Mannose Binding Protein Deficiency: Immunochemistry and Mutation Analysis

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Richard John Lipscombe

Institute of Child Health
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Abstract

Mannose Binding Protein (MBP) is a serum lectin believed to play a role in innate immunity by specifically recognising foreign carbohydrate structures, and evolutionary studies have shown that MBP is an ancient molecule found in species as diverse as shark and man. Previous work has linked human MBP deficiency with a common opsonisation defect that predisposes to frequent infections during infancy, and associated low protein levels with a point mutation in codon 54 of the MBP gene.

The investigations described here extend our knowledge of MBP deficiency from its original discovery in Caucasians into other population groups using both functional and quantitative ELISA procedures. MBP deficiency was found in the Chinese and was associated with the codon 54 mutation, whereas in Gambians (West Africa) a new point mutation was discovered in codon 57. Both mutations are predicted to disrupt the structure of the collagenous region of MBP. The frequencies of the structural MBP gene mutations were investigated in these populations by PCR followed by restriction enzyme digestion, oligospecific hybridisation, or direct sequencing, and compared with serum protein concentrations for each group. This showed conclusively that the Eurasian and African mutations account for most of the observed low MBP levels. The geographical distribution of the mutations was further investigated by studies on a range of populations including Xhosa (South Africa), San Bushmen (Namibia), and inhabitants from Papua New Guinea and Vanuatu (South West Pacific).

A physicochemical evaluation of the effects of the mutations on circulating MBP was also undertaken. Protein forms within sera were separated on the basis of charge and size by electrophoresis, gel filtration and sucrose density centrifugation, and analysed by immunoblotting. This suggested that the major serum forms of wild type MBP are dimers, trimers and tetramers of three-chain subunits, whilst the mutant phenotypes are predominantly of lower molecular mass.
To my family and friends

"There is something fascinating about science. One gets such wholesome returns of conjectures out of such trifling investment of fact."

Mark Twain

[1835-1882]
Acknowledgements

For his guidance and encouragement I am indebted to Prof. Mac Turner. The countless hours of enjoyable and fruitful discussions, together with the opportunity to experience research as many hope it should be performed will be amongst my fondest memories of these studies.

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There are so many others who have also contributed during this time that acknowledging all by name is not practicable. However, I hope that those involved will see themselves below.

The inhabitants of the Immunochemistry Laboratory, both past and present, have always made working enjoyable and provided help whenever needed. Their humour - loud, subtle and pathetic - has never failed.

Others who I would like to thank are my colleagues from Coram Fields, not least the indomitable centre forward, my numerous office companions, and those ever present individuals that have made life on the 5th floor tick (most of the time!).

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Finally, thank you Niamh for being you.
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<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>AA</td>
<td>acrylamide</td>
</tr>
<tr>
<td>Asp (D)</td>
<td>aspartic acid</td>
</tr>
<tr>
<td>Arg (R)</td>
<td>arginine</td>
</tr>
<tr>
<td>B.C.</td>
<td>binding coefficient</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BP</td>
<td>before present</td>
</tr>
<tr>
<td>Bis</td>
<td>bisacrylamide</td>
</tr>
<tr>
<td>C1qR</td>
<td>C1q receptor (collectin receptor)</td>
</tr>
<tr>
<td>CS2/54/57</td>
<td>codon 52/54/57</td>
</tr>
<tr>
<td>Cys (C)</td>
<td>cysteine</td>
</tr>
<tr>
<td>CRD</td>
<td>carbohydrate recognition domain</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>C.V.</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotidetriphosphate</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethenediamine tetra-acetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>FPLC</td>
<td>fast protein liquid chromatography</td>
</tr>
<tr>
<td>g α r</td>
<td>goat anti-rabbit immunoglobulin</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetyl-D-glucosamine</td>
</tr>
<tr>
<td>Gly (G)</td>
<td>glycine</td>
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<tr>
<td>Glu (E)</td>
<td>glutamic acid</td>
</tr>
<tr>
<td>Hib</td>
<td><em>Haemophilus influenzae</em> type b</td>
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<tr>
<td>HRP</td>
<td>horse-radish peroxidase</td>
</tr>
<tr>
<td>IgE/G/M</td>
<td>immunoglobulin E/G/M</td>
</tr>
<tr>
<td>MAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MAC</td>
<td>membrane attack complex</td>
</tr>
<tr>
<td>MASP</td>
<td>MBP associated serine protease</td>
</tr>
<tr>
<td>MBP</td>
<td>Mannose binding protein</td>
</tr>
<tr>
<td>NC</td>
<td>nitrocellulose</td>
</tr>
<tr>
<td>OPD</td>
<td>O-phenylene diamine</td>
</tr>
<tr>
<td>P</td>
<td>probability</td>
</tr>
<tr>
<td>PAb</td>
<td>polyclonal antibody</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS(-T)</td>
<td>phosphate buffered saline (plus Tween-20)</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PNG</td>
<td>Papua New Guinea</td>
</tr>
<tr>
<td>R</td>
<td>correlation coefficient</td>
</tr>
<tr>
<td>RaRF</td>
<td>Ra-reactive factor</td>
</tr>
<tr>
<td>RMM (Mr)</td>
<td>relative molecular mass</td>
</tr>
<tr>
<td>s20,w</td>
<td>sedimentation coefficient</td>
</tr>
<tr>
<td>s α m</td>
<td>sheep anti-mouse immunoglobulin</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic Lupus Erythematosus</td>
</tr>
<tr>
<td>SP-A</td>
<td>Surfactant protein A</td>
</tr>
<tr>
<td>SP-D</td>
<td>Surfactant protein D</td>
</tr>
<tr>
<td>SSC</td>
<td>standard saline citrate</td>
</tr>
<tr>
<td>T1/2</td>
<td>half-life</td>
</tr>
<tr>
<td>TAE</td>
<td>tris acetic acid EDTA</td>
</tr>
<tr>
<td>TBE</td>
<td>tris boric acid EDTA</td>
</tr>
<tr>
<td>TE</td>
<td>tris EDTA</td>
</tr>
<tr>
<td>VBS(++)</td>
<td>veronal buffered saline (+ MgCl2, + CaCl2)</td>
</tr>
</tbody>
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Mannose Binding Protein Deficiency: Immunochemistry and Mutation Analysis
Overleaf: A representation of dimeric and trimeric fibrils of wild type MBP, together with a mutant form described in this thesis
CHAPTER 1

INTRODUCTION

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AIMS OF INVESTIGATIONS DESCRIBED IN THIS THESIS .......... 43
The human immune system is a complex array of many components, each acting to provide the host with defence against infection. The elements which have been studied in most detail are those constituting the adaptive system, that is to say the soluble immunoglobulins produced by B lymphocytes, and the T lymphocytes. These produce a specific response adapted to each infectious agent that the host encounters. However, before this system is engaged many important non-adaptive immunological mechanisms must be breached. These together constitute the innate, or inborn, immune system, which is the first line of defence against foreign organisms and includes components such as the skin barrier, soluble complement proteins, lysozyme and phagocytes.

Many infectious agents (for example bacteria and viruses) contain carbohydrate structures that can be recognised by a particular class of proteins known as lectins. Furthermore, these carbohydrate moieties are characterised by subtle variations which distinguish them from the native carbohydrates found in the host. Mannose binding protein (MBP), the subject of this thesis, is a lectin believed to play a part in the innate arm of host defence by recognising these foreign carbohydrate patterns, and then interacting with other components of the innate response system. In humans MBP is secreted into the serum by the liver and binds to carbohydrate structures in a calcium dependent manner. Additionally, MBP is a member of a family of proteins, termed the collectins (Malhotra et al., 1992), which contain a lectin domain and a collagenous segment (reviewed in Holmskov et al., 1994), and constitute a subdivision of the broader C-type lectin superfamily (Drickamer, 1989).

In order to facilitate an understanding of the biological role of MBP its fundamental characteristics will now be considered. These essentially divide into two branches that cover structural and functional aspects of the protein. The following sections, therefore, focus on the properties of lectins and specifically collectins, and
Introduction

subsequently, the components of the innate immune system which interface functionally with MBP, namely the complement system and an MBP-specific cell surface receptor. Finally, a clinical defect in innate immunity relating to the role of MBP in host defence will be introduced together with the initial accounts of MBP deficiency.

As a preface to these topics some of the terminology and evolutionary aspects of MBP will first be considered.

**History, Nomenclature and Phylogeny of Mannose Binding Protein**

Human MBP was first reported in 1983 both in a preparation from liver (Wild *et al.*, 1983) and also in serum (Kawasaki *et al.*, 1983). Prior to that identical or analogous molecules had been described on several occasions in rabbit liver (Kawasaki *et al.*, 1978), rabbit serum (Kozutsumi *et al.*, 1980), rat liver (Townsend & Stahl, 1981; Mizuno *et al.*, 1981; Maynard & Baenziger, 1982) and mouse serum (Ihara *et al.*, 1982). Subsequently, the existence of two similar yet discrete mammalian forms was first reported in the rat (Drickamer *et al.*, 1986); there is no evidence of a second human form.

As a consequence of these discoveries the molecule is known by several names. It was initially termed mannan binding protein (Kawasaki *et al.*, 1978) and is also frequently called mannose binding protein (first used by Wild *et al.*, 1983). Furthermore, it has been referred to as the core-specific lectin (Brownell *et al.*, 1984), as part of the complex Ra-reactive factor (RaRF) (Ihara *et al.*, 1982b), and has been given the code MBL (mannose binding lectin) in genetic databases. Additionally, it is likely that MBP is one of the β-inhibitors of influenza A virus - heat labile serum factors, first reported in the 1940's and found to neutralise viral infection (Anders *et al.*, 1990). The traditional abbreviation as "MBP" is a simple way of avoiding the mannan/mannose conflict, and will be used throughout.

Much of the phylogeny of MBP has been detailed through study on RaRF, named as the reactive factor found to bind the Ra chemotype strains of salmonella (Ihara *et al.*, 1982b). Isolation of the major component of RaRF from the mouse and subsequent cDNA and amino acid analysis showed it to be homologous to rat MBP (Ihara *et al.*, 1991; Kuge *et al.*, 1992), and further studies on human isolates confirmed their identity (Matsushita *et al.*, 1992).
In functional studies RaRF has been shown to be present in all vertebrates higher than Cyclostomata (Kawakami et al., 1984). This group includes all mammals, birds, reptiles and amphibians, and both bony (e.g. trout) and cartilaginous (e.g. shark) fishes. However, more ancient fish, such as the lamprey and hagfish of the class Agnatha, and the more primitive Protocordates (a division of the Chordata - distinguished by having no cranium, vertebral column, or specialised anterior sense organs) and Arthropods are excluded. This analysis shows MBP to be over 300 million years old and apparently one of the ancestral molecules of the vertebrate immune system.
INTERPRETING THE CARBOHYDRATE CODE

Lectins

Nature has three basic building blocks: amino acids, nucleotides and monosaccharides. Of these it is the sugars (monosaccharides) that provide by far the greatest variation in assembly of higher order structures. The large number of different saccharides can combine via isomeric linkages into complex branched structures offering an enormous diversity in carbohydrate composition. For example, two units of a single monosaccharide can assemble in 11 different configurations, whereas in the same circumstances a single dipeptide or dinucleotide would result. Furthermore, 4 different monosaccharides could produce 35,560 unique molecules (Sharon & Lis, 1989).

The presence of carbohydrates, either as independent structures or in the form of glycosylated proteins, is now considered an important feature of many living organisms. These molecular forms are known to occur both intra- and extra-cellularly in animal and plant life (Barondes, 1984; Drickamer, 1988; Weir, 1989).

The variety of structure and occurrence strongly imply that polysaccharides must encode some form of information. The class of molecules that can decode this is known as the lectins.

The term lectin was first used by Boyd in 1954 and is derived from the latin "lectus", meaning chosen or selected. An early definition was proteins that agglutinate cells and/or precipitate polysaccharides and glycoproteins (Goldstein et al., 1980). Additionally, the requirement for a non-immune origin was specified to distinguish this group from the immunoglobulins. As time has progressed and the field broadened the definition has become less restricting. Lectins are now perceived as proteins which selectively bind to specific carbohydrate structures without being enzymes or antibodies (Barondes, 1988). In contrast to the early ideas several lectins are now known to play important roles in the action of the mammalian immune system.

The earliest lectin was described by Hermann Stillmark in 1888, being derived from castor oil seeds, and was notable for its ability to agglutinate erythrocytes (Sharon & Lis, 1987). At the turn of the century conglutinin from ox serum became the first vertebrate lectin reported (Bordet & Gay, 1906). The importance of lectin-carbohydrate interactions in higher order life forms was then largely neglected until reports of the clearance of desialylated glycoproteins from the circulation of mammals resulted in the
isolation of the membrane bound asialoglycoprotein receptor, also referred to as the galactosyl receptor or rat hepatic lectin (Ashwell & Morell, 1974). Meanwhile the first vertebrate lectin to be characterised had been described in the eel (Springer & Desai, 1971) and far ranging research was under way to uncover the importance of the carbohydrate code. Since these studies a widespread role for lectins has been found and postulated, ranging for example, from bacteria and protozoa, where carbohydrate binding sites on the surface of the organism are known to aid infection of the host through adherence to mammalian cells (Sharon & Lis, 1989), to additional roles in cell clearance, lymphocyte migration, and cell differentiation and growth in mammals (Feizi, 1985).

To assist classification of the highly specific types of lectin that have been described several categories have been introduced, and will now be considered in more detail.

C-type Lectins

The distinguishing feature of this class of proteins is their dependence on calcium for their ability to bind sugars. The carbohydrate recognition domain (CRD) is characterised by 14 invariant and 18 highly conserved amino acids (Drickamer, 1988). The whole domain contains between 115 and 130 residues and its conformation is maintained by important disulphide bonding and the chelated $\text{Ca}^{2+}$ ions. There are normally two calcium binding sites and four disulphide bonded cysteines per domain. The structural details of the CRD will be considered in depth in a later section. The functional CRD is attached to a variety of effector domains, which can be used as a basis for classification (Bezouska et al., 1991; Drickamer & Taylor, 1993). The principal categories are the transmembrane receptors, the soluble collectins, the surface bound selectins, and the free CRDs.

The transmembrane group, typified by the asialoglycoprotein receptor of hepatocytes mentioned above, feature a membrane anchor with an internal amino-terminal signal sequence (Type II transmembrane). In this case the hepatic receptor is providing a clearance system for serum glycoproteins, which are continually desialylated in the circulation, thereby exposing galactose. The avian homologue, the chicken hepatic receptor acts similarly, endocytosing N-acetylglucosamine (GlcNAc) terminated glycoproteins following removal of galactose. Interestingly the recently isolated alligator hepatic lectin is specific for terminal D-mannose or L-fucose. By coincidence or design the hierarchy of the species and their sugar specificity therefore reflect the order of
assembly of the complex-type polysaccharides from the core outwards: mannose (reptiles), GlcNAc (birds) and galactose (mammals) (Lee et al., 1994). A further human hepatic receptor is found on the resident liver macrophages (Kupffer cells) and is specific for fucose and galactose.

Other transmembrane receptors with the CRD consensus sequence are found on natural killer cells, but their binding characteristics have yet to be resolved accurately. Additionally, there is CD23, the low affinity IgE Fc receptor found on lymphocytes. The interaction of CD23 with IgE is not considered to be mediated by the CRD, however, a new ligand for the receptor, CD21, has recently been shown to implicate the CRD (Aubry et al., 1992). The macrophage mannose receptor could also be included here, although it contrasts with the other proteins by having a multiple number of CRD-like domains in one single polypeptide chain whereas all the other proteins have one. Moreover, it is the carboxy-terminus of this receptor that is situated inside the membrane (Type I transmembrane). The receptor is functionally similar to serum MBP in its specificity for mannose and ability to bind yeasts and parasites directly, but in addition it can signal the secretion of a number of inflammatory mediators. There are eight CRD-like modules in total, the fourth and fifth having the highest sequence consensus with the CRD of MBP, and it is the combination of them all that gives the receptor its binding affinity (Ezekowitz et al., 1990).

The collectins, of which MBP is one, encompass a subfamily of C-type soluble polymeric lectins where the CRD is linked to a collagenous domain. They are considered in detail below.

The most intensively studied animal lectins at present are the selectins, of which three are well characterised. These are termed E-, L- and P-selectin (formerly known as ELAM-1, LAM-1, and PADGEM/GPM140 based on their discovery in the endothelium, leukocytes and platelets respectively, and now given the designations CD62 E, L and P (Schlossman et al., 1994)). They are cell adhesion molecules in which an amino-terminal CRD is connected by an epidermal growth factor (EGF)-like domain, a variable number of complement regulatory repeats, and a membrane spanning region to the carboxy-terminal cytoplasmic domain (Type I transmembrane) (Drickamer & Taylor, 1993). The selectin genes are highly conserved and the proteins are functionally similar. They are involved in the initial "rolling" step of the interaction between endothelia and leukocytes, and all bind the carbohydrate structure sialyl Lewis X (Feizi, 1991).

The independent action of the separate regions within these multi-domain proteins
was illustrated by X-ray crystallography of E-selectin. Here, in crystals which contained both the EGF domain and the CRD very little interaction between the two regions was found (Graves et al., 1994).

An additional group of multi-domain proteins that do not fit into any of the above categories are the proteoglycans, formerly called mucopolysaccharides, which are structural constituents of the extracellular matrix and rich in polysaccharide. There are three which are known to possess a CRD: aggrecan found in cartilage, versican produced by fibroblasts, and neurocan found in the brain. These also contain EGF-like domains and complement regulatory repeats, plus the specialised regions for the covalent attachment of glycosaminoglycans (repeating disaccharide units involved in the retention of water within the tissue). The role of the proteoglycan CRD is not understood, but all of these proteins have an amino acid sequence consistent with galactose binding and aggrecan may interact with the galactose substituents of collagen type II (Day, 1994).

The free CRDs are a diverse group of proteins containing just the isolated binding domain. In invertebrates such lectins have been isolated from the sea urchin - echinoidin (Giga et al., 1987), acorn barnacle - lectins 1 and 2 (Muramoto & Kamiya, 1986; Muramoto & Kamiya, 1990), and fly (Takahashi et al., 1985). This group is distinct in evolutionary terms from the vertebrate free CRDs found principally in the pancreas and related to the HIP/PAP protein (over-expressed in liver cancer)(Lasserre et al., 1994). Free CRDs have also been found in snake venom, as antifreeze protein in arctic fish serum (Ng & Hew, 1992) and as other unclassified mammalian forms, such as tetranectin, a serum protein that binds plasminogen of the inflammation enzyme system (Fuhlendorff et al., 1987). The specificity of these CRDs is diverse, reflecting the different environments in which they must function.

The vertebrate free CRDs are all encoded by four exons and contrast with the single exon coding for the CRD in the collectins and selectins and the three exons found for the membrane bound receptors. This implies independent evolution of these CRDs.

**S-type Lectins**

These can be fundamentally distinguished from C-type lectins in that they are calcium independent. The binding specificity is predominantly for lactose, in particular beta-galactosides, and this has led to the term "Galectins" being proposed for this family of proteins (Barondes et al., 1994). Early ideas on the S-type lectins were summarised by Barondes (1984), and more recent data is reviewed by Drickamer & Taylor (1993).
The common structural feature of these lectins is a consensus sequence in the carbohydrate recognition domain of 19 invariant and 36 conserved residues and this has been found in several species (Drickamer & Taylor, 1993). The name S-type lectins was derived from the stability brought to this group by the presence of thiol groups, since oxidative conditions generally inhibit their binding activity. They have also been referred to as the soluble tissue lectins and any membrane association is principally due to carbohydrate binding of membrane components such as glycolipids or glycoproteins. S-type lectins tend to be multivalent, readily forming dimers as illustrated by recent X-ray crystallographic analyses (Bourne et al., 1994), and most have similar molecular masses, in the 14-16kDa range (Drickamer, 1988).

Lectins of this class are widely distributed, being detected in tissues as diverse as muscle and brain (Barondes, 1984). They are extensively found in intracellular locations, however, some functional S-type lectins have recently been found in the oxidising extracellular matrix, e.g. galectin-3 (formerly known as IgE-binding protein), which may be involved in immune regulation (Liu, 1993). No traditional signal transport sequence has been reported for any S-type lectin so the extracellular activity of the 31kDa galectin-3 has been proposed to follow a vesicle exporting process.

It is likely that soluble lectins mediate cellular activity, either through interactions within the cytoplasm, or by binding cell surface glycoproteins.

Other Types of Lectin

The pentraxins, serum amyloid P and C-reactive protein (CRP), which form pentameric structures, have CRD's which are not C- or S-type, yet both are involved in immune clearance through binding to carbohydrates on bacteria, fungi and parasites (Pepys, 1981). CRP was named for its ability to bind the C-polysaccharide of pneumococcus, and SAP binds several carbohydrate structures including agarose, phosphomannans and zymosan (Tennent & Pepys, 1994). Additionally, carbohydrate binding by SAP is calcium dependent and binding avidity is probably enhanced by the multimeric structure of the protein. In humans CRP is a major acute phase reactant, with levels increasing 1000-fold; SAP behaves analogously in other species (Steel & Whitehead, 1994). Furthermore, CRP and SAP activate complement via binding to the collagen-like region of C1q (Jiang et al., 1991). The pentraxins are not only widely distributed in vertebrates but have also been found in homologous forms in more primitive species such as the invertebrate horseshoe crab (Marchalonis & Schluter, 1990).
The mannose 6-phosphate receptors, classified as P-type lectins again contain a novel CRD (Drickamer & Taylor, 1993). As with the C-type macrophage mannose receptor there are multiple recognition sites on one molecule, in the form of 15 calcium independent and one calcium dependent site, thus giving an appearance analogous to the collectins.

The existence of several structurally independent sets of animal lectins, plus the apparently unrelated plant lectins and the bacterial carbohydrate binding proteins (Drickamer, 1988) implies that there has been strong pressure for lectins to evolve multiple times. Furthermore, as typified by C-type lectins, the variety of environments in which they function does provide an impressive display of the ability of organisms to adapt and utilise carbohydrate interactions.

Collectins

MBP is a member of a unique class of C-type lectins with a complex structure based on collagen helices connected to CRDs. As a consequence of these features they have been termed collectins (Malhotra et al., 1992) and are structurally similar to the well characterised complement protein C1q (reviewed in Reid, 1983). Additionally, the collectins interact with the collectin or C1q receptor, which is discussed in a later section. The current members of this family are the lung surfactant proteins SP-A and SP-D, and the serum proteins MBP, conglutinin and CL-43; the first three have been found in several mammalian species, however, the latter two are known only in bovine forms.

All collectins are polymeric structures based on assemblies of a three chain subunit as detailed below for MBP. Figure 1.1 illustrates their polypeptide chains - an initial cysteine rich region is followed by a series of Gly-X-Y repeats (X and Y can be any amino acid), a so-called neck region and the CRD. The Gly-X-Y triplets allow formation of a triple helix characteristic of collagens in which close packing of the chains ensures that glycine is positioned exclusively at the helix centre (Traub & Piez, 1971). The domain is also rich in proline and hydroxyproline, and each chain is considered to form a left handed polyproline II-like helix that then coils into a right handed 3 chain fibril (Bella et al., 1994). These fibrils assemble forming quaternary structures which are regarded as bouquet- or cross-like (Figure 1.2), although electron microscopy studies

1 fibril - a triple helical arrangement of 3 polypeptide chains
show them to be far from rigid (Lu et al., 1993).

Figure 1.1 Polypeptide Structure of the Collectins
The single collectin polypeptide chains have similar structural features that include a cysteine (C) rich N-terminus, a sequence of repeating Gly-X-Y triplets and the C-terminal carbohydrate recognition domain (CRD). The amino acid (aa) length of each region is indicated. (Adapted from Thiel & Reid, 1989).

Figure 1.2 Molecular Structure of the Collectins
Three polypeptide chains associate to form a subunit or fibril, which then combine to form higher order polymers. The structures are drawn to scale, with IgG shown for size comparison. The collagenous regions are illustrated by wavy lines and each CRD by a tulip shaped head. An alternative view of the MBP molecule is that the fibrils diverge at the start of the collagenous helix and commonly contains only three or four fibrils; see next Section. (From Holmskov et al., 1994).
The similarity in primary structure of the proteins is of interest. Conglutinin and bovine SP-D show 78% identity (Lim et al., 1993), whereas between bovine and human SP-D there is 67% identity, and between bovine and rat 65% identity. It is possible that conglutinin could represent a recent duplication of the SP-D gene found only in cows (Kölble & Reid, 1993). CL-43 also displays close similarity to bovine SP-D and conglutinin, showing 70% and 74% identity respectively (Lim et al., 1994).

The Structure of MBP

Human MBP is a homopolymer based on identical 30kDa polypeptide chains, which contain 228 amino acids, plus a 20 residue signal peptide removed prior to secretion (Summerfield & Taylor, 1986; Taylor et al. 1989). As mentioned above, three of these chains associate into a subunit, with a collagenous triple helical fibril (composed of 19 Gly-X-Y repeats from each chain) and a carboxy-terminal globular head containing three carbohydrate recognition domains (CRDs). The lectin domain provides the specificity for oligosaccharide structures on microorganisms, particularly D-mannose, N-acetyl glucosamine and L-fucose (Summerfield & Taylor, 1986; Weis et al., 1992), whereas the collagenous domain is believed to be responsible for interactions with other proteins involved in host defence.

The 3-chain subunits are held together by strong non-covalent interactions as well as disulphide bonding at the amino-terminus. These further combine to give higher order polymers of 6 - 18 chains, with the units being arranged in a bouquet-like manner (Lu et al., 1990; 1993). The tertiary structure of MBP and the method by which it forms are a matter of some debate. The interruption of the Gly-X-Y sequence at the interface of the first and second exons gives a protein sequence of Gly-Gln-Gly which is analogous to that found in two of the three chains of C1q (the A and C chains) (Reid, 1979). In that case the result is a bend or kink in the assembled helix. For MBP, however, the aberration is symmetrical, occurring in all three chains of the collagenous helix. Electron microscopy photographs have been interpreted in two ways, initially in favour of a bend at this point (Lu et al., 1990) and subsequently without, the structure being presumed to be merely more flexible at the interruption, and the fibrils to diverge at the start of the collagenous region (Lu et al., 1993). This latter model is also supported by studies from Kurata et al. (1993) on recombinant MBP in which site directed mutagenesis was used to remove the Gly-Gln-Gly interruption; despite this the protein was expressed and found to polymerise normally.
The Biochemistry and Genetics of MBP

Studies on MBP secreted from rat hepatocytes have shown that it is synthesised in the endoplasmic reticulum and then transported to the golgi prior to secretion. The half-life ($T_{1/2}$) of secretion is 4 hours, which is long compared to other secretory proteins ($T_{1/2} = 1$ hour for albumin). Several processes occur in the golgi - hydroxylation of proline and lysine residues within the collagen region, followed by glycosylation of a hydroxylysine that increases molecular mass by 2kDa, polymerisation and attainment of carbohydrate binding activity (Colley & Baenziger, 1987a; b). These modifications do not affect assembly, but do affect secretion (Colley & Baenziger, 1987c), and have been shown in part to occur in human MBP (Colley et al., 1988).

Once in the circulation turnover of serum MBP in the rat is relatively fast, with a $T_{1/2}$ of 1.6 days compared to the average of 3.0 days for serum proteins. The dissociation constant for carbohydrate binding has been measured at $2 \times 10^{-9}$M to mannan and $6 \times 10^{-9}$M to E. coli (assuming $M_w = 600,000$)(Oka et al., 1988; Kawasaki et al., 1989). This binding shows an interdependence on both the pH and the $Ca^{2+}$ concentration (Loeb & Drickamer, 1988).

The serum and liver forms of rat MBP (MBP- A and C) show 56% homology, and human MBP has features of both - the 3 cysteines and acute-phase nature of the serum MBP, and the larger N-terminus and interruption at the 8th Gly-X-Y repeat of liver MBP (Sastry & Ezekowitz, 1993). The overall homology of the rat forms with human MBP is 52% (serum) and 61% (liver)(Taylor et al., 1989).

The gene for human MBP is located on chromosome 10 (Taylor et al., 1989; Sastry et al., 1989). The intron/exon structure of the 7kb human gene closely resembles that of rat liver MBP (MBP C), and comprises four exons, each encoding a different domain of the protein. These regions can be separated into the N-terminal cysteine-rich domain (exon 1), the collagen domain (exons 1 and 2), the neck region (exon 3), and the C-terminal carbohydrate recognition domain (exon 4) (Taylor et al., 1989)(Figure 1.3).

In situ hybridisation studies have mapped MBP to chromosome 10q11.2-q21 (Sastry et al., 1989; Schuffenecker et al., 1991). This location is particularly interesting as a number of collectin genes cluster in this region: SP-D, has been mapped to chromosome 10q22.2-23.1 (Crouch et al., 1993; Kölble et al., 1993), and approximately 400kb away SP-A has been broadly assigned to 10q21-24 (Bruns et al., 1987), itself forming a cluster of two SP-A genes and one pseudogene (Kölble & Reid, 1993). The SP-A and SP-D genes lie 500-2000kb from the MBP locus. There is also some evidence
of an MBP pseudogene (MBP-B) in the rat, showing strong identity to exons 3 and 4 of the serum and liver forms (Drickamer & McCreary, 1987), although this may merely represent CRD homology with the corresponding domains of other C-type lectins. The probability of gene duplication and shuffling giving rise to these similar proteins seems high.

![Intron/Exon Structure of the Human MBP Gene](image)

**Figure 1.3** *Intron/Exon Structure of the Human MBP Gene*

The gene contains four exons, represented by boxes, with the length of each segment shown in base pairs (Sastry et al., 1989). The RNA contains a large untranslated (UT) region and codes for the protein domains indicated.

The upstream regulatory region of the gene contains several consensus sequences consistent with MBP being an acute phase protein. These include a region at -592bp similar to the heat-shock element first described in Drosophila (Pelham, 1982), and also found in C-reactive protein, the concentration of which can rise 1000-fold during an inflammatory response. There are also three regions similar to glucocorticoid-responsive elements, at -245bp, -656bp, and -736bp (Taylor et al., 1989). Levels of glucocorticoid hormones such as cortisol are released by the adrenals in response to stress; they regulate sugar metabolism and have anti-inflammatory action. Additionally, studies using a human hepatoma cell line (Hu H-7) have shown that gene transcription is enhanced by the cytokines interleukin-6 (IL-6), dexamethasone and heat-shock, although it is inhibited by IL-1 (Arai et al., 1993). In the mouse it has been shown that the liver form of MBP is not an acute phase reactant, whilst the serum form behaves like human MBP (Sastry et al.,
The regulatory motifs and genetic data suggesting an acute phase nature are supported by a study showing increased serum levels of MBP following either surgery or severe malaria, where MBP concentrations were found to rise 2 to 3-fold (Thiel et al., 1992).

**Surfactant Proteins A (SP-A) and D (SP-D)**

The surfactant proteins are an integral part of the lipid-protein layer that controls surface tension at the air-fluid interface of the lung. In addition some of these components may play a role in pulmonary host defence. The properties of SP-A and SP-D have recently been reviewed by Reid & Turner (1994) and Haagsman (1994).

SP-A has been found in several mammalian species, and in humans, at a concentration of 3mg/100mg phospholipid, is the most abundant lung surfactant protein. It has a structure similar to that of hexameric MBP, although SP-A differs in that it is composed of two polypeptide chain types. It is predominantly produced by the surfactant secreting alveolar epithelial type II cells and its primary role is likely to be in the maintenance of the surfactant layer (surfactant homeostasis). In the presence of Ca^{2+} SP-A can self aggregate, possibly by binding interactions involving the CRD, and cause the aggregation of phospholipid vesicles.

The principally tetrameric and much larger SP-D is present in the surfactant at approximately one tenth the level of SP-A. In purified form it has been demonstrated to assemble into higher order polymers, with up to 32 globular heads emanating from the central hub (Crouch et al., 1994). SP-D is less surfactant-like than SP-A, showing less affinity for phospholipids, and appears more involved in defence against inhaled pathogens (Hartshorn et al., 1994; Takayama et al., 1994).

