Discovering the Role of Mannose-Binding Lectin (MBL) in Innate Immunity: The Early History

Malcolm Turner

Introduction

The so-called lectin pathway of complement activation was the third such molecular sequence to be described which results in enzymatic cleavage of the C3 component of complement. However its discovery was a gradual process arising from observations in different areas of the biological sciences. Three such strands of research predominated and these were pursued essentially independently of each other with little or no “cross talk” until 1989 when links began to be established. These areas of study were (1) Biochemical studies (especially in the field of liver biochemistry); (2) Identification of an immunodeficiency disorder and (3) Microbiology research.

The study of a relatively common immunodeficiency disorder will be used here to illustrate the clinical relevance of one of the initiating recognition molecules of the lectin pathway, namely mannose-binding lectin (MBL).

Early Studies on Defective Opsonisation

The most significant contributor to the early history of this particular narrative was John Soothill (1925–2004). His early research life was spent in John Squire’s Department of Experimental Pathology in the University of Birmingham where one of his responsibilities was the co-ordination of the MRC trial of replacement IgG in patients with Bruton’s agammaglobulinaemia. This background clearly facilitated his appointment in 1968 as the first Professor of Immunology at Great Ormond Street Hospital and the associated Institute of Child Health (Fig. 1).
After moving to London Soothill set about building a department with a major focus on immunodeficiency disorders involving both the cellular and humoral arms of the immune system. The author joined the team after a period as a postdoctoral research fellow in the Biochemistry Institute of the University of Uppsala in Sweden. In the decades after the 1930s the Biochemistry Institute had acquired a world leading reputation in the study of plasma proteins. One important result of this training experience which was to prove critical was the acquisition of a large −70°C freezer for the storage of serum in the Institute of Child Health at a time when many hospital laboratories still relied heavily on −20°C storage.

Always receptive to reports of potentially interesting patients Soothill was quick to follow up a paper in The Lancet describing a girl with frequent infections and a phagocytic defect (Miller et al. 1968). Accordingly, he began a systematic search for similar patients and over time collected some 40 serum samples, duly stored at −70°C, from patients with frequent unexplained infections. It is also worth noting that because Great Ormond Street Hospital is a tertiary referral centre it inevitably concentrates subsets of patients with the most severe symptoms.

Soothill, ably assisted by his technician Betty Harvey, then tested these stored samples using the assay described by Miller and colleagues. This involved incubating heat killed baker’s yeast with diluted serum and polymorphonuclear cells. The uptake of yeast particles was evaluated microscopically after appropriate staining and some 25% of the patients were found to have defective uptake (Soothill and Harvey 1976). Furthermore, it could be shown that this defect was due to poor opsonization by the serum rather than a defect of phagocytic function, precisely as demonstrated initially by Miller and colleagues.

As shown in Fig. 2, using sera with normal opsonic function resulted in the phagocytes ingesting three or more yeast particles whereas using sera with defective function was associated with little or no uptake of particles.
The microscopic assay was, however, time consuming and in 1978 my late colleague Roland Levinsky developed an automated version of the test based on Coulter Counter technology which was then used to study a cohort of apparently healthy school children (Levinsky et al. 1978). Surprisingly, this revealed that some 5–7% of such individuals had poor opsonic function, an observation which was consistently confirmed in a range of assays subsequently developed for routine use.

In the early 1980s the author was encouraged to investigate the immunochemical basis of this defect since there was some scepticism among the wider immunological community regarding the significance of observations made using baker’s yeast as targets in the assays. It was already well established at this time that two distinct families of opsonic molecules were important in antimicrobial immunity. These were (1) certain immunoglobulin molecules and (2) various cleaved fragments of complement component C3. Since the levels of immunoglobulin classes and subclasses had already been investigated and found to be normal in most of the patients with defective opsonization it was unlikely that these molecules were implicated in the disorder. Therefore C3 derived opsonins were assumed to be those playing a role in the development of the defect and an assay was devised to directly
measure the deposition of C3 related molecules on various surfaces (Turner et al. 1981). In brief, serum diluted in VBS buffer containing calcium and magnesium was initially incubated with a suspension of the target organism or yeast zymosan before quenching the reaction with ice cold VBS-EDTA buffer. After centrifugation and washing steps, trypsin was incubated with the target preparations at 37 °C and the released C3c fragments were quantified in single radial diffusion plates containing anti-C3. The site of trypsin cleavage of a cell bound C3b molecule is illustrated in Fig. 3.

