Mannan-binding lectin (MBL) production from human plasma

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Abstract

Individuals with low levels of mannan-binding lectin (MBL) appear to be susceptible to infectious diseases. This suggests that substitution therapy with MBL might be a beneficial treatment of patients with MBL deficiency. A production process for an MBL product has been developed from a fraction II + III precipitate obtained by ethanol fractionation of plasma. The MBL process includes three chromatographic steps, where the first and key step is affinity chromatography on a cross-linked agarose matrix selecting for oligomeric, carbohydrate-binding MBL. The yield from the production process is about 25% of the plasma MBL content, and the purity is about 65%. The MBL product shows mannan-binding activity and complement-activating ability. A safety study has shown this plasma-derived MBL to be safe and well tolerated in adult MBL-deficient volunteers.

Introduction

Mannan-binding lectin (MBL) is an important constituent of innate immunity. It was first observed as an opsonic defect in serum from children suffering from frequent, unexplained infections [1–3]. This defect was corrected by plasma infusions, which improved the clinical outcomes. The cause of the opsonic defect was discovered 20 years later to be MBL deficiency [4].

About 4% of the population are homozygous for structural mutations impairing the formation of MBL oligomers necessary for functional activity, and constitute the group with total MBL deficiency [5,6]. However, about 40% of the population carries genotypes associated with decreased MBL concentrations, which might cause MBL insufficiency [6,7]. Individuals with low MBL levels appear to be susceptible to recurrent infections, particularly in childhood [8,9], and especially with co-existing immune deficiencies [10,11]. MBL insufficiency has also been linked to several clinical disorders, not only according to susceptibility but also to progression of disease [12,13]. Substitution therapy with a plasma-derived MBL therefore appears a promising treatment of diseases associated with MBL insufficiency.

The mean concentration of MBL in a plasma pool is 1.0 µg/ml, making an affinity step necessary in a purification process to obtain sufficient purity and recovery. It has been natural to use the lectin characteristic of MBL for purification by calcium-dependent carbohydrate affinity chromatography, and affinity purification on mannan- or mannosylated materials, e.g. Sepharose, or underivatized Sepharose has been widely used with subsequent desorption of MBL by EDTA, mannose or N-acetylglucosamine [14–17]. In general, purification schemes for MBL have included sequential affinity chromatography on identical or alternating carbohydrate-conjugated resins and chemicals. Furthermore, for establishing production, access to an adequate starting material is needed. This material can be a fraction obtained from conventional ethanol fractionation of plasma, ideally a waste fraction [20,21]. Cohn fraction III or a similar fraction that is discarded from the production of intravenous immunoglobulin (IVIG) has been shown to be enriched with MBL [22,23]; in addition to MBL, fraction III contains other proteins of the innate immune system, i.e. serum amyloid P component (SAP) and C-reactive protein [22]. MBL has been purified to homogeneity from Cohn fraction III by sequential chromatography on a mannan-conjugated resin on laboratory scale [22], and the first MBL product for therapeutic use was also purified from fraction III-like starting material by affinity chromatography on a mannosylated matrix [24]. This product was given twice to an MBL-deficient adult as one infusion of 6 mg of MBL and 2 years later as three infusions of 6 mg. A 2-year-old MBL-deficient girl suffering from frequent infections received treatment as three daily infusions of 2 mg of MBL to be repeated 2 weeks later, with good clinical improvement and no adverse effects [24].

The positive result of these MBL treatments provided the ground for further process development aimed at a process...
Figure 1 | Ethanol fractionation of plasma at SSI by the method of Kistler and Nitschmann [21]

The diagram shows where fractions II + III are harvested for IVIG and MBL.

![Diagram of ethanol fractionation of plasma]

compatible with large-scale production of a plasma-derived MBL. Here we describe the production process, characteristics and clinical use of such an MBL product from plasma, named MBL SSI [23].

**Production of the starting material**

The initial starting material for the MBL production at Statens Serum Institut (SSI) is fraction II + III precipitated during ethanol fractionation of plasma [21] (see Figure 1). After extraction of the immunoglobulins for the IVIG production, the MBL-containing fraction III-like residual paste is solubilized in Tris-buffered saline (TBS), whereby MBL is extracted. After serial filtrations through depth and delipid filters to remove filter aid and non-dissolved material the MBL extract is concentrated about 10-fold by ultrafiltration (cut-off value of 300 kDa). The MBL content of this extract is enriched about 14-fold relative to plasma, and the mean recovery is 60%, equivalent to 0.6 mg of MBL/kg of plasma. Before the subsequent purification process the MBL extract is solvent/detergent (S/D)-treated by the addition of 1% Tween-80 and 0.3% tri(n-butyl)phosphate (TNBP).