SP-A and SP-D can bind to alveolar macrophages, an interaction possibly mediated by the collectin receptor. This has been shown to lead to phagocytosis and may result in microorganism killing through oxygen radical production. The specificity of the CRD of SP-A is for mannose, fucose and to a lesser extent galactose and glucose, but not GlcNAc. SP-D binds glucosyl sugars such as maltose and glucose. Such specificity does imply a role in pulmonary defence and indeed SP-A and SP-D have been shown to bind to the terminal and core regions, respectively, of lipopolysaccharide from gram negative bacteria (Haagsman, 1994).
Bovine Conglutinin and CL-43

Conglutinin (reviewed in Jensenius et al., 1994) is the structural homologue of SP-D, although the tetrameric subunits differ slightly in having a slight kink early in their collagenous regions. Conglutinin is known to play a role in host defence through its ability to agglutinate erythrocytes, and this interaction is mediated via the complement fragment iC3b deposited on cells, specifically by carbohydrate on the α-chain of iC3b. This binding only occurs once the C3 molecule has been catalytically cleaved to this form, thus illustrating the importance of the folding of the carrier protein in lectin-carbohydrate interactions. Conglutinin is known to interact with phagocytic cells during the immune response, and it is again possible that this occurs via the collectin receptor.

CL-43 has only been found as single subunits and, to date, no functional purpose has been described, although it is known to bind the collectin receptor in vitro. Both of these bovine proteins show sugar binding specificity similar to MBP (Loveless et al., 1989; Holmskov et al., 1993).

The Structure of the Carbohydrate Recognition Domain

The tertiary structure of a C-type CRD was first determined in 1991 by X-ray crystallography of the isolated domain of rat serum MBP (MBP-A) produced in a bacterial expression system (Weis et al., 1991b). Further studies in which the CRD was crystallised complexed with carbohydrate, and subsequent mutational analysis of the residues located in the vicinity of the binding pockets have established the critical residues involved in carbohydrate recognition, and shown why the observed consensus sequence is important (Weis et al., 1992; Iobst et al., 1994). Figure 1.4 shows two views of the sugar binding and second calcium chelation sites. Here the 3-OH of mannose is hydrogen bonded to MBP residues Glu185 (carbonyl group) and Asn187 (amine group), while the 4-OH sugar group binds to Glu193 (carbonyl) and Asn205 (amide). Both hydroxy groups also interact with the Ca^{2+}, which is additionally coordinated by the carbonyl groups from side chains of the four residues mentioned above and by Asp 206. Mutagenesis studies have also shown that His189 is important for mannose binding, since the loss of the His189 β-carbon which interacts by van der Waals forces with the C-4 of mannose causes a 5-fold loss of binding affinity (Iobst et al., 1994). The interaction shown between the C61 of Ile207 and mannose C-6 is due to weak van der Waals forces and is not of major importance (Ile is replaced by Val in human MBP and rat liver MBP). All of these residues fall within the last 25 of the 115 amino acids comprising the
domain.

Figure 1.4 Sugar and Calcium Binding to the MBP Carbohydrate Recognition Domain

The two orientations show the critical residues that are involved in interactions with the oligosaccharide (mannose) and Ca\(^{2+}\) (large grey sphere). White, black and light grey spheres represent carbon, oxygen and nitrogen, respectively. In the view on the left the lines show calcium coordination (long dash) and hydrogen bonds (short dash), and in the right view, van der Waals interactions (dotted). (From Weis et al., 1992 and lobst et al., 1994).

These structures for rat MBP provide a model for the CRDs of all C-type lectins, however, numerous modifications are to be expected given the variations in protein sequence found within the domain. By way of example, the subsequent crystallisation of the CRD domain of E-selectin has detected some differences in the coordination of Ca\(^{2+}\) (Graves et al., 1994). Selectins have only one calcium binding site, and this shows exact sequence homology with that of site two in rat serum MBP (the MBP coordinating residues Glu185, Asn187, Glu193, Asn205 and Asp206 equate to E-selectin Glu80, Asn82, Glu88, Asn106 and Asp106) (Weis et al., 1991b). Consequently it was predicted that the selectins would bind sugars in an identical manner to MBP (Weis et al., 1992). However, despite the similarity, slight changes in protein folding allowed one residue Asn83 (MBP equivalent Asp188) that binds the second Ca\(^{2+}\) in MBP to act as a coordination site in place of Glu88 (MBP equivalent Glu193) (Graves et al., 1994).

Furthermore, distortions can arise from forcing dynamic proteins to assume a rigid crystal form, exemplified by artefacts such as the third Ca\(^{2+}\) anomalously found binding to the rat MBP CRD (Weis et al., 1992). It would not be surprising, therefore, if all
CRDs showed some differences.

It is noteworthy that the C-type CRD in which the cation directly interacts with the ligand is fundamentally different from that of other known lectins. As already discussed, the S-type lectins are cation independent, and in the legume lectins, such as concanavalin A, the bound metal cations (calcium and manganese, or another transition metal) act to hold the ligand binding site together and position amino acids for binding, but do not interact directly with the sugar (Sharon, 1993).

The Control of Carbohydrate Specificity

MBP binds to a variety of sugars, and as the origin of its name suggests the preferred ones are the primary components of mannann, namely D-mannose and N-acetyl-D-glucosamine (GlcNAc) (Kawasaki et al., 1978). The structures of these and related sugars are illustrated in Appendix A. All the collectins bind monosaccharides containing equatorial 3- and 4- OH groups and consequently other sugars to which MBP will bind are D-glucose and N-acetyl-D-mannosamine, plus L-fucose via its 2- and 3- hydroxy groups (Colley et al., 1988; Holmskov et al., 1994). The binding of L-fucose is possible because on rotation its hydroxy groups align directly with those of the D-pyranose groups (Weis et al., 1992).

In the case of human MBP the binding is believed to be directed at the terminal (non-reducing) residues and can be blocked by capping the terminal group with galactose (this has an axial 4-OH group and is known not to bind MBP) (Childs et al., 1989). The two forms of rat MBP have different specificity, the serum form (MBP-A) behaves like human MBP, whilst the hepatic form (MBP-C) preferentially binds core mannose residues, although it also binds terminal fucose (Childs et al., 1990). The specificity of the liver form of MBP explains the early description of the core-specific lectin prepared from rat hepatocytes (Brownell et al., 1984), however, the specificity of the serum form does not agree with the binding characteristics reported for RaRF. This complex, and therefore MBP, has been widely identified in the sera of many species and the binding site reported as the core GlcNAc residues of the lipopolysaccharide of Ra-strain salmonella (Ihara et al., 1982a). Additionally, there are other contradictory reports of human MBP binding core rather than peripheral sugars (Summerfield & Taylor, 1986).

An explanation for the RaRF study may be that the sugar binding preference of MBP does vary between species (Holmskov et al., 1994), perhaps reflecting the different self and non-self carbohydrate structures that the molecule encounters. Alternatively, the
carrier protein may influence carbohydrate presentation and therefore affect biological specificity (Solis et al., 1994).

The detailed crystal structures now available have permitted the precise binding sites of the monosaccharides to be modelled and provide an explanation of why a particular ring system is preferred. Structural differences between some key sugar residues are highlighted in Appendix A. The critical importance of individual residues within the CRD for sugar binding was shown by the engineering of a new ligand specificity for the rat MBP domain (Drickamer, 1992). Site directed mutagenesis was used to replace two of the conserved residues found in the mannose binding lectins (in rat MBP Glu185 and Asn187, shown in Figure 1.4) with corresponding residues conserved in C-type CRDs known to preferentially bind galactose (Gln and Asp). This successfully generated galactose binding activity, which was subsequently improved by further changes - mutating His189 to Trp increased affinity, and a further 5 amino acid insertion led to an actual specificity for galactose. This type of analysis permits an understanding of how all the C-type CRDs function, since the final mutant amino acid sequence and specificity was analogous to the rat hepatic lectin, and the Trp intermediate resembled the proteoglycan aggrecan (Jobst & Drickamer, 1994).

Assembly of the Subunits

Protein folding frequently occurs following the formation of disulphide bonds, as shown in the assembly of the triple-helical domain of procollagen (Freedman, 1987). Furthermore, the collagen helix is normally assembled from the C- to N- terminus (Sykes, 1992). C1q is synthesised in this manner with the N-terminal disulphide bonds anchoring the structure (Brodsky-Doyle et al., 1976). For the collectins to polymerise from the cysteine rich domain they would have to assemble in the reverse direction, i.e. from the N- to C- terminus. This is intuitively unlikely and there was early evidence for SP-A that oligomerisation would occur despite disruption of disulphide bonding (Kuroki et al., 1988a). The method of assembly and the long speculation as to the true purpose of exon 3 (first raised by Drickamer & McCreary, 1987) were resolved when the neck region was shown to be the initiation site for assembly of SP-D (Hoppe et al., 1994). Nevertheless there does appears to be some variation in the site of nucleation for the different collectins.

In SP-D studies by nuclear magnetic resonance on recombinant sections of the neck domain have shown that this will spontaneously trimerise, and furthermore, can
reassemble after heat denaturation (Hoppe et al., 1994). In contrast, corresponding sections of MBP will not assemble (Weis & Drickamer, 1994), although the neck plus CRD does form stable trimers in solution (Schweinle et al., 1993). Examination of the amino acid sequence that would result from a α-helical coil for SP-D, conglutinin and CL-43 shows that strong hydrophobic interactions are possible between the residues on the inside of the coil. The same alignment is not as favourable for MBP, with some hydrophobic residues pointing outwards, which would explain the lack of stability of the isolated neck region (Hoppe & Reid, 1994). Crystallisation of the trimeric head plus neck unit has shown, however, that these hydrophobic residues on the outside of the coil are stabilised by contact with a hydrophobic region in the CRD of the adjacent anticlockwise chain (Sheriff et al., 1994). The resulting structure shown in Figure 1.5 has the binding sites of the three CRDs separated by between 45Å (human) (Sheriff et al., 1994) and 53Å (rat) (Weis & Drickamer, 1994). The distance between the sites suggests that extended carbohydrate structures would be required for multi-point attachment of the heads. Such structures are found on many pathogens.

Figure 1.5 The Trimeric Head of an MBP Fibril
Axial view of a globular head of the MBP molecule containing three CRD’s, looking towards the amino terminus. The chelated calcium ions (1 and 2) are shown by dark spheres (the 3rd calcium ion (white sphere) is probably an artefact of the crystallisation). Carbohydrate binds at site 2. (From Weis & Drickamer, 1994).
ASPECTS OF INNATE IMMUNITY

The Complement System

Complement is a major part of the human immune system that is responsible for defence against foreign particles and the clearance of immune complexes. Its actions "complement" the role of antibodies in the destruction of invading microorganisms. The complement system which was first described in the 1890s consists of about thirty plasma proteins, with new members still being discovered. Complement is fundamentally activated by either of two distinct enzyme cascade systems called the Classical and Alternative pathways (reviewed in Law & Reid, 1988; Walport & Lachmann, 1993). However, the interaction of MBP with complement, initially discovered through its ability to activate the classical pathway, could be considered as introducing a new route of activation - the Lectin pathway.

These pathways fulfil several roles, enhancing opsonisation of alien particles and later merging as the complement cascade follows the single lytic (or terminal) pathway that ultimately leads to cell lysis. The term complement cascade arises from the chain reaction-like process where each step initiates the next, but importantly, this process is strictly controlled by a number of regulatory molecules. Significantly, the complement cascade interacts with other enzyme cascades which control coagulation, kinin generation (involved in the regulation of vascular permeability), and fibrinolysis (in which wound healing is promoted and inflammatory regulators such as plasmin are produced)(Male & Roitt, 1989).

The Classical Pathway

Activation of the classical pathway principally involves recognition of aggregated immunoglobulin in immune complexes by C1q. Specifically, C1q binds through ionic interactions to the Fc region (C1q2 domain) of IgG. This is a high affinity interaction when multiple Fc sites are presented together (Reid, 1983). Alternatively C1q binds to the C1q3 domains of the pentameric IgM molecule once it has bound antigen and assumed a non-planar, arched configuration. It is also possible for C1q to interact directly with certain bacterial or viral envelopes, for example lipopolysaccharides in the outer layer of a bacterial capsule (Law & Reid, 1988; Rook, 1989).

C1q is the recognition part of a circulating five component complex, C1, which
also contains a catalytic subunit - two molecules of each of the serine proteases C1r and C1s, arranged C1s-C1r-C1r-C1s (Arlaud et al., 1990; 1993). The binding of C1q leads to activation of these proenzymes, and subsequently, to cleavage of the next complement component C4, which exists as two isotypes, C4A and C4B that have slightly different activity. This process involves autoactivation of C1r to its active form, which then activates C1s, and that in turn acts on C4 and subsequently C2. The process of cleavage of C4 produces C4a (an anaphylatoxin) and the labile C4b containing an active thioester which can react with amine (mainly the C4A isotype and peptides) or hydroxy groups (mainly the C4B isotype and carbohydrates) on the surface of the complement activator (Law & Dodds, 1990). C2 in the presence of active C1s, is cleaved forming the fragment C2a which contains a serine esterase catalytic site. Association of C2a with the bound C4b leads to the formation of the "C3 convertase" C4b2a, which cleaves C3 via the action of the catalytic site on C2a. C3, like C4, contains a thioester and following the action of a C3 convertase is converted to C3b which binds to any suitable nearby surface. The action of C1 is controlled by two proteins: the C1-inhibitor, which binds and inactivates C1r and C1s; and factor J, which dissociates the C1 complex into its subunits (Nicholsen-Wellar et al., 1991).

The Alternative Pathway

Activation of the alternative pathway depends on a constant low level turnover of C3 in body fluids (Barnum & Volanakis, 1990). This can occur by the action of serum proteases, low level hydrolysis, or any disturbance of the C3 molecule that results in the exposure of its internal thioester bond. Under physiological conditions the thioester has a half life ($T_{1/2}$) of 230 hours and slow hydrolysis to C3(H$_2$O) leads to conformational and functional changes in C3. The new species can form a complex with factor B that is magnesium cation dependent. It is this procedure that constitutes the initiation step in the pathway. Next factor D (another serine protease) catalyses cleavage of factor B releasing the N-terminal, 30kDa, Ba fragment. The C3(H$_2$O)Bb complex has C3 convertase activity. Once C3b is formed this associates with factor B, which is again cleaved by factor D to form C3bBb. This can be stabilised by properdin and is able to bind to activator surfaces (e.g. bacterial polysaccharides or parasites). The C3bBb complex is the main C3 convertase of the alternative pathway and amplifies the whole mechanism by producing further C3b.

The alternative pathway is down regulated by a number of molecules. In serum
factor H influences the stability of C3bBb, accelerating its dissociation into C3b and Bb. Factor H also acts as a cofactor for factor I, promoting cleavage of C3b to yield an inactive form, iC3b, that is no longer able to bind to factor B. To prevent attack of host cells both the classical and alternative pathways are inhibited by three membrane bound regulatory molecules, complement receptor type 1 (CR1; C3b/C4b receptor; CD35), decay accelerating factor (DAF; CD55) and membrane cofactor protein (MCP; CD46). These all behave analogously to factor H by suppressing the action of the C3 convertases (Morgan & Meri, 1994).

**Destruction of Foreign Particles**

The classical and alternative pathways converge at this activation of C3, the most abundant complement component (concentration ca 1mg/ml). The splitting of C3 produces C3a, a small 77 residue anaphylatoxin fragment and the much larger C3b (approximately 175kDa), in which a large conformational change occurs, exposing the highly labile thioester ($T_{1/2} = 60\mu s$ in serum). Many interactions are possible at this point, but to progress towards cell lysis the classical and alternative pathway complexes, C4b2a and C3bBb, must covalently bind C3b. This results in a new enzyme specificity - the C5 convertases, the actions of which are also inhibited by the membrane bound regulatory proteins. It is currently considered that C3b forms a covalent dimer with the surface bound C4b or C3b of the C3 convertases (Kinoshita et al., 1988). C5 binds to these dimers and is catalytically cleaved by the complex releasing the C5a anaphylatoxin and C5b, the first member of the terminal or lytic pathway.

C5b binds non-covalently to C6 and C7, producing a complex, C5b-7, which is hydrophobic in nature and can insert itself into lipid bilayers. The binding of C8 further enhances this process and leads to the polymeric assembly of C9 within the membrane. This final membrane attack complex (MAC), C5b-9, causes lesions in the lipid membrane and ultimately results in cell lysis. To prevent attack of host cells there are two known inhibitors of MAC assembly: CD59 and homologous restriction factor (HRF).

An alternative and frequently more common method for eliminating pathogens involves the deposition of large quantities of opsonic C3b fragments on the surface of the microbe. These are then recognised by receptors on phagocytic cells.

**The Lectin Pathway**

The ability of MBP to activate complement has been established by a number of
groups and is believed to be via the classical pathway (Ikeda et al., 1987; Lu et al., 1990; Super et al., 1990), however, it is also claimed that MBP can activate the alternative pathway (Schweinle et al., 1989). Additional work with RaRF has further suggested that the classical pathway is involved (Ji et al., 1988).

It is known that the serine protease components of C1, C1r and C1s, can interact with MBP bound to a mannan coated surface and initiate cleavage of C4, in the absence of C1q (Ohta et al., 1990; Lu et al., 1990). The affinity of C1r/s for MBP appears similar to that for C1q, although it is only the larger polymeric forms of MBP that will activate C1r efficiently (Lu et al., 1990). The relative biological importance of this mechanism has been brought into question by the discovery of a novel C1s-like molecule, termed MBP associated serine protease (MASP) (Matsushita & Fujita, 1992). MASP appears to circulate with MBP, bound in a calcium dependent manner, and autoactivates upon binding of the lectin. The stoichiometry of this interaction and the actual serum concentration of MASP remain to be established. Further evidence for this novel activation pathway for MBP comes from studies on RaRF in the mouse. The minor component of this complex, referred to as P100, has been shown to cleave C4 (Ji et al., 1993), and has now been isolated and found to be a new serine protease similar to C1r and C1s (Takahashi et al., 1993; Takayama et al., 1994). It seems probable that murine P100 and human MASP are equivalent, and given the phylogenetic diversity established for RaRF (Kawakami et al., 1984) MASP activation of MBP appears to be universal amongst vertebrates.

The recognition of a new lectin pathway for the independent activation of the complement system by MBP appears to be gaining acceptance (Thiel, 1992; Reid & Turner, 1994), but the ability of the other collectins to act through this or an analogous pathway awaits investigation.

A summary of the pathways of complement activation is shown in Figure 1.6.

The Collectin Receptor

Formerly referred to as the C1q receptor (C1qR), the term collectin receptor was coined in 1993 (Malhotra et al., 1993b). This receptor has been shown to bind several members of the collectin family including MBP, conglutinin and SP-A (Malhotra et al., 1990). The existence of a cell surface receptor for C1q was first suggested by Dickler
Introduction

A lternative Classical LECTIN Pathway

Foreign Surface

CARBOHYDRATE

C-Rcactive Protein

SP-A, SP-D, Conglutinin

M B P

C lq

COLLECTIN Receptor

Phagocytic cells

C1r,s

C2, C4

C3 activation

Receptors : CR1, CR2, CR3 for C3 fragments

+ Lytic Pathway

Figure 1.6 Pathways of Complement Activation and Opsonisation

The alternative, classical and lectin pathways of complement merge at the activation of C3 and ultimately lead to opsonophagocytosis or cell lysis. Some of the principal components of the pathways are illustrated above. (The properties of the complement receptors (CR) are reviewed in Lachmann et al. 1993).

& Kunkel (1972), and it was later shown that binding was via the collagenous region of the molecule (Arviex et al., 1984). Given the homologous collagen domains found in the collectins it is presumed that they interact with the receptor in the same manner. The majority of work with the receptor has utilised C1q and since the relative interactions of MBP, SP-A and C1q with the receptor are similar (Malhotra et al., 1990), any properties reported for the C1qR are presumed to apply directly to interactions involving the collectins.

The receptor is widely distributed (reviewed in Tenner, 1993), with activity found on most leukocytes, endothelial cells, platelets, smooth muscle and epithelial cells. For leukocytes most positive cells are contained in the B cell and macrophage populations.
The murine distribution of the C1qR is similar to that found for humans (Erdei, 1990). The effects of receptor-ligand interactions include enhanced phagocytosis and immunoglobulin secretion, and neutrophil mediated cytotoxicity (oxygen radical stimulation), although there is evidence that the latter is not triggered by MBP or SP-A (Goodman & Tenner, 1992).

The characterization of the collectin receptor has been complicated by two factors: primarily the anomalous behaviour of the receptor when analysed by different procedures (Erdei & Reid, 1989), and additionally, by the existence of a further receptor for C1q, now known to bind to the globular head of this molecule (Ghebrehiwet et al., 1994). The purified collectin receptor has an apparent molecular mass of 56kDa under reducing conditions, and contains 15-20% carbohydrate (Malhotra & Sim, 1989). Moreover, C1qR shares a high degree of sequence homology and immunological cross-reactivity with calreticulin, a Ca^{2+} storage protein (Malhotra et al., 1993). Calreticulin is released from neutrophils during inflammation and is able to bind to MBP (and more favourably C1q). There is speculation that this interaction may cause immune complex formation and a subsequent stimulation of the immune response (Eggleton et al., 1994).

The bonding between the receptor and the collectins is thought to be ionic, since lower salt strengths are preferred. The precise point of interaction has been proposed as the "bend" region, as evidenced by the presence of a cluster of charged residues at this point in C1q, MBP and SP-A. In conglutinin this cluster is near the N-terminus and is lost in a truncated form of the molecule which does not bind the receptor (Malhotra et al., 1993a).

The Common Opsonic Defect

An insight into the clinical importance of serum MBP was established in 1989 when low concentrations of the protein were associated with a common defect of opsonisation (Super et al., 1989). The normal process of opsonisation entails the coating of particles with proteins that enhance their ingestion by phagocytic cells. Wright and Douglas (1904) first reported a failure of this procedure, detailing the inability of certain individuals to opsonise staphylococci for phagocytosis by polymorphonuclear leukocytes. The common opsonic deficiency was first described by Miller et al. (1968) in a child with frequent infections and failure to thrive. These authors also demonstrated a familial
linkage and showed that the defect could be corrected by plasma infusion. Further studies showed the abnormality was present in approximately 5-8% of random populations (Soothill & Harvey, 1976; Levinsky et al., 1978; Yamamura & Valdimarsson, 1978; Kerr et al., 1983; Wyke et al., 1983) and confirmed that there was a defective factor in these individuals, although the primary cause was still not established. The defect appeared to be more common in children with frequent unexplained infections and atopy (Soothill & Harvey, 1976; Candy et al., 1980; Richardson et al., 1983). Almost all these investigations used baker's yeast (Saccharomyces cerevisiae), or its cell wall extract zymosan, in order to measure phagocytic function, and subsequent studies were able to link suboptimal deposition of complement fragments C3b and C3bi on the yeast surface with the opsonic defect (Turner et al., 1981; 1986) and the absence of an essential cofactor (Turner et al., 1985a; b). When mannan, a predominant polysaccharide in zymosan, was used in complement binding assays to study a population of blood donors, defective opsonisation was associated with low levels of MBP and the role of the novel lectin pathway of complement activation was appreciated (Super et al., 1989; 1990). The importance of MBP deficiency as a newly recognised complement defect was detailed by Turner (1991) and Turner et al. (1991).
THE ROLE OF MBP IN HOST DEFENCE

As detailed in the above sections MBP has a structure that enables it to recognise particular foreign organisms and facilitate their clearance from the circulation both by activating the complement system and interacting directly with a specific cell surface receptor. The importance of these processes was illustrated by the linking of the clinical common opsonic defect with low levels of MBP (Super et al., 1989), and a concurrent study in which the protein was shown to function as an opsonin in the phagocytosis of gram-negative bacteria bearing mannose rich polysaccharides (Kuhlman et al., 1989). Several subsequent investigations have addressed the function of MBP in host defence.

MBP has been shown to play a role in the complement mediated neutralisation of influenza virus (Anders et al., 1994). This protection was lost for a mutant viral strain in which a glycosylation site for high-mannose carbohydrate was missing; MBP was proposed to act by binding this oligosaccharide chain and thereby blocking access to the adjacent cell binding domain (Anders et al., 1990). Fellow collectins SP-D and conglutinin have similarly been shown to function as opsonins for influenza (Hartshorn et al., 1993; Anders et al., 1994). Complement activation has also been observed following MBP binding to HIV envelope glycoproteins (Haurum et al., 1993) and inhibition of viral infection has been reported (Ezekowitz et al., 1989). However, a role in host defence is unclear since the later in vitro study used concentrations of MBP far in excess of physiological levels, and another investigation has shown that carbohydrate-mediated interactions may actually be the basis of viral infection (Larkin et al., 1989). A similar enhancement of infection by MBP has also been reported for herpes simplex virus in mice (Fischer et al., 1994).

Further in vitro studies on yeasts have shown MBP to have no effect on the phagocytosis of Cryptococcus neoformans (Levitz et al., 1993) and to actually inhibit the phagocytosis of Candida albicans by murine macrophages (Kitz et al., 1992). Set against this is the complement mediated bactericidal properties of MBP against Ra strains of Salmonella (Ihara et al., 1982a).

MBP Deficiency

In 1991 the molecular cause of low serum MBP levels and therefore the basis of
the common opsonic defect was established (Sumiya et al., 1991). Family studies, centred on individuals presenting with frequent infections and the opsonic defect, suggested that a point mutation at base 230 of exon 1 of the MBP gene was associated with low concentrations of the protein (Figure 1.7). The mutation changed codon 54 from GGC to GAC and at the protein level resulted in the replacement of glycine, from the fifth Gly-X-Y collagenous repeat, with the much larger aspartic acid. It was further suggested that this substitution would disrupt the secondary structure of the protein, thereby preventing its secretion, or making it vulnerable to degradation once in the circulation, and consequently lead to low concentrations of serum MBP.

![Figure 1.7 MBP Levels and Genotype in Families of Patients with the Common Opsonic Defect](image)

Protein levels were measured by ELISA and genotype determined by DNA sequencing (Sumiya et al., 1991). * indicates probands, Gly/Gly - wild type sequence, Gly/Asp - heterozygous for the codon 54 mutation, Asp/Asp - homozygous for the codon 54 mutation. (Adapted from Sumiya et al., 1991 after revision of MBP serum levels (Super et al., 1992)).
AIMS OF INVESTIGATIONS DESCRIBED IN THIS THESIS

The following Chapters describe investigations aimed at furthering our knowledge on various aspects of human MBP and its deficiency. These include:

► The development of ELISA procedures for measurement of human MBP
► A study of the common opsonic defect in the Chinese population
► An evaluation of the frequencies of mutations in the MBP gene in several population groups
► A physicochemical analysis of MBP from individuals of differing genotype
► Selected studies of MBP deficiency and possible clinical associations
CHAPTER 2

METHODOLOGY

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# REAGENTS AND MATERIALS

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<td>Sulphuric acid, H₂SO₄</td>
<td>BDH</td>
<td>10249</td>
</tr>
<tr>
<td>Name, chemical formula, formula weight (where appropriate)</td>
<td>Company</td>
<td>Code</td>
</tr>
<tr>
<td>----------------------------------------------------------</td>
<td>------------------</td>
<td>--------</td>
</tr>
<tr>
<td>T4 polynucleotide kinase</td>
<td>Promega</td>
<td>M4101</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>Bioline</td>
<td>M55801 B</td>
</tr>
<tr>
<td>Temed (N,N,N',N' tetramethylethylenediamine), C_6H_{16}N_2</td>
<td>Bio-Rad</td>
<td>1610800</td>
</tr>
<tr>
<td>Thimerosal (Thiomersal), C_2H_3Hg.S.C_6H_4CO_2Na, FW 404.8</td>
<td>BDH</td>
<td>30416</td>
</tr>
<tr>
<td>Tris (hydroxymethyl) aminomethane, C_4H_{10}NO_3, FW 121.1</td>
<td>Sigma</td>
<td>T6791</td>
</tr>
<tr>
<td>tRNA, yeast</td>
<td>Boehringer Mannheim</td>
<td>109495</td>
</tr>
<tr>
<td>Tween 20 (polyoxyethylene(20)sorbitan monolaurate)</td>
<td>BDH</td>
<td>66368</td>
</tr>
<tr>
<td>Urea, CH_4N_2O, FW 60.1</td>
<td>Sigma</td>
<td>U5378</td>
</tr>
</tbody>
</table>

**Antibodies**

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Company</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-human C3c HRP (mouse)</td>
<td>Serotec</td>
<td>AHP031P</td>
</tr>
<tr>
<td>Anti-human MBP, clone 131-1 (mouse)</td>
<td>Cymbus Bioscience</td>
<td>CBL 303</td>
</tr>
<tr>
<td>Anti-mouse IgG HRP (sheep)</td>
<td>Sigma</td>
<td>A6782</td>
</tr>
<tr>
<td>Anti-rabbit IgG HRP (goat)</td>
<td>Sigma</td>
<td>A4914</td>
</tr>
<tr>
<td>Murine IgG1, kappa (MOPC-21)</td>
<td>Sigma</td>
<td>9269</td>
</tr>
<tr>
<td>Anti-human MBP antibodies</td>
<td>Monoclonals M35, M37</td>
<td>Prof. J. Summerfield London</td>
</tr>
<tr>
<td>Monoclonals #3 HRP, #6</td>
<td>Dr. R.A.B. Ezekowitz</td>
<td>Boston</td>
</tr>
<tr>
<td>Polyclonal (Oxford)</td>
<td>Dr. J. Lu</td>
<td>Oxford</td>
</tr>
<tr>
<td>Polyclonal (Boston)</td>
<td>Dr. R.A.B. Ezekowitz</td>
<td>Boston</td>
</tr>
</tbody>
</table>

* All reagents were Analar grade or equivalent

* See Appendix B for full addresses of suppliers
BUFFERS AND SOLUTIONS

Carbonate/Bicarbonate buffer
(ELISA coating):

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Working Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaHCO₃</td>
<td>2.93g/l 35mM</td>
</tr>
<tr>
<td>Na₂CO₃</td>
<td>1.59g/l 15mM</td>
</tr>
<tr>
<td>pH 9.6 +/- 0.2</td>
<td></td>
</tr>
</tbody>
</table>

Citrate/Phosphate buffer
(ELISA substrate) for 100ml:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>48.5 ml 0.1M citric acid</td>
<td></td>
</tr>
<tr>
<td>51.5 ml 0.2M di-sodium hydrogen phosphate (Na₂HPO₄)</td>
<td></td>
</tr>
<tr>
<td>pH 5.0</td>
<td></td>
</tr>
</tbody>
</table>

Citric acid (C₆H₈O₇.H₂O) 21.0g/l 0.1M
Na₂HPO₄ 28.4g/l 0.2M

ELISA Substrate
(made up fresh)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>20ml citrate/phosphate buffer, pH 5.0</td>
<td></td>
</tr>
<tr>
<td>10mg o-phenylene diamine (OPD) 0.5mg/ml</td>
<td>27mM</td>
</tr>
<tr>
<td>10µl 30%(v/v) hydrogen peroxide 0.5µl/ml</td>
<td>0.05%(v/v)</td>
</tr>
</tbody>
</table>

Imidazole buffer
(MBP/mannan ELISA):

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>imidazole</td>
<td>2.72g/l 40mM</td>
</tr>
<tr>
<td>HCl adjust to pH 7.8</td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>73.1g/l 1.25M</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>5.55g/l 50mM</td>
</tr>
<tr>
<td>or {CaCl₂ . 2H₂O</td>
<td>7.35g/l}</td>
</tr>
</tbody>
</table>

Immunoblot transfer buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>5.82g/l 48mM</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.93g/l 39mM</td>
</tr>
<tr>
<td>Methanol</td>
<td>20% (v/v)</td>
</tr>
</tbody>
</table>

Phosphate buffer
(general purpose):

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>di-sodium hydrogen phosphate (Na₂HPO₄)</td>
<td>28.4g/l 0.2M</td>
</tr>
<tr>
<td>sodium di-hydrogen phosphate (NaH₂PO₄.2H₂O)</td>
<td>31.2g/l 0.2M</td>
</tr>
<tr>
<td>(coating buffer; Na₂HPO₄ 265ml : NaH₂PO₄ 735ml, pH 6.5)</td>
<td></td>
</tr>
</tbody>
</table>
Phosphate buffered saline (PBS) (general purpose):

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>1.15 g/l</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.2 g/l</td>
</tr>
<tr>
<td>NaCl</td>
<td>8.0 g/l</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2 g/l</td>
</tr>
<tr>
<td>pH</td>
<td>7.3</td>
</tr>
</tbody>
</table>

Working Concentration

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>14.4 g/l</td>
</tr>
<tr>
<td>Tris</td>
<td>3.03 g/l</td>
</tr>
<tr>
<td>SDS</td>
<td>0.1% (w/v)</td>
</tr>
</tbody>
</table>

SDS-PAGE running buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>3.03 g/l</td>
</tr>
<tr>
<td>SDS</td>
<td>0.1% (w/v)</td>
</tr>
</tbody>
</table>

Standard saline citrate (SSC) (general purpose):

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8.76 g/l</td>
</tr>
<tr>
<td>Trisodium citrate</td>
<td>4.41 g/l</td>
</tr>
<tr>
<td>pH</td>
<td>7</td>
</tr>
</tbody>
</table>

Tris acetic acid EDTA (TAE) (agarose electrophoresis buffer) stock solution 50x:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>242 g/l</td>
</tr>
<tr>
<td>acetic acid</td>
<td>57.1 ml/l</td>
</tr>
<tr>
<td>EDTA (0.5M, pH 8.0)</td>
<td>100 ml/l</td>
</tr>
</tbody>
</table>

working solution 1x:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris acetate</td>
<td>40 mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>1 mM</td>
</tr>
</tbody>
</table>

Tris boric acid EDTA (TBE) (DNA electrophoresis buffer)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>12.1 g/l</td>
</tr>
<tr>
<td>boric acid</td>
<td>5.56 g/l</td>
</tr>
<tr>
<td>sodium EDTA</td>
<td>0.74 g/l</td>
</tr>
<tr>
<td>pH</td>
<td>8.3</td>
</tr>
</tbody>
</table>

Tris EDTA (TE) (DNA buffering) pH 7.6:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris HCl (pH 7.6)</td>
<td>10 mM</td>
</tr>
<tr>
<td>EDTA (pH 8.0)</td>
<td>1 mM</td>
</tr>
</tbody>
</table>

Methodology
Veronal buffered saline (VBS)
stock solution x5:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>42.5g/l</td>
</tr>
<tr>
<td>barbituric acid</td>
<td>2.86g/l</td>
</tr>
<tr>
<td>sodium barbitone</td>
<td>1.88g/l</td>
</tr>
</tbody>
</table>

VBS\(^{++}\) (veronal buffered saline, + MgCl\(_2\), + CaCl\(_2\))
(complement fixation/mannan ELISA):

- 20ml VBS x5
- 1ml 0.5M MgCl\(_2\)
- 1ml 0.5M CaCl\(_2\)
- 78ml distilled water

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgCl(_2), 6H(_2)O</td>
<td>2.03g/20ml</td>
</tr>
<tr>
<td>CaCl(_2), 2H(_2)O</td>
<td>1.47g/20ml</td>
</tr>
</tbody>
</table>

working solution:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>145mM</td>
</tr>
<tr>
<td>barbituric acid</td>
<td>4.4mM</td>
</tr>
<tr>
<td>sodium barbitone</td>
<td>1.8mM</td>
</tr>
<tr>
<td>MgCl(_2)</td>
<td>5mM</td>
</tr>
<tr>
<td>CaCl(_2)</td>
<td>5mM</td>
</tr>
</tbody>
</table>
SUBJECTS AND SAMPLES

Caucasian

Samples were obtained from adult British blood donors attending the West End Donor Centre in London (98 individuals; 66 male, 33 female). Whole blood was taken into potassium-EDTA and frozen at -70°C until used for DNA extraction. Separated serum was also stored at -70°C until required.