The development of this procedure, which was termed the C3c elution assay, provided an important investigative tool which clearly established that abnormal C3 deposition was implicated in the opsonic disorder. For example, dose response curves over a 30 min incubation period consistently indicated increasing deposition of C3b fragments from sera found to be normal in the Coulter Counter assay whereas there was always minimal deposition from sera found to be defective in the Coulter Counter assay. The assay was also used to show that adding small aliquots of normal sera could correct the defect but no serum with defective opsonization was ever shown to correct other sera with the defect. In later studies (Turner et al. 1986) the assay was employed to compare the deposition of C3b on the surfaces of *Candida albicans*, *Staphylococcus aureus* and *E. coli* using sera from five individuals with normal deposition on yeast particles with five sera having defective deposition on yeast. This study showed convincingly that the opsonic defect was a general phenomenon and not confined to yeast targets.

Despite anchoring the phenomenon of defective opsonization firmly in the complement system it was proving impossible to pinpoint the precise cause within the two known complement activation pathways. Total haemolytic complement values were normal in sera with the defect and immunochemical levels of C3, C4, Factor B, Properdin and Factor H were all normal as was functional Factor H (Turner et al. 1985). At this stage the data appeared to suggest that there was some previously unidentified co-factor required for full opsonic potential but this idea was met with some scepticism in some quarters when the data was presented at the sixth International Congress of Immunology held in Toronto in July 1986. It was argued that no additional factors or regulators were required for the expression of full complement potency. Despite this negativity we were fortunate to obtain further funding from Action Research in 1987 which permitted the investigation of various possibilities such as non functional variant alleles of critical complement components e.g., properdin. Later that year Mike Super was recruited as a graduate student to work on this project and our plan included the exploration of such possibilities in association with colleagues at the MRC Immunochemistry Unit in Oxford.
Biomedical research in the 1980s was underpinned by access to the world output of peer reviewed publications exactly as it is now but with one important difference. The retrieval of papers of relevance required visits to the library and the scouring of the weekly publication Current Contents. The choice of which journals were to be perused was made by the reader and the numbers of potentially relevant titles increased year on year. This was a particular problem with papers on aspects of the complement system which could be found in a wide variety of journals covering the medical and biochemical literature. However, a revolution in the retrieval process occurred in the late 1980s. Libraries began to install computer terminals and the output of individual journals collated by Current Contents on a floppy disk could be viewed on screen and, critically, it became possible to type in key words to ensure that papers of relevance were not missed whichever journal had accepted the submission. Accordingly, in 1988 such a literature search (see Fig. 4) identified a paper by Ikeda and colleagues working in Kyoto and published a few months previously in 1987. This described a lectin called mannan-binding protein (MBP) isolated from rat serum which had been found to activate complement through the classical pathway (Ikeda et al. 1987).

Already in 1988 it was known that C-reactive protein was an alternative initiator of complement activation by the so-called classical pathway but it did not seem likely to be involved in yeast opsonization since it does not bind to yeast mannan. However the rat lectin did appear to be worthy of further consideration as a potential candidate. As part of the follow up to this we would need to have access to both purified human MBP and preferably also antiserum to the protein. By happy

Fig. 4  Graduate student Michael Super undertaking an early computer based literature search in the library of the Institute of Child Health in 1988
coincidence we discovered shortly afterwards in discussions with Ken Reid and colleagues from the Oxford MRC Immunochemistry Unit that their group was also beginning to take an interest in the same area and that visiting scientists Steffen Thiel and Jinhua Lu were already purifying human MBP and intended to raise a rabbit antiserum in due course. It was agreed in principle that when these materials became available we would be able to have access to them on a collaborative basis.

With the availability of the rabbit anti-MBP antibody and an MBP standard it was possible to develop an ELISA procedure for the measurement of the protein in stored sera including samples from ten children known to have the opsonic defect, 59 healthy babies and 186 adult blood donors. Whereas both the adult blood donors and the healthy babies were found to have a wide range of MBP values, each of the ten children with the opsonic defect had extremely low levels of the serum lectin. Moreover it was possible to titrate in increasing levels of MBP into serum from an individual with the opsonic defect and demonstrate a dose dependent correction of the deficiency (Super et al. 1989).

A Question of Nomenclature

Once it was possible to ascribe a predominantly immunological role for the protein known up to then as mannan- (or mannose-) binding protein it created a potential confusion with the usage of MBP as an abbreviation. MBP was already used extensively in the immunological literature to denote three other proteins, namely myelin basic protein, major basic protein of eosinophils and maltose binding protein. Accordingly, at least in the immunological sphere, the abbreviation of MBL is now practically universal.

