THE MOLECULAR BASIS OF A COMMON DEFECT OF OPSONISATION

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Summary

A failure of serum to opsonise baker's yeast *Saccharomyces cerevisiae* is a defect found in 5-7% of the general population but at a higher frequency in paediatric patients with repeated, unexplained infections. Previous work had suggested an abnormality of the complement system and in the present studies assays were devised to measure the deposition of complement moieties on yeast zymosan and on the surface of microtitre plates coated with the yeast cell wall component - mannan. The latter was found to be technically superior and was used for all the subsequent studies. C4 fragments, properdin and Factor B were bound to the mannan-coated plates as well as the expected opsonic fragments C3b and C3bi. Analysis of 179 sera from healthy adult blood donors revealed that the binding levels of C3b, C4, properdin and Factor B were highly significantly correlated. When the assays were carried out at the same dilution in MgEGTA, there was no detectable binding of complement proteins to the mannan surface, confirming that no alternative pathway activation was occurring at this serum concentration in this experimental system. The levels of bound anti-mannan antibodies (IgG, IgA and IgM and the IgG subclasses IgG1, IgG2 and IgG3) were found to be completely unrelated to the C3bi levels previously observed.

In parallel with these investigations affinity chromatography of serum with normal opsonisation on a mannan-Sepharose column suggested that a calcium dependent macromolecule with a molecular weight of 600-700 kDa was able to correct the opsonic defect. A candidate molecule having these physicochemical characteristics and known to be complement activating in the rat was the serum lectin mannose binding protein (MBP). Using a polyclonal monospecific rabbit anti-human MBP the protein was assayed in the blood donor population both by antibody capture ELISA procedure and the previously described mannan capture procedure. The levels of MBP bound in the latter correlated with the levels of C4, C3b, properdin and Factor B but not with the levels of anti-mannan immunoglobulins bound. The results suggest that, in this experimental system using low concentrations of serum, mannose binding protein initiates an antibody independent mechanism of cleavage of the classical pathway component C4 which subsequently regulates the degree of cleavage of C3 and binding of alternative pathway proteins. In a study of 10 paediatric patients previously shown to have the functional opsonic defect the median MBP concentration was 4.9μg/l (range 2.5-35μg/l) compared to 142μg/l for a paediatric control group (range 2.5-880μg/l). Purified MBP was shown to correct the opsonic deficiency in a dose dependent fashion in an *in vitro* assay measuring deposition of complement moieties on a mannan coated surface. Thus it appears that low levels of serum MBP underlie the common opsonic defect.

Cross species functional correction assays were performed with sera from both eutherian mammals and marsupials. Partial or complete correction was observed in every case suggesting that MBP is a widely distributed conserved molecule with a major role in antigen non-specific immune responses to pathogens.

Acknowledgements

I am very grateful to Dr Mac Turner for his advice, enthusiasm and constructive criticism during the past three years. I feel that I have gained much from this experience. I would also like to thank Professor Roland Levinsky for providing laboratory space in his department to allow me to carry out the experiments described here and for his interest in the research.

I would like to thank the staff of the Immunochemistry and Clinical Immunology Laboratories at the Institute of Child Health, particularly Mr Nigel Seymour, for their help and advice.

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I wish to thank my parents for their continued encouragement. Finally, I wish to thank my wife Ruth, for her limitless drive and enthusiasm which have prevented me from being side-tracked from the production of this thesis despite getting married, moving house (twice), setting up a new computer (twice), and even the birth of our first child !

I dedicate this thesis

to

RUTH AND DINAH

Publications

The following publications contain part of the work presented in this thesis:

Super M; Thiel S; Lu J; Levinsky RJ; Turner MW
Association of low levels of mannan-binding protein with a common defect of opsonisation.
Lancet; 1989 Nov 25; 2(8674); P 1236-9

Super M; Levinsky RJ; Turner MW
The level of mannan-binding protein regulates the binding of complement-derived opsonins to mannan and zymosan at low serum concentrations.
Clinical and Experimental Immunology; 1990 Feb; 79(2); P 144-50

Turner MW; Super M; Singh S; Levinsky RJ
Molecular basis of a common opsonic defect.
Proceedings of the European Academy of Allergology and Clinical Immunology
Clinical and Experimental Allergy (Suppl.) ;1990(a); (in Press)

Turner MW; Super M; Levinsky RJ; Summerfield J
The molecular basis of a common defect of opsonisation.
Royal Society of Medicine International Congress and Symposium Series
1990 (b); (in Press)

Declaration

All the work which is presented in this thesis is that of the candidate with the following exceptions which are found in **Chapters 3 and 4**:

(i) Mr Nigel Seymour of the Immunochemistry Laboratory ICH measured the CH50 levels shown in **Table 3.1**, the total C3 and C4 levels illustrated in **Figure 3.1(e)& (f)** and the functional opsonisation (C3c elution assay) of paediatric patients (**Figure 4.3**).