Chinese

Several groups of samples were collected from the Chinese population of Hong Kong by Dr. Y.L. Lau (University of Hong Kong). Adults were recruited from commercial organisations (123 individuals; 63 male, 60 female). The children studied were attending health clinics, kindergartens and schools (100 individuals divided into age ranges as follows: 0-1 (n=20), 1.1-4 (n=20), 4.1-5 (n=10), 5.1-6 (n=10), 6.1-9 (n=20), 9.1-16 years (n=20)). Newborn cord blood samples were obtained at the Queen Mary Hospital (60 individuals). Whole blood was taken into potassium-EDTA and frozen at -70°C. Separated serum was also stored at -70°C. All samples were subsequently air freighted to London in dry ice and stored at -70°C until required.

Gambian

Blood samples from two population groups were collected at the Royal Victoria Hospital, Banjul, The Gambia by Dr. A. Hughes. The first comprised adult male blood donors (100 individuals) and the second consisted of cord blood samples collected at delivery in the Hospital (100 individuals, 41 males, 59 females). HIV positive samples were removed prior to commencement of the study (this represented 4 adults and 3 newborns). Whole blood (collected into lithium heparin) and plasma were frozen at -30°C and then air-freighted to the UK in dry ice where they were stored at -20°C. DNA was prepared by Dr. A.V.S. Hill and colleagues (John Radcliffe Hospital, Oxford) and transported to London. Plasma was subsequently stored at -70°C.

Nigerian

Serum samples, collected from 120 donors in Rigacikun, near Kaduna, North-Central Nigeria, were provided by Dr. D. Jordan, Department of Palaeoanthropology,
Cambridge University. The samples had been stored at -20°C.

**Papua New Guinea**

Samples were collected, and DNA isolated, from inhabitants of the coastal region of the island by Dr. D. Lalou (50 individuals comprising 25 known to have malaria and 25 matching controls). DNA was transported to the UK in dry ice and stored at -20°C until required.

**San Bushmen**

Sekele San DNA (N=59) with matching plasma, collected by Dr. A. Spurdle and Prof. T. Jenkins (South African Institute for Medical Research, University of Witwatersrand, Johannesburg), were air freighted to London in dry ice and stored at -70°C until required.

**Vanuatu**

Sets of umbilical cord blood were collected by Dr. M. Ganczakowski (John Radcliffe Hospital, Oxford) from two islands, the main clinic at Kerepei, Maewo (53 individuals) and the Northern District Hospital, Santo (59 individuals). These represent a random group from a total of 1592 consecutive births collected over a two year period (1989-91). Whole blood was taken, stored at 4°C overnight, separated the next day and frozen at -70°C. All samples were subsequently air freighted to London in dry ice and stored at -20°C until required.

**Xhosa population (S.Africa)**

Samples of whole blood and serum (N=55) were collected from blood transfusion donors by Dr. E Goddard, Department of Paediatrics and Child Health, Red Cross War Memorial Hospital, University of Cape Town. Separated serum and blood, collected into EDTA tubes, were frozen and shipped to London in dry ice, and stored at -70°C.

**Patient groups**

Samples were obtained from a number of individuals with different infections, and these are detailed in Chapter 8.
METHODS

2.1 Antibodies

The anti-MBP murine monoclonal antibodies used in this study were clones M35 and M37 (Sano & Summerfield, 1989)(IgG1 subclass), clone 131-1 (IgG1 subclass) [Cymbus Bioscience, Southampton, UK], and clone #6 (IgG1 subclass, specific for the collagenous domain) and horse-radish peroxidase (HRP) conjugated clone #3 (an IgG2a antibody specific for the carbohydrate recognition domain)(Schweinle et al., 1993)[gifts from Dr. R. Ezekowitz, Children’s Hospital, Boston]. MOPC-21, a mineral-oil induced plasmacytoma (IgG1 subclass) [Sigma, St. Louis, USA] was used as a control murine monoclonal antibody. In addition two anti-MBP rabbit polyclonal antibodies (monospecific as determined by immunoelectrophoresis against normal human serum) were used [kindly supplied by Dr. J. Lu, MRC Immunocchemistry Unit, Oxford and Dr. R. Ezekowitz]. Antibodies were biotinylated for use as required (see Section 2.4).

2.2 Antibody Purification

Anti-MBP monoclonal antibody (clones M35 and M37 from Prof. J. Summerfield, St. Mary’s Hospital Medical School) was prepared from ascites fluid by anion exchange chromatography, using a Mono Q column (Pharmacia, Uppsala, Sweden)[Clezardin et al., 1984]. The column was equilibrated in 20mM Tris, pH 7.7, and then ascites, diluted 1/2 in equilibration buffer and 0.2μm filtered, was loaded onto the column and eluted with the same buffer containing a gradient of sodium chloride (0-0.35M). Fractions of 0.5ml were collected and tested for anti-MBP activity by ELISA. The maximum antibody signal eluted at approximately 150mM NaCl.

2.3 Determination of Monoclonal Antibody Subclass

Monoclonal antibodies were tested by a double diffusion assay to determine their subclass (Ouchterlony & Nilsson, 1978). The unknown antibody and an array of anti-mouse subclass specific antibodies were allowed to react in agar gels following diffusion at 4°C overnight. Lines of precipitation were used to establish the specific subclass.

2.4 Biotinylation of Proteins

Antibodies were dialysed against NaHCO₃ 0.1M, pH 8.2 until all inhibiting agents
(e.g. azide or Tris) were removed. The biotinylating agent was biotinamidocaproate N-hydroxysuccinimide ester [Sigma] which binds covalently to amine groups and features a bridging component which spatially separates the biotin molecules from the protein thus making them more sterically accessible. The biotin succinimide ester was dissolved in DMSO at a concentration of 1mg/ml, and 75μl of this solution added per milligram of antibody. The reagents were mixed and incubated at room temperature for 4-6hr. The product was dialysed extensively against PBS pH 7.3 (at least a 1/1000 dilution equilibrated 3 or 4 times) and stored at -70°C or 4°C prior to use.

2.5 Assay for Mannan Binding C3b Opsonins

Complement components binding to yeast mannan were measured by a functional ELISA essentially as described by Super et al., 1990. Dynatech Immulon 2 flat bottomed assay plates were coated with mannan [Sigma] at a concentration of 5μg/ml in carbonate/bicarbonate coating buffer (due to variations in commercial batches of mannan an alternative coating system used 1mg/ml mannan in 0.2M phosphate buffer, pH 6-6.5) and incubated at 4°C overnight. A reaction volume of 100μl per well was used, with the plate washed three times (approximately 3min each) in PBS-T after each stage. Serum was diluted to 5% in VBS+ (See Buffers and Solutions) and duplicate aliquots incubated at 37°C for 30min. Control sera known to give high (HB) and low (LB) levels of binding were included in quadruplicate on each plate. Bound levels of complement fragments C3b/C3bi were measured using HRP conjugated sheep anti-human C3c specific antibody [Serotec, Oxford, UK] diluted 3/2000 in PBS-T and incubated at 37°C for 60min. Substrate solution (100μl/well) was added and absorbance values measured at 490nm using the Mikrotek/Dynatech [Dynatech Laboratories, Billinghurst, UK] plate reading system.

Results were calculated to give binding coefficients as follows:

\[
\text{Binding Coefficient (\%)} = \frac{(\text{Sample}_{490} - \text{LB}_{490})}{(\text{HB}_{490} - \text{LB}_{490})} \times 100
\]

\[
\text{(Sample/HB/LB}_{490} = \text{Sample absorbance at 490nm)}
\]

The average daily interplate coefficient of variation was 3.8% (calculated from a single standard tested in duplicate dilutions on each of five plates).

2.6 Assays for Mannose Binding Protein

These are detailed in Chapter 3.
2.7 Polyacrylamide Gel Electrophoresis (PAGE)

2.7.1 Native PAGE. Non-denaturing electrophoresis was performed using variations on the method of Ornstein (1964). The technique employed used a continuous gel of 5% acrylamide (AA), 0.13% bisacrylamide (Bis) [T (total amount monomer (w/v))=5.1%, C (percentage of crosslinker)=2.5%] and a Tris-Glycine buffer system (25mM Tris, 192mM Glycine, pH 8-9). In the experiments detailed in Chapter 7 whole serum (5μl) was loaded directly on to the gel and electrophoresed for 900 Vhr.

2.7.2 SDS-PAGE. Modifications of the methods of Ornstein (1964), Davis (1964) and Laemmli (1970) were used for two discontinuous electrophoresis systems (i.e. the buffer in the matrix differed from the electrode buffer). The slab gels were electrophoresed in vertical tanks [Bio-Rad, Richmond, USA].

Stacked gels. Differing concentrations of acrylamide (AA) were used in separating gels with a low porosity stacking gel cast on top. The recipes and solutions used were as follows:

stock solutions: a] acrylamide 30% (w/v), bisacrylamide 0.8% (w/v); b] Tris/HCl 0.75M pH 8.8; c] Tris/HCl 1.0M pH 6.8; d] SDS 10% (w/v); e] ammonium persulphate 10% (w/v)

for x% AA gels, volume 60ml (2 gels)
[a] 2x ml, [b] 30ml, [d] 0.6ml, [e] 0.6ml, H₂O (28.6-2x) ml, TEMED 200μl
stacking gel (5% AA), volume 18ml
[a] 3ml, [c] 2ml, [d] 0.16ml, [e] 0.16 ml, H₂O 12ml, TEMED 100μl

Gradient gels. Gels with a continuous gradient from 3 to 10% AA (0.08-0.27% Bis; T=3-10%, C=2.6%) were cast a minimum of 5hr before use. In the experiments detailed in Chapter 7 samples were diluted 1/2 in sample buffer (120mM Tris-HCl, pH 6.75, 20% (v/v) glycerol, 4% (w/v) SDS), and in specific analyses 55mM iodoacetamide was added. Samples were then loaded on to the gel, either directly or after incubation at 100°C for 5min, and electrophoresed for 600 Vhr.

The following biotinylated standards were used for size determinations: myosin (200kDa), β-galactosidase (116kDa), phosphorylase B (97.4kDa), bovine serum albumin (66kDa), and ovalbumin (45kDa) [Bio-Rad]. In addition, non-biotinylated standards [Sigma] and rainbow markers [Amersham International, Amersham, UK] were used to assist the assignment of molecular masses.

Protein was detected by Coomassie blue staining (0.025% (w/v) in 50% (v/v)
methanol, 5% (v/v) acetic acid) followed by destaining in 7.5% (v/v) methanol, 5% (v/v) acetic acid, silver staining according to the manufacturer's instructions [Bio-Rad], or immunoblotting.

2.8 Immunoblotting

Electrophoresed proteins were transferred to nitrocellulose (NC) using a semi-dry electrophoresis apparatus (Trans-Blot SD [Bio-Rad, Richmond, USA]; Towbin et al., 1979) according to the manufacturer’s instructions. Transfer was performed for 40min at 25V (buffer: 48mM Tris, 39mM Glycine, 20% (v/v) methanol, pH 9.2) onto Hybond-C extra [Amersham]. The membrane was then incubated with 3% skimmed milk in PBS for 2hr in order to block free binding sites, and washed in PBS-T for 20min. MBP was detected using labelled monoclonal antibodies and visualised by enhanced chemiluminescence (ECL). Two procedures were used; membranes were incubated overnight with a), HRP conjugated anti-MBP MAb, or b), biotinylated anti-MBP antibody, washed for 30min in PBS-T then developed directly (a), or after a 3hr incubation with Streptavidin HRP (b). A mixer-roller [Denley, Billingshurst, UK] was found to be the optimum apparatus for incubations. After treatment with ECL reagents according to the manufacturer's instructions [Amersham] the membranes were exposed to film (X-OMAT AR [Kodak, Rochester, USA]) for between 15s and 30min as necessary. All stages were performed at room temperature. Signal strength was quantified using an LKB Pharmacia 2210 scanning densitometer.

2.9 Gel Filtration

Serum samples were fractionated on a Superdex 200 column (300 x 10mm) [Pharmacia] equilibrated in phosphate buffered saline (Na_2HPO_4 8.1mM, KH_2PO_4 1.5mM, NaCl 140mM, KCl 2.7mM, pH 7.3), using the FPLC system [Pharmacia]. The column was calibrated using a range of standards (blue dextran 2000kDa, thyroglobulin (bovine) 669kDa, apoferritin (horse spleen) 443kDa, β-amylase (sweet potato) 200kDa, alcohol dehydrogenase (yeast) 150kDa, transferrin (bovine) 81kDa, ovalbumin 43 kDa, and carbonic anhydrase 29kDa [Sigma]) and operated at a flow rate of 0.5ml/min. The void volume of the column was 8.3ml. Figure 2.1 shows a plot of standard relative molecular mass (RMM) against elution position (V elution/V void); regression coefficient, R = 0.997. Sera were diluted 1/2 in PBS, filtered (0.2μm) and an aliquot of 200μl loaded onto the column; 0.5ml fractions were collected and 30μl of each analysed
Methodology

by SDS-PAGE and immunoblotting as appropriate.

Figure 2.1 Superdex 200 Column Calibration
The elution volumes of a range of standards (Ve) relative to the void volume of the column (Vo) were plotted against relative molecular mass (exact masses are given in the text). The equation of the regression line shown is: \( \log y = 4.6 - 1.6x \)

2.10 Sucrose Density Centrifugation
Sucrose density gradient centrifugation of whole serum was performed on a Beckman L8-70M Ultracentrifuge with an SW41Ti rotor. Proteins of known sedimentation coefficient, thyroglobulin \( (s_{20,w} 19.3) \), catalase \( (s_{20,w} 11.3) \), C1q \( (s_{20,w} 10.5) \) [a gift from Dr. J. Lu, MRC Immunochmistry Unit, Oxford], immunoglobulin G \( (s_{20,w} 7.0) \), and human serum albumin (HSA) \( (s_{20,w} 4.6) \) were used as standards. Linear gradients of 10-40\% (w/v) sucrose in PBS were established in Beckman ultra-clear 13.2ml tubes by pre-spinning at 151,200g \( (r_{av}=110\text{mm}) \) for 3hr at 12\^\circ C. Serum (100\mu l) and standards (200\mu g) were made up in 0.5ml 10\% sucrose/PBS and applied to the linear
gradients. These were centrifuged at 151,200g for 20hr. After centrifugation the samples were separated into 15-22 fractions (approximately 0.6-0.8ml) and analysed by SDS-PAGE and immunoblotting.

2.11 DNA Isolation

Genomic DNA was prepared from whole blood by two methods.

(i) 1ml of EDTA blood was centrifuged at 10,000g for 15min. The supernatant was removed and the pellet resuspended in 200μl of water followed by boiling for 10min. The suspension was then centrifuged for a further 10min and the supernatant stored at -20°C until required.

(ii) 10ml of EDTA blood were lysed by the addition of 50ml ice-cold water and centrifuged at 10,000g for 20min. The supernatant was decanted and the pellet resuspended in 25ml ice-cold nonidet P40 and further centrifuged at 10,000g for 20min. The pellet was homogenised with 7ml 6M guanidine hydrochloride and 0.5ml 7.5M ammonium acetate followed by the addition of 0.5ml 20% sodium sarkosyl and 150μl (10mg/ml) proteinase K. The mixture was then incubated at 60°C for 90min. DNA was precipitated by the addition of 2.5 volumes of ethanol and dissolved in 1ml Tris-EDTA, pH 7.6. The DNA was reprecipitated by the addition of 1/10th volume of 3M sodium acetate and 2 volumes of ethanol, redissolved in 1ml TE buffer (10mM Tris-HCl, pH 7.6, 1mM EDTA) and stored at -20°C. Assuming the concentration of DNA in whole blood to be approximately 1mg/ml this gave a preparation of genomic DNA of 10mg/ml.

Samples of genomic DNA obtained from external laboratories were prepared by the alternative method of phenol-chloroform extraction (Old, 1986).

2.12 Polymerase Chain Reaction (PCR)

Exon 1 of the MBP gene was amplified by PCR. A 328 base pair fragment was prepared using the 20mer oligonucleotide 5’-dGTAGGACAGAGGCGATGCTC-3’ (bases -16 to 4 of the upper strand) and the 21mer 5’-dCAGGCAGTTTCCTCTGGAAGG-3’ (bases 312 to 292 of the lower strand) as primers.

The following PCR reaction mixture was used: 50mM KCl, 10mM Tris/HCl pH 8.3, 1.5mM Mg²⁺ [Bioline, London, UK], with the addition of 200μM of each deoxynucleotide triphosphate (dNTP)[Pharmacia], 25pmol of each primer, and 2.5 Units Taq polymerase [Bioline]; total reaction volume was 50μl.

Using a thermal cycler [Hybaid, Teddington, UK] genomic DNA (approximately
1-10μg) was initially denatured at 94°C for 4min in the reaction mixture (or 99°C for 10min, followed by the addition of Taq) and then subjected to 35 cycles of a denaturing step (94°C, 1min), an annealing step (61°C, 1min) and polymerisation (72°C, 1min). Five microlitres of the final reaction mix were electrophoresed using a TAE buffer on 2% agarose gels and stained with ethidium bromide to confirm the expected amplification product.

2.13 Enzyme Digestion of PCR Product

The PCR product was precipitated by the addition of 1 volume of 4M ammonium acetate and 2 volumes of propan-2-ol, followed by washing in 70% EtOH, and left to air dry. Each product was then digested with 5U of Ban I [Boehringer Mannheim, Mannheim, Germany] at 50°C for 70min, or 3.5U of Mbo II [New England Biolabs, Beverly, USA] at 37°C for 90min. Alternatively, 5μl of PCR product were digested directly with 5U of Ban I (reaction volume 30-50μl). The fragments resulting from the digest were visualised by electrophoresis on 2% agarose gels.

2.14 Sequencing of DNA

Single stranded DNA template was generated by asymmetric PCR amplification using the reaction mix of Section 2.12, but with 50pmol of the upper primer and 1pmol of the lower. After precipitation of the product (as detailed in Section 2.13) dideoxy-mediated sequencing was performed incorporating 35S according to the manufacturer’s instructions [United States Biochemicals, Cleveland, USA]. The reaction products were run on 6% acrylamide (0.32% bisacrylamide) gels in the presence of 8.3M urea with TBE buffer at 200mA for 2hr. Gels were then dried and exposed to film (X-ray RX [Fuji, Tokyo, Japan]) as necessary.

2.15 Oligonucleotide Labelling

Radiolabelled phosphor (γ-32P) was used to tag the oligospecific probes, according to the method of Sambrook et al. (1989). Oligonucleotide (20μM) was incubated with bacteriophage T4 polynucleotide kinase [Promega, Southampton, UK] (8U/μl) and [γ-32P]ATP (5000Ci/mole; 10mCi/ml) in the buffer supplied at 37°C for 30min. Unbound isotope was removed by passage down a column of diethylaminoethyl cellulose (DE52) [Whatman, Maidstone, UK], followed by elution with 0.5M NaCl/TE.
2.16 Oligonucleotide Specific Hybridisation

Aliquots of the final PCR amplification mixture were rendered single stranded prior to transfer to nitrocellulose (Hybond N, 0.45μ [Amersham]). The PCR product (15μl) was mixed with denaturing buffer (86μl Tris 10mM, EDTA 0.5mM, pH 7.5; 8μl NaOH 6M; 6μl EDTA 0.5M, pH 7.5) in microtitre plates on ice for 10min, neutralised with 110μl NH₄OAc 2M, and then transferred to nitrocellulose filters using a Biorad manifold. The filters were prewetted in water, and each well washed with 200μl of NH₄OAc 1M before and after transfer (Wordsworth et al., 1990).

Filters were washed briefly in 6xSSC and exposed facedown to UV for 3min to ensure that DNA was bound. Duplicate filters were incubated for 10min at 31°C in 1.8xSSC, 0.2% (w/v) Ficoll, 0.2% (w/v) polyvinylpyrrolidone, 0.2% (w/v) BSA, 0.5% (w/v) SDS and yeast tRNA (400μg/ml) and then hybridised with 32P end labelled oligonucleotide probes (activity 1 e 8 counts/min/mg) in 1.8xSSC for approximately 8hr at 31°C.

The filters were then washed for 10min in 6xSSC before exposing to film for 1hr at -70°C to establish a baseline prior to high stringency washes - used to remove any probe not completely matched to the product sequence. The wash temperature was based on the theoretical melting temperature of the oligonucleotide probe \[T_m=4(G+C)+2(A+T)°C\]. After the high temperature washes the filters were exposed to film (X-ray RX) as appropriate for the strength of signal.

Filters were stripped using a solution of NaOH 50mM, Tris 2mM pH 7.5, NaCl 0.5M, followed by 3x5min washes in 6xSSC, checked by autoradiography and then hybridised with the next probe.

2.17 Statistical Analysis

The various population data were compared by the non-parametric Mann-Whitney rank sum test using the Minitab software package [Minitab Inc., State College, PA, USA]. Correlation coefficients (R), Students t-tests, and graphical analyses were performed using the Sigmaplot graphics package [Jandel Scientific, Erkrath, Germany]. The statistical significance of R values was derived from t tables using the formula \[t=R\{(n-2)/(1-R^2)\}^{1/2}\]. Differences between observed and expected values were determined by Chi-square distribution analysis.
2.18 Miscellaneous

Water was obtained from a Millipore RO/Q purification system [Millipore, Molsheim, France].

Optical densities at 260 and 280nm were measured on a Philips PU8620 Series single beam spectrophotometer.

Polyacrylamide gels were dried using a Model 583 gel dryer [Bio-Rad].
CHAPTER 3

ENZYME LINKED IMMUNOSORBENT ASSAYS FOR MBP: DEVELOPMENT AND COMPARISON

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Enzyme Linked Immunosorbent Assays for MBP: Development and Comparison

Introduction

Molecules of mannose binding protein (MBP) are polymers of a single peptide encoded by the MBP gene. The number of separate chains in a molecule is thought to range from 6 to 18, and so it is likely that any particular antigenic epitope will be repeated several times in the final structure. This is an important feature when considering the format of immunoassays used to detect MBP. In earlier work a variety of different enzyme linked immunosorbent assay (ELISA) systems have been used to measure concentrations of MBP (Super et al., 1989; Ezekowitz et al., 1989; Super et al., 1990; Lu et al., 1990). Some of these assays were developed from the original opsonisation studies that employed baker's yeast or the yeast wall extract zymosan, which consists mainly of β-D-glucan and α-D-mannan carbohydrate polymers (Super et al., 1990). Consequently purified mannan was frequently used together with anti-MBP antibodies in the ELISA's. In order to evaluate MBP levels in a variety of population groups further assays have been developed and all results obtained have been correlated with existing formats and also with each other.

ELISA Format

The types of assay used fall into three categories: a) functional, involving interactions with mannan; b) asymmetric antibody sandwich, using monoclonal and polyclonal or two different monoclonal antibodies; and c) symmetric antibody sandwich, where a single monoclonal antibody is used throughout. For functional assays either the capture (Super et al., 1989) or the detection (Lu et al., 1990) stage relies on the affinity of MBP for mannan (Kawasaki et al., 1983), and binding occurs in the presence of a buffer containing calcium ions. In all studies described herein mannan was only used in the capture stage. In the case of the symmetric assays the polymeric nature of the MBP molecule ensures that particular epitopes are repeated, thus giving a monoclonal antibody
a binding site in both the capture and detection phases.

The basic characteristics of the ELISA's used are shown in the following flow diagram:

**CAPTURE**

Moieties to which MBP will bind are coated onto immunoassay plates

\[ \downarrow \]

**LIGAND BINDING**

Serum containing MBP is diluted and incubated in an appropriate buffer

\[ \downarrow \]

**DETECTION**

Bound MBP is recognised and levels measured by colorimetric analysis

---

**Methodology and Results**

**Reagents and Materials**

Dynatech Immulon 2 flat bottomed assay plates were used in all ELISA's, with the outer wells left empty to minimise intra-plate variations.

Purified mannan was obtained from Sigma. (The binding of MBP to a fixed concentration of mannan was found to be lot dependent, and investigations revealed that this reflected the differing protein content of each mannan batch. This can be explained in terms of these protein impurities being necessary to bind the mannan to the immunoassay plate since the carbohydrate will not bind directly - a batch with a higher protein content would coat more mannan to the assay plate in the capture stage and hence result in greater MBP binding).

The anti-MBP antibodies used were: murine monoclonal antibodies, M37 and M35 (Sano & Summerfield, 1989), prepared as described in Chapter 2: Section 2.2, and found to be of IgG1 isotype (Section 2.3); monoclonal antibody (MAb) clone 131-1 (subclass IgG1,\(\kappa\)) [a gift from Dr. S. Thiel, Denmark or purchased from Cymbus Bioscience, Southampton]; rabbit polyclonal antibodies were kindly provided by Dr. J. Lu (Oxford, England), and Dr. R.A.B. Ezekowitz (Boston, USA). Antibodies were biotinylated as required according to the procedure detailed in Section 2.4.

The detection antibody was recognised using horse-radish peroxidase (HRP) conjugated sheep anti-mouse immunoglobulin (s\(\alpha\) m) [Sigma], goat anti-rabbit
immunoglobulin (g α r) [Sigma] or streptavidin [Amersham] as appropriate.

An incubation volume of 100μl per well was used in all stages, with serum samples diluted 1/20 for the mannan capture assays and 1/30 for the antibody capture. All samples were tested in duplicate.

Buffers

The coating buffer used for all reagents was carbonate/bicarbonate (Na₂CO₃ 15mM, NaHCO₃ 35mM, pH 9.6). All results presented in this thesis were obtained using lot number 16F-3847 and carbonate coating buffer. However, in the case of more recent batches of mannan (from lot number 19F-3913) 0.2M phosphate buffer pH 6.5 was found to give optimum results. In functional assays involving the binding of MBP to mannan the sera were diluted in a buffer of imidazole 40mM, pH 7.8, containing NaCl 1.25M and CaCl₂ 50mM.

All other stages were performed using phosphate buffered saline, plus Tween-20 (PBS-T)(Na₂HPO₄ 8.1mM, KH₂PO₄ 1.5mM, NaCl 140mM, KCl 2.7mM, pH 7.3, Tween-20 0.05% (v/v)) as the diluting buffer. After each stage the assay plates were washed three times (approximately 3min per wash) in PBS-T.

Assay Types and Conditions

Assay formats were as shown in the previous flow diagram, with the detection stage normally consisting of three steps: i) primary antibody recognition of bound MBP, predominantly using biotinylated antibody, ii) secondary recognition of this bound antibody by conjugated reagent (HRP), and iii) addition of substrate solution (citrate/phosphate buffer pH 5.0, o-phenylene diamine 0.5mg/ml, H₂O₂ 30%(v/v) 0.5μl/ml). The incubation time for the substrate was between 5 and 30min depending on the assay, and the reaction was stopped by the addition of 4N H₂SO₄. Absorbance was then measured at 490nm on a Dynatech MR4000 plate reader.

The different combinations of reagents, with optimum working concentrations and incubation times are shown in Table 3.1. For ease of reference ELISA’s will henceforth be referred to by their capture/detection stages, e.g. the assay using mannan capture and PAb(Oxford) as the primary detection antibody becomes the mannan/PAb(Oxford) system.

To test for absorbance derived from non-specific interactions (produced, for example, by human rheumatoid factors cross-reacting with animal immunoglobulin) a
proportion of samples were measured using a non-MBP specific antibody as capture. The murine monoclonal antibody used was mineral oil induced and of the same subclass as M37 and MAb 131-1 (MOPC-21 [Sigma]). In this case the normal detection steps were used.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Time (mins)</th>
<th>CAPTURE</th>
<th>LIGAND BINDING</th>
<th>DETECTION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>i</td>
<td></td>
<td>mannan</td>
<td></td>
<td>PAb(Oxford)</td>
</tr>
<tr>
<td></td>
<td>ii</td>
<td></td>
<td>5μg/ml</td>
<td></td>
<td>1μg/ml</td>
</tr>
<tr>
<td></td>
<td>iii</td>
<td></td>
<td>o/n</td>
<td></td>
<td>120</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1μg/ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>i</td>
<td></td>
<td>mannan</td>
<td></td>
<td>M37 or M35</td>
</tr>
<tr>
<td></td>
<td>ii</td>
<td></td>
<td>1-5μg/ml</td>
<td></td>
<td>5μg/ml</td>
</tr>
<tr>
<td></td>
<td>iii</td>
<td></td>
<td>o/n</td>
<td></td>
<td>110</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1μg/ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>i</td>
<td></td>
<td>mannan</td>
<td></td>
<td>PAb(Boston)</td>
</tr>
<tr>
<td></td>
<td>ii</td>
<td></td>
<td>1μg/ml</td>
<td></td>
<td>0.2μg/ml</td>
</tr>
<tr>
<td></td>
<td>iii</td>
<td></td>
<td>o/n</td>
<td></td>
<td>110</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>i</td>
<td></td>
<td>M37</td>
<td></td>
<td>PAb(Boston)</td>
</tr>
<tr>
<td></td>
<td>ii</td>
<td></td>
<td>1μg/ml</td>
<td></td>
<td>0.2μg/ml</td>
</tr>
<tr>
<td></td>
<td>iii</td>
<td></td>
<td>o/n</td>
<td></td>
<td>110</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1μg/ml</td>
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<td></td>
<td></td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>i</td>
<td></td>
<td>M37</td>
<td></td>
<td>M37</td>
</tr>
<tr>
<td></td>
<td>ii</td>
<td></td>
<td>1μg/ml</td>
<td></td>
<td>0.3μg/ml</td>
</tr>
<tr>
<td></td>
<td>iii</td>
<td></td>
<td>o/n</td>
<td></td>
<td>90-110</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1μg/ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>i</td>
<td></td>
<td>MAb 131-1</td>
<td></td>
<td>MAb 131-1</td>
</tr>
<tr>
<td></td>
<td>ii</td>
<td></td>
<td>1μg/ml</td>
<td></td>
<td>0.3μg/ml</td>
</tr>
<tr>
<td></td>
<td>iii</td>
<td></td>
<td>o/n</td>
<td></td>
<td>90</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1μg/ml</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>i</td>
<td></td>
<td>M37</td>
<td></td>
<td>MAb 131-1</td>
</tr>
<tr>
<td></td>
<td>ii</td>
<td></td>
<td>1μg/ml</td>
<td></td>
<td>0.5μg/ml</td>
</tr>
<tr>
<td></td>
<td>iii</td>
<td></td>
<td>o/n</td>
<td></td>
<td>90</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1μg/ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>60</td>
</tr>
</tbody>
</table>

i) Reagent  ii) Working concentration  iii) Incubation time (minutes)

Abbreviations:
o/n - overnight; g α r - goat anti-rabbit immunoglobulin; s α m - sheep anti-mouse immunoglobulin

Table 3.1  Reagents and Conditions for Human MBP ELISA Systems
Standardisation of Assays

In order to compare sample values each plate contained a nine point doubling dilution standard curve and three spot standards. The curve was derived from an in house serum pool (SSL or SSH) that had been calibrated against an external standard of purified MBP (quantified by amino acid analysis and kindly provided by Dr. S. Thiel, Denmark). The day-to-day coefficient of variation for three calibrations performed over a three month period was 2.8%, and the concentration of this pool was determined to be 1660ng/ml. The spot standards represented values at the top (HB), bottom (LB) and middle (SSL or SSH) of the standard curve. The high and low standards could also be used to determine binding coefficients (see next Section) for comparison with C3b opsonin measurements, and each were assayed in quadruplicate.