**The purification process**

MBL constitutes between 0.02 and 0.03% of the total protein content in the starting material for the purification process, shown in Figure 2, making affinity chromatography a necessary step. Commonly used materials for affinity purification are agaroses on which mannan or mannose is conjugated. Such materials are expensive, show low chemical resistance and leak ligands, which might cause adverse reactions in patients. I have found that underivatized cross-linked agarose can be used for affinity purification of MBL from the recovered extract. Cross-linked agarose fulfils the requirements for a matrix to be used for purification of a plasma product, as it shows high chemical stability necessary for efficient cleaning, does not leak ligands, has good flow properties, and is inexpensive. Therefore, affinity chromatography on a cross-linked Sepharose resin is the first purification step and the key step of the process. This basic
agarose matrix binds MBL with high specificity, and selects for oligomeric, carbohydrate-binding MBL. CaCl$_2$ is added to the S/D-treated MBL extract before loading it on to the Sepharose resin equilibrated with TBS/5 mM CaCl$_2$, pH 7.3. After loading, the column is washed extensively with TBS/0.5 mM CaCl$_2$, pH 7.3, whereby structurally impaired MBL is eliminated with the major part of applied proteins, before MBL is desorbed from the column with 30 mM mannose. The mean recovery from the affinity step is 60%, and the purity is about 60%; by this means, a purification of about 2500-fold is obtained. Besides constituting a purification step the affinity chromatography serves as a virus-removal step.

Next, virus inactivation by S/D treatment of the mannose eluate is performed, again with addition of 1% Tween-80 and 0.3% TNBP, and incubation for 6 h at 25°C.

The second chromatography step is anion-exchange chromatography on a Q-Sepharose matrix equilibrated with 15 mM Tris/25 mM NaCl, pH 8.0. The primary purpose of this step is to wash out solvent and detergent while
retaining MBL on the column. MBL is subsequently eluted with NaCl.

Prior to the last process step – gel filtration on a Superose 6 column – the volume of the fraction eluted from the Q-Sepharose column is reduced. The gel-filtration step has two functions, being a polishing and a buffer-exchange step, by using PBS as equilibration buffer. MBL elutes as a symmetrical peak from the column and is collected as the final MBL fraction.

MBL SSI is formulated as a liquid product containing from 250 to 500 μg of MBL/ml depending on batch size, and is stabilized by addition of nanofiltered albumin to 5 mg/ml. The final product is filled as portions of 3 mg of MBL.

The yield from the production process constitutes about 25% of the MBL content in the plasma pool, equalling about 275 mg of MBL from 1100 kg of plasma.

**Viral safety**

Two steps in the process have been validated as virus-reduction steps according to EMEA (The European Agency for the Evaluation of Medicinal Products) guidelines, the affinity and the S/D-treatment steps [25]. Studies have been conducted to validate the viral removal capacity of the affinity chromatography using the small non-enveloped viruses hepatitis A virus, poliovirus and canine parvovirus as a model for parvovirus B19. The studies showed the following removal: $3.2 \log_{10} \leq \text{hepatitis A virus} \leq 6.7 \log_{10}$, $3.8 \log_{10} \leq \text{canine parvovirus} \leq 7.5 \log_{10}$ and $3.6 \log_{10} \text{poliovirus}$. The S/D treatment has been validated in studies employing three enveloped viruses: HIV, bovine viral diarrhoea virus as a model for hepatitis C and pseudorabies virus as a large DNA virus. All were shown to be inactivated efficiently; $\text{HIV} \geq 5.6 \log_{10}$, bovine viral diarrhoea virus $\geq 6.9 \log_{10}$ and pseudorabies virus by $6.3 \log_{10}$.

**Characteristics of MBL SSI**

The purity before adding the stabilizer is shown in Figure 3(A) (lanes 3 and 7) and 3(C). Figure 3(B) (lane 1) shows that MBL SSI is composed mainly of higher-oligomeric forms, i.e. $\geq 290$ kDa. The purity is estimated to be at least 65%, from the ratio between the MBL concentration, determined by a sandwich ELISA, and total protein concentration from $A_{280}$ measurements; this was confirmed by densitometric scanning of lane 7 on the SDS/PAGE gel (Figure 3A). The major contaminants are IgM (11%), SAP (7%), α2-macroglobulin (3%) and IgA (1%).