A Decade of Biochemical Studies

As the 1980s came to an end it was clear that there was strong evidence that the protein known as MBP had an important immunological function but for most of the decade MBP had been a protein in search of a function. The existence of serum lectins was first predicted by Robinson et al. (1975) and shortly afterwards Kawasaki et al. (1978) described the isolation and characterization of mannan-binding protein from rabbit liver. The same group subsequently isolated the protein from human serum (Kawasaki et al. 1983) from bovine serum (Kawasaki et al. 1985) and from rat serum (Oka et al. 1988). Progress was made on the basic structure of the protein (notably by Weis et al. 1991 and Weis and Drickamer 1994 and by Sheriff et al. 1994) and the structure of the gene encoding human serum mannose-binding protein was independently published by Sastry et al. (1989) and Taylor et al. (1989). Some of the key steps in this biochemical chronology are shown schematically in Fig. 5.
In fact we now know that there are two human MBL genes but \textit{MBL-1} is a pseudo-gene. The functional \textit{MBL-2} gene located on chromosome 10 encodes the protein product found in serum and comprises four exons. Exon 1 encodes the signal peptide, a cysteine rich region and part of a glycine rich collagenous region. Exon 2 encodes the remainder of the collagenous region whilst exon 3 encodes the so-called neck region characterized by an alpha-helical coiled coil structure. Finally, exon 4 encodes the C terminal globular carbohydrate recognition domain (CRD). In the assembly of a functional MBL molecule three such polypeptide chains come together with the collagenous regions forming a triple helix stabilized by hydrophobic interactions and interchain disulphide bonds in the cysteine rich region. In serum the circulating MBL consists of a series of oligomers of this basic subunit ranging from dimers to hexamers but expression of functional binding to microbial surfaces appears to require higher order structures (tetramers to hexamers). The reason for this is that a single CRD binds only weakly to its target sugar group and high functional affinity (avidity) is only achieved through multiple CRD binding as is also the case with IgM antibody interactions with its target antigens. X-ray crystallographic studies reveal that the high order MBL oligomers adopt a sertiform or bouquet like structure facilitated by an interruption in the collagenous region which gives rise to a kink or hinge.

In 1990 it was possible to construct a model of the probable functional role of MBL in the activation of the complement system. In essence the hypothesis was that MBL acted as a mimic of C1q in the classical complement cascade. This model required MBL to interact with both C1r and C1s subcomponents of the C1 complex and the binding of the CRD domains of MBL to multiple sugar groups on a target

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**Fig. 5** Chronology of studies in three biomedical areas which were pursued essentially in isolation until 1989 when links began to be established between the various studies. Key findings in the biochemical area are highlighted in this scheme.

| Biochemistry & Molecular Biology | Immunodeficiency | Microbiology |
|---------------------------------|------------------|--------------|
| 1968                            | Plasma associated phagocytic defect |
| 1975                            | Serum lectins predicted |
| 1978                            | MBL isolated from rabbit liver |
| 1983                            | Human serum MBL isolated |
| 1987                            | Rat MBL activates classical pathway |
| 1989                            | Human MBL gene cloned | Opsonic defect linked to low serum MBL |
surface was believed to induce the autocatalytic activation of C1s leading in turn to cleavage of both C4 and C2 and the assembly of the C1-esterase enzyme. This model received powerful support from the study of Lu et al. (1990) but was destined to be challenged by the description in 1992 of an alternative model in which MBL was postulated to interact with a completely novel C1s-like serine protease (Matsushita and Fujita 1992). Although initially it appeared odd that the authors had sought to establish the involvement of another serine protease in the expression of MBL induced complement activation the explanation was provided in earlier studies that both Dr. Fujita and Dr. Matsushita had co-authored with Dr. Kawakami. These provide a link to the third strand of research which contributed significantly to our understanding of the role of MBL in immunity, namely microbiological research.

Microbiological Studies in the 1980s

In parallel with the previously described biochemical studies on serum MBL Masaya Kawakami and colleagues were publishing a series of papers on a family of bactericidal factors called RaRF present in the sera of a range of vertebrates. In particular, RaRF was found to react specifically with Ra chemotype strains of Salmonella and the R2 strains of E. coli. The authors proposed that these factors were necessary to resist infection by rough strains of enterobacteria. Furthermore the authors were able to demonstrate that, after binding to bacteria, RaRF was able to activate complement thereby killing the organisms. In 1988 the authors presented evidence that RaRF had a polypeptide structure which resembled C1q and was partially collagenous (Ji et al. 1988). In the same paper the authors noted the close similarity between RaRF and the rat lectin described by Ikeda et al. (1987) and, significantly, they reported that whereas C4 and C2 components were required for complement activation by RaRF, C1 apparently was not required. Both Dr. Fujita and Dr. Matsushita were coauthors on this publication and these RaRF studies led them to question the precise mechanism of complement activation by MBL and describe the first of the three MBL associated serine proteases (MASPS) (Matsushita and Fujita 1992). In the same year Matsushita and Fujita also co-authored the paper which definitively demonstrated that RaRF was indeed identical to MBL (Matsushita et al. 1992).