(ii) Ms Lesley Alterman of the Immunochemistry Laboratory ICH measured the functional opsonisation (C3c elution assay) of healthy adults (**Figure 4.2**).

(iii) Dr Susan Cross of the Department of Genetics Oxford performed C3 and Factor B allotyping (see **Table 3.2**).

Abbreviations

AP	alternative pathway of complement
C 1-9	complement components 1-9
C1 INH	C1 inhibitor
C3a	a fragment of C3
C3b	b fragment of C3
C3bi	inactive b fragment of C3
C3(H_2O)	fluid phase activated C3
C4A	A isotype of C4
C4B	B isotype of C4
C4 bp	C4 binding protein
CP	classical pathway of complement
CR	complement receptor
CRD	carbohydrate receptor domain
DAF	decay acceleration factor
DFP	di-isopropyl fluorophosphate
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethylether)N,N,N',N',-tetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
Fab	antigen binding portion of immunoglobulin
Fc	constant portion of immunoglobulin
FITC	fluorescein isothiocyanate
GPER	guinea pig erythrocyte
HRPO	horseradish peroxidase
IC	immune complex
Ig	immunoglobulin
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
kb	kilobases
kDa	kilodaltons
LPS	lipopolysaccharide
MAC	membrane attack complex
MBP	mannose binding protein
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PMN	polymorphonuclear leucocytes
PSAP (SP-A)	pulmonary surfactant apoprotein
r_p	Pearson correlation
r_s	Spearman rank correlation
SCR	short consensus repeat
SDS	sodium dodecyl sulphate
UTR	untranslated region

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Phagocytosis

Phagocytosis is one of the body's most important lines of defence against invading pathogens. The major phagocytic cells are (i) the mononuclear tissue macrophages and circulating monocytes and (ii) the polymorphonuclear circulating polymorphonuclear leucocytes (PMN) or neutrophils. The mononuclear cells are mainly involved in immune responses to intracellular parasites e.g. viruses, while the polymorphonuclear cells are involved in the control of infection by extracellular bacteria and fungi. Together these cells constitute a major, antigen non-specific, innate immune mechanism which interfaces with the complement system and with specific antibodies.

Phagocytosis is a complex process which can be divided into four stages:

(i) Chemotaxis (circulating phagocytes migrate to sites of infection in response to chemotactic factors),

(ii) Recognition and binding to particles coated with opsonins

(iii)Ingestion (opsonised particles are internalised by endocytosis)

(iv)Killing (lysosomes fuse with phagosomes and release lytic enzymes).

The second stage, in which phagocytes **recognise and bind to opsonised micro-organisms**, constitutes the major complement/phagocyte axis, at which the innate cellular immune mechanisms interact with both innate and specific humoral immune mechanisms.

ii) Recognition and binding to particles coated with opsonins

In the late 19th century there was much controversy over the relative importance of phagocytic cells and serum factors in defence against extracellular pathogens. Emil von Behring, who had first described antibodies in 1890, strongly favoured a role for the humoral (molecular) components while Metchnikoff was equally convinced that phagocytic cells were the most important element. It was Wright and Douglas (1903), studying the interaction between staphylococci, leucocytes and separated serum, who established that phagocytosis of bacteria resulted from **co-operation between serum and cellular factors**. The key difference between these studies and those that preceded them is that they separated cells from serum and treated the fractions differently, for example, they heat treated the serum before adding this to the staphylococci and leucocytes. Wright and Douglas coined the term "opsonic effect" (derived from the Greek opsono - "I cater for; I prepare victuals for") to describe the binding of serum factors to particles such as micro-organisms, thereby increasing their susceptibility to phagocytosis.

Wright and Douglas established that :

The effect of opsonisation was on the bacteria.

The opsonic capacity of non-immune sera was heat labile.

Opsonic capacity was not specific to one organism.

Immunisation boosted the levels of opsonins.

These studies suggested the existence of at least two opsonic ligands; subsequent work showed that the quantitatively important opsonins in human sera are antibody (especially the class immunoglobulin G) and complement (especially the major split products of the complement component C3). Phagocytes have specific receptors on their surfaces which mediate binding and phagocytosis of particles which have been opsonised by complement fragments or complexed immunoglobulin (Schreiber et al 1982, Kurlander and Batker 1982).