The purification process contains two steps – affinity chromatography and gel filtration – selecting for functionally active and oligomeric MBL; the MBL product is therefore expected to possess functional activity. Results from a ligand-binding assay, performed as described in [26], confirm that the mannan-binding activity equals that of a normal human plasma standard, ranging from 80 to 96%.

The presence of MASPs in complex with MBL is measured by densitometric scanning of lane 7 on the SDS/PAGE gel (Figure 3A). The major contaminants are IgM, SAP and α2-macroglobulin. The carbohydrate-specific antibodies and SAP will probably contribute to the immune defence against micro-organisms in co-operation with MBL during treatment.

Circulating MBL consists of several oligomers ranging from dimers to hexamers of the basic trimeric subunit [30]. The higher-oligomeric forms induce C4 activation more efficiently than the lower oligomers [31]. MBL SSI is predominantly composed of higher oligomers, i.e. $\geq 290$ kDa, with which complement-activating capacity is associated. However, the production process results in a loss of about 40% of the ability to cleave C4, associated with MASP-2, through activation and/or dissociation of the protease from the MBL–MASP complex. Activation of MASPs during purification is consistent with the early observations of associated MASPs becoming partly activated, unless protease inhibitors were added [17,19]. Some activation can probably not be avoided when producing a plasma-derived MBL with omission of protease inhibitors; however, activated MASPs are expected to be neutralized by their specific inhibitors. The observation of α2-macroglobulin as a contaminant with subsequent C4b deposition on mannan-coated wells, by the method of Petersen et al. [27]. In this assay the plasma standard was arbitrarily assigned a C4-depositing activity of 1000 m-units/ml corresponding to a specific activity of 400 m-units/μg of MBL. The mean specific activity of 13 batches was 246 m-units/μg, indicating that about 40% of the complement-activating ability of MBL was lost during the purification from plasma to final product.

Finally, MBL SSI is stable. A programme including three consecutive batches has shown 3 years’ stability under cold-room storage, without changes in the product’s characteristics.

**Clinical studies**

A phase I study has been conducted in collaboration with Professor Helgi Valdimarsson at the National University Hospital, Reykjavik, Iceland, involving 20 healthy MBL-deficient volunteers. This study has shown the MBL product to be well tolerated and safe [28]. Several patients have also been treated with MBL SSI, including a patient with end-stage cystic fibrosis [29], with no observation of serious side effects and showing good clinical efficacy. Phase II and III clinical studies are being planned.

**Conclusions**

A process has been developed for large-scale manufacture of an MBL product from a fraction III-like material discarded in the production of IVIG. The key step of the process is affinity chromatography on underivatized cross-linked agarose, selecting for oligomeric, functionally active MBL. The mean yield of the process is 0.25 mg of MBL/kg of plasma for fractionation, and the purity of the product is about 65% MBL, a purification factor of about 38 000 from plasma to product. The major contaminants are IgM, SAP and α2-macroglobulin. The carbohydrate-specific antibodies and SAP will probably contribute to the immune defence against micro-organisms in co-operation with MBL during treatment.
indicates that activated MASPs, most probably MASP-1, have bound α2-macroglobulin, with loss of proteolytic activity; and complexes of MBL–MASP and α2-macroglobulin have been observed by others [32]. The C1-inhibitor expected to be specific for MASP-2 cannot be detected in the MBL product. MBL SSI is well tolerated, indicating that if activated MASPs are present they do not result in adverse events. MBL oligomers which have lost their MASPs during the purification are expected to bind free MASPs from the circulating pool of the patient during treatment; by this means complement-activating ability is gained, as has been shown in vitro [31].

Virus-validation studies have shown that the production process removes and effectively inactivates possible viral contaminants in plasma, and a clinical study has confirmed that MBL SSI is safe and well tolerated. Treatment of selected patients has indicated that the MBL product might be beneficial for some patients with active disease. However, in line with current evidence, MBL therapy might be most effective as prophylactic treatment, especially in immunocompromised patients. Therefore, further clinical studies with MBL SSI will focus on such patient groups. Should clinical benefit be demonstrated, it will be possible to produce enough plasma-derived MBL for treatment of these patients from fraction III, which today is discarded in fractionation plants worldwide.

References

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