The above standard was introduced in mid-1992 and represented an approximate ten fold increase on previously determined values (Super et al., 1992). Results obtained prior to this date were scaled to allow for the change. Quantifications based on binding coefficients were not affected.

The algorithms "four parameter" and "Logit-log" were applied for calculating concentrations from the standard curves, using the Mikrotek software package [Dynatech]. Typical curves obtained for different assay systems are shown in Figure 3.1.

For the principal assays used the coefficients of variation (C.V.) (calculated from n measurements taken over period t) for the mid-range concentration standard were as shown in Table 3.2.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Average daily inter-plate C.V.</th>
<th>Day-to-day C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>M37/M37</td>
<td>4.1% (n=14, t=5 months)</td>
<td>3.1% (n=16, t=5 months)</td>
</tr>
<tr>
<td></td>
<td>with average intra-plate C.V.'s of HB=2.3%, LB=3.2% (n=15, t=2 months)</td>
<td></td>
</tr>
<tr>
<td>MAb 131-1/ 131-1</td>
<td>8.3% (n=18, t=6 months)</td>
<td>11.3% (n=20, t=6 months)</td>
</tr>
<tr>
<td></td>
<td>with average intra-plate C.V.'s of HB=3.3%, LB=4.2% (n=10, t=1 month)</td>
<td></td>
</tr>
<tr>
<td>Mannan/PAb(Oxford)</td>
<td>14.4% (n=4, t=1 month)</td>
<td>12.7% (n=4, t=1 month)</td>
</tr>
<tr>
<td></td>
<td>with average intra-plate C.V.'s of HB=7.9%, LB=5.1% (n=12, t=1 month)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2 ELISA Coefficients of Variation
Figure 3.1  ELISA Standard Curves

Levels of MBP in samples were calculated from curves of the in house serum pool SSL (MBP concentration 1660ng/ml). This was incubated in doubling dilutions and is shown for the M37/M37 (upper panel) and MAb 131-1/131-1 (lower panel) systems.
**Binding Coefficients**

Results were calculated to give binding coefficients as follows:

\[
\text{Binding Coefficient (\%)} = \left\{ \frac{\text{Sample}_{490} - \text{LB}_{490}}{\text{HB}_{490} - \text{LB}_{490}} \right\} \times 100
\]

(Sample/HB/LB \(_{490}\) = Sample absorbance at 490nm)

This method of expressing results was used primarily in the mannan/PAb(Oxford) assay, and gave coefficients of variation for the binding coefficient of SSH of: inter-day C.V. = 9.4\% (n = 8, t = 1 month); average daily inter-plate C.V. = 4.9\% (n = 6, t = 1 month). Figure 3.2 shows that the relative MBP levels obtained from binding coefficient calculations correlate closely with those determined quantitatively using the M37/M37 assay system.

![Graph showing correlation of measurements used to quantify MBP levels](image)

**Figure 3.2** Correlation of Measurements used to Quantify MBP Levels

The level of MBP was measured for 98 individuals and expressed as a binding coefficient (y) or in ng/ml (x), using the M37/M37 assay system as detailed in the text. For values with a concentration less than 2000ng/ml the coefficient of regression, R = 0.987, and the line equation is \(y = 0.034x + 0.027\).
Correlation between Assays for MBP

The assays used in these studies and detailed in Table 3.1 evolved from the functional mannan capture ELISA described by Super et al. (1989). Subsequent variations were employed to increase the accuracy of MBP determinations. The initial change in ELISA format involved a switch in the detection stage to the use of an antibody raised internally. Included in Table 3.3 are a series of line equations and correlation coefficients obtained from a comparison of selected samples measured with the original mannan/PAb(Oxford) format and various alternative assays. The comparison with the preferred replacement configuration of mannan/M37 is illustrated in Figure 3.3, and is shown in terms of concentrations (a) and binding coefficients (b). To assist comparison of results with other laboratories the internal antibodies (M35 and M37) were also compared with another external polyclonal antibody (PAb(Boston)) and this is shown in Figure 3.3(c), and Table 3.3. All correlations were highly significant (P < 0.0005).

In order to improve the sensitivity and reproducibility of the MBP measurements the assay was subsequently changed to use a MAb (M37) in the capture stage. The difficulties already mentioned with the chemical consistency of the commercial preparations of mannan, coupled with the increased specificity of a monoclonal detection system also favoured the switch to the use of M37. The correlation of MBP levels for a group of 25 samples measured with the mannan/M35 + M37 and M37/M37 systems are shown in Table 3.3.

As greater interest developed in the quantification of MBP levels it became appropriate to change the internal MAb for a commercially available reagent in order to facilitate inter-laboratory comparisons. The MAb clone 131-1 was switched directly for M37 and the resulting correlation between MBP measurements is shown in Figure 3.4. The sensitivity limit for detection of MBP by these symmetrical systems was 10ng/ml (calculated from the concentration given by the mean blank sample absorbance plus 3 standard deviations). Additional information is provided in Table 3.3, including a comparison of the asymmetric MAb system, using M37 and MAb 131-1, with the M37 symmetrical ELISA.
Figure 3.3 Comparison of Mannan Capture Assay Systems

The levels of MBP were determined for 15 samples using assays based on mannan capture and a variety of detection antibodies. The original mannan/PAb(Oxford) system is compared with its replacement, the mannan/M37 system by concentration (a) and binding coefficient (b). Panel (c) illustrates one of the other correlations shown in Table 3.3, comparing the mannan/PAb(Boston) system with the M35 and M37 antibodies.
### Table 3.3  Correlations between ELISA Systems used to Measure MBP

<table>
<thead>
<tr>
<th>Comparison</th>
<th>units</th>
<th>line equation</th>
<th>correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>mannan/PAb(Oxford)</td>
<td>mannan/M35</td>
<td>conc.</td>
<td>y = 1.8x-190</td>
</tr>
<tr>
<td></td>
<td>mannan/M37</td>
<td></td>
<td>y = 1.5x+46</td>
</tr>
<tr>
<td></td>
<td>mannan/M35+M37</td>
<td></td>
<td>y = 1.4x-72</td>
</tr>
<tr>
<td></td>
<td>mannan/PAb(Boston)</td>
<td></td>
<td>y = 1.5x-150</td>
</tr>
<tr>
<td>mannan/PAb(Boston)</td>
<td>mannan/M35</td>
<td></td>
<td>y = 1.2x-52</td>
</tr>
<tr>
<td></td>
<td>mannan/M37</td>
<td></td>
<td>y = 0.94x+230</td>
</tr>
<tr>
<td></td>
<td>mannan/M35+M37</td>
<td></td>
<td>y = 0.91x+96</td>
</tr>
<tr>
<td>mannan/M37</td>
<td>mannan/M35</td>
<td></td>
<td>y = 1.2x-260</td>
</tr>
<tr>
<td></td>
<td>mannan/M35+M37</td>
<td></td>
<td>y = 0.94x-80</td>
</tr>
<tr>
<td>mannan/PAb(Oxford)</td>
<td>mannan/M35</td>
<td>B.C.</td>
<td>y = 1.2x-6.5</td>
</tr>
<tr>
<td></td>
<td>mannan/M37</td>
<td></td>
<td>y = 1.0x-5.5</td>
</tr>
<tr>
<td></td>
<td>mannan/M35+M37</td>
<td></td>
<td>y = 1.1x-4.6</td>
</tr>
<tr>
<td></td>
<td>mannan/PAb(Boston)</td>
<td></td>
<td>y = 1.1x-12</td>
</tr>
<tr>
<td>mannan/PAb(Boston)</td>
<td>mannan/M37</td>
<td></td>
<td>y = 0.92x+5.3</td>
</tr>
<tr>
<td></td>
<td>mannan/M35+M37</td>
<td></td>
<td>y = 0.95x+7.5</td>
</tr>
<tr>
<td>mannan/M37</td>
<td>mannan/M35</td>
<td></td>
<td>y = 1.2x+0.2</td>
</tr>
<tr>
<td>mannan/(M35+M37)</td>
<td>M37/M37</td>
<td>B.C.</td>
<td>y = 0.84x+5.9</td>
</tr>
<tr>
<td>M37/M37</td>
<td>MAb 131-1/131-1</td>
<td>conc.</td>
<td>y = 0.85x+260</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>all concentrations</td>
</tr>
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<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>y = 1.1x-12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>conc. &lt; 3000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N = 35</td>
</tr>
<tr>
<td>M37/M37</td>
<td>M37/MAb 131-1</td>
<td>conc.</td>
<td>y = 0.58x+560</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>all concentrations</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>N = 74</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>y = 1.1x+150</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>conc. &lt; 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N = 60</td>
</tr>
</tbody>
</table>

B.C. = Binding Coefficient
Figure 3.4  Comparison between the Symmetrical M37 and MAb 131-1 Systems
MBP levels determined by the two assay formats are compared for 44 individuals over the whole concentration range, and in expanded form for those with values less than 3000ng/ml.
Discussion

The use of a number of MBP binding reagents in a variety of combinations provides the potential for large differences in detection sensitivity and specificity. Despite this the assay systems described here generally showed high correlation and therefore appear to be behaving in a similar manner. This is obviously important when comparing MBP measurements obtained in different studies or laboratories. However, the quantification of MBP is more complex than it might appear.

The fundamental question for all the assay formats is "What is actually being measured?". The initial assays based on a mannan capture stage recognise functional, i.e. sugar binding MBP. These molecules must be tightly bound to the carbohydrate surface since they do not dissociate in the later stages of the assay when buffers that lack Ca$^{2+}$ are used. In the symmetrical monoclonal antibody (MAb) systems the presence of repeating epitopes is a prerequisite for the detection of MBP, and such assays must be detecting protein of a polymeric nature. Any assay in which a polyclonal antibody or two different MAbs are used can theoretically detect any form of MBP, including single MBP fibrils and perhaps even single polypeptides. The interactions of MBP with antibodies or mannan will also be subject to steric influences. For example, a MAb specific for the carbohydrate recognition domain of the protein may be unable to access its epitope in the case of MBP that is functionally attached to mannan. In this respect the mannan/PAb assays should be less susceptible to steric hindrance than mannan/MAb systems.

The heterogeneity of polymeric MBP is also of concern - the molecules can consist of two to six subunits (6 to 18 polypeptide chains). As such it is a matter of debate whether a dimeric molecule would give rise to the same signal as the hexameric form. Both may lead to an equivalent response, or alternatively the latter could bind several detector antibodies, leading to an amplified signal. Obviously steric hindrance of any binding is likely to be important in this case (see Figure 1.1 in which the relative sizes of an MBP fibril and an antibody molecule are illustrated). Related to this difficulty is the fact that the polymeric composition of standard MBP preparations may not be exactly mirrored in the samples being measured. For certain assay formats the protein present in the standard and sample preparations may behave differently, and the relatively poor correlation obtained for the comparison of the M37/M37 and M37/MAb 131-1 systems may be a reflection of this.

The higher polymeric forms of MBP have higher binding affinities and are likely
to be more important in any host response mediated by the protein. The functional assays that detect these MBP molecules are inherently less sensitive than antibody based systems, and for accuracy, the symmetric MAb assays that detect only MBP polymer may be the most appropriate ELISA detection systems.
CHAPTER 4

THE COMMON OPSONIC DEFECT - AN INTERNATIONAL PHENOMENON

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The Common Opsonic Defect
- An International Phenomenon

Introduction

MBP deficiency was first established in British Caucasian children presenting with frequent infections (Super et al., 1989) and was subsequently linked to a point mutation in the MBP gene (Sumiya et al., 1991). An initial aim of this thesis was to extend such investigations to another population, and hence examine the influence of a different environment on serum MBP levels. To facilitate this investigation samples were obtained from Hong Kong Chinese donors; these comprised three groups, a) children under the age of sixteen, b) newborns, and c) a cohort of adult donors. The aim was then to compare and relate MBP concentrations to opsonic capacity, and to ascertain whether the same point mutation was present in the population.

Results

Chinese Children: C3b Opsonic Capacity and MBP Levels

Sera from a group of 100 Chinese children (detailed in Chapter 2: Subjects and Samples) were assayed for their levels of C3b opsonins to mannan and for serum MBP levels using appropriate ELISA techniques (Chapter 2: Section 2.5 and Chapter 3: mannan/PAb(Oxford) system). Complement fragments were measured in serum diluted to 5% in imidazole buffer containing 5mM magnesium and calcium ions. Under these conditions the activation of the alternative complement pathway is minimal and, therefore, the values reflect only classical pathway activation (Super et al., 1990). All results are expressed as binding coefficients (B.C.'s), with the values determined by reference to two sera previously identified as having high and low binding characteristics and defining the 100% and 0% levels respectively (see calculation of Section 2.5 and Chapter 3: Binding Coefficients). The results are shown together in Figure 4.1 (a) and (b). Both distributions are clearly bimodal and their appearances similar to that seen in the Caucasian population (Super et al., 1989; 1990).
Figure 4.1  Comparison of Functional C3b Opsonic Capacity and MBP Levels in Chinese Children
Sera from 100 healthy Hong Kong Chinese children were studied and the results expressed as binding coefficients. (a) Binding of C3b/C3bi complement fragments to mannan coated ELISA plates. Sera previously determined to have high and low binding characteristics defined the 100% and 0% values respectively. (b) Levels of MBP binding to mannan coated ELISA plates (mannan/PAb(Oxford) assay system). Individuals with low levels of MBP are highlighted by cross-hatching.
Nine percent of Chinese children were found to have low MBP concentrations (B.C. <20%) and the sera of all these individuals were shown to generate low levels of C3b opsonins. The overall correlation between MBP levels and opsonin generation is shown in Figure 4.2, and is highly significant with regression coefficient, \( R = 0.77\) (probability, \( P < 0.001 \)). This data agrees with that previously reported for Caucasians.

It was considered possible that for some of the children studied opsonin generation might be impaired if the complement system was not yet fully mature. Hence an association was sought between poor C3b opsonic capacity and age. Of the nine individuals with coexisting low levels of MBP and C3b opsonins only one was under six months of age, and for children under 1 year only 3 (of 20) had C3b binding coefficients below the 30% level. Therefore, for this study group the maturity of the complement system appears not to be an important factor.

![Figure 4.2](image)

**Figure 4.2** Correlation between MBP Levels and Functional C3b/C3bi Opsonic Capacity in Chinese Children
Levels were measured in 100 Chinese children and expressed as binding coefficients (B.C.%)(See Figure 4.1). The overall correlation was found to be highly significant (regression coefficient, \( R = 0.77, P < 0.001 \)).
The levels of MBP in Chinese cord samples were assayed using the mannan/PAb(Oxford) system and expressed as binding coefficients. Individuals classified as having low levels of MBP are indicated by cross-hatching. The distribution is similar to that found for older Chinese children (inset).

**Figure 4.3 Distribution of MBP Concentrations in Chinese Newborns**

Newborn MBP levels were measured in serum obtained from 60 Chinese cord blood samples (Chapter 2: Subjects and Samples), and an assessment of their C3b opsonic function was also made. As expected the immature complement system known to be present at birth was associated with a reduced response in the functional opsonisation assay: the mean level of C3b opsonins in newborns was 26% compared to 68% in older children. However, Figure 4.3 shows that the distribution of MBP concentrations in newborns was broadly similar to that found in children, with 13% having low levels. Furthermore, the amounts of C3b opsonin generated again correlated well with MBP levels \(R=0.64, P<0.001\), Figure 4.4).

The levels of serum MBP in the population of children and newborns were analysed according to age and this data is shown in Table 4.1. Although there was some variation in MBP concentration with age there was no significant difference between any
group when compared by Mann-Whitney non-parametric analysis of medians (P > 0.1 in all cases). Nor was there any significant difference between the median concentrations of newborns (2000ng/ml) and that of all children (2070ng/ml). Comparison of the data expressed as binding coefficients gave similar results.

![Graph](attachment:image.png)

**Figure 4.4  Correlation between MBP Levels and Functional C3b/C3bi Opsonic Capacity in Chinese Newborns**

Levels were measured for 60 Chinese cord samples and expressed as percentage binding coefficients (B.C.%). The overall correlation was found to be highly significant (regression coefficient, R = 0.64, P < 0.001).

<table>
<thead>
<tr>
<th>Age Range (years)</th>
<th>Number</th>
<th>MBP Concentration (ng/ml) mean</th>
<th>median</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>60</td>
<td>2430</td>
<td>2000</td>
</tr>
<tr>
<td>0-1</td>
<td>20</td>
<td>2740</td>
<td>2060</td>
</tr>
<tr>
<td>1-4</td>
<td>20</td>
<td>1820</td>
<td>1910</td>
</tr>
<tr>
<td>4-6</td>
<td>20</td>
<td>2760</td>
<td>2290</td>
</tr>
<tr>
<td>6-9</td>
<td>20</td>
<td>3040</td>
<td>2040</td>
</tr>
<tr>
<td>9-16</td>
<td>20</td>
<td>2720</td>
<td>1280</td>
</tr>
</tbody>
</table>

Concentrations were determined using the mannan/PAb(Oxford) assay system

**Table 4.1  MBP Concentrations in Chinese Children**
Pilot Investigation of Reduced MBP Levels in Chinese Donors

In order to determine whether the point mutation discovered in the Caucasian population was also responsible for the low MBP levels found in the Chinese, nucleotide sequencing was performed on exon 1 of the MBP gene. As detailed in the Introduction the Caucasian mutation is located at base 230 in the middle of codon 54 (GGC), and involves the substitution of adenosine (A) for guanine (G).

From a group of 30 Chinese adults the individuals with the three highest and three lowest serum concentrations, as determined by ELISA, were identified and DNA isolated in each case (Section 2.11). The polymerase chain reaction (PCR) was performed as described in Sections 2.12 and 2.14. The three individuals with high MBP levels had the published nucleotide sequence, however, all three individuals with low concentrations were found to be heterozygous for the previously described point mutation at codon 54 (GGC/GAC).

Discussion

In this study measurement of the opsonic capacity of dilute serum has been linked unequivocally to MBP levels in a second population. In addition, MBP deficiency has also been identified in the Chinese population and the cause of the reduced protein levels is again associated with a point mutation in codon 54 of exon 1 of the gene. These results raise questions concerning the origin of the gene mutation and the importance of the deficiency. Both these subjects are addressed in later Chapters.

From the data presented here there appears to be no significant variation in MBP levels for the HK Chinese population during the childhood years. Other analyses of MBP concentrations in infants have been conducted and their conclusions are summarised below.

Perinatal studies on a cohort of Japanese newborns (N=50) showed an increase in MBP concentrations from day 0 to day 5, with mean levels rising from 1.0μg/ml to 2.5μg/ml (Terai & Kobayashi, 1993). In a smaller paired sample group MBP levels increased in each individual from day 2 after a 1 day time lag. Additionally, MBP levels on day 5 were similar to those reported for children in the 3-9 year age range (mean concentration 2.4μg/ml), before decreasing in adult life (mean concentration 1.6μg/ml for age >20yrs)(Terai et al., 1993).

A further study on Hong Kong Chinese newborns (N=200) showed an
approximate doubling in mean MBP concentration between 25 weeks and 40 weeks from conception (normal term). In the subsequent 20 week period levels remained virtually unchanged (Dr. Y.L. Lau, University of Hong Kong, personal communication).

In an investigation of the ontogeny of MBP in 41 Danish Caucasian children there was a statistically significant increase in serum levels during the first 3 months of life (mean MBP concentration of 1.3μg/ml at birth, 2.6μg/ml at 3 months; P < 0.001). There was no significant change in subsequent MBP levels up to 12 months of age (Thiel et al., 1995).

All these studies show slight differences in the development of MBP levels, however, the data for the HK Chinese, Japanese and Danes is consistent with relatively stable, high concentrations of MBP through childhood. The discrepancies between newborn and later levels may reflect the wide concentration variations between individuals which result in large population standard deviations, thus making comparisons difficult. Alternatively, there may be minor differences in protein expression between these groups. Since MBP is an acute phase protein the trauma associated with birth might be expected to produce elevated serum MBP levels in newborns. Whether this would manifest itself at the time of birth, or after a short delay is open to speculation.

A functional complement system is known to be present early in the first year of life. It has been proposed that MBP could play a role in host defence during the period of vulnerability that occurs following the decrease in maternal antibodies (6 months) and before the infant’s own antibody repertoire has matured (2 years)(Turner et al., 1991). The fact that MBP levels are near their optimum and correlate with opsonic capacity from birth implies that the protein may be a key element in the immune system from the first days of life.
PARALLEL SELECTION OF MUTATIONS IN THE MBP GENE

Introduction

The codon 54 (C54) point mutation in the MBP gene was first identified for Caucasians, and in Chapter 4 evidence was presented for its existence in a different racial group - the Hong Kong Chinese. Furthermore, the mutation was associated with reduced serum MBP concentrations in each case. Consequently a more detailed investigation of these Eurasian populations was undertaken to ascertain the gene frequency of the mutation together with its effect on serum levels for each group. In addition, the serum concentrations and molecular genetics of MBP in Gambians from West Africa was examined. It was intended that such a study would complement the Eurasian groups, providing insights into the evolutionary history of MBP and the effects of environment on protein levels.

Results

Eurasian MBP Concentrations

Serum samples were obtained from Caucasian (N=98) and Hong Kong Chinese (N=123) donors as detailed in Chapter 2 (Subjects and Samples), and levels of MBP were determined using the M37/M37 ELISA system and the procedure outlined in Chapter 3 (Table 3.1). The MBP levels determined for the populations are illustrated in the bar charts of Figure 5.1. The wide range of concentrations detected, which cover three orders of magnitude, have been plotted using a logarithmic abscissa in order to assist comparison of the data. The overall distribution can then be seen to fall into a bimodal, or possibly trimodal pattern. The median level of serum MBP in Caucasians was 990ng/ml, with 95% confidence intervals for the median of 642 and 1410ng/ml, whereas for the Chinese population the levels were 1650 (confidence intervals 1390-2070)ng/ml. Comparison of these values using the non-parametric Mann-Whitney rank sum test showed them to be significantly different (P=0.007).
Figure 5.1 Distribution of MBP Concentrations in Eurasian Populations

Serum MBP levels were measured using the M37/M37 ELISA system for 98 Caucasian (upper panel) and 123 Hong Kong Chinese (lower panel) blood donors. The distributions cover a wide range of concentrations and are plotted using a logarithmic abscissa in order to assist comparison of the data.
MBP Deficiency in the Gambian Population

To broaden the population base from which information was collected MBP levels were measured in two groups of Gambian donors, namely newborns (N = 100) and adults (N = 100) (Chapter 2). Analysis using the M37/M37 assay showed that a deficiency of MBP again existed in some of these individuals, however, preliminary examinations did not reveal the presence of the C54 mutation. For the populations as a whole the median protein levels found in the adults and newborns were 550 (95% confidence intervals 334-877) ng/ml and 760 (377-1560) ng/ml, respectively. A comparison between the two Gambian groups showed that the levels were not significantly different (P = 0.41). However, the adult levels were significantly lower than those of the Caucasian adults (990 ng/ml) (P = 0.011). The population profiles illustrated in Figure 5.2 show a striking similarity to those of the Eurasians (Caucasian data inset), and implied the presence of a similar gene mutation to that already discovered. Sequencing of DNA from Gambian individuals with low concentrations of MBP did indeed reveal a new point mutation of the MBP gene at base 239, changing codon 57 from GGA to GAA (Figure 5.3). This codon change when translated again altered one of the repeating glycine (G) residues of the collagenous helix, this time substituting glutamic acid (E).

In those cases where MBP was undetectable (<10 ng/ml) samples were re-assayed using alternative ELISA procedures. The systems used were mannan capture with detection by PAb(Boston), PAb(Oxford) or M37, and M37 capture with PAb(Boston) detection. Using M37 detection several samples, including a serum depleted of MBP by mannone affinity chromatography (kindly supplied by Dr. S. Thiel, University of Aarhus, Denmark) gave no signal. However, using the polyclonal antibody detection systems a weak optical response was detected in these samples that was marginally above background. The depleted serum had been independently shown to have no detectable MBP and it was considered possible that the polyclonal antibodies were cross-reacting with non-MBP material. Therefore, the signal in the depleted serum was regarded as being the revised background level for these assays, and as such MBP remained undetectable in several samples. Subsequent analysis with highly sensitive detection techniques (Chapter 7) suggests that trace levels of MBP related material were being observed in these samples.
Figure 5.2  Distribution of MBP Concentrations in Gambian Populations
Serum MBP levels were measured using the M37/M37 ELISA system for two groups of Gambian donors: 100 newborns (upper panel) and 100 adults (lower panel). The distribution profile found in Caucasians is inset for comparison.
Parallel Selection of MBP Mutations

A  C  G  T

Figure 5.3 DNA Sequencing of the MBP Codon 57 Mutation
Gambian individuals with high (a) and low (b) levels of serum MBP were sequenced as described in Section 2.14. This revealed the presence of a new mutation in the MBP gene, changing the sequence of codon 57 (shown in bold) from GGA (a) to GAA (b). Nucleotides adenine (A), cytosine (C), guanine (G) and thymine (T) are indicated below each lane. (Gels kindly provided by Dr. M. Sumiya, St. Mary’s Hospital Medical School, London).

Mutation Analysis

Two similar point mutations in the MBP gene had been identified and a simple technique was sought to ascertain their presence. Inspection of the modified gene sequences showed that both could be detected by restriction fragment length polymorphisms. The C54 mutation leads to the loss of a Ban I restriction enzyme site, and the codon 57 (C57) mutation introduces an Mbo II restriction enzyme site (Figure 5.4).

The populations were examined at the genetic level by PCR followed by restriction enzyme digestion using DNA extracted according to the methods described in Section 2.11 and analysed as described in Section 2.13 (Chapter 2). Ban I and Mbo II enzyme digestion of the 328 base pair PCR product was visualised on agarose gels with ethidium bromide staining, as shown in Figure 5.5.
Parallel Selection of MBP Mutations

<table>
<thead>
<tr>
<th>codon</th>
<th>53</th>
<th>54</th>
<th>55</th>
<th>56</th>
<th>57</th>
<th>58</th>
</tr>
</thead>
<tbody>
<tr>
<td>product length (base pairs)</td>
<td>3' uncut 328</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wild type sequence</td>
<td>5' GAT GGC ACC AAG GGA GAA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>codon 54 mutant sequence</td>
<td>5' GAT GAC ACC AAG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ban I recognises and cuts</td>
<td>5' GGC ACC CCG TGG 5'</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>WT 246 + 82 mutant 328</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>codon 57 mutant sequence</td>
<td>AAG GAA GAA 3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mbo II recognises and cuts</td>
<td>5' GAA GA(N)g WT 328 CTT CT(N)7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT 328 mutant 266 + 62</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 5.4 Restriction Enzyme Digestion Sites of the MBP Mutations

The polymorphisms introduced by the codon 54 and 57 mutations can be differentially recognised by the restriction enzymes Ban I (codon 54) and Mbo II (codon 57). PCR product obtained as described in Section 2.12 is cut into the fragments indicated.

<table>
<thead>
<tr>
<th>bp</th>
<th>M</th>
<th>54 het</th>
<th>WT</th>
<th>WT</th>
<th>WT</th>
<th>57 hom</th>
</tr>
</thead>
<tbody>
<tr>
<td>506</td>
<td>396</td>
<td>344</td>
<td>298</td>
<td>220</td>
<td>154</td>
<td>134</td>
</tr>
</tbody>
</table>

Figure 5.5 Ban I and Mbo II Digestion of the PCR Product

Left panel: Ban I cuts the 328bp wild type sequence into fragments of 246 and 82bp (lanes WT). Introduction of the codon 54 mutation removes the cut site, hence homozygotes for the mutation give a single band of 328bp (not shown) and heterozygotes give all three fragments (lane 54 het).

Right panel: Mbo II will not cut the wild type sequence (lane WT), but in the presence of the codon 57 mutation fragments of 266 and 62bp result (lane 57 hom; 62bp fragment not visible).

The size in base pairs (bp) of the markers (lanes M; 1kb ladder [Gibco]) are shown on the left.
Frequency of the Mutations

The observed frequencies for the C54 mutation in the Eurasian population and the C57 mutation in the Gambian populations are shown in Table 5.1, along with the expected distribution of the mutations as calculated from the Hardy-Weinberg equation (Vogel & Motulsky, 1986). Figures 5.6 and 5.7 show the serum MBP population profiles replotted according to genotype, and clearly illustrate the dramatic effect of the point mutations on protein levels.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Wild Type WT (GGC/GGC)</th>
<th>Heterozygous G54GD (GGC/GAC)</th>
<th>Homozygous G54DD (GAC/GAC)</th>
<th>Gene Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caucasian</td>
<td>Observed: 66</td>
<td>26 (27%)</td>
<td>0</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>&quot;Expected&quot;: 68 (71%)</td>
<td></td>
<td>3 (3%)</td>
<td></td>
</tr>
<tr>
<td>Chinese</td>
<td>Observed: 95</td>
<td>25 (20%)</td>
<td>0</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>&quot;Expected&quot;: 97 (78%)</td>
<td></td>
<td>2 (2%)</td>
<td></td>
</tr>
<tr>
<td>Gambian (Adults)</td>
<td>Observed: 57</td>
<td>41 (41%)</td>
<td>14</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>&quot;Expected&quot;: 51 (51%)</td>
<td></td>
<td>8 (8%)</td>
<td></td>
</tr>
<tr>
<td>Gambian (Newborns)</td>
<td>Observed: 62</td>
<td>35 (35%)</td>
<td>9</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>&quot;Expected&quot;: 59 (60%)</td>
<td></td>
<td>5 (5%)</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.1 Gene Frequencies for the Codon 54 and Codon 57 Mutations

In Figure 5.6 Caucasian and Chinese individuals heterozygous for the C54 mutation (G54GD) are shown in the two lower panels, and those with the wild type sequence are shown above. For the Caucasian and Chinese populations the observed distribution of genotypes did not differ significantly from the expected values when compared using Chi-squared analysis (P > 0.1). The overall gene frequency of the C54 mutation was 0.16 in Caucasians and 0.11 in Chinese, and in these populations no individuals homozygous for the C54 mutation were found. When the median levels for wild type (WT) and heterozygote (G54GD) were compared in the Caucasian and Chinese groups, the wild type levels were found to be significantly higher (P < 10⁻⁴: Mann-Whitney non-parametric analysis, Table 5.2). Additionally, the median level of serum
Figure 5.6  Relationship between Serum MBP Concentrations and Codon 54 Genotype in Eurasian Populations

Protein levels were determined by ELISA (see Figure 5.1) and genotype assigned on the basis of Ban I restriction enzyme analysis for the Caucasian (left panels) and Hong Kong Chinese (right panels). G54GD individuals are heterozygous for the mutation (lower panels). No individuals homozygous for the mutation were found; WT = Wild type gene sequence (upper panels). One Caucasian was typed G57GE and is not included above.
Figure 5.7  Relationship between Serum MBP Concentrations and Codon 57 Genotype in West African (Gambian) Populations

Protein levels were determined by ELISA (see Figure 5.2) and genotype assigned on the basis of Mbo II restriction enzyme analysis for the Newborns (left panels) and Adults (right panels). G57EE individuals are homozygous for the mutation (lower panels), and G57GE individuals are heterozygous for the mutation (middle panels); WT = Wild type gene sequence (upper panels). One newborn was typed G54GD and is not included above.
Parallel Selection of MBP Mutations

MBP in wild type Chinese was significantly higher than the corresponding level in wild type Caucasians (P=0.0074).