1.2 Receptors on phagocytic cells

1.2.1 Fc receptors

Although the binding of immunoglobulins to phagocytic cells has been recognised as a function of the Fc region for many years, it is only the recent advances in the fields of membrane biochemistry and molecular biology which have permitted a detailed picture to emerge of the interactions between complexed immunoglobulins and phagocytic receptors. The Fc γRI, Fc γRII and FcγRIII receptors (Anderson 1989) recognise the constant Fc portion of immunoglobulin G (IgG) whereas Fcα R recognises IgA (Albrechtsen et al 1988). There are two IgE receptors, Fcε RI and Fcε RII (Ishizaka et al 1970, Bettler et al 1989), however the high affinity FcεRI is not found on phagocytes and the low affinity FcεRII is only expressed on monocytes following interleukin 4 (IL4) stimulation.

Fcγ RI is a glycosylated protein of approximately 72kDa which has a high affinity for both monomeric and complexed IgG, especially for the subclasses IgG1 and IgG3 (Kurlander and Batker 1982). Fcγ RI is constitutively expressed by monocytes and macrophages (Perussia et al 1983) and is inducable on PMN by treatment with γ IFN (Shen et al 1987). Resting PMN constitutively express Fcγ RII and Fcγ RIII, (Kulczycki et al 1981, Fleit et al 1982) both of these are low affinity receptors and only bind to complexed IgG, mainly that of subclasses IgG1 and IgG3 (Kurlander and Batker 1982, Duncan et al 1988). Fcγ RII (CD32), a 40kDa single chain structure, is also found on monocytes, platelets and B lymphocytes, whereas Fcγ RIII/Fcγlo,

(CD16) (50-70kDa) which is linked to the cell membrane by glycosyl-phosphatidylinositol (GPI), (Hogg 1988) is also found on monocytes, macrophages and natural killer cells (Fleit 1982, Clarkson and Ory 1988). The Fc γ receptors are trypsin resistant and bind to the amino-terminal portion of the Fc regions of IgG, (Duncan et al 1988) in the absence of divalent cations (Huber and Fudenberg 1968). A common structural feature of the Fc receptors is possession of immunoglobulin domains in the extracellular regions (see **Figure 1.1 (a)**).

FcαR (60kDa) is a heavily glycosylated protein found on neutrophils, monocytes and granulocytes (Albrechtsen et al 1988, Monteiro et al 1990) as well as on non-phagocytic cells. FcαR is a transmembrane glycoprotein,composed of 32 and 36kDa domains which binds monomeric or polymeric IgA subclasses 1 and 2 (Monteiro et al 1990). The expression of this receptor is constitutive, but it can be upregulated with polymeric IgA or phorbol esters.