In addition to the RaRF studies there was another area of microbiological research which should be acknowledged. In 1946 Sir Frank Macfarlane Burnet and John McCrea described three inhibitors in mammalian serum (called alpha, beta and gamma) which were able to inactivate influenza virus (Burnet and McCrea 1946). By 1969 the beta-inhibitors were known to be calcium dependent, nonsialylated, heat–labile glycoproteins present in mouse, guinea-pig, ferret and rabbit serum (Krizanova and Rathova 1969) but it was not until the 1990s that the beta-inhibitors in these species were finally shown to be mannose–binding lectins (Anders et al. 1990, 1994). The chronology of some of the key discoveries in the microbiology area are summarized schematically in Fig. 6.
Determination of the Underlying Cause of the Opsonic Deficiency

The association of low levels of MBL with the opsonic deficiency still begged the question of what was causing the low levels. A genetic explanation was clearly one possibility and the cloning of the human MBL gene in London by Taylor et al. (1989) provided ready local access to the technology required. As discussed previously the MBL-2 gene comprises four exons arranged as follows. Exon 1 encodes a signal peptide, a cysteine rich region and part of the glycine-rich collagenous region, exon 2 encodes the remaining portion of the collagenous region and exon 3 encodes the so-called neck region characterized by an alpha-helical coiled coil structure. Finally, exon 4 encodes the carbohydrate recognition domain (CRD) which has a globular structure.

However, there were no observations to suggest where a putative mutation might be located and Mike Super commenced the nucleotide sequencing of individuals from three British families with the opsonic defect beginning at the C-terminal end of exon 4. This proved to be an unfortunate decision for him since, after working through exons 4, 3 and 2, but finding no evidence of any mutations, he had to leave London to take up a post doctoral position in the US and it was Michiko Sumiya’s good fortune to take over the search and discover the first MBL mutation in exon 1 (Sumiya et al. 1991). This was a single point mutation at base 230 in codon 54 which resulted in a change of the translated amino acid in the peptide chain from a glycine to an aspartic acid. This has significant consequences because the integrity
of all collagenous proteins is based on assembling a triple helix in which every third residue is the smallest amino acid, glycine, situated exactly along the axis of the helix. Three aspartic acid residues, each having a bulky side chain, would not be readily accommodated in the axial position of the helix. Such disruption was already documented in the structural collagen molecules of patients with osteogenesis imperfecta (Sykes 1989) and is illustrated schematically in Fig. 7.

In essence the defect is not a primary genetic deficiency but rather a secondary functional deficiency in which individual 3-chain MBL subunits are unable to assemble into higher order oligomers with the capacity to make multipoint attachments to target sugar groups and hence overcome the intrinsic low affinity of such binding by an individual subunit of three CRDs. SDS-PAGE analysis of MBL from an individual homozygous for the codon 54 mutation shows most protein is indeed of low molecular weight compared to the MBL of an unaffected individual which is characterized by a spectrum of higher order oligomers (see Fig. 8).

Following Mike Super’s departure the author recruited a new graduate student, Richard Lipscombe, to continue the early work on structural and functional aspects of MBL. In the first of a series of fruitful collaborative publications with former Institute of Child Health Research Fellow Yu Lung Lau, Richard initially confirmed the presence of the codon 54 mutation in a small group of Hong Kong Chinese

Fig. 7  Schematic showing the presumed effect of the codon 54 mutation in the MBL-2 gene on the expressed protein product. A critical axial glycine residue is replaced with an aspartic acid having a bulky side chain and this disrupts the assembly of higher order oligomers essential for proper biological function.
This suggested that the mutation had probably originated more than 40,000 years ago in a population ancestral to both modern day Asians and Europeans. Subsequently a series of population studies were undertaken, one of which included the identification of a second codon 1 mutation in a cohort from The Gambia in West Africa (Lipscombe et al. 1992b). The latter mutation in codon 57 results in a glutamic acid substitution for an axial glycine and, since it is associated with reduced levels of serum MBL, is presumed to result in disrupted assembly of high order oligomers of MBL protein as is the case with the codon 54 mutation.