<table>
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<tr>
<th>Population</th>
<th>Genotype</th>
<th>Number of Individuals</th>
<th>Median MBP (ng/ml)</th>
<th>Range (90% confidence)</th>
<th>Significance (P value) *</th>
</tr>
</thead>
<tbody>
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<td>Caucasian</td>
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<td></td>
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<tr>
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<td>1920</td>
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<tr>
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<td>-</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

* all comparisons made with corresponding wild type median; Mann-Whitney non-parametric analysis

Table 5.2 Serum MBP Concentrations in Caucasian, Chinese and Gambian Subpopulations

In the breakdown of the Gambian groups according to the presence of the C57 mutation (Figure 5.7), the heterozygotes (G57GE) are now shown in the middle panels, with the homozygous mutant individuals (G57EE) below. These populations showed a different distribution for the C57 mutation to that of the corresponding C54 mutation in the Eurasian examples. A fundamental distinction was the increased mutant gene frequency (0.26 for both groups combined), which was highlighted by the detection of 23 (of 200) Gambians homozygous for the mutation.

The observed genotype frequencies for the Gambian newborns (Table 5.1) did not differ significantly from the expected pattern, however, for the adults the two distributions were significantly different ($\chi^2=8.7$, P<0.02). For both adults and newborns the median wild type MBP levels were significantly higher than the median heterozygote levels (P<10^{-4}, Table 5.2), which in turn were significantly higher than the
median homozygote (G57EE) levels. Comparison of wild type median Gambian levels with the Eurasian populations showed that Gambian adult concentrations were not significantly different from those of Caucasians (P=0.81), but were significantly lower than the Chinese (P=0.009).

**Relationship between MBP Genotype and Phenotype**

The Chinese population separates neatly into two subgroups with no wild type individuals (WT) having low serum MBP, and no heterozygotes (G54GD) having high levels (above 1000ng/ml). However, two individuals in the British Caucasian group were found to have low MBP concentrations and, furthermore, four Gambian adults that did not have the C57 mutation also had undetectable levels.

Screening of the Eurasian populations by oligonucleotide specific hybridisation (Section 2.16 and Chapter 6) revealed no evidence of the C57 mutation in the Chinese, although one Caucasian individual with undetectable MBP was found to be G57GE. Complementary analysis of the Gambian populations with Ban I restriction enzyme digestion detected only one sample having the C54 mutation - a heterozygote in the newborn population (these mutation analyses were performed by Dr. M. Sumiya). The HLA DR haplotype patterns of this donor, determined by oligonucleotide specific hybridisation, were those frequently found in this population (Hill et al., 1992) and provided no evidence to suggest a recent admixture of Caucasian genes.

Parallel work by Madsen et al. (1994) has revealed the existence of a relatively rare third mutation in the MBP gene. A point mutation at base 223 changes codon 52 (C52) from CGT to TGT, and at the protein level substitutes cysteine (C) for arginine (R) in the central residue of the fourth Gly-X-Y repeat. Subsequent analysis of the three populations using oligonucleotide specific hybridisation revealed gene frequencies for the C52 mutation in Caucasians, Chinese and Gambians of 0.023, 0.010 and 0.010, respectively (Caucasian and Gambian analyses were performed by Dr. M. Sumiya, St. Mary’s Hospital Medical School, London). No individuals were found who were homozygous for the C52 mutation.

Some of the individuals having low serum MBP but neither of the two glycine substitutions were identified as being heterozygous for the codon 52 mutation. For the Caucasian population the mean MBP concentration for the C52 heterozygotes (R52RC) was 560ng/ml. The only two Gambians genotyped R52RC were also heterozygous for the C57 mutation and had average serum levels of 310ng/ml. In the Chinese one R52RC
individual had an MBP concentration of 800ng/ml and the other was also genotyped as G54GD and had a level of 140ng/ml.

**Genotype and Opsonic Capacity**

In order to confirm the relationship between the MBP mutations and the original opsonisation studies the two Caucasian genotypes were compared with respect to their ability to generate C3b opsonins. Studies using the mannan opsonisation assay (Section 2.5) for the two groups gave median C3b opsonin binding coefficients for wild type individuals of 85% and for heterozygotes of 55%. Non-parametric analysis showed this difference to be highly significant (P = 0.0003), implying that the original studies probably identified individuals with an MBP mutation.

The Chinese serum samples were also tested with the mannan opsonisation ELISA. There again appeared to be a concentration of the C54 heterozygotes among those individuals having lower C3b opsonin binding coefficients. However, all of these samples had been through at least one freeze/thaw cycle during shipment to London. This may have affected complement activation, and so quantitative comparisons would not be accurate. The median binding coefficients obtained were 6.8% for heterozygotes and 53% for wild type. This compared to a median C3b binding coefficient of 75% in HK Chinese children, apparently confirming some sample degradation had occurred.

The Gambian sera had been stored extensively at -20°C and in a preliminary investigation of 40 individuals the highest B.C. detected was 30%, suggesting that C3b opsonin binding coefficients could not be measured accurately in these samples.

**Discussion**

The MBP codon 54 mutation in which a glycine residue is replaced by an aspartic acid has been identified in two Eurasian populations. In this investigation of large numbers of healthy donors from these British Caucasian and Hong Kong Chinese groups the frequency of the MBP C54 mutation was high, with values of 0.16 and 0.11, respectively. Moreover, the presence of the mutation resulted in a dramatic reduction in the serum levels of the protein. Surprisingly no individuals homozygous for the C54 mutation (G54DD) were observed. This contrasts with the original reports on the discovery of the mutation when the G54DD state was found in 5 of 19 individuals from three British families. However, these homozygous individuals were identified from
tertiary hospital referrals. The absence of the G54DD state in the healthy populations is not statistically significant and may merely be a reflection of sample size. In addition, the codon 54 mutation has been reported in Danish Caucasians and Eskimos at a frequency of 0.13 (Garred et al., 1992a; b).

Low concentrations of MBP were also found in samples from the Gambian population of West Africa. Interestingly these individuals had the wild type gene sequence for C54, but further analysis led to the discovery of a new mutation in C57 of the gene. In a manner that is directly analogous to the C54 mutation a di-carboxylic acid (glutamic acid) replaces a glycine residue that occupied a core location in the collagenous helix of the protein. The occurrence of the C57 mutation in the Gambian adult and newborn populations was surprisingly high, with gene frequencies of 0.29 and 0.23, respectively. Additionally, many individuals homozygous for the mutation (G57EE) were found, and in all such cases serum MBP was undetectable by ELISA. These results were subsequently confirmed by a study on an East African population from Kenya, where the C57 mutation was found at a gene frequency of 0.25 (Madsen et al., 1994a).

The codon 57 mutation presumably has the same effect on the secondary structure of MBP as the aspartic acid substitution, leading to the disruption of the collagenous triple helix as illustrated in Figure 5.8. A theoretical consideration of this model would suggest that for heterozygotes only 1/8 of the polypeptide chains would form an undisrupted triple helix. This is based on a probability of selecting one normal chain of 1/2, and hence for the required three normal chains the probability is (1/2)^3. Any disrupted protein may be vulnerable to degradation and readily removed from the circulation, thereby resulting in reduced concentrations of protein. For each population group the median level of serum MBP in heterozygotes was compared with the median level of those having the wild type sequence. The observed ratios for Caucasians of 1/5, Chinese 1/6, Gambian adults 1/8 and Gambian newborns 1/10, were consistent with the above prediction.

As expected the examination of complement activation and genotype showed that the presence of the C54 mutation in Caucasians corresponded with a significant reduction in the ability of this group to generate C3b opsonins to mannan. A similar effect was also observed for Chinese C54 heterozygotes, suggesting that the original mannan opsonisation assays (Super et al., 1989) were essentially a functional identification of individuals carrying an MBP mutation. Despite the correlation between the three exon 1 mutations and low MBP levels there were some individuals with the published wild type sequence
and low serum protein concentrations. Furthermore, there was a broad range of protein levels within the heterozygous and wild type genotypic groups. In part this is probably explained by the existence of sequence variations in the non-translated, regulatory region of the MBP gene which were recently been reported by Madsen et al. (1994b).

Comparison of the observed distribution of the mutations with that expected from the Hardy-Weinberg equilibrium showed that the populations conformed in the case of the Eurasian adult and the Gambian newborn populations. However, the Gambian adult frequencies differed significantly from the expected values. This may be explained by a
number of factors such as inbreeding (which is known to increase the number of homozygotes, as seen in this population), recent migrations, or a population based on a mixture of subpopulations that do not completely interbreed. However, there was no evidence of unexpected skewing in the tribal groups comprising this population. An alternative explanation was presented by Madsen et al. (1994a) for a Kenyan cohort that also exhibited non-conformity in respect of the C57 mutation. An analysis of the population as a four allelic system (wild type, C57, C54 and C52 mutations) led to a distribution in line with Hardy-Weinberg expectations. Despite this manipulation the Kenyan population still contained fewer heterozygotes than expected, and this discrepancy becomes significant if the less frequent alleles are combined together to remove small subgroups.

The presence in different population groups of two independent MBP mutations, both at high frequencies is somewhat curious. The C54 and C57 mutations produce identical phenotypes and it is tempting to speculate that parallel selection processes may have occurred to drive down protein levels. From the clinical observations leading to the discovery of the C54 mutation it appears that the homozygous mutant state is disadvantageous in the Caucasian population (Sumiya et al., 1991). This would be consistent with MBP having a critical role in innate immunity during the window of vulnerability in early life (Turner et al., 1991). However, MBP would retain the ability to activate complement in response to mannose rich organisms in later life. Thus the increased risk of infection in childhood may be balanced by a heterozygous advantage manifesting as a reduced capacity for complement activation and possible associated host damage. Such an idea has been proposed to explain why complement deficient individuals experience lower mortality from meningococcal disease despite a higher susceptibility - the lower mortality possibly reflecting reduced endotoxin activation of complement (Ross & Densen, 1984). Similarly the high frequency of C6 deficiency in black and mixed race populations from S. Africa may be beneficial in reducing the incidence of septicaemic shock in infancy (Orren et al., 1987). Comparison of MBP serum levels in the three ethnic groups studied showed the Gambians (adults) to be lowest (550ng/ml), followed by the Caucasians (990ng/ml) and the Chinese (1650ng/ml); all differences were significant. It is tempting to speculate that the lower concentrations of MBP in the hostile African environment may help to reduce the severity of host damage following the release of inflammatory mediators.

An alternative, but not necessarily unrelated, hypothesis is that MBP deficiency
decreases the infectivity of some intracellular micro-organisms that are dependent on opsonisation (Garred et al., 1992a). For example, certain bacteria and parasites are known to become coated with C3b opsonins and then gain access to phagocytes via complement receptors (Mosser & Edelson, 1987; Payne & Horwitz, 1987; Schlesinger et al., 1990). Those individuals with high MBP levels could be more susceptible to infection from any organism which contains carbohydrate structures that are recognised by MBP.

The two main MBP mutations appear to be almost mutually exclusive for the populations investigated. Additionally, the C54 mutation has remained largely confined to Eurasia, and the C57 mutation to Africa. The presence of the latter in West and East Africans raises the question of whether or not this is a general finding for African populations from the sub-Saharan regions. This and other related issues are the subject of the next Chapter.
CHAPTER 6

AN INSIGHT INTO THE EVOLUTION OF MUTATIONS IN THE MBP GENE

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AN INSIGHT INTO THE
EVOLUTION OF MUTATIONS IN THE MBP GENE

Introduction

The studies of MBP in British Caucasian, Hong Kong Chinese and West African Gambian populations described in earlier Chapters represent individuals from diverse areas of the globe. The three structural mutations found in codons 54, 57, and 52 of the MBP gene are present at widely differing frequencies in these groups. Furthermore, the codon 54 mutation has also been reported in the Eskimo and Danish populations (Garred et al., 1992a; b), and the codon 57 mutation in East Africans, with the codon 52 mutation present at low frequency in the two latter groups (Madsen et al., 1994a). These findings are of interest in relation to the evolution of the mutations and the MBP concentrations of people living in different environments. Accordingly the studies were extended to other groups from around the world. These populations ranged from Central and Southern Africa to the South Pacific, and comprised South African Xhosa, Nigerians, San Bushmen from Namibia, and islanders from Vanuatu and Papua New Guinea.

Nomenclature

The following abbreviations will be used throughout this Chapter for genotypic variants:

WT Individual with the wild type gene sequence
G54DD Individual homozygous for the codon 54 mutation
   (both alleles code for aspartic acid (D) instead of glycine (G))
G54GD Individual heterozygous for the codon 54 mutation
G57EE Individual homozygous for the codon 57 mutation
   (both alleles code for glutamic acid (E) instead of glycine (G))
G57GE Individual heterozygous for the codon 57 mutation
R52RC Individual heterozygous for the codon 52 mutation
   (one allele codes for arginine (R), one for cysteine (C))
Techniques for DNA Mutation Analysis

In order to improve the efficiency of genotype screening for large populations the technique of oligonucleotide specific hybridisation was used. DNA probes specific for the wild type and each mutant gene sequence were designed (Table 6.1), radiolabelled (Section 2.15) and used to evaluate samples as described in Section 2.16. The washing conditions used for filters screened by this method were 10 min at 37°C, followed by further washes at temperatures appropriate for each mutation: codon 54 - 38, 42 and 44°C; codon 57 - 37°C; codon 52 - 42, 46, 48, 51°C (determined by Dr. M. Sumiya).

The assignments of genotype were made on the basis of inspections of different exposures of the filters, and was performed by two observers independently, with >95% concordance. In some cases the results obtained by this method ("dot blotting") were difficult to interpret and consequently all assignments for codon 54 and 57 genotype were independently confirmed by restriction enzyme digestion or sequencing (Sections 2.13 and 2.14).

Results
MBP Levels and Genotype in Populations from the South West Pacific

Vanuatu (formerly the New Hebrides): MBP concentrations were determined for
inhabitants of the islands of Maewo and Santo (Chapter 2: Subjects and Samples) using the MAb 131-1/131-1 assay system (Chapter 3). The populations were also analysed for the presence of the three known mutations, as described above (screening of the Maewo population is illustrated in Figure 6.1).

Figure 6.1  Mutation Analysis of DNA in Populations from the South West Pacific
DNA was probed by oligonucleotide specific hybridisation (as described in the text and Section 2.16) and is illustrated for a) 49 samples from Maewo and Papua New Guinea analysed for the presence of the codon 54 mutation, and b) 21 samples from Maewo analysed for the presence of the codon 52 mutation; left panels show probing for the wild type DNA sequence and the right panels for the mutant sequence. No samples were positive for the C52 mutation. The positive signals observed for the codon 54 mutation (circled top right) were confirmed by Ban I restriction enzyme digestion (described in Section 2.13 and Figures 5.3 and 5.4) and selected samples are shown in (c). The samples positive for the C54 mutation by dot blotting typed G54GD by Ban I analysis; the G54DD example is a control sample.
Figure 6.2  Relationship between Serum MBP Concentrations and Codon 54 Genotype in the Vanuatu Population
Protein levels were determined by ELISA (MAb 131-1/131-1 system) and genotype assigned on the basis of oligonucleotide specific hybridisation and Ban I restriction enzyme analysis for newborns from Maewo (N=53, upper panels) and Santo (N=59, lower panels). No individuals homozygous for the mutation were found.
The codon 52 and 57 mutation was not found in any individual, however 3 of 112 were genotyped G54GD after restriction enzyme analysis. The resulting MBP concentration profiles are shown in Figure 6.2. Genotype data on these and all other populations is presented in Table 6.2.

Papua New Guinea: Individuals from the coastal region of the island were genotyped as above, but no sera were available for measurement of MBP concentrations. G54GD was again the only non-wild type gene sequence found, with an even distribution between the controls (3/25) and the malaria infected (4/24) groups (Figure 6.1 and Table 6.2).

MBP Levels and Genotype in African Populations

Xhosa: MBP concentrations and genotype were determined for this population of Black South Africans (Chapter 2: Subjects and Samples; Chapter 3: MAb 131-1/131-1 assay system). The codon 57 mutation was found at a frequency of 0.27 (Table 6.2), comparable to that in the Gambians, and a representative dot blot analysis is shown in Figure 6.3. The codon 54 and 52 mutations were absent. The concentration distribution of MBP in each genotypic group is shown in Figure 6.4.

Figure 6.3  Codon 57 Mutation Analysis of DNA from the Xhosa (S. Africa)
DNA was probed by oligonucleotide specific hybridisation for the presence of the codon 57 mutation (as described in the text and Section 2.16) and is illustrated for 24 Xhosa samples. The upper panel shows probing for the wild type DNA sequence and the lower panel for the codon 57 mutant sequence. The positive signals observed for the codon 57 mutation were confirmed by Mbo II restriction enzyme digestion (described in Section 2.13 and Figures 5.3 and 5.4). The typings indicated are those determined by such restriction enzyme analysis.
Figure 6.4 Relationship between Serum MBP Concentrations and Codon 57 Genotype in the Xhosa (S. Africa) Population
Protein levels were determined by ELISA (MAb 131-1/131-1 system) and genotype assigned on the basis of oligonucleotide specific hybridisation and Mbo II restriction enzyme analysis for the Xhosa (N=45).
Figure 6.5  Distribution of MBP Concentrations in the Nigerian Population  
Serum MBP levels were measured using the M37/M37 ELISA system for 120 Nigerian donors. The distribution profile found in Gambian adults (Chapter 5) is inset for comparison.

**Nigerians:** MBP levels were assayed in a large group of donors from West-Central Africa using the M37/M37 system (Chapter 3). The profile of protein concentrations, illustrated in Figure 6.5, was very similar to that found in the Gambian population when considered as a whole. This resemblance, taken with the high frequency of the codon 57 mutation in East Africans (Madsen et al., 1994a) strongly implies that the codon 57 mutation is present in Nigerians at a level similar to that seen in other resident populations of Africa, however, no DNA was available for genotype study.

**San Bushmen:** Examination of samples from this African hunter-gatherer population revealed a unique pattern of genotypes. The codon 57 mutation was present at a much lower frequency than that seen in all other African populations and, in addition, the codon 54 mutation was found in a significant number of individuals. The genotype frequencies of the two mutations are shown in Table 6.2; the codon 52 mutation was not present. Measurement of MBP concentrations (Chapter 3: MAb 131-1/131-1 system) gave an overall distribution similar to those from other African populations (Figure 6.6).
## Table 6.2 Frequencies of Codon 54 and Codon 57 Mutations in all Populations Studied

<table>
<thead>
<tr>
<th>Population</th>
<th>N</th>
<th>Distribution</th>
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</thead>
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<td></td>
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<td>codon 54</td>
</tr>
<tr>
<td>Vanuatu</td>
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<td>109 3 - - -</td>
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</tr>
<tr>
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<td>58 1 - - -</td>
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<td>Papua New Guinea</td>
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<tr>
<td>Newborns</td>
<td>100</td>
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</table>

All distributions conform to the Hardy-Weinberg Equilibrium (except for the Gambian Adults discussed in Chapter 5)
Figure 6.6  Relationship between Serum MBP Concentrations and Genotype in the San Bushmen

Protein levels were determined by ELISA (MAb 131-1/131-1 system) and genotype assigned on the basis of oligonucleotide specific hybridisation and restriction enzyme analysis (N = 58).

Comparison of MBP Concentrations in all Populations Studied

The levels of MBP for each subgroup investigated are shown in Table 6.3. With one exception the median protein concentrations found in the heterozygote groups (G54GD or G57GE) were significantly lower than the wild type levels (P < 10^-4: Mann-Whitney comparison). The exception was the San Bushmen population, where the G54GD median was not significantly different from wild type (P = 0.204). In the Xhosa, which was the only new population that contained individuals homozygous for a gene mutation, the median G57EE levels were also significantly lower than the G57GE median. It is of interest that the median wild type MBP concentration in the San Bushmen was lower than
the median wild type levels in the other corresponding African groups; this was significant for the Xhosa (P = 0.0024), but not for the Gambians (see Table 5.2)(P = 0.11). There was no significant difference between MBP levels found in Vanuatu and those of Hong Kong Chinese newborns.

<table>
<thead>
<tr>
<th>Population</th>
<th>Genotype</th>
<th>N</th>
<th>Median (ng/ml)</th>
<th>Range (90% confidence)</th>
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<td>G54DD</td>
<td>n.f.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n.f. not found

Table 6.3 MBP Concentrations in Xhosa, San and Vanuatu Sub-populations

Overall, the median concentrations of MBP show wide variations between peoples (Table 6.4), and appear to decrease between birth and adulthood (although this is not significant for the Gambians or Chinese). When the adult populations are considered together the median MBP level for Africa (720ng/ml; N = 324) is significantly lower than the median Eurasian level (1410ng/ml; N = 221), P < 10^-4: Mann-Whitney analysis.
<table>
<thead>
<tr>
<th>Population</th>
<th>N</th>
<th>Median (ng/ml)</th>
<th>95% confidence interval for median</th>
<th>Range (90% Confidence)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xhosa (S.Africa)</td>
<td>45</td>
<td>830</td>
<td>630-1460</td>
<td>120-5500</td>
</tr>
<tr>
<td>Nigerian</td>
<td>120</td>
<td>460</td>
<td>310-650</td>
<td>&lt;10-3730</td>
</tr>
<tr>
<td>San Bushmen</td>
<td>58</td>
<td>930</td>
<td>810-1190</td>
<td>170-2490</td>
</tr>
<tr>
<td>Gambian (Adult)</td>
<td>100</td>
<td>550</td>
<td>340-820</td>
<td>&lt;10-3640</td>
</tr>
<tr>
<td>Gambian (Cord)</td>
<td>100</td>
<td>760</td>
<td>380-1560</td>
<td>&lt;10-3210</td>
</tr>
<tr>
<td>Vanuatu (Cord)</td>
<td>112</td>
<td>2410</td>
<td>2150-2800</td>
<td>900-6000</td>
</tr>
<tr>
<td>Santo</td>
<td>59</td>
<td>2650</td>
<td>2220-3080</td>
<td>1120-5190</td>
</tr>
<tr>
<td>Maewo</td>
<td>53</td>
<td>2270</td>
<td>1950-2580</td>
<td>1190-6000</td>
</tr>
<tr>
<td>Chinese (Cord)</td>
<td>60</td>
<td>2000</td>
<td>1640-2440</td>
<td>80-7000</td>
</tr>
<tr>
<td>Chinese (Adult)</td>
<td>123</td>
<td>1650</td>
<td>1400-2070</td>
<td>45-4960</td>
</tr>
<tr>
<td>Caucasian (British)</td>
<td>98</td>
<td>990</td>
<td>640-1410</td>
<td>&lt;10-4470</td>
</tr>
</tbody>
</table>

Table 6.4 Median Population Levels of MBP

Discussion

During the evolution of mankind two common mutations have occurred in the human MBP gene. The data presented in Chapter 5, and by others (Garred et al., 1992a; b) showed that the frequency of the codon 54 mutation ranged from 0.11 to 0.16 in four Eurasian peoples, and the codon 57 mutation from 0.23 to 0.29 in three African cohorts. The results of this Chapter generally confirm the geographical location of the mutations, with the codon 57 mutation essentially restricted to Africa, and the codon 54 mutation found in peoples of Eurasian descent. Furthermore, these additional analyses show that the codon 52 mutation is uncommon and provide further interest by identifying the first examples of groups having low frequencies of the codon 54 and 57 mutations.

Overall, these population studies support an origin for the codon 57 mutation in
sub-Saharan Africa and of the codon 54 mutation in the Near/Middle East. This analysis fits comfortably with current theories suggesting that *Homo sapiens* first appeared in Africa and then migrated to other areas of the world (Vigilant *et al.*, 1991; Cavalli-Sforza, 1991; Bowcock *et al.*, 1994). The divergence of African and non-African populations is thought to have occurred *ca* 100,000 years before present (BP) (Stringer, 1990), and this appears to provide an upper limit for the age of both mutations. Other evidence both from the fossil record and genetic analysis has been used to construct mankind’s evolutionary tree (see Figure 6.7) and provide an explanation for the colonisation of the globe. This suggests that the ancestral European and Chinese populations separated around 40,000 years BP, and so it appears that the codon 54 mutation predates this event.

![Figure 6.7 Tree of Human Relatedness](image)

Figures indicate the approximate number of years before present that the branching occurred. (Adapted from Long *et al.*, 1990; Bowcock *et al.*, 1991; Cavalli-Sforza, 1991).

In addition, the low mutation frequencies for codon 54 of 0.071 in Papua New Guinea (PNG) and 0.013 in Vanuatu can be tailored to ideas on the colonisation of the Pacific rim. An early migration of South-East Asian peoples into New Guinea and Australia may have occurred 50–60,000 years BP (Brown, 1992). This was then followed much later (6,000 years BP) by peoples of the so-called Lapita culture from South China and Taiwan who swept down into Melanesia, reaching Vanuatu as recently as 3,000 years BP, before going on to colonise Polynesia and even Madagascar (Bellwood, 1991; Gibbons, 1994). Thus the codon 54 mutation may have occurred after the separation of
ancestral Australasians and the first migration into Papua New Guinea ca 50,000 years ago. These early settlers could then have moved on to occupy the nearby Solomon Islands and Vanuatu. The more recent waves of dispersal from South-East Asia may have brought the codon 54 mutation to the coastal regions of PNG and to a lesser extent to the other islands of Eastern Melanesia. This hypothesis is partly supported by the observation that inhabitants of Vanuatu speak languages related to the rest of Polynesia, whilst having a physical appearance similar to that of peoples from coastal New Guinea (Hill et al., 1989). It would thus be fascinating to investigate the frequency of the codon 54 mutation in more isolated peoples from the highlands of PNG and the more recently settled (1-3,000 years BP) islands of the South and Central Pacific. If this theory of migration is correct then the codon 54 mutation would be absent in Papuan highlanders, but be present at a similar frequency to South-East Asians in Polynesians, and even Madagascans.

An alternative explanation for the low frequencies of the codon 54 mutation is that it has drifted downward by chance in these isolated populations. However, this would contrast with the Greenland Eskimos who have maintained a mutant allele frequency of 0.13 since their migration out of Asia between 15,000 and 30,000 years BP (Cavalli-Sforza, 1991).

The above model is apparently confounded by the analysis of the San Bushmen of South West Africa, a population uniquely different from the other African populations investigated. Although Homo sapiens are generally thought to have arisen in Africa approximately 200,000 years BP (Mellars et al., 1992), much of the present culture of sub-Saharan Africa can be traced back to early agriculturalists, who became established as recently as 3,000 years ago in a zone between five and fifteen degrees north latitude. From their start point in Nigeria and the Cameroon these Niger-Kordofanian language speakers spread east and south, assimilating the Bantu, and populating regions previously occupied by more ancient hunter-gatherer peoples such as the San (Bellwood, 1991; Cavalli-Sforza, 1991). These Bushmen contrast with the other peoples in maintaining a continuous, distinct geneline that originated in the late Pleistocene age, up to 130,000 years ago (Deacon, 1992). It should therefore not be surprising that the presence of MBP mutations in the latter population is rather distinct. This manifests itself in two ways, namely the codon 57 mutation, which is common to all African populations, is present at a much reduced frequency (Table 6.2) and the codon 54 mutation, which is essentially absent elsewhere in Africa, is present at significant levels.

In Chapter 5 it was suggested that a heterozygous advantage associated with the
structural gene mutations would act to drive up the frequency of the codon 57 mutation. Assuming the mutation did occur in the ancestral African population, then it seems likely that different selection pressures have acted on the San. Their hunter-gatherer lifestyle would mean they have been exposed to different levels of risk from bacterial, viral and parasitic infections than those of settled agro-pastoralists (farmers!).

The existence of the codon 54 mutation in 4 of 58 San Bushmen is extremely interesting. This allele is present, but rare in other African populations and its occurrence in Gambians (1/200) and in Kenyans (4/66) may result from an admixture of European genes in historical times. However, the San, together with the Pygmies of the Central African Republic and Zaire, represent ancient foraging peoples who have evolved in relative cultural isolation. It would thus be of interest to investigate whether the Pygmies also have the codon 54 mutant allele. It is also noteworthy that the median wild type levels of MBP found in the San are lower than in any other population investigated.

The San findings invite speculation that the codon 54 mutation occurred before the "Out of Africa" migration 100,000 years ago. It was then carried to Eurasia where selective pressures pushed the mutation frequency higher, but not before the mutation had been carried to Australasia. The codon 54 mutation was then retained at low frequencies in the hunter-gatherers of Southern Africa and the S.W. Pacific, but was subsequently lost in the other sub-Saharan African peoples. At some point during this process the codon 57 mutation arose in Africa, perhaps in the ancestors of the Kordofanian language speakers. Early interactions between these agriculturalists and the foragers may have introduced the codon 57 mutation to the forefathers of the San. As suggested above, differing selection pressures would consequently lead to the different gene frequencies observed today, and these are summarised, along with possible migration routes in Figure 6.8.

In addition to these evolutionary theories there is also the possibility that the presence of the codon 52, 54 and 57 mutations in such close proximity is more than coincidental. This region of the MBP gene may be highly susceptible to mutational events, although there are no traditional DNA sequence motifs in the region that would make this likely (Cooper & Krawczak, 1990). If a mutation hotspot does exist then it is possible that any of the allelic variations could have arisen on more than one occasion during human evolution. Appropriate haplotype studies would help to resolve such questions.

Throughout the population groups studied the presence of a mutation in the MBP
Figure 6.8 Global Occurrence of Common MBP Mutations and Possible Dispersion Routes of Homo Sapiens

Upper panel: The frequencies of the codon 54 and 57 mutations in various populations (includes data from Madsen et al., 1994a).

Lower panel: One hypothetical dispersion route for the codon 54 (continuous lines) and codon 57 (dashed lines) MBP mutations superimposed on a theoretical dispersion of human populations (from Cavalli-Sforza, 1991). The earliest migration into Australasia (dotted lines) may have pre-dated the codon 54 mutation.
gene has always resulted in reduced serum levels of the protein. The model proposed in Chapter 5 predicted that heterozygous individuals would have protein concentrations close to 1/8 normal levels, and this has been further substantiated in each of the new groups analysed. The original model also proposed that there would be a failure of efficient oligomerisation of MBP in those individuals with a structural gene mutation and this aspect is explored in the next Chapter.
CHAPTER 7

PHYSICOCHEMICAL ANALYSIS OF PHENOTYPIC VARIANTS OF MBP

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PHYSICOCHEMICAL ANALYSIS
OF PHENOTYPIC VARIANTS OF MBP

Introduction

The three mutations identified in exon 1 of the MBP gene are in the region that encodes for the first part of the collagenous domain of the protein. In Eurasian populations the point mutation in codon 54, when translated, substitutes aspartic acid (D) for glycine (G), and in Gambians a comparable mutation in codon 57 substitutes glutamic acid (E) again for glycine. In individuals homozygous for either mutation serum MBP was undetectable by ELISA (<10ng/ml), whereas in those heterozygous for a mutation serum MBP levels were profoundly reduced (e.g. in Caucasians median levels of 360ng/ml compared to 1630ng/ml in wild type individuals). The less frequent mutation in codon 52 of the gene that substitutes cysteine (C) for arginine (R) is also associated with reduced protein levels. The codon 54 and 57 mutations disrupt the fifth and sixth collagenous Gly-X-Y repeats respectively, by inserting a bulky di-carboxylic acid in place of a glycine, which is the key axial residue in the collagenous helix. It was predicted that these disruptions would lead to instabilities in the secondary structure and render the protein vulnerable to degradation, and hence to low MBP levels in the serum.

Two studies using artificial cell expression systems (Super et al., 1992; Kurata et al., 1993) have suggested that recombinant protein with the codon 54 mutation can assemble into normal polymers, and it was proposed in these reports that the mutations do not destabilise MBP sufficiently to account for the observed low protein levels in serum.