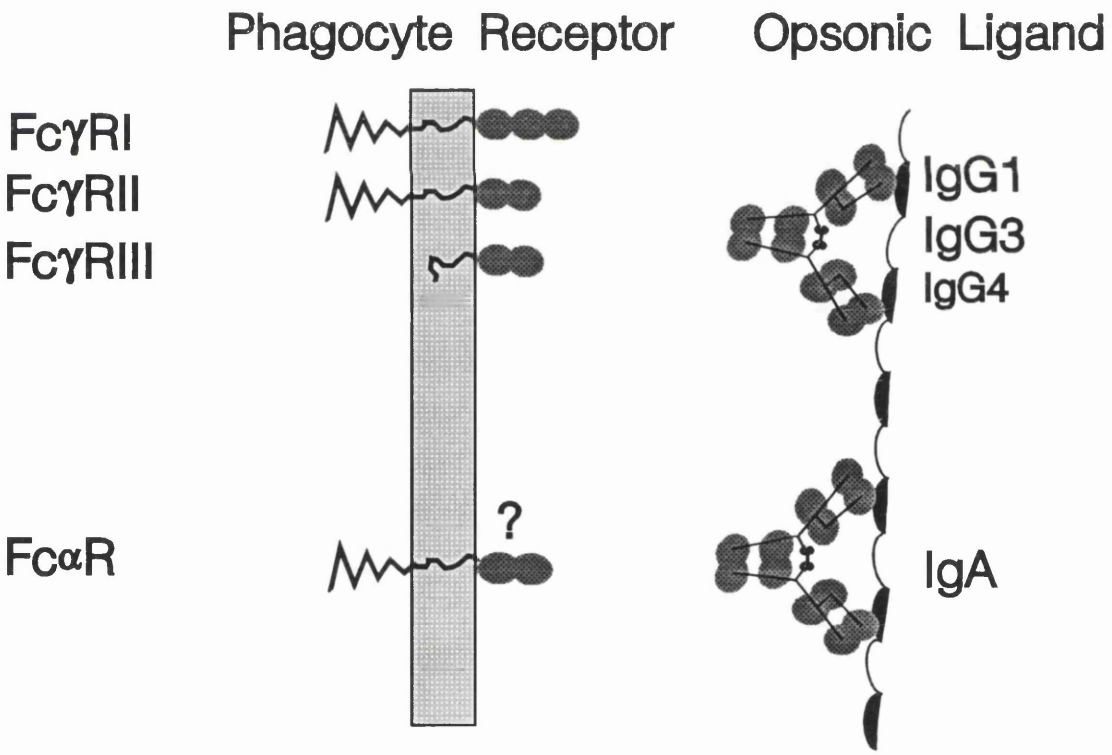


Figure 1.1 (a) Immunoglobulin opsonins and the phagocyte receptors FcγIR, FcγIIR, Fc γIIIR and Fc α R. Subclasses IgG1 and IgG3 are the major opsonins.

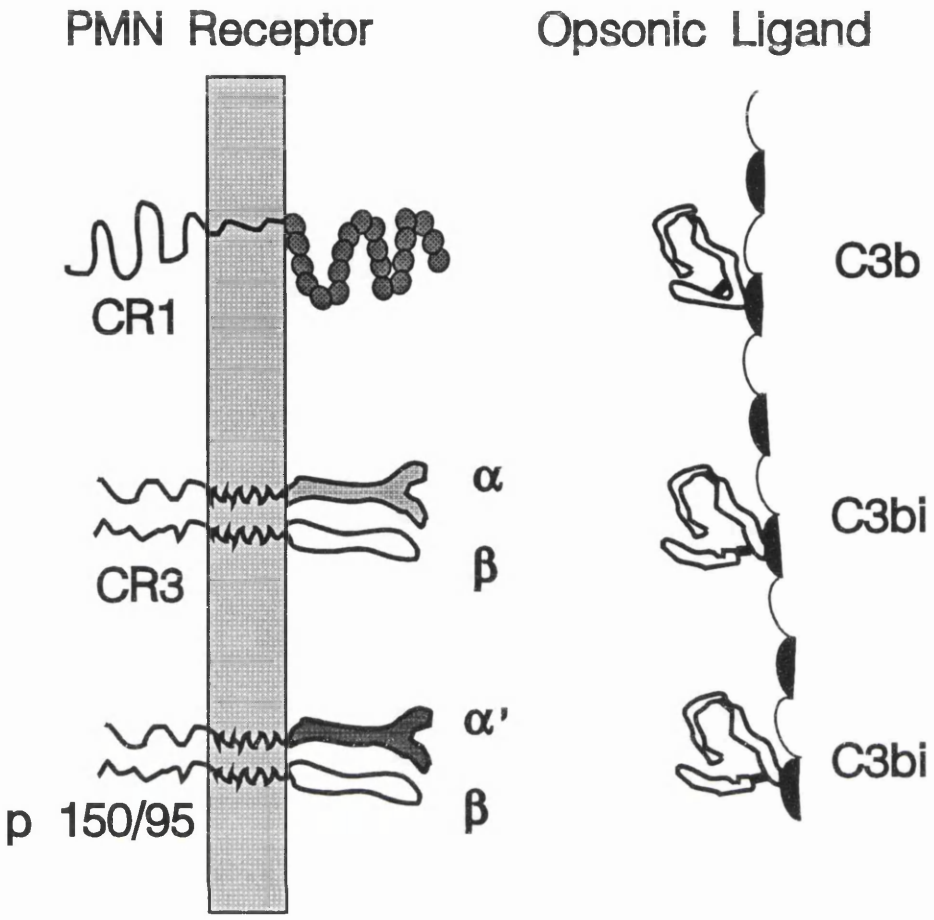


Figure 1.1 (b) Complement receptors and the opsonic ligands recognised.

1.2.2 Complement receptors

There are 4 or 5 complement receptors found on phagocytic cells (reviewed by Lambris 1988)(see **Figure 1.1 (b)**). The complement receptor 1 (CR1) (CD35) recognises C3b, C4b and to a lesser extent C3bi (Dykman et al 1985, Lambris 1988). There are 4 allotypic variants of this protein each differing in molecular weight: 160kDa, 190kDa, 220kDa and 250kDa(Dykman et al 1985). The major phagocytic cells expressing CR1 are PMN, monocytes and tonsil macrophages, and all appear to bind dimeric C3b far more avidly than monomeric C3b (Ross and Medof 1985). The C3b binding site of CR1 has been localised to the amino-terminus of the α' chain of C3b (Becherer and Lambris 1988), this may be the same site at which Factors H and B bind to C3b (Koistinen 1989) and hence may explain why CR1 bound to C3b inhibits the complement cascade (Iida and Nussenzweig 1981).

Complement receptor 