37 °C for 90 min before washing and development with biotin-labelled anti-C4 antibody and Eu-labelled streptavidin. pMBL was analysed independently four times and rMBL was analysed eight times.

(B) The stability of the biological activity was evaluated by subjecting samples of rMBL stored at 4 °C (■) and −20 °C (○) for 12 months, and comparing them with the reference standard stored at −80 °C (▲). The assay was carried out as in (A). The figure illustrates one of three independent assays giving similar results. pMBL from the same preparation was analysed independently four times whereas the rMBL preparation was analysed eight times.

to evaluate stability, a parameter of great importance for a recombinant protein aimed at therapeutic use. A contemporary pharmaceutical formulation buffer was developed without any protein-stabilizing excipients.

Essentially identical mass spectra were obtained for pMBL and rMBL when examined by matrix-assisted laser desorption-ionization MS (MALDI-MS; Figure 1). The heterogeneity of MBL at the polypeptide level is caused by variations in post-translational modifications in the collagenous region, i.e. hydroxylation of proline and lysine, and the O-linked glycosylation of hydroxylysine. The separate peaks correspond to different glycoforms of the polypeptide. The variation in the masses of identical glycoforms between pMBL and rMBL is due to rMBL containing more hydroxyprolines. The overall glycoprofiles of pMBL and rMBL are identical. This indicates that the transfected cells in vitro make similar post-translational modifications as are made in vivo by hepatocytes and are present on purified pMBL. The very high stability of the MBL polypeptide chain is illustrated in Figure 1(B) where MALDI-MS was performed after long-term storage at 4 °C and −20 °C.

Stability was also examined by gel-permeation chromatography in order to judge the protein in its native state. No change was apparent after storage at −20 °C for 12 months (Figure 2).

Biological activity was evaluated by the capacity to promote MASP activation and thus C4b deposition on to a mannan-coated surface. Purified rMBL was shown to have biological activity comparable with that of MBL purified from donor plasma by mannan-affinity chromatography (Figure 3A). The stability of this biological activity is illustrated in Figure 3(B). No change can be observed after storage for 1 year at 4 °C or −20 °C.

Toxicological investigations on mice have shown the rMBL to be without side effects at a dose 400 times the stipulated clinical dose.

Discussion
We are happy to report that we have moved quickly towards the goal of being able to provide a clinical formulation of rMBL. Obviously, several further investigations are required before the rMBL can be made widely available, including phase I, II and III trials. Several patient groups are being considered for phase II trials, including leukaemia patients undergoing chemotherapy. All currently available information indicates a substantial role for rMBL treatment in the armamentarium for preventing or fighting infections. The increasing worries about multi-resistant micro-organisms alone warrant investment in new treatments. An altogether different and unresolved issue is the role of MBL in regulating immune responses, which may indicate that MBL could find uses outside the primary patient groups. The formulation work has shown promising results for developing a stable liquid formulation for pharmaceutical use.
References


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