In order to investigate further the real effect of the mutations whole serum has been analysed by a variety of size separation techniques, followed by detection of MBP using immunoblotting and enhanced chemiluminescence.
Nomenclature

The following abbreviations will be used throughout this Chapter when considering protein produced by an individual of a particular genotype:

- **MBP<sub>WT</sub>** MBP produced by an individual with the wild type gene sequence  
- **MBP<sub>G54DD</sub>** MBP produced by an individual homozygous for the codon 54 mutation  
  (both alleles code for aspartic acid (D) instead of glycine (G))  
- **MBP<sub>G54GD</sub>** MBP produced by an individual heterozygous for the codon 54 mutation  
- **MBP<sub>G57EE</sub>** MBP produced by an individual homozygous for the codon 57 mutation  
  (both alleles code for glutamic acid (E) instead of glycine (G))  
- **MBP<sub>G57GE</sub>** MBP produced by an individual heterozygous for the codon 57 mutation  
- **MBP<sub>R52RC</sub>** MBP produced by an individual heterozygous for the codon 52 mutation  
  (one allele codes for arginine (R) one for cysteine (C))

Results

Native PAGE

In previous studies, using an ELISA procedure with a sensitivity of 10ng/ml, no MBP was detected in the serum of individuals homozygous for the codon 54 mutation (Sumiya <i>et al.</i>, 1991). However, when the serum of such individuals was electrophoresed under non-denaturing conditions and the proteins immunoblotted, as described in Chapter 2: Sections 2.7.1 and 2.8, it was possible to detect MBP reactive material by enhanced chemiluminescence (Figure 7.1). This material migrated more rapidly than did MBP<sub>WT</sub>, and pooled serum (containing both wild type and heterozygous forms of MBP) produced both bands. Serum absorbed on a mannose affinity column and shown by ELISA (M37/M37 and MAb 131-1/131-1 systems) to be apparently depleted of MBP (<10ng/ml) also showed traces of the same faster migrating material present in samples containing MBP<sub>G54DD</sub> or MBP<sub>G54GD</sub>. (This sample was kindly supplied by Dr. S. Thiel, University of Aarhus, Denmark).
Analysis of MBP Phenotypes

**Figure 7.1 Native PAGE and Immunoblotting Analysis of Whole Sera**

Proteins separated by Native PAGE (5% AA) were transferred to nitrocellulose and MBP moieties visualised by immunodetection (MAb clone #3) and ECL (Sections 2.7.1 and 2.8). 5\(\mu\)l of whole serum from different individuals (genotype as indicated) were applied to each lane. Pooled serum contained material from >20 different Caucasian donors and was known to contain a mixture of MBP\(_{WT}\) and MBP\(_{G54GD}\). "Depleted serum" was pooled serum that had been passed through a mannose affinity column.

**SDS-PAGE**

To investigate further the differences illustrated in Figure 7.1 selected samples were electrophoresed under non-reducing SDS denaturing conditions (Section 2.7.2). Two experimental approaches were used: standard Laemmli conditions in which the whole serum samples were boiled in sample buffer for 5 minutes prior to analysis by PAGE, and alternatively analysis without sample boiling (Section 2.7.2). The MBP moieties were then specifically detected by immunoblotting (Section 2.8), and the results for the respective conditions are illustrated in Figure 7.2 (with boiling: lanes A-E, without boiling: lanes F-J; two exposures of the immunoblot are shown in order to highlight differences in signal intensity between the samples). Under both conditions the presence of a quantitatively reduced amount of rapidly migrating MBP-related material in the sera of individuals having MBP mutations was confirmed. Traces of this material were also detected in the sera of wild type individuals.
Figure 7.2 **SDS-PAGE and Immunoblotting Analysis of Whole Sera**

Proteins were separated by SDS-PAGE (3-10% AA gradient) and MBP moieties visualised by immunodetection (MAb clone #3; see Sections 2.7.2 and 2.8). The upper panel shows a long exposure of the immunoblot and the lower panel a short exposure. Serum samples were diluted 1/2 in sample buffer, boiled (lanes A-E) or incubated at room temperature (lanes F-P) for 5min, and 4μl aliquots loaded on to the gel. Individual MBP genotypes (see Nomenclature) or source of sera are indicated above each lane (lanes K-P are control animal sera: CM1 & 2 - control mice, TM1 & 2 - human MBP transgenic mice, NRS - normal rabbit serum, NGS - normal goat serum; see text). Molecular masses are indicated on the left of each panel.
The less denaturing conditions were favoured for a number of reasons. Firstly one of the primary objectives was to examine the form of naturally expressed mutant MBP with minimal physicochemical disruption. Furthermore, Super et al. (1992) had previously found that there was no difference in the banding patterns of recombinant MBP when analysed by SDS-PAGE with or without boiling, and Figure 7.2 shows that the banding patterns obtained using partial denaturation were better defined than those observed after sample boiling. The migration of the protein standards and higher order MBP forms under these conditions was unaffected, however, there were minor differences in the mobility of low molecular mass MBP (see also Figure 7.3). In addition, the recognition of MBP in whole serum, after electrophoresis and transfer to a membrane, by monoclonal antibodies (MAbs) was potentially problematic (for example M37 did not to recognise reduced MBP). The mild denaturing conditions ensured maximum epitope recognition in each antibody system.

A representative analysis of sera with differing physicochemical forms of MBP, developed using MAb #3, is also shown in Figure 7.2 (lanes F-J). Apparent molecular masses were calculated from standard curves, and represent the average of at least five experiments (for protein <220kDa, coefficient of variation (C.V.) <5%; for protein >220kDa, C.V. <10%).

MBP<sub>WT</sub> showed a ladder of higher order MBP polymers. A band of apparent molecular mass 210kDa probably corresponding to a dimer of 3-chain subunits (6 chains), was followed by a 300kDa band corresponding to a trimer and other higher mass forms (largest apparent molecular mass ≈700kDa). In the case of MBP<sub>GS4DD</sub>, however, predominantly low molecular mass material was detected - a doublet at apparently 120/130kDa, with trace amounts of some larger polymeric forms also present. In addition, the serum absorbed on a mannose affinity column also contained the 120/130kDa material. Pooled serum and MBP<sub>GS4GD</sub> exhibited both high molecular mass polymers and the low molecular mass material. It is important to note that the intensity of the MBP signal detected from MBP<sub>WT</sub> was considerably greater than from MBP<sub>GS4DD</sub> or MBP<sub>GS4GD</sub> (see also Figure 7.4).

The above results obtained with Mab #3 were confirmed using PAb(Boston) and both the clone #6 and 131-1 monoclonal antibodies. There were, however, differences in the clarity of the banding patterns in the low molecular mass region (Figure 7.3). The monoclonal antibody M37 (not included in Figure 7.3) detected MBP of high apparent molecular mass but not the low molecular mass moiety.
The specificity of the banding patterns observed was confirmed in two additional ways. In one experiment the sera of two different transgenic mice expressing the wild type human MBP gene (kindly supplied by P. Tabona, St. Mary’s Hospital Medical School, London) were compared after SDS-PAGE and immunoblotting with the sera of two control mice. The normal banding pattern with traces of the low molecular mass moiety were identified only in the two transgenic animals expressing the human protein. Furthermore, no cross reactivity of the anti-human MBP antibodies was observed in the low molecular mass region for whole goat or rabbit serum (Figure 7.2 lanes K-P). Secondly, non-specific binding of anti-MBP antibodies to serum proteins was also determined in the presence of a 20 fold excess of a control antibody of the same subclass (MOPC-21); this had no effect on the banding patterns observed.

Figure 7.3  Comparison of Antibodies used to Detect MBP following SDS-PAGE
To help confirm the identity of the material detected in Figures 7.1 and 7.2 multiple aliquots of sera were electrophoresed under partially denaturing conditions (* fully denaturing conditions) and immunoblotted (Sections 2.7.2 and 2.8). Panels were then screened with different anti-human MBP antibodies: (a) MAb clone #3; (b) MAb clone 131-1; (c) PAb(Boston); (d) MAb clone #6. Individual MBP genotypes are indicated above each lane, and molecular masses are indicated on the left.
Gel Filtration

To confirm the differences seen by PAGE and investigate further the size of the material detected, whole sera containing either MBP<sub>WT</sub> or MBP<sub>G54DD</sub> were fractionated on a Superdex 200 column using the FPLC system (Pharmacia; Section 2.9) and the fractions analysed by SDS-PAGE and immunoblotting. The column was calibrated with a range of standards as shown in Figure 2.1. The profiles obtained are shown in Figure 7.4 and the lower panels illustrate the differences in size and relative amounts of MBP from individuals of differing genotype.

The upper panels (Figure 7.4a and b) illustrate the serum fractionation profiles for the two individuals, with the arrows indicating the elution points of known globular protein standards. The different intensities of the signals obtained from MBP<sub>WT</sub> and MBP<sub>G54DD</sub> are highlighted by Figures 7.4c-f. The principal MBP forms derived from the two genotypes are illustrated in short exposures of the immunoblot (Figures 7.4c and d). Only low concentrations of smaller mass forms of MBP were detectable in fractions from MBP<sub>G54DD</sub> (Figure 7.4d), whereas the expected ladder of bands was visible in the MBP<sub>WT</sub> fractions (Figure 7.4c). When long exposures (Figures 7.4e and f) were used trace amounts of low mass material could be detected in MBP<sub>WT</sub>, and some additional high and low mass forms in MBP<sub>G54DD</sub>.

Assessment of size by comparison with the known gel filtration standards showed that MBP<sub>G54DD</sub> eluted from the column in a region corresponding to an apparent molecular mass of 240kDa (Fraction 9), with additional components in elution positions corresponding to 380kDa (Fraction 7) and 130kDa (Fraction 12)(Figure 7.4f). For MBP<sub>WT</sub> (Figure 7.4c and e) the first material to elute had an apparent molecular mass of 900kDa (Fraction 4) with levels peaking around the 500kDa elution position (Fraction 6).

Thus all forms of MBP eluted earlier than would be predicted from the masses determined by SDS-PAGE. Similar observations have been reported with other collagenous proteins (Holmskov et al., 1994).
Figure 7.4  Superdex 200 Gel Filtration of Whole Serum Followed by SDS-PAGE Analysis and Immunoblotting with Anti-MBP MAb
(a) and (b) Absorption profiles of serum from individuals expressing MBP<sub>WT</sub> and MBP<sub>GMDD</sub> respectively, with elution positions of gel filtration standards indicated above; (c) and (d) 30 second film exposures of the immunoblots (determined with MAb clone #3) for MBP<sub>WT</sub> and MBP<sub>GMDD</sub>; (e) and (f) 3 min film exposures for MBP<sub>WT</sub> and MBP<sub>GMDD</sub>. Lane S shows the banding pattern of unfractionated whole serum. SDS-PAGE molecular mass markers are indicated on the right of the panels.
a) Absorption Units (AU)

b) Elution Volume (ml)

c) Fraction Number

S 2 3 4 5 6 7 8 9 10 11 12 13 14 15

d) kDa

200
116
97
66

e) kDa

200
116
97
66
Analysis of MBP Phenotypes

b) Absorption Units (AU)

Fraction Number

S 2 3 4 5 6 7 8 9 10 11 12 13 14 15

f) kDa

200 116 97 66
Sucrose Density Centrifugation

In order to assess more accurately the native state of the molecular forms of MBP, serum containing MBP<sub>WT</sub> or MBP<sub>G54DD</sub> (two individuals of each phenotype), and pooled Caucasian serum (presumed to contain predominantly MBP<sub>WT</sub> and MBP<sub>G54GD</sub>) were analysed by sucrose density centrifugation (Section 2.10). A panel of proteins of known sedimentation coefficient were run in parallel and all fractions were analysed by SDS-PAGE. Figure 7.5 illustrates the separation obtained for some of the standards which were used in the construction of the calibration plot shown in Figure 7.6.

![Calibration of Sucrose Density Centrifugation Gradients](image)

**Figure 7.5** Calibration of Sucrose Density Centrifugation Gradients

Standards of known sedimentation coefficient were centrifuged on a sucrose density gradient (Section 2.10), which was separated into fractions (lanes 4-19) and analysed by SDS-PAGE (5% AA; Section 2.7.2). To assist identification the standards were also loaded directly onto the gel (lanes 1-3) and those analysed above under partially denaturing conditions are thyroglobulin (<sub>20</sub>, M<sub>669</sub>kD; lanes 1 & 7), C1q (<sub>20</sub>, M<sub>400</sub>kD; lanes 2 & 11), and human serum albumin (<sub>20</sub>, M<sub>65</sub>kD; lanes 3 & 15). SDS-PAGE standards are shown on the left. Notes: lane 14 shows the elution of dimeric forms of albumin; under the electrophoresis conditions the collagenous C1q molecule is migrating with an anomalously high apparent molecular mass.
Figure 7.6 Separation of MBP Moieties by Sucrose Density Centrifugation

Upper panel: Calibration of the sucrose gradient using proteins of known sedimentation coefficient (the second order regression line was calculated using the Sigmaplot graphics package [Jandel Scientific, Erkrath, Germany]). Lower panel: SDS-PAGE separation and immunodetection of fractions from a separation of an aliquot of pooled Caucasian serum known to contain both MBP_{GGQ} and MBP_{WT}. ECL detection was performed with MAb clone 131-1. Lane WS shows the banding pattern of whole serum prior to centrifugation. The fractions are aligned with their respective radial distance in the upper panel.
Figure 7.7 Separation of $\text{MBP}_{\text{WT}}$ and $\text{MBP}_{G54DD}$ by Sucrose Density Centrifugation

SDS-PAGE separation and immunodetection of fractions from a separation of an aliquot of $\text{MBP}_{\text{WT}}$ (upper panel) and $\text{MBP}_{G54DD}$ (lower panel). Fraction numbers are shown across the top and lane WS indicates the banding pattern of whole serum prior to centrifugation. ECL detection was performed with MAb clone #3.
The separation illustrated in Figure 7.6 (lower panel) shows pooled serum detected with MAb clone 131-1 containing MBP oligomers ranging from presumed dimers to pentamers (s\textsubscript{20,w} values 10.3, 11.9, 13.6 and 14.6, respectively) and, in addition, the apparent 120/130kDa moieties of s\textsubscript{20,w} 6.7, with trace amounts of material migrating at 70kDa (s\textsubscript{20,w} 5.8). This figure combines the features seen individually when the sera containing MBP\textsubscript{WT} and MBP\textsubscript{G54DD} were analysed (Figure 7.7). The near uniform increase in sedimentation coefficient of the higher mass material is supportive of the assumption of a ladder of oligomers based on a constant sized subunit.

**MBP protein patterns in individuals having mutations in codons 54, 57 and 52 of the MBP gene**

A selection of samples from individuals reflecting the three MBP mutations and having different serum MBP concentrations as measured by ELISA were examined by SDS-PAGE with ECL detection of MBP. As is illustrated in Figure 7.8 a complex array of molecular forms of MBP were detected. These divide broadly into two groups - the higher apparent mass forms (bands labelled d to k) and the lower apparent mass material (bands a to c).

For MBP\textsubscript{WT} (lanes A,B,G,H) bands corresponding to higher mass polymers of MBP ranging from dimers of subunits (presumably 6 chains) to octamers of subunits (presumably 24 chains) were detected (calculated apparent molecular masses are shown in Table 7.1). In addition traces of low molecular mass material (150kDa - band c) seen in Figure 7.2 lane F were sometimes present. The banding patterns for individuals having codon 54 mutations (lanes C,D,E,F) were characterised by a range of low molecular mass forms: doublet bands a (70kDa), b (120/130kDa) and c (≈150kDa). Differing amounts of higher apparent molecular mass material were also detected with a band (e) at approximately 250kDa being the most prominent. This was intermediate in size between the dimeric and trimeric polymers of MBP\textsubscript{WT}. Heterozygotes (C) also showed the normal high molecular mass forms, but there was wide variation in MBP expression within a genotype and higher mass forms were not always found (e.g. lane D).

In the analysis of all samples from individuals with the codon 57 mutation (lanes I,J,K,L,O,P) the 120/130kDa doublet (band b) was again detected. As expected MBP\textsubscript{G57GE} from an individual with serum MBP detectable by ELISA (lane I) also showed evidence of the higher order polymers. However, another heterozygote (MBP\textsubscript{G57GE}, lane
J) with undetectable MBP by ELISA yielded a relatively faint ladder of bands (predominantly the e and f components). For MBP<sub>R52RC</sub> samples (lanes M and N) the 130kDa band was present, although the 120kDa band was absent. Band e (250kDa) was very faint in these two samples but was more prominent in samples containing both the codon 52 mutation and either the codon 57 or codon 54 mutations (lanes O,P,Q).

Figure 7.8  MBP Mutation Analysis by SDS-PAGE and immunoblotting
Samples were obtained from Caucasian (lanes A-F,M,N), Gambian (lanes G-L,O,P) and Chinese (lane Q) donors. The MBP expressed by each individual is indicated at the top of each lane, with serum MBP concentrations (ng/ml) as determined by ELISA underneath. Molecular sizes are indicated on the right and the bands labelled a-k (at left) are referred to in the text.
Scanning Densitometry

The relative proportions of MBP moieties in MBP<sub>WT</sub>, MBP<sub>G54GD</sub> and MBP<sub>G54DD</sub> (Figure 7.8 lanes A, C and F) were calculated by scanning densitometry of the films and are shown in Table 7.1 and Figure 7.9. As can be seen from this analysis there is a higher percentage of MBP in the lower mass forms when the mutation is present. Expressing the total signal detected in each lane as a percentage of the total signal in a serum of average concentration (as determined by ELISA) gives values of MBP<sub>WT</sub> (lane A) 170%, MBP<sub>G54GD</sub> (lane C) 30%, and MBP<sub>G54DD</sub> (lane F) 5%.

<table>
<thead>
<tr>
<th>Band (as defined in Figure 7.8)</th>
<th>Apparent Molecular Mass (SDS-PAGE) [kDa]</th>
<th>Sedimentation coefficient [s&lt;sub&gt;20,w&lt;/sub&gt;]</th>
<th>Proportion of MBP in each molecular form [%] (Scanning densitometry analysis)</th>
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<tbody>
<tr>
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<td>70</td>
<td>5.8</td>
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</tr>
<tr>
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<td>6.7</td>
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</tr>
</tbody>
</table>

Table 7.1 Analysis of MBP banding patterns
Figure 7.9  Scanning Densitometry Analysis of SDS-PAGE Separations of MBP

Lanes from the immunoblot shown in Figure 7.8 were analysed by scanning densitometry. The profiles obtained for (a) MBP_WT (lane A) and (b) MBP_GDP (lane F) are shown, and the percentage of MBP represented by each peak is detailed in Table 7.1 (the relative signal intensities illustrated are not on the same scale).
Discussion

Sensitive techniques have been used to characterise the MBP circulating in the serum of individuals with different mutations in the MBP gene. The genotypes investigated were those arising from point mutations in the collagenous region of the gene that would translate into protein substitutions likely to affect the secondary structure of the protein. The results presented illustrate that there are clear physicochemical differences between the MBP phenotypes when they are observed in an environment more closely resembling the natural state than any so far described.

Individuals genotyped as homozygous for mutations at codon 54 or 57 of the gene were found to produce MBP that could be detected readily in their serum when analysed by immunoblotting and enhanced chemiluminescence. As has been shown in Chapter 5 and also by others (Garred et al., 1992a) the serum MBP of individuals homozygous for the codon 54 or 57 mutations has previously been undetectable or found at trace levels only when measured by ELISA (sensitivity 10ng/ml, representing a physical limit of 30pg protein). The present findings can be explained in terms of the increased sensitivity of the detection technique (1pg of protein) but may also reflect the reliance of the ELISA procedures on the polymeric nature of MBP. In this respect it is important to note that the mutant material detected is primarily of low apparent molecular mass and therefore comprises few repeating antigenic epitopes in contrast to the MBP produced by individuals having the wild type gene sequence.

The presence of the low molecular mass material in the serum absorbed on a mannose affinity column is also likely to reflect differences in the binding affinity of the different polymeric forms of MBP. The larger polymers with higher avidity would be readily removed by the affinity chromatography preparative procedure whereas the smaller forms with fewer carbohydrate recognition domains would not bind so efficiently to the matrix and hence would not be removed.

Previous data have indicated that the serum concentrations of MBP are far higher in MBP\textsuperscript{WT} subjects than in individuals with the mutations. The ELISA procedure gave median levels of 1630ng/ml for MBP\textsuperscript{WT}, 360ng/ml for MBP\textsubscript{G54GD} and <10ng/ml for MBP\textsubscript{G54DD}, and this data compares favourably with densitometric analysis showing MBP\textsubscript{G54GD} levels to be 30% and MBP\textsubscript{G54DD} levels to be approximately 5% of average MBP\textsuperscript{WT} levels.

Individuals who were heterozygous for the point mutations at codons 52, 54, or
57 produced MBP that usually showed characteristics of both the wild type and homozygous mutant forms. In these cases the proportion of MBP of low apparent molecular mass accounted for only a small percentage of the total amount of protein, and it was not possible to determine whether such protein comprised exclusively mutant peptide chains or was a mixture of mutant and normal chains.

Size analysis of MBP\textsubscript{WT} found circulating in serum shows material to be present in several polymeric forms. Gradient SDS-PAGE detected a ladder of subunits starting at ca 200kDa (presumably two subunits) with several steps at ca 100kDa increments, possibly up to an octamer of subunits (24 chains). Under non-reducing conditions SDS did not disrupt this ladder, implying that the structures were held together by intra- and inter-subunit disulphide bonding. These moieties could be resolved into discrete fractions by sucrose density centrifugation and showed a constant value change in sedimentation coefficient, which would substantiate a fixed unit increase in size (See Figure 7.7 upper panel and Table 7.1). Scanning densitometry of the banding patterns showed circulating MBP to be composed of 25% dimeric, 25% trimeric and 20% tetrameric subunits with less than 20% pentamer/hexamer. Earlier studies on human forms of MBP have led to estimates of molecular mass ranging from 200kDa to 700kDa (Kawasaki \textit{et al.}, 1987; Colley \textit{et al.}, 1988; Schweinle \textit{et al.}, 1989; Lu \textit{et al.}, 1990). In the latter study trimers and tetramers accounted for approximately 80% of the MBP obtained after extensive purification, but the possibility exists that the concentration procedures used may have encouraged the formation of higher order polymers. The studies presented here of serum MBP without the use of such purification procedures may reflect more closely the \textit{in vivo} situation.

In individuals with the codon 54 mutation the ratios of the polymeric forms was different from that of wild type individuals. In heterozygotes over 80% of MBP was dimer/trimer and only 1% hexamer. In mutant homozygotes less than 10% of the detected MBP was assembled into the expected polymeric forms, with most material being of low molecular mass.

The major moiety detected in the serum of an MBP\textsubscript{G54DD} individual has an apparent size of 120/130kDa by SDS-PAGE under partially denaturing non-reducing conditions, with a sedimentation coefficient similar to that of IgG. The doublet may reflect disulphide interchange or post-translational modifications of the polypeptide, such as glycosylation (Colley & Baenziger, 1987c). Other bands are present at apparent positions of 70, 150 and 250kDa, although when analysed by gel filtration or density
Analysis of MBP Phenotypes

In the case of mutant MBP molecules steric hindrance may therefore inhibit incorporation of the third chain and two chain fibrils may be the basic assembly unit for the small amount of protein detected. This would migrate close to 64kDa (band a in
Figure 4), with dimers (i.e. 4 chains) expected to migrate at 120/130kDa and tetramers (8 chains) at 250kDa (band e). The doublet band c which was widely observed in Figure 4 may represent a partial degradation product or an artefact caused by the electrophoretic conditions.

The present data indicates that only small amounts of mutant MBP can be detected in serum and that this is essentially of low molecular mass and low binding affinity. In other recent studies of these mutations using recombinant MBP and artificial expression systems Super et al. (1992) and Kurata et al. (1993) claimed that the mutations did not explain low serum levels. The former group used HPLC to analyse recombinant MBP and found no difference between the mutant and wild type forms, both existing as 20% pentamer/hexamer and 80% 270kDa material. However, it should be stressed that this was a serum-free system and their SDS-PAGE studies did suggest that recombinant mutant MBP dissociated more readily than wild type. Similarly Kurata et al. (1993) found evidence of impaired oligomerisation of recombinant MBP<sub>GS4DD</sub>. The findings presented here, which are likely to closely reflect the physiological state, clearly show that failure of polymerisation is associated with low circulating levels of mutant protein. It is of interest that Super et al. (1992) also reported failure of complement activation by serum immunochemically depleted of MBP and reconstituted with mutant MBP. This suggests that in serum the protein may not have been appropriately polymerised since complement activation is believed to be dependent on high order structure.

Assuming normal translation of the mutant polypeptide (as suggested by Kurata et al., 1993) the data suggests that approximately 95% of mutant MBP cannot be accounted for in the serum. Whether this material has been degraded in the hepatocyte before secretion, or has been subject to rapid intravascular catabolism remains to be established.
CHAPTER 8

CLINICAL ASPECTS OF MBP DEFICIENCY

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CLINICAL ASPECTS OF MBP DEFICIENCY

Introduction

The common opsonic defect has been associated with a variety of infections since it was first described (See Chapter 1). Furthermore, low concentrations of MBP and hence the genetic mutations causing them can be related back to these earlier studies. Thus a link between MBP deficiency and susceptibility to disease is implied, although the deficiency itself has not been related directly to a higher risk of infection. In addition, certain pathogens and disease states present themselves as possible candidates for the involvement of MBP, and some of the investigations that have been carried out into these interactions are detailed in the Introduction (see: The Role of MBP in Host Defence). In contrast to this, the findings presented in some earlier chapters have suggested that there may be an advantage to having reduced serum MBP concentrations. The following results represent analyses of various pilot studies addressing several clinical states and the associated serum MBP levels.

The investigations focus on a number of patient groups, and in each case the underlying hypothesis and aims are detailed at the start of each section, followed by the assay system used to measure MBP concentrations, the results obtained and a short commentary.

Investigations

Primary Immunodeficiency

_Hypothesis and Aims:_ Caucasian individuals homozygous for the MBP gene mutations appear to be susceptible to severe infections (Sumiya _et al._, 1991; Summerfield _et al._, 1995). Therefore, a preliminary investigation was undertaken into whether MBP deficiency is increased in patients referred to a primary immunodeficiency referral centre.

_Patient Group:_ Samples were collected from 18 children (age range 2 weeks - 10 years) by Dr. D. Webster at the Immunodeficiency Referral Laboratory, Northwick Park
Hospital, Harrow. All patients were diagnosed as severely hypogammaglobulinemic, with a minority having X-linked agammaglobulinemia and the remainder common variable immunodeficiency.

**Assay type:** MBP levels were determined by an asymmetrical sandwich ELISA procedure (assay type mannan/PAb(Oxford); Chapter 3).

**Results:** 33% of immunodeficiency referrals had reduced levels of MBP (<300ng/ml), which is approximately twice the frequency in the population of healthy Caucasian adults (Chapter 5)(P=0.06: Chi-square analysis).

**Comment:** This association is supported by a study of Danish patients screened for immunodeficiency disease, where MBP homozygosity was found to be an increased risk factor (Dr. P. Garred, Rigshospitalet University Hospital, Copenhagen, personal communication).

**Haemophilus Influenzae Meningitis and Osteitis**

**Hypothesis and Aims:** MBP binds to a wide range of micro-organisms and facilitates their removal by opsonophagocytosis. The aim of this study was to investigate whether MBP deficiency might be a risk factor predisposing to either meningitis or osteitis in children having *H. influenzae* type b (Hib) infections.

**Patient Group:** Children (mean age 1 year) with bacteriologically proven Hib meningitis or Hib osteomyelitis admitted to the Red Cross War Memorial Children's Hospital, Cape Town. For patients with bacterial meningitis specimens were collected on admission (sample 1; N=80), 7-10 days later (sample 2; N=47) and 6 weeks later at a follow-up appointment (sample 3; N=26). Hib osteitis patient specimens were collected on admission (N=33) and 3-4 weeks later at a follow-up visit (2nd sample; N=20). Control samples were adult Black S. Africans (Xhosa, N=45; Chapter 2). All samples were provided by Dr. E. Goddard.

**Assay type:** MBP levels were determined by a symmetrical sandwich ELISA procedure (MAb clone 131-1/131-1; Chapter 3).

**Results:** For the Hib meningitis patient group the median MBP levels at the respective sampling points were 1) 2370, 2) 2660 and 3) 2050ng/ml (Figure 8.1). There was no significant difference between these levels when compared by non-parametric Mann-Whitney analysis, nor was there any significant change in paired sample MBP concentrations during the sampling period (samples 1 & 2: N=47, 2 & 3: N=26; Student’s t-test). The Hib osteitis median MBP levels were 1) 1200 and 2) 2880ng/ml.
This difference was again not significant (P=0.18: Mann-Whitney analysis) reflecting the broad distribution of MBP concentrations within each group.

There was no significant difference in the frequency of MBP deficient individuals for either study compared to controls, however, there were differences in the median MBP levels. The serum MBP levels of the Hib meningitis cohort were significantly higher (Mann-Whitney analysis) than those of the adult control group (median MBP concentration 830ng/ml) on admission (P=0.011) and for the 2nd sampling (P=0.004), but not at the time of the 3rd sampling (P=0.083). In the Hib osteitis patients only the median follow-up levels were significantly higher than controls (P=0.006).

The patient samples were a mixture of black, coloured and white children and only limited information on the composition of the cohorts was available. The meningitis group (1) contained 27 blacks and 49 coloureds, with median MBP levels of 2070 and 2390ng/ml, respectively. In the osteitis group (1) there were 16 blacks and 17 coloureds, with median MBP levels of 680 and 2700ng/ml, respectively.

Comment: Since MBP concentrations can differ considerably between different ethnic groups it is difficult to interpret the above data accurately. Nevertheless, it appears possible that high MBP levels are associated with an increased risk of admission to hospital with *H. influenzae* infection. An alternative explanation is that low levels of MBP predispose to rapidly fatal meningococcal disease and, consequently, in the surviving hospitalised population there is an excess of individuals with higher levels of the protein.

![Figure 8.1](image_url)

**Figure 8.1** Distribution of MBP Levels in *H. Influenzae* Meningitis Patients

Matched serum samples were obtained from South African children on hospital admission (1), 7-10 days later (2)(N=47), and 6 weeks later (3)(N=26). MBP levels were measured using the MAb clone 131-1/131-1 system.
Atopy

_Hypothesis and Aims:_ Two early studies measuring the common opsonic defect in relatively small cohorts of atopic patients suggested that there was an association (Turner et al., 1978; Richardson et al., 1983). The aim of this study was to investigate larger cohorts using MBP concentrations as the definitive end point.

**Patient group:** 71 individuals collected by Dr. R. Coleman, Institute of Child Health, London and classified in two independent centres as having atopic eczema.

**Assay type:** MAb clone 131-1/131-1 system.

**Results:** The median MBP concentration of the atopy cohort was 1730 ng/ml. This was compared with the control population of healthy adult Caucasian blood donors (N=98, median MBP level 990 ng/ml). Mann-Whitney non-parametric analysis showed that the atopy patient median was significantly higher (P=0.027). However, when genetically related members of the study were removed the new median was not significantly different (N=32, median MBP concentration 780 ng/ml; P=0.71).

**Comment:** These results do not confirm the earlier studies which found an increased frequency of defective yeast opsonisation in patients who presented with atopic eczema before 2 years of age (Turner et al., 1978; Richardson et al., 1983). However, the numbers studied are still low and larger prospective studies should be undertaken to address this question.

Systemic Lupus Erythematosus (SLE)

_Hypothesis and Aims:_ MBP is able to activate the classical pathway of complement in an antibody independent manner, and it might be expected to share some of the characteristic features of other early acting components of this pathway. It is believed that the classical pathway is critically involved in the removal of immune complexes, and deficiencies of C1r, C1s, C1q, C2 and C4 have all been associated with an increased risk of immune complex diseases such as SLE (Lachmann, 1993). The aim of this study was to examine whether there was a similar association between MBP deficiency and SLE.

**Patient group:** 46 individuals collected by Dr. N. Snowden, Hope Hospital, Manchester and diagnosed as having SLE.

**Assay type:** MAb M37/M37 system.

**Results:** The patient population had a median MBP level of 910 ng/ml which was not significantly different from the Caucasian control group (median 990 ng/ml; P=0.62: Mann-Whitney non-parametric analysis).
Comment: A recent study at the genetic level on a larger Caucasian population, involving 102 SLE patients and 136 controls, found 41% of patients had the codon 54 mutation compared to 30% of controls (P=0.08: Chi-square analysis) (Davies et al., 1995), and it was concluded that the mutant allele was a minor risk factor for SLE. However, the wide variations in MBP concentration found within each genotype (Chapters 5 and 6), coupled with the above findings, suggest that such analysis based solely on DNA may be unsuitable.

Otitis Media

Hypothesis and Aims: Previous studies have suggested that the common opsonic defect predisposes to otitis media in early infancy (Richardson et al., 1983). The aim of this study was to investigate whether MBP deficiency was associated with otitis media in childhood.

Patient group: 93 Caucasian children suffering from otitis media and collected by Dr. G. Scadding, Ear, Nose and Throat Hospital, London.

Assay type: MAb clone M37/M37.

Results: The median MBP level in the patient group was 1670ng/ml. This is significantly higher than the median adult level (P=0.0095: Mann-Whitney comparison), but does not differ significantly from the reported median level in healthy children (1400ng/ml: Super et al., 1989).

Comment: A similar study measuring protein concentrations and genetic polymorphisms of MBP in Danish children (mean age 51 months) also found no significant difference from control levels (Garred et al., 1993).

Malnutrition

Hypothesis and Aims: Many components of the immune system are affected by nutritional status, and malnutrition is associated with increased levels of infection. Although it is unclear which is the primary factor, it is known that each influences the other (Keusch, 1990). Since there is a high frequency of MBP deficiency in African populations (Chapters 5 and 6) in whom malnutrition is common the aims of this investigation were, a) to ascertain whether MBP deficient individuals were more prone to malnutrition, and b) to investigate the affects of malnutrition on MBP levels.

Patient group: This comprised severely malnourished children, aged 6-36 months, who had been admitted to the MRC Hospital, Fajara, The Gambia. The definition of
malnourished was <60% of the World Health Organisation standard mean weight for age. Matched serum samples were taken from 68 individuals on admission (V1), after three weeks of antimicrobial and nutritional treatment, i.e. when infection free (V2), and three months after discharge (V3). As a control group samples were collected from 59 children of the same age, who were not severely malnourished, but attending the outpatient clinic of the hospital for various mild skin or eye conditions. All samples were provided by Dr. G. Morgan, Great Ormond St. Hospital for Sick Children, London.

*Assay type:* MAb clone 131-1/131-1.

*Results:* The distribution of MBP levels at the three measurement points is shown in Figure 8.2a. There was a large variation in concentrations within each treatment group, as indicated by the wide confidence intervals (see also the healthy Gambian profiles of Figure 5.2). In order to clarify the relative differences in MBP levels between the groups all measurements for each patient were expressed as a percentage of the level found in that individual’s fully healthy state (i.e. MBP level at V2 = 100%) and replotted (Figure 8.2b).

In both cases the levels were compared using paired t-tests and Mann-Whitney non-parametric analyses as appropriate (Table 8.1).

<table>
<thead>
<tr>
<th>Group</th>
<th>V1</th>
<th>V2</th>
<th>V3</th>
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<tr>
<td>Median concentration (ng/ml)</td>
<td>430</td>
<td>1220</td>
<td>800</td>
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<tr>
<td>Mean concentration (ng/ml)</td>
<td>1120</td>
<td>2440</td>
<td>2030</td>
</tr>
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<td>Mean percentage level (%)</td>
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<td>100</td>
<td>91</td>
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<th>Comparison</th>
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</tr>
<tr>
<td>V2 vs V3</td>
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</tr>
<tr>
<td>V1 vs V3</td>
<td>7.4 x10^-7</td>
</tr>
<tr>
<td>Paired t-test (percentage level)</td>
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</tr>
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<td></td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>3.1 x10^-5</td>
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<tr>
<td>Mann-Whitney (median concentrations)</td>
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<tr>
<td></td>
<td>0.14</td>
</tr>
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<td></td>
<td>0.0058</td>
</tr>
</tbody>
</table>

V1 - on presentation, V2 - after treatment, V3 - 3 months after discharge

Table 8.1 Comparison of MBP Levels in Malnourished Gambian Children
Figure 8.2 Distribution of MBP Levels in Malnourished Gambian Children

Matched serum samples were obtained from 68 severely malnourished children (V1), after treatment (V2), and three months after discharge (V3). MBP concentrations were measured using the MAb clone 131-1/131-1 system (a), and expressed as a percentage of the level of MBP in the same individual when fully healthy (b).
The median level of MBP on presentation was significantly lower than that of the control group (710ng/ml, P=0.059: Mann-Whitney analysis), but there was no significant difference between the V2 and V3 values. The proportion of individuals with MBP deficiency was not significantly different between the control and malnourished groups (comparison made with MBP level at V2).

The serum levels of a number of other acute phase proteins were also measured for these children and are presented in Table 8.2 (Drs. N. Hadzic and G. Morgan, Great Ormond Street Children’s Hospital, personal communication). The observed levels of acute phase proteins in these patients suggest that virtually all the severely malnourished children were infected. One explanation of these findings is that most malnourished children become infected and that the infection then leads to severe malnutrition. After hospital treatment the children are infection free, but exposure to pathogens back in the local community leads to mild re-infection.

<table>
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<th>Parameter</th>
<th>Group</th>
<th>Comparison of V1 vs Control P*</th>
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<tr>
<td></td>
<td>V1</td>
<td>V2</td>
</tr>
<tr>
<td>Albumin (g/l)</td>
<td>32.1</td>
<td>42.9</td>
</tr>
<tr>
<td>Ferritin (µg/l)</td>
<td>48.5</td>
<td>29.5</td>
</tr>
<tr>
<td>Vitamin A (µM)</td>
<td>0.57</td>
<td>1.68</td>
</tr>
<tr>
<td>α1-antichymotrypsin (g/l)</td>
<td>0.95</td>
<td>0.51</td>
</tr>
<tr>
<td>MBP (ng/ml)</td>
<td>430</td>
<td>1220</td>
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</tbody>
</table>

V1 - on presentation, V2 - after treatment, V3 - 3 months after discharge
(Data kindly supplied by Dr. N. Hadzic and Dr. G. Morgan)

Table 8.2 Median Levels for some Nutritional and Acute Phase Parameters

Comment: The serum concentrations of MBP contrast with those of the other positive acute phase proteins, ferritin and α1-antichymotrypsin, but resemble more closely the response of the negative acute phase protein, albumin. This is a surprising finding, and may reflect the consumption of MBP during the infected state, or a down-
regulation of protein synthesis by the liver because of the malnutrition.

Discussion

The investigations presented here do not clarify the implied connections between serum MBP concentrations and susceptibility to infections. It appears that both high and low levels of MBP may be observed in populations in different situations and there are a variety of factors which may help to account for this. Firstly, the acute phase nature of MBP means that concentrations in any infected individual may be elevated at the time of sampling. This would obviously affect median protein levels for the population as a whole. Secondly, there is the possibility that MBP is consumed during infection. This is a possible explanation for the changing levels in both the Gambian and Southern African studies, and has also been reported in bovine infections (Akiyama et al., 1992).

MBP levels are governed by both structural gene mutations and polymorphisms of the promoter region (Madsen et al., 1994b) and differ widely between individuals. Analysis of changes in MBP concentrations for a population as a whole is complicated by this non-normal distribution. Furthermore, there is some support from the data that high MBP levels may be a risk factor in certain infections, such as *Haemophilus influenzae*. If both high and low MBP levels have the capacity to increase the risk of infection depending on the nature of the organism any median based analysis may be uninformative. In addition, the small numbers in each patient group investigated hinder distribution based comparisons such as Chi-squared analysis.

It is important to consider that there are other branches of the immune system which may be compensating for reduced MBP levels. In this scenario a coexisting deficiency may be required before clinical effects are observed. One situation when MBP deficiency may be particularly important is suggested by an early report describing the opsonisation deficiency as a transient phenomenon of infancy (Richardson et al., 1983). In this case MBP may play an important host defence role during the window of vulnerability present in early childhood (Turner et al., 1991). This period arises following the loss of protection afforded by maternal antibodies (6 months) and before the maturation of the infant's own antibody repertoire (2 years). At this time the early antibody responses to carbohydrate antigens are commonly of low affinity and confined to the IgG1 subclass, whereas in older children higher affinity antibody responses of IgG2 subclass (associated with clinical protection) are detected (Freijd et al., 1984). Since
MBP baseline levels are near their maximum at this time (Chapter 4) and the complement system is fully functional it is possible that MBP provides a defence against carbohydrate coated organisms while the adaptive immune response is poor. The failure of the present studies of patients with atopy and otitis media to confirm the original associations with opsonisation deficiency may be a consequence of not targeting children in this age group.

The fact remains that the deficiency was first identified in children who suffered frequent infections that resulted in their referral to a tertiary medical centre (Sumiya et al., 1991). In these cases low MBP levels appear to have been an important factor. Furthermore, with the high frequencies of the two primary mutations the possibility remains that heterozygotes may enjoy an advantage. It appears that finding associations between MBP levels and susceptibility to infection will require precise targeting of groups in large numbers. Additionally, measurements may be complicated by any state of malnutrition. Therefore, it would be preferable to obtain patient samples at several time points so that the kinetics of MBP can be followed throughout the infection. Screening for MBP gene mutations would supplement this data, but would be incomplete by itself.
CHAPTER 9

GENERAL DISCUSSION AND FUTURE PERSPECTIVES
GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The human immune system is a complex array of many components, each acting to provide the host with defence against infection. This reiterates the opening line of this thesis and sets the scene for one such component, mannose binding protein (MBP), which because of its structural characteristics and ability to interact with the immune system has received much attention in recent years. A protein ancestral to this lectin appears to have evolved at least 300 million years ago (Kawakami et al., 1984) since RaRF functional activity has been described in most vertebrate families including fish. The expression "ante-antibody" immunity has been coined for molecules such as MBP and the pentraxin CRP (Ezekowitz, 1991), both of which appear to be ancient in an evolutionary sense. Consequently, it is possible to speculate that MBP plays a role in host defence akin to the immunoglobulins, but specialising in binding to carbohydrate structures such as mannan.

Many groups have targeted MBP for investigation, the principal areas of interest being the structure and binding specificity of the carbohydrate recognition domain, and the interaction of MBP with the collectin receptor. More recently the novel serine protease, MASP, specific for MBP, and able to activate the complement cascade directly has also attracted attention. An important area that has not been pursued as vigorously is the biological role of MBP and the implications for the host of MBP deficiency.

The investigations described in this thesis have (i) explored the occurrence of low levels of MBP in different populations, through the measurement of protein concentrations and the analysis of genetic mutations, and (ii) examined more closely the effect of the structural gene mutations on the expression of protein.

Before the present studies commenced it had already been established that a mutation in codon 54 of the MBP gene, resulting in the substitution of aspartic acid (D) for glycine (G), was associated with the deficiency of MBP in the serum of Caucasian families (Sumiya et al., 1991). The initial studies presented in this thesis, showing that
the common opsonic defect and MBP deficiency were also present in the Chinese population, led to an investigation of various populations throughout the world. Importantly, this resulted in the discovery of a new point mutation in the MBP gene that was directly analogous to the codon 54 mutation. First found in the Gambians, this mutation at codon 57, when translated, substitutes glutamic acid (E) for glycine and was also associated with low serum concentrations of MBP (Chapter 5). Population analysis of these independent mutations showed that they were widespread, with both manifesting themselves as a profound protein deficiency and establishing these structural mutations as the principle cause of low MBP levels. The much rarer codon 52 mutation (substituting cysteine (C) for arginine (R)) also appears to cause a reduction in serum levels of MBP.

It is of considerable interest to find the codon 54 and 57 mutations at high frequencies in several different populations, ranging from Africa to Asia. Since the mutations are not neutral it seems unlikely that they would both drift to the elevated frequencies found, but more plausible that parallel selection processes have occurred. At this point a biological contradiction arises - the high mutation frequencies in different populations suggest that high concentrations are, in some way, disadvantageous. However, the deficiency was discovered through the hospital admission of individuals homozygous for the codon 54 mutation and having undetectable levels of the protein (Sumiya et al., 1991). We (Lipscombe et al., 1992) and others (Garred et al., 1992a) have speculated that those who are heterozygous for an MBP mutation enjoy some biological advantage similar to the well known protection against malaria exhibited by those heterozygous for the sickle cell and thalassaemia mutations (see for example Flint et al., 1986).

As discussed in Chapter 5 high levels of MBP may predispose to septic shock, or assist infection by pathogenic organisms, and the study on *H. influenzae* (Chapter 8) may support this. It would be interesting to pursue these ideas by studying appropriate patient groups but there are a number of factors which will make this difficult. The interactions of MBP with a multi-factorial immune system mean that any heterozygous advantage will be difficult to detect because levels of MBP may be compensated for by other components of the immune system. Furthermore, changing social and behaviour patterns in the modern world may mean that the serum concentrations of MBP are less important now than they were for our ancestors. It is certainly the case that the fast moving frontiers of medicine, particularly in the Western world, have dramatically reduced the incidence of many diseases caused by pathogens which might interact with MBP (e.g.
tuberculosis). On the other hand, because the reduction in MBP levels for heterozygotes is so pronounced (concentrations range from 1/5th to 1/10th of wild type) it may be more practicable to detect any effects than it usually is for other proteins (where a heterozygous mutation would only reduce levels by one half). Additionally, a more hostile environment such as sub-Saharan Africa may highlight differences. As was observed in the malnutrition study (Chapter 8) MBP concentrations appear to be influenced dramatically by the environment and general state of health in the Gambia. Here, there would be a greater risk of infection and so the influence of MBP levels could be more readily determined.

Another aspect of the mutations in the MBP gene and their frequencies in different populations relates to the question of human evolution and the migration of ancestral populations. The occurrence of the mutations can be interpreted in terms of the movement of *Homo sapiens* out of Africa and the subsequent colonisation of the globe (Chapter 6). In particular, because the mutations are not neutral, but apparently catastrophic for the structural integrity of the circulating protein, the frequencies of the mutations may also give information about environmental factors and lifestyle. In the light of the coexistence of the codon 54 and codon 57 mutations in the San Bushmen it would be intriguing to investigate further peoples believed to be close to the ancestral stock. It would also be appropriate to check for any admixture of foreign genes where other anomalous results have occurred and determine if each mutation has arisen only once, or several times. The latter explanation would raise the question of the possible reasons for such a genetic hotspot. There is no obvious explanation in terms of the flanking DNA sequence and there are no other known parallels in the other collectins or C1q.

The evolutionary conservation of MBP in different species, ranging from primitive vertebrates through to mammals, is also of considerable interest. Indeed, in apes such as the chimpanzee and the gorilla the carbohydrate recognition domain of MBP has a protein sequence which is highly homologous to the human form, with only two amino acid differences (T. Jensen, University of Aarhus, Denmark, personnel communication). If apes and humans are vulnerable to similar pathogens and the MBP mutations do bestow a heterozygous advantage, then perhaps apes also have structural mutations in the collagenous region of their MBP. The diversification of humans from the great apes is considered to have taken place 8-10 million years ago, and from chimpanzees approximately 6 million years ago (Morell, 1994). It would, therefore, be of interest to compare the MBP levels and genes of the three chimpanzee species found in Western,
Central and Eastern Africa, with the lowland and mountain Gorillas (of West and East Africa) and the Asian Orangutans. There is already evidence that MHC polymorphisms predate speciation (Klein et al., 1993) and similar investigations into MBP polymorphisms could provide a fascinating incite into human evolution and mammalian immunity.

To pursue any investigations at the genetic level new techniques for the detection of MBP mutations will need to be developed. The methods used in this thesis are restricted by their ability to only screen for one mutation at a time. To improve efficiency a means of examining the whole region of interest simultaneously would be preferable. The popular techniques for general mutation detection are denaturing gradient gel electrophoresis (DGGE), and the use of single stranded conformational polymorphisms (SSCP). A more recent method is universal heteroduplex generation (UHG) (Bidwell et al., 1994). UHG is the simplest method of the three, although it is only sensitive to mutations very close to the target region (a range of approximately 20 base pairs). SSCP and DGGE would perhaps be more appropriate for screening the whole collagenous region. Recently it has been reported that there are additional mutations in the regulatory region of the gene (Madsen et al., 1994b), and these influence the levels of protein that are expressed. In this respect different populations such as the San, who have the lowest median wild type MBP levels of any group examined, might be expected to show additional promoter region mutations. Thus a complete DNA analysis of MBP must now include not only the three structural gene mutations but also the polymorphisms of the regulatory region.

Since biology and/or pathology ultimately depends on the phenotypic expression of normal or low levels of the serum protein it is of fundamental importance to measure protein levels. Unfortunately, because of the polymeric nature of MBP (discussed in Chapter 3) this is not without complications. The collagenous domain of the protein should ensure that all forms of MBP present in the circulation are based on subunits composed of three polypeptide chains. The number of subunits, or fibrils, that associate remains an open question. The model for the quaternary structure of MBP has been, and in many publications still is, the hexameric structure typified by C1q. However, this model of MBP appears to be somewhat mythical. The results presented in Chapter 7 suggested that the predominant forms of the protein are dimers, trimers and tetramers, and many other workers have regularly acknowledged the presence of such moieties. Higher order polymers do exist but no researchers have shown that they are major forms.
This type of structural heterogeneity has also been reported for another collectin, SP-D, where up to 32 fibrils were observed in purified material (Crouch et al., 1994). Additional support for dimers and trimers being the predominant species comes from studies on Ra-Reactive Factor (RaRF). In this complex, widely found in vertebrates, the molecular mass reported for the MBP component has always corresponded to between six and ten peptide chains (Kawakami et al., 1984; Kuge et al., 1992), i.e. almost certainly a dimer or trimer of fibrils. Further work on the nature of the interactions between fibrils within the MBP molecule is also necessary. The stability of the ladder of MBP bands resolved by PAGE under non-reducing denaturing conditions (Chapter 7) implies that there is disulphide bonding between subunits, but the stoichiometry of such interactions remains unresolved. This consideration of the polymeric forms of MBP is important from the perspective of its interactions with other molecules. Functionally, the greater the number of CRDs in the molecule the stronger the binding to a prospective pathogen and hence a hexameric structure ought to be preferable to a dimer or trimer. Resolution of this oligomerisation issue is urgently needed both for a better understanding of MBP biology and also for standardisation purposes.

The structure and specificity of MBP is of key importance when considering which carbohydrate-protein interactions are possible. Numerous studies detailed in the Introduction have claimed a particular binding specificity for MBP, although there is not complete agreement between them. The minor structural differences in the spatial configuration of sugar groups that will, or will not, bind MBP are illustrated in Appendix A, and imply that the actual specificity is extremely precise. The form of presentation is also important and the size of any potential carbohydrate ligand must also be considered. The three carbohydrate recognition domains (CRD’s) within each MBP fibril span a distance of 4.5-5.3nm (Sheriff et al., 1994; Weis & Drickamer, 1994) suggesting that for high affinity multi-point attachment a large oligosaccharide surface will be required (by comparison the size of a bacterium is approximately 1μm, and viral particles range from 20-80nm). When further defining the biological function of MBP following interactions with particular organisms it will be necessary to address the issue of the surface carbohydrate structures in much more detail. In addition, the affinity of the different oligomeric forms of MBP for antigen await further investigation.

The precision of the CRD binding, coupled with the quaternary structure of MBP give an indication of why host oligosaccharides are not targeted in vivo, even though they often contain sugars known to bind the CRD in vitro. Despite this some host-MBP
interactions have been reported, such as the binding of IgM (Nevens et al., 1992) and agalactosyl forms of IgG analogous to those found in rheumatoid arthritis (Malhotra et al., 1995). It will be of interest to establish whether these effects are merely manifestations of a routine clearance mechanism similar to that performed by hepatic lectins, or whether MBP is involved in some forms of autoimmunity.

The introduction of structural mutations into collagenous proteins has dramatic consequences, as exemplified by the bone disease osteogenesis imperfecta. It is not surprising, therefore, that the MBP structural gene mutations which affect critical glycine residues in the collagenous region of the protein should lead to dramatically reduced serum levels. The physicochemical analyses presented in this thesis show that high order polymeric forms of mutant MBP do not exist in the circulation. These studies wholly contradict the conclusions of researchers working with recombinant mutant MBP in vitro. There is also support for the instability of the mutant forms from X-ray crystallographic studies of short lengths of collagenous helix (Figure 9.1). In this case an alanine for glycine substitution causes a relaxation of the helical structure in the area of the mutation (Bella et al., 1994). In the future it would be of interest to analyse the molecular structure of the sterically more severe mutations present in MBP by X-ray crystallography or perhaps NMR. Furthermore, the existence of similar mutations in other members of the collectin family should also be considered.

The effect of the mutations on MBP await further investigation. Firstly, there is the issue of whether the mutant protein is catabolised in the serum or fails to be secreted. Given the naturally high turnover of serum MBP and the in vitro stability of the mutant form, it seems more likely that the protein is secreted and then rapidly degraded. A second question which needs to be addressed is the nature of the protein detected in heterozygotes. Based on the median concentrations of MBP found in the serum of such individuals, it appears that the incorporation of any mutant peptide into a polymeric form results in the loss of that molecule from the circulation. It remains to be established whether a solitary glycine mutation is sufficient to compromise the formation of an entire polymer.

Interactions of MBP with other molecules of the innate immune system may also be influenced by its structure. The binding of MBP to the collectin receptor, which is thought to proceed via the amino-terminal end of the collagenous domain, may be stronger for the larger polymers of MBP. Additionally, the binding of MASP to MBP remains to be determined, both in terms of the interacting region of MBP and the overall
The most common triplet in collagen is proline, 4-hydroxyproline, glycine which accounts for about 10% of total sequence. The peptide (Pro-Hyp-Gly)_10 forms a very stable triple helix and the substitution of alanine for glycine in the centre of each peptide chain disrupts this structure. In the upper space filling model the alanine residues (yellow) are packed inside the triple helix causing a twist relaxation, or local unscrewing of the helix, as illustrated in the lower ribbon diagram. The middle structure shows a native collagen fibre for comparison. It is of interest that all the alanine residues are exposed to the solvent. (From Bella et al., 1994).

An indication of the molecular ratio of MASP:MBP is again provided by studies on RaRF. In the mouse the minor component of RaRF, P100, is equivalent to MASP (Takahashi et al., 1993). Assuming that the MBP component is a dimer, the interaction that could be expected in humans is two MBP fibrils to one MASP molecule.

It will also be of interest to investigate whether other molecules are able to interact with MBP, for example other cell surface receptors, and regulatory molecules such as the C1-inhibitor. Further areas that merit investigation are the roles played by fellow members of the collectin family in host defence, and how they may interact with accessory molecules. The existence of other, as yet undiscovered, collectins also remains a distinct possibility. In addition, with the carbohydrate recognition domain having arisen...
multiple times during evolution there may also be collagenous lectins with a non-C-type CRD awaiting discovery.

The investigations described in this thesis have provided information on MBP deficiency in humans - its measurement and occurrence in different populations together with the underlying immunochemistry - and have been far reaching in the global sense. At present the field of lectin chemistry continues to grow rapidly as more molecules with the ability to bind carbohydrates are discovered, and the importance of the information carried in these structures is realised. The diversity of function expressed by these lectins is already vast and will undoubtedly expand further. Thus it would appear that nature has long appreciated the information contained in the carbohydrate code, even if mankind has not.
Appendices

A

CARBOHYDRATES: STRUCTURE AND ISOMERISM ............... 160

B

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**Carbohydrates: Structure and Isomerism**

The asymmetry of the carbon atoms found in sugars leads to the existence of many stereoisomers (by definition, each having the same structural formula). For example, glucose has 16 isomeric forms, which include mannose and galactose, and a variety of naming systems exist to help their identification.

D and L forms are related as structure and mirror image and the terms derive from the ability of purified stereoisomers to rotate plane-polarised light. The abbreviations originate from dextrorotatory (clockwise rotation; from the Latin *dexter* meaning right) and laevorotatory (anticlockwise rotation). Mammalian monosaccharides are normally of the D configuration.

![D-glucose and L-glucose](image)

In this case the D isomer is determined by the -OH group of the carbon adjacent to the terminal primary alcohol carbon lying on the right. The linear forms shown above only exist temporarily and are in equilibrium with the more stable closed ring hemiacetal forms derived from pyran (6 membered rings) and furan (5 membered rings).

![Pyran and Furan](image)

In solution glucose exists as >99% pyranose rings and <1% furanose. Once the ring is formed the structure is in a fixed configuration, normally the chair conformation, and is highly rigid. The α and β isomers are formed because ring closure at the carbonyl group can occur in two ways:

![α and β isomers](image)
Glucose exists as 38% α- and 62% β- forms in solution.

Variation of the configuration of the hydroxy groups of 2-, 3- or 4- carbons of glucose results in epimers, such as mannose and galactose (epimers are defined as stereoisomers differing in configuration at one asymmetric centre in a compound with two or more such centres). These and other sugars of interest in relation to MBP are shown below. The groups where there is a different spatial orientation from that found in mannose are highlighted.

Abbreviations used: man, α-D-mannose; glu, α-D-glucose; glcNac, β-N-acetyl-D-glucosamine; manNac, α-N-acetyl-D-mannosamine; gal, α-D-galactose; fuc, α-L-fucose (6-deoxy-α-L-galactose).

In the crystal structures of MBP shown by Drickamer and colleagues (Weis et al., 1992, Iobst et al., 1994) the equatorial 3- and 4- OH groups of D-mannose are involved directly in interactions with the carbohydrate recognition domain. D-galactose which lacks
the equatorial 4-OH group is thus unable to bind MBP. The binding reported for L-fucose arises because its OH groups at the 2- and 3- carbons can be directly aligned with the 3- and 4- OH groups of D-mannose.

Polysaccharides are formed by linking rings together as illustrated in the two examples below. Maltose consists of two α-D-glucose residues united by 1→4 linkages. Lactose is formed by β-D-galactose 1→4 linked to β-D-glucose. Highlighted groups again reflect a different orientation from that found in D-mannose.

The saccharide selectivity for human MBP has been reported as:
GlcNAc > Fuc, Man, ManNAc > Mal > Glu >> Gal, GalNAc, Lac (Holmskov et al., 1994). These studies confirm the importance of the equatorial 3- and 4- OH groups in D isomers for binding to MBP. However, several structures shown above have these features yet appear to have low affinity for MBP. This would suggest that the binding interactions are more subtle than so far described.

Information in this appendix is taken in part from Murray et al. (1993).
### Addresses of Suppliers

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Schweinle, J. E., Nishiyasu, M., Ding, T. Q., Sastry, K., Gillies, S. D., and Ezekowitz, R. A. 175


Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from


The following publications have arisen from work described in this thesis:


Identical point mutation leading to low levels of mannose binding protein and poor C3b mediated opsonisation in Chinese and Caucasian populations

R. J. Lipscombe, Y. L. Lau, R. J. Levinsky, M. Sumiya, J. A. Summerfield and M. W. Turner

*Institute of Child Health, University of London, London, UK; ‡Department of Paediatrics, University of Hong Kong, Hong Kong; and 'St. Mary's Hospital Medical School, London, UK

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1. Summary

A common opsonic defect occurring in 7% of the Caucasian population is associated with low serum levels of the lectin mannose binding protein (MBP). This study sought to determine whether the deficiency was also present in a Chinese population using sera obtained from 100 healthy Chinese children (age range 6 weeks – 16 years). The distribution profiles of MBP levels and C3b/C3bi fragments binding to mannan coated plates were both bimodal and similar to the corresponding Caucasian profiles. Serum MBP levels were low in 9% of the Chinese children and all of these sera generated low levels of C3b/C3bi fragments. Overall there was a high significant correlation between MBP levels and C3b opsonin generation (r = 0.77; P < 0.001). By analogy with similar findings in a Caucasian population we believe this correlation to be a reflection of antibody independent complement activation by MBP. In a pilot study of DNA obtained from three adult Chinese with low MBP levels the point mutation causing MBP deficiency in Caucasians was identified in all three cases.

2. Introduction

We have previously shown that an opsonic defect which impairs phagocytosis by neutrophils in approximately 7% of the Caucasian population is associated with low serum levels of mannose binding protein (MBP) [1]. The frequency of this opsonic defect is increased in children with frequent unexplained infections [2], chronic diarrhoea of infancy [3] and otitis media in the first year of life [4].

MBP is a calcium dependent serum lectin, secreted by the liver, which binds mannose- and N-acetylgalcosamine-terminated glycoproteins [5, 6] and occurs as a mixture of oligomers based on 9-18 identical polypeptide chains of 32 kDa [7]. The protein activates the classical pathway of complement through an antibody independent mechanism [7-9] and, at low serum concentrations, MBP may be a major regulator of C3b deposition on mannose-rich surfaces. Extrapolating from earlier work on the opsonic defect it is probable that the protein binds to a wide range of microorganisms [10] and there is also evidence that MBP can function directly as an opsonin [11, 12].

In man the MBP gene has been shown to comprise four exons which encode discrete collagen-like domains (exons 1 and 2) and a carbohydrate recognition domain (exon 4) [13, 14]. We have recently reported that the molecular basis of the deficiency is a point mutation at base 230 in codon 54 of exon 1 [15]. In the translated protein this replaces a glycine with an aspartic acid and presumably...
prevents the formation of a normal collagenous triple helix. Nothing is known of the incidence of this immunodeficiency in other racial groups and the aims of the present study were: (i) to evaluate opsonic function in the sera of healthy Chinese children; (ii) to relate such opsonic capacity to the serum levels of mannose binding protein; and (iii) to determine whether low serum MBP levels were associated with the same point mutation in the MBP gene as that found in Caucasians.

3. Materials and Methods

3.1. Subjects and samples

Blood samples were obtained, with ethical approval, from 100 healthy Chinese children attending health clinics, kindergartens and schools in Hong Kong. The age ranges and numbers of children were as follows (years): 0 - 1 (n = 20); 1.1 - 4 (n = 20); 4.1 - 5 (n = 10); 5.1 - 6 (n = 10); 6.1 - 9 (n = 20); 9.1 - 16 (n = 20).

In addition, serum and potassium EDTA-blood samples were obtained from 32 healthy Chinese adults. All sera were stored at -70°C until required. The EDTA-blood samples were used as a source of genomic DNA for nucleotide sequence analysis.

3.2. Measurement of serum levels of mannan binding C3b opsonins and MBP

The levels of C3b opsonins and MBP in serum were measured using modifications of the ELISA procedures described in detail by Super et al. [9]. Dynatech “Immulon” micro ELISA plates were coated overnight at 4°C with mannan (Sigma) diluted to 5 µg/ml in carbonate-bicarbonate buffer (15 mM Na2CO3, 35 mM NaHCO3, pH 9.6). After incubation, and after each subsequent stage, the plates were washed three times with Dulbecco “A” phosphate-buffered saline, pH 7.3, containing 0.05% (v/v) Tween 20 (PBS-T).

To measure levels of C3b/C3bi opsonins the serum samples were diluted to 5% in veronal-buffered saline (145 mM NaCl, 4.4 mM diethylbarbituric acid, 1.8 mM sodium barbitone, pH 7.2) containing 5 mM MgCl2 and 5 mM CaCl2. Duplicate aliquots (100 µl) were added to the plates which were then incubated at 37°C for 30 min.

Bound levels of complement C3b/C3bi fragments were measured using horseradish peroxidase-labelled sheep anti C3c specific antibody (Sero-tec) diluted 1/1000 in PBS-T.

To measure MBP in serum the samples were diluted to 5% in imidazole buffer (40 mM imidazole – HCl pH 7.8 with 1.25 M NaCl) containing 50 mM CaCl2, added to the mannan coated wells and incubated at 37°C for 120 min. Rabbit antibody to human MBP (kindly provided by Jinhua Lu, MRC Immunochimistry Unit, Oxford), diluted 1/1000 in PBS-T, was added to the plate and incubated at 30°C for 120 min. The levels of bound antibody were detected using horseradish peroxidase-labelled sheep antibody to rabbit IgG (Sero-tec), diluted 1/1000 in PBS-T, and incubated for 60 min at 37°C, followed by addition of substrate solution (10 µg O-phenylenediamine in 20 ml citrate-phosphate buffer, pH 5.2 (prepared from 10.5 g citric acid, 14.2 g Na2HPO4 dissolved in 1 l) plus 10 µl 30% hydrogen peroxide). Absorbance values were measured at 490 nm using a Mikrotek/Dynatech plate reading system.

Sera previously shown to given high (HB) and low (LB) levels of binding in both assay systems were included on each plate and were used to calculate a binding coefficient for each serum sample:

\[
\text{Binding coefficient (%) = } \left( \frac{\text{HB}_{490} - \text{Test}_{490}}{\text{HB}_{490} - \text{LB}_{490}} \right) \times 100
\]

\([A_{490} = \text{sample absorbance at } 490 \text{ nm}].\) This approach facilitates comparisons between two assay systems measuring ligands for which there are no agreed international standards.

Correlation coefficients were determined using the SigmaPlot (4.1) graphics package.

3.3. Nucleotide sequence analysis of MBP gene

Genomic DNA was isolated from the EDTA-blood samples of six adult Chinese donors shown (by the MBP ELISA) to have high binding coefficients \((n = 3)\) and low binding coefficients \((BC < 10\%; n = 3).\) Exon 1 of the MBP gene (site of the point mutation at codon 54 which is known to be associated with low levels of MBP) was ampli-
fied using primers derived from the genomic sequence [13] by the asymmetric polymerase chain reaction (PCR) [16] and sequenced directly. The 20-mer oligonucleotide 5′-dGTAGGACAGAGG-GCATGCTC-3′ (bases -16 to 4 of the upper strand) and the 21-mer 5′-dCAGGCAGTTTCC-TGGAAGG-3′ (bases 312 to 292 of the lower strand) derived from the sequence of exon 1 [13] were used as primers to produce a single stranded DNA product of 328 nucleotides. The PCR product was purified by isopropanol precipitation and sequenced using a complementary primer and Sequenase (Cambridge Bioscience).

4. Results

4.1. Levels of mannan binding C3b opsonins and serum MBP in Chinese children

Fig. 1 (a, b) shows the distribution profiles for mannan binding C3b opsonins and MBP using serum from the Chinese children at a concentration of 5%. Both distributions are clearly bimodal and similar to the profiles previously observed with a Caucasian population [8].

9% of the Chinese children were found to have a low serum MBP binding coefficient and all of these sera also generated low levels of C3b opsonins in the experimental system used. It is possible that certain components of the complement system would not have reached adult values in some of the younger children and evidence of an association between age and low levels of C3b was sought. However, only one of the nine individuals with coexisting low levels of MBP and C3b was under six months of age whereas seven other children aged less than six months had C3b binding coefficients above 30%. The overall correlation between MBP level and C3b opsonin generation was found to be highly significant ($r = 0.77, P<0.001$), as illustrated in Fig. 2, and as previously described for a Caucasian population [8].

4.2. Nucleotide sequencing of exon 1 of the MBP gene from Chinese adults

In all three Chinese adults with a high serum MBP binding coefficient the nucleotide base sequence GGC (encoding Gly) was identified at codon 54. However, each of the individuals with a low serum MBP binding coefficient was heterozygous for codon 54 (GGC/GAC) and therefore resembled Caucasian individuals heterozygous for
the mutation who have low levels of MBP.

5. Discussion

The use of mannan-coated ELISA plates for the assay of C3b/C3bi opsonins and mannose binding protein levels has been validated in our previous study [9]. This showed that the population profiles of various anti-mannan immunoglobulins binding to the mannan were completely different from those obtained for C3 fragments, C4, Factor B, properdin and MBP. Moreover, irrelevant proteins such as transferrin show no significant binding. Our observations together with those of Ikeda et al. [8] and Lu et al. [7] suggest that these assays measure the deposition of various complement moieties following antibody independent activation of the classical pathway of complement by mannose binding protein.

The population profiles observed for both C3b/C3bi opsonins and MBP in the Chinese children were bimodal and there was a highly significant correlation between the two measurements (P < 0.001). These observations parallel those previously made in Caucasians. The frequency of functional opsonic deficiency in several studies of Caucasian groups of various ages has ranged from 5.3 to 7.3% (see Table 1) and since MBP levels regulate the generation of C3 derived opsonins at low concentrations of serum it is believed that each of these functional studies was essentially an indirect measurement of low MBP levels. In the Chinese populations 9% were found to have low levels of the protein. It remains to be established whether in Chinese children the deficiency is associated with the increased incidence of infection that has been reported in Caucasian children [1–4].

The similar frequencies of low serum MBP levels in both Chinese and Caucasian populations prompted a pilot investigation of the MBP DNA sequence in individuals having low or high binding coefficients of serum MBP. All three individuals with low levels were heterozygous for precisely the same mutation previously associated with MBP deficiency in Caucasoids [15]. In the latter no other abnormality was detected in the exons of MBP but this possibility was not excluded in the samples of Chinese DNA. Nevertheless, the point mutation identified is, for the reasons discussed below, a sufficient explanation of the observed low levels of MBP.

In the normal assembly of MBP the collagenous regions of the molecule which ultimately associate into a triple helix are encoded by exons 1 and 2 and the translated peptide chains are characterised by so-called Gly-Xaa-Yaa sequence repeats. Exon 1 encodes seven of these repeats and exon 2 encodes a further 12 repeats. A Gly-Gln-Gly interruption between the two stretches appears to correspond to a bend in the collagenous fibrils which can be visualised by electron microscopy [21]. The mutation involves the fifth Gly-X-Y repeat of exon 1 and we have suggested [15] by analogy with the mutations which have been described in the condition osteogenesis imperfecta (brittle bone disease) [22] that the replacement of a small glycine residue by a bulky aspartic acid residue would profoundly disturb the secondary structure of the protein and inhibit formation of a triple helix. The association of an identical mutation with low levels of MBP in

TABLE 1

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Method</th>
<th>Number with deficiency in group studied</th>
<th>Frequency of deficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[17]</td>
<td>Radiometric assay</td>
<td>3/39 healthy adults</td>
<td>7.8</td>
</tr>
<tr>
<td>[18]</td>
<td>Coulter counter assay</td>
<td>7/112 healthy children (aged 11 + 12 years)</td>
<td>6.2</td>
</tr>
<tr>
<td>[19]</td>
<td>Coulter counter assay</td>
<td>3/41 healthy laboratory staff (aged 18–40 years)</td>
<td>7.3</td>
</tr>
<tr>
<td>[20]</td>
<td>Coulter counter assay</td>
<td>3/45 healthy young adults</td>
<td>6.6</td>
</tr>
<tr>
<td>[21]</td>
<td>Coulter counter assay</td>
<td>4/57 elderly individuals (aged 65–88 years)</td>
<td>7.0</td>
</tr>
<tr>
<td>[22]</td>
<td>Radiometric assay</td>
<td>3/56 healthy individuals</td>
<td>5.3</td>
</tr>
</tbody>
</table>

* Yeaman, G. and Kerr, M. A., personal communication.
a Chinese population is of considerable interest and raises a number of questions. In particular, it is unclear whether this finding results from two completely independent mutations occurring in the two racial groups or is a reflection of an ancient, highly conserved mutation. Although the origin of modern races remains highly controversial the paleoanthropological data supports a European-Asian separation approximately 35,000–40,000 years ago [23]. A further point of interest concerns the frequency of the mutation in the two population groups. Although the measurements of serum MBP suggest that the mutation occurs at a similar frequency in Chinese and Caucasoids it will be important to establish the frequency of the mutant allele in both populations. Such studies are currently under way.

Acknowledgements

We are grateful to Action Research for essential financial support and Jinhua Lu (MCR Immunochrometry Unit, Oxford) for providing the polyclonal anti-human MBP antiserum.

References

MUTATIONS IN THE HUMAN MANNOSE BINDING PROTEIN GENE: THEIR FREQUENCIES IN THREE DISTINCT POPULATIONS AND RELATIONSHIP TO SERUM LEVELS OF THE PROTEIN

M.W. TURNER¹, R.J. LIPSCOMBE¹, R.J. LEVINSKY¹, Y.L. LAU², A.V.S. HILL³,⁴, J.A. SUMMERFIELD⁵ and M. SUMIYA⁵

¹The Molecular Immunology Unit, Institute of Child Health, London, UK, ²Department of Paediatrics, University of Hong Kong, ³Molecular Immunology Group, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, ⁴MRC Laboratories, Fajara, The Gambia, ⁵Department of Medicine, St. Mary's Hospital Medical School, Imperial College of Science, Technology and Medicine, London

KEY WORDS: Mannose binding protein, mutations, common opsonic defect, Caucasian population, Chinese population, Gambian population.

INTRODUCTION

Mannose binding protein (MBP) is a serum lectin able to bind mannose and N-acetylglucosamine terminated glycoproteins¹ and activate the classical pathway of complement independent of antibody²,³. Low serum levels of MBP have been associated with a common opsonic defect presenting as frequent unexplained infections and otitis media in the first year of life⁴. We have recently shown that the molecular basis of this deficiency is a point mutation in codon 54 of exon 1 of the MBP gene which results in the substitution of aspartic acid for glycine in the collagenous region of the protein⁵. The consequent disruption to the secondary structure is believed to render the protein vulnerable to degradation. We now report the frequency of this mutation in three distinct populations and the identification of a second mutation in Gambians from West Africa.

MATERIALS AND METHODS

Whole blood (for DNA extraction) and plasma were obtained from: (i) 98 adult British Caucasian blood donors (ii) 123 Hong Kong Chinese donors (iii) 100 adult male Gambian blood donors (iv) 99 cord samples from newborn Gambians.

DNA was extracted from each sample and exon 1 of the MBP gene amplified by PCR. Mutations were evaluated by a combination of sequencing and restriction
enzyme digestions. Serum levels of MBP were measured by appropriate ELISA procedures.

RESULTS

Frequency of the Codon 54 Mutation and MBP Levels in British Caucasian and Chinese Populations

In the Caucasian population of 98 individuals 65 were wild type (GGC/GGC at codon 54) and 33 were heterozygous (GGC/GAC). No individual homozygous for the mutation (GAC/GAC) was found although the Hardy Weinberg law predicts a frequency of 3%. Overall the frequency of the mutant gene was 0.17.

In the Chinese population 95 of the 123 donors were wild type and 28 were heterozygous. Again no individual homozygous for the mutation was found (expected frequency 2%) and the overall frequency of the mutant gene was 0.11.

As expected, the median values for serum MBP levels of wild type Caucasian and Chinese were significantly higher than the corresponding levels of heterozygous individuals (p<0.0001).

Identification of a New MBP Mutation in Gambians

The codon 54 mutation was extremely rare in Gambians. 198 samples were wild type and only one donor was typed as heterozygous. Nevertheless, 39 individuals were identified in whom MBP could not be detected (<10 ng/ml) in the serum. Sequence analysis of exon 1 of the MBP gene in these individuals revealed another point mutation in codon 57. In the translated protein this would result in a glycine→glutamic acid substitution.

Considering the codon 57 mutation in the cord samples from newborn Gambians the frequencies of wild type, heterozygous and homozygous mutant individuals were 62, 28 and 9 respectively (gene frequency 0.23). In the samples from adult Gambians the frequencies of wild type, heterozygous and homozygous mutant individuals were 57, 29 and 14 respectively (gene frequency 0.29).

MBP was undetectable in the serum of all Gambians homozygous for the codon 57 mutation serum and, as with Caucasian and Chinese donors heterozygous for the codon 54 mutation, the median level of MBP in Gambians heterozygous for the codon 57 mutation was significantly lower than the median level of wild type individuals.

CONCLUSIONS

This study has revealed the existence of a point mutation (GGA→GAA) in codon 57 of exon 1 of the MBP gene of Gambians from West Africa. This mutation was very common in this African population as was the codon 54 mutation in British Caucasians and Chinese. In contrast, the codon 54 mutation was extremely rare in the West Africans (gene frequency 0.003) suggesting that the two mutations have arisen independently since the division between African and Non-African peoples occurred some 100–150,000 years ago. However, both are now expressed at such
high frequencies that some biological advantage associated with lower serum levels of the protein appears likely.

Acknowledgement

The support of Action Research is gratefully acknowledged.

References

High frequencies in African and non-African populations of independent mutations in the mannose binding protein gene

R.J. Lipscombe, M. Sumiya, A.V.S. Hill, Y. L. Lau, R. J. Levinsky, J. A. Summerfield and M. W. Turner*

The Molecular Immunology Unit, Institute of Child Health, 30 Guilford Street, London WC1N 1EH, 1Department of Medicine, St Mary's Hospital Medical School, Imperial College of Science, Technology and Medicine, London, 2Molecular Immunology Group, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, UK, 3MRC Laboratories, Fajara, The Gambia and 4Department of Paediatrics, University of Hong Kong, Hong Kong

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ABSTRACT

We have previously identified, in three British families having an index child with frequent infections, a point mutation (GGC—GAG) in codon 54 of exon 1 of the gene for the human lectin mannose binding protein (MBP). This was associated with low serum levels of this complement activating protein and would be anticipated to impair opsonization of mannose rich microorganisms. We now report a second point mutation (GGA—GAA) in Gambians from West Africa, involving codon 57 of exon 1. By substituting carboxylic acids for axial glycines in the translated proteins both mutations would be expected to disrupt the secondary structure of the collagenous triple helix of the 96 kDa MBP subunits. In the Gambians the codon 57 mutation was studied by PCR, sequence analysis and restriction analysis and found to be remarkably common (frequency of the mutant gene 0.29 in adults and 0.23 in newborns) whereas the codon 54 mutation was very rare (frequency 0.003). However, the codon 54 mutation was frequent in both a British Caucasian and a Hong Kong Chinese population (frequency of the mutant gene 0.17 and 0.11 respectively). It was predicted that both homozygous and heterozygous individuals would have profoundly reduced serum levels of the protein and this was confirmed by immunoassay as was the reduced capacity of such sera to activate complement through the MBP initiated classical pathway. Our data indicate that the two mutations have arisen independently since the divergence of African and non-African populations and both have attained high frequencies.

INTRODUCTION

Mannose binding protein (MBP) is a calcium dependent serum lectin secreted by the liver which binds mannose and N-acetylglucosamine terminated glycoproteins [1,2] and is capable of activating the classical complement pathway independent of antibody [3,4,5]. Identical MBP peptide chains of 32kDa associate to form trimers of 96kDa and these assemble into higher oligomers (trimers—hexamers) which circulate in serum. In man the MBP gene has been cloned [6,7] and shown to comprise four exons which encode a cysteine rich region (exon 1), a collagenous region (exons 1 and 2), a 'neck' region (exon 3) and a carbohydrate recognition domain (exon 4).

We have previously shown that a common opsonic defect presenting as frequent unexplained infections [8], chronic diarrhoea [9] and otitis media in the first year of life [10] is associated with low serum levels of MBP [11]. Recently we reported that the molecular basis of the deficiency in 3 British families was a point mutation at base 230 in codon 54 of exon 1 [12]. This substitutes an aspartic acid for an axial glycine in the collagenous region of the translated protein and by analogy with homologous collagen mutations in osteogenesis imperfecta [13] is predicted to disrupt formation of the normal triple helix. Proteins with such disordered secondary structure would be vulnerable to enzymic degradation and this is presumed to underly the low serum levels. In a pilot study of three Chinese individuals with a low serum MBP level the same mutation was identified [14]. We now report a study of the frequency of this mutation in three populations (Caucasians, Chinese and Gambians) and the relationship of genotype to both protein level and complement activating function. The identification of a second mutation, of similar disruptive potential, in the Gambian population suggests that the two mutations have been selected independently in African and non-African populations, the codon 54 mutation probably occurring more than 40,000 years ago.

RESULTS

A new MBP mutation identified in the Gambian population

When newborn Gambian serum MBP levels were assayed, 10 individuals were identified in whom the protein was undetectable (<10ng/ml). Exon 1 of the MBP gene was sequenced in these individuals and revealed the presence of another point mutation (GGA—GAA) in codon 57. This results in a glycine — glutamic acid substitution and would be expected to resemble the codon 54 mutation in its effect on the secondary protein structure.

* To whom correspondence should be addressed
Table 1. (a) Genotype frequencies for the codon 57 mutation (GGA→GAA) in the MBP gene of Gambians.

<table>
<thead>
<tr>
<th>Wild type (GGA/GGA)</th>
<th>Heterozygous (GGA/GAA)</th>
<th>Homozygous (GAA/GAA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAMBIAN (Newborn)</td>
<td>Observed 62</td>
<td>28</td>
</tr>
<tr>
<td>(n = 99)</td>
<td>'Expected' 59 (60%)</td>
<td>35 (35%)</td>
</tr>
<tr>
<td>GAMBIAN (Adult)</td>
<td>Observed 57</td>
<td>29</td>
</tr>
<tr>
<td>(n = 100)</td>
<td>'Expected' 51 (51%)</td>
<td>41 (41%)</td>
</tr>
</tbody>
</table>

The 'expected' numbers (with percentages in brackets) are calculated using the Hardy–Weinberg Law.

(b) Genotype frequencies for the codon 54 mutation (GGC→GAC) in the MBP gene of Caucasians and Chinese.

<table>
<thead>
<tr>
<th>Wild type (GGC/GGC)</th>
<th>Heterozygous (GGC/GAC)</th>
<th>Homozygous (GAC/GAC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAUCASIAN (n = 98)</td>
<td>Observed 65</td>
<td>33</td>
</tr>
<tr>
<td>(Adult)</td>
<td>'Expected' 68 (69%)</td>
<td>27 (28%)</td>
</tr>
<tr>
<td>CHINESE (n = 123)</td>
<td>Observed 95</td>
<td>28</td>
</tr>
<tr>
<td>(Adult)</td>
<td>'Expected' 97 (78%)</td>
<td>25 (20%)</td>
</tr>
</tbody>
</table>

The substitution of adenine for guanine in codon 57 leads to the introduction of an MboII restriction site between bases 250 and 251. DNA from both adult and cord blood samples were screened and individuals classified as GGA/GGA wild type, GGA/GAA heterozygotes or GAA/GAA homozygotes. The observed and expected frequencies are given in Table 1(a) together with those expected if the Hardy–Weinberg equation [15] is to be satisfied. The frequencies derived for the cord samples did not differ significantly from the expected values but those observed for the adult population were significantly different from the expected frequencies ($\chi^2=8.7$, p<0.02).

The median levels and 95% confidence intervals of serum MBP in the cord and adult Gambian populations were 825 (383–1596)ng/ml and 550 (334–877)ng/ml respectively. These were not significantly different (p = 0.18) but the levels of MBP in Gambian adults were significantly lower than those in Caucasian adults (median 995ng/ml) (p=0.011).

The relationship of genotype to serum MBP level is illustrated in Figure 1. MBP was undetectable (<10ng/ml) in all Gambians who were genotypically homozygous for the codon 57 mutation. The median values for MBP levels of adult wild type (GGA/GGA), and heterozygous (GGA/GAA) Gambians differed significantly (p<0.0001) (Table 2(a)) as did the corresponding median values for newborn Gambian MBP (p<0.0001). Three individuals in the adult population were genotypically wildtype but no MBP was detectable in their serum. DNA sequencing of exons 1 and 2 of the MBP gene in these subjects failed to reveal any further abnormalities. Exon 1 was sequenced in 22 Caucasian and Chinese subjects (including all those with low serum MBP levels) but none of these individuals had the codon 57 mutation.

DNA isolated from both newborn and adult Gambian blood donors was screened for the codon 54 mutation using Ban I (see next section). 198 samples typed as GGC/GGC wild type and a single cord sample was found to be GGC/GAC. The HLA DR haplotype patterns of this donor were those frequently found in this population (DRB1*1304/1302) [16] and provided no evidence of a recent admixture of Caucasian genes.
Table 2. (a) Serum MBP levels (medians and 5–95 percentiles) of Gambian populations genotyped for the codon 57 mutation in the MBP gene.

<table>
<thead>
<tr>
<th>Codon 57 Genotype</th>
<th>No. of individuals</th>
<th>Median MBP (ng/ml)</th>
<th>Range (5–95 percentiles)</th>
<th>Significance* (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAMBIAN (Adults)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GGA/GGA</td>
<td>57</td>
<td>1790</td>
<td>&lt;10–5310</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>GGA/GAA</td>
<td>29</td>
<td>218</td>
<td>&lt;10–758</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>GAA/GAA</td>
<td>14</td>
<td>&lt;10</td>
<td>-</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>GAMBIAN (Newborn)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GGA/GGA</td>
<td>62</td>
<td>1915</td>
<td>173–3360</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>GGA/GAA</td>
<td>28</td>
<td>186</td>
<td>&lt;10–529</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>GAA/GAA</td>
<td>9</td>
<td>&lt;10</td>
<td>-</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

(b) Serum MBP levels (medians and 5–95 percentiles) of Caucasian and Chinese genotyped for the codon 54 mutation in the MBP gene.

<table>
<thead>
<tr>
<th>Codon 54 Genotype</th>
<th>No. of individuals</th>
<th>Median MBP (ng/ml)</th>
<th>Range (5–95 percentiles)</th>
<th>Significance* (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAUCASIAN</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GGC/GGC</td>
<td>65</td>
<td>1630</td>
<td>64–4470</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>GGC/GAC</td>
<td>33</td>
<td>358</td>
<td>&lt;10–3030</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CHINESE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GGC/GGC</td>
<td>95</td>
<td>2180</td>
<td>790–5120</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>GGC/GAC</td>
<td>28</td>
<td>370</td>
<td>&lt;10–655</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

* All comparisons made with corresponding homozygous wild type

Figure 2. Relationship between serum levels of mannose binding protein and MBP codon 54 genotype in British Caucasians (left panel) and Hong Kong Chinese (right panel). Protein levels were determined by an ELISA. Genotype was assigned on the basis of sequence analysis and Ban I restriction analysis. GGC/GGC individuals are wild type; GGC/GAC individuals are heterozygous for the mutation. No GAC/GAC homozygous individuals were identified.

Frequency of the codon 54 mutation and MBP levels in Caucasian/Chinese populations

The substitution of an adenine for guanine at base 230 in codon 54 of the human MBP gene leads to the loss of a Ban I restriction site. Amplified exon 1 DNA from individuals of differing genotype gave distinct gel electrophoretic patterns after Ban I digestion and these differences were used to genotype the two populations. The observed frequencies are summarized in Table 2(b). The observed and expected values did not differ significantly (p > 0.05). In these populations no individuals homozygous for the mutation (i.e. of GAC/GAC genotype) were observed, although we had previously identified five homozygotes in the three families studied [12]. Serial serum levels of mannose binding protein were determined for all of these individuals and this data is related to genotype in Figure 2. The median level of serum MBP in Caucasians was 995 ng/ml (confidence intervals 642 and 1410 ng/ml) whereas for...
the Chinese population the values were 1645 (1395–2065)ng/ml; this difference was significant (p=0.007). The median values for MBP levels of wild type (GGC/GGC) Caucasians and Chinese were significantly higher than the corresponding levels of heterozygous (GGC/GAC) Caucasians and Chinese (p < 0.0001) (see Table 2(b)). Furthermore, the median level of serum MBP in wild type Chinese was significantly higher than the corresponding levels of wild type Caucasians (p=0.0074) and wild type adult Gambians (p=0.0088). The median MBP levels of wild type Caucasians and Gambians did not differ significantly (p=0.81).

Serum MBP levels were broadly distributed and 3 wild type (GGC/GGC) Caucasian individuals were found to have no detectable MBP (protein result independently confirmed using three different assay formats). Both exon 1 and exon 2 of the MBP gene of these individuals were sequenced and shown to be identical to the published sequence. In addition, exon 1 sequencing was undertaken in a further 19 individuals with low serum MBP levels in order to confirm the genotypic assignment.

Complement activation and genotype

Serum samples from both the Caucasian and Chinese populations were also studied for their ability to generate C3b opsonins through MBP activation of the classical pathway of complement. In the Caucasian population there was a significant correlation between serum MBP level and the generation of C3b opsonins (r=0.90 for wild type individuals and r=0.78 for heterozygotes). The median value for MBP initiated C3b opsonisation by individuals who were genotypically heterozygous (55 BC%) was significantly lower than the median value for wild type individuals (85 BC%) (p=0.0003). Similar results were obtained for the Chinese population (not shown).

DISCUSSION

In our previous study of nineteen individuals from three British families [12] mannose binding protein deficiency was clearly linked with the GGC–GAC mutation in codon 54 of exon 1 of the MBP gene. Furthermore there was a striking association between susceptibility to severe recurrent infections and homozygosity (GAC/GAC) for the mutation (four out of five homozygous individuals were referred to hospital with such infections in early infancy). We have postulated a major role for MBP in immunity to mannose rich organisms between 6–18 months of age since maternally transferred antibody levels are then low and the infant’s own antibody repertoire is still immature [17]. MBP is able to promote opsonisation either by activation of the classical pathway of complement and generation of C3b fragments [4,5] or directly as an opsonin [18] able to interact with Clq receptors on neutrophils [19].

In a surprisingly large number of Gambian individuals serum MBP was undetectable (<10ng/ml) and yet genotypically they were clearly GGC/GGC at codon 54. This discrepancy led to the discovery of the codon 57 mutation which involves a substitution of glutamic acid for glycine in the Gly-Xaa-Yaa repeat immediately C terminal to codon 54 and which should, theoretically, have a similar effect on the secondary structure of the triple helix (see Figure 3).
Super et al [20] have recently expressed the MBP gene encoding the codon 54 mutation in an in vitro system and report that mutant chains were able to assemble into higher polymeric forms. However, secondary structure was clearly compromised since mutant peptides dissociated more readily than did normal MBP peptide chains and complement activation was abrogated. These findings appear to be at variance with our observations of undetectable MBP in homozygous mutant individuals but we suggest that this hybridoma expression system may not be an appropriate model for the hepatocyte. It remains to be established by in vivo studies whether mutant MBP with disordered secondary structure is usually degraded intracellularly or after secretion.

The frequency of the codon 57 mutation in the two Gambian populations was surprisingly high although only the newborn data satisfied the Hardy-Weinberg law. Various factors might be invoked to explain why the adult population showed a significant deviation from that expected [15]. These include a high frequency of consanguinity (which is known to increase the number of homozygotes, as seen in this population), recent migrations and a population sample based on a mixture of subpopulations that do not completely interbreed. These possibilities require further investigation but preliminary analysis of the tribal groups represented in the two populations revealed no unexpected skewing. Furthermore, the codon 57 mutation was present in all six major tribes although small sample sizes precluded frequency analysis. It will also be important to undertake in selected Gambian family linkage studies similar to those recently reported in British families [12].

The median values of serum MBP levels in newborn and adult Gambians heterozygous for the codon 57 mutation were approximately 1/10 and 1/8 respectively of the corresponding median values of individuals lacking the mutation. This is consistent with our prediction of a 1/8 probability of three normal 32kDa peptide chains associating into a stable triple collagenous helix in heterozygotes [12]. Nevertheless, there was considerable variation in the level of protein detected and this may reflect the fact that MBP is an acute phase protein [6,21].

The frequency of the codon 54 mutation was high in both British Caucasian and Chinese populations, the proportions of wild type and heterozygous individuals approximating to the expected Hardy Weinberg distributions. The median values of serum MBP levels in Caucasians and Chinese heterozygous for the codon 54 mutation were approximately 1/5 and 1/6 of the corresponding median values of individuals lacking the mutation. Similar data has also recently been obtained for a population of Danish blood donors [22, P. Garred, unpublished data]. However, whereas no homozygous (GAC/GAC) individuals were detected in either of our populations (n=221), 2/123 of the Danish population were homozygous for the mutation. The absence of homozygous individuals in the British Caucasian and Chinese donors may be a reflection of the sample size.

It remains to be established whether the MBP mutation in Gambians is representative of other African peoples or is confined to West Africa and (presumably) the American Black descendents of West Africans. The single Gambian individual with the codon 54 mutation may represent an admixture of European genes although we could find no evidence for this in the background MHC haplotype of this donor. The codon 54 mutation now identified in both British and Danish populations probably has a single origin [23] but haplotype analysis will be required to determine whether the mutation detected in the Chinese is the same or the result of two independent events.

Seven of the 420 individuals studied (1.6%) were found to have undetectable serum MBP and no evidence of a mutation in the exons encoding the collagenous regions of the molecule. This may indicate other causes for low MBP levels, such as abnormalities in the 3’ and 5’ untranslated regions of the gene which are thought to have control and regulatory functions.

![Figure 4](image-url)
The data available for mutations in the human MBP gene fits readily into one of the current models of recent human evolution (see Figure 4). This draws on relationships between nuclear DNA patterns of various populations and points to a separation between the ancestors of modern African and non-African peoples (perhaps by 100,000 years ago). Present day Chinese and European peoples may have diverged some 40,000 years BP although subsequent gene flow across Asia may have occurred. Thus, the presence of the codon 54 mutation in British, Danish and Chinese populations at approximately similar gene frequencies suggests that it may have arisen more than 40,000 years ago. If its occurrence in a single individual in the Gambia does not reflect recent admixture, the mutation may, in fact, be much older. Maintenance of the codon 54 mutant allele over such a long time period suggests some associated biological advantage and the presence of a comparable but distinct mutation in Gambians (at an even higher frequency) provides further strong support for this view. With the evolution of the adaptive antibody response the major role for MBP in immunity may be restricted in man to the 'window of vulnerability' in early life [17]. Nevertheless, throughout the lifetime of the individual the protein would retain the capacity to activate complement following interactions with a wide range of mannose rich organisms. Thus the increased susceptibility to paediatric infection of a small number of homozygotes with MBP mutations may be outweighed by an advantage enjoyed by both homozygotes and heterozygotes of a reduced capacity for complement activation and possible immunopathologically mediated host-damage. Ross and Densen [24], in a review of complement deficiency states and infection, noted the low mortality associated with meningococcal disease in complement deficient individuals and suggested that tissue damage resulting from activation of complement by endotoxin may be reduced in such individuals. Similarly, Orren et al [25] have reported a high frequency of C6 deficiency in the mixed race and black populations of the Cape (S. Africa) and suggested that the deficiency might be beneficial in infancy by limiting the incidence of septicaemic shock. In the relatively hostile African environment deletion of MBP associated immune mechanisms may also substantially reduce the incidence of host damage arising from release of inflammatory mediators and this, we speculate, may explain the high frequency of the mutant allele.

**Materials and Methods**

**Subjects**

Two Gambian populations were studied. The first group comprised 100 adult male blood donors attending the Royal Victoria Hospital, Banjul. HIV positive individuals (n=4) were eliminated before the study commenced. The second group consisted of cord blood samples (40 males; 59 females) obtained at delivery in The Gambia and the 21mer 5'-dCAGGCAGTTTCCTCTGGAAGG-3' (bases 312 to 292 of the lower strand) derived from the sequence of exon 1 [6] were used as primers to produce a DNA product of 328 bp. This was digested with 5U of Ban I [Boehringer Mannheim] at 50°C for 60 min and/or 3.5U of Mbo II (New England Biolabs, Beverly, MA, U.S.A.) at 37°C for 90 min. Digest fragments were visualised by electrophoresis in 2% agarose gels. DNA sequencing of selected samples was performed following asymmetric PCR [27] to produce single stranded DNA using a complementary primer and Sequenase (Pharmacia).

**Analysis of Mutations**

Exon 1 of the MBP gene was amplified by PCR. The 20mer oligonucleotide 5'-gtGAGCACAGGAGGCTC3'- (bases 16 to 4 of the upper strand) and the 21mer 5'-cAGGAGTTCCCTCAGAGG-3' (bases 312 to 292 of the lower strand) derived from the sequence of exon 1 [6] were used as primers to produce a DNA product of 328 bp. This was precipitated by the addition of 3.5M sodium acetate and 2 volumes of ethanol, redissolved in 1ml Tris-EDTA and stored at -20°C.

**DNA Isolation**

Genomic DNA was prepared from Gambian blood by phenol-chloroform extraction [26]. DNA from Caucasian and Chinese donors was prepared from whole EDTA blood by two different methods. In the first method 1ml EDTA blood was centrifuged at 10,000g for 15min. The supernatant was removed and the pellet resuspended in 200ul of water or Tris EDTA buffer. The suspension was boiled for 15min and then centrifuged for a further 10min at 10,000g. The resultant supernatant was stored at -20°C until required.

In the second method 10ml EDTA blood were lysed by the addition of 50ul ice-cold water and centrifuged at 10,000g for 20min. The supernatant was decanted and the pellet resuspended in 25ul ice cold 0.1% Nonidet P40 and centrifuged for 20min at 10,000g. The pellet was homogenised with 7ml 6M guanidine hydrochloride and 0.5ml 7.5M ammonium acetate followed by the addition of 0.5ml 20% sodium sarkosyl and 150ul (10mg/ml) proteinase 'K' (Boehringer Mannheim UK). The mixture was then incubated at 60°C for 90min. DNA was precipitated by the addition of 2.5 vols of absolute ethanol and dissolved in 1ml Tris EDTA. The DNA was reprecipitated by the addition of 1/10th volume of 3M sodium acetate and 2 volumes of ethanol, redissolved in 1ml Tris EDTA and stored at -20°C.

**MATERIALS AND METHODS**

**Subjects**

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**Samples**

Gambian. Whole blood (taken into lithium heparin) and plasma were stored frozen at -30°C and then air-freighted to London in dry ice.

Caucasian/Chinese. Whole blood was collected into potassium EDTA and frozen at -70°C until used for DNA extraction. Separated serum was also frozen at -70°C until required. Samples from the Hong Kong Chinese donors were air-freighted to London in dry ice and subsequently stored at -70°C.

**Statistical Analyses**

MBP and C3b opsonin data for various population groups were compared using the non parametric Mann Whitney rank sum test. Correlation coefficients between the levels of C3b opsonin bound and MBP levels were determined using the Sigma-Plot (4.1) graphics package. Differences between observed and expected values for the Hardy Weinberg Equilibrium were analysed using chi-square tests.
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ABBREVIATIONS

MBP: Mannose binding protein
ELISA: Enzyme linked immunosorbent assay
C3b: Large, opsonic, cleavage fragment from complement component C3.

REFERENCES