Fig. 8 SDS-PAGE analysis of human MBL from a subject with no codon 1 mutation and showing a spectrum of oligomeric forms (upper profile). In contrast, MBL from a subject homozygous for the codon 54 mutation is predominantly of low molecular weight (lower profile). See Lipscombe et al. (1995) for more details.
Some Concluding Thoughts

The studies described in this chapter provided a reasonable model of the biological function of MBL as viewed in the mid 1990s. This is summarized schematically in Fig. 9.

Here the tetrameric form of MBL is shown with each of the four subunits composed of three identical chains terminating in a cluster of three carbohydrate recognition domains. Binding of these domains to repeating sugar groups on a microbial surface were assumed to bring about activation of the MBL-associated serine protease −2 molecules located near the “kink” in the collagenous regions. Nearby C4 and C2 molecules would be cleaved and biologically active C3 convertase generated. The formation of C3b fragments able to bind covalently to the microbial surface would then complete the first stage of the opsonic process. For an update on the latest findings regarding MBL-MASP activation mechanisms the reader should look elsewhere in this volume.

The initial association of frequent infections with the opsonic deficiency state suggested an important role for MBL in the innate immune system. However, the apparent maintenance of the codon 54 mutation over several 1000 years suggest some associated biological advantage and the presence of high frequencies of a similar independent mutation in Sub-Saharan Africa provides strong support for this view. Two hypotheses have been proposed and these may not be mutually exclusive. Firstly, the reduced levels of complement activating MBL could help to reduce the risk of host damage from the excessive release of inflammatory mediators particularly in tropical environments (Lipscombe et al. 1992b). Alternatively, it has been proposed that MBL deficiency may help to protect the host against intracellular parasitic infections which depend upon C3 opsonization and C3 receptor uptake in order to breach cellular defence mechanisms (Garred et al. 1992).

![Fig. 9](image-url) Functional human MBL binds to sugar groups on microbial surfaces in association with MASP-2 moieties (shown in light green) to generate C4b2a complexes with C3 convertase activity. This leads to the deposition of C3b opsonins (shown in red) on the nearby surface.
Many groups, including the author’s, have undertaken various disease association studies with respect to serum MBL levels and these are the subject of another chapter within this volume.

In evaluating the importance of MBL deficiency in human disease the author has always advocated the need to seek evidence of effects beyond simple disease associations. For example, co-existing partial immunodeficiencies are by no means rare. Some 8% of the Caucasian population lack two of the four possible functional C4 genes and we know that 5% of the same ethnic group have very low MBL levels which means that one in 250 individuals would present with both partial deficiencies (Turner 1996). Similarly we reported that polymorphisms in exon-1 of the MBL-2 gene, resulting in low plasma levels of MBL, were significantly overrepresented in patients with primary antibody deficiency and mycoplasma infections (Hamvas et al. 2005).

In support of the concept of MBL playing a role as a disease modifier we showed that individuals deficient in MBL were at risk of developing sepsis and systemic inflammatory response syndrome (SIRS) (Fidler et al. 2004).

The initial association of the opsonic deficiency with a clinical presentation of frequent infections in young children suggested that MBL must be particularly important in early life after maternally derived antibodies have decayed and the host is relatively antibody deficient, the so-called “window of vulnerability” (Super et al. 1989). However, it has also been suggested that MBL plays an important role in the early phase of many primary encounters with sugar rich pathogens, acting as an “ante-antibody” (Ezekowitz 1991). A unique opportunity to test this hypothesis arose when a novel human pathogen emerged in China in 2003. The coronavirus which caused severe acute respiratory syndrome (SARS) infected some 8098 individuals and resulted in 774 deaths. In a case controlled study of 569 patients Ip et al. (2005) found that serum levels of MBL were significantly lower in patients with SARS than in control subjects suggesting that MBL did indeed contribute to first-line host defence against SARS-CoV. We have shown that a wide range of different microorganisms are able to bind to MBL (Neth et al. 2000) and in a later study we also demonstrated that the MBL-MASP system can significantly enhance other complement activating pathways (Neth et al. 2002). It is not unreasonable to conclude that MBL is a major player in the initial immune response to many pathogens throughout life but is of particular importance in young infants.

Acknowledgments The author gratefully acknowledges the considerable input of many former colleagues involved in the development of this research area and Dr. Jonathan Turner for help with the preparation of this chapter.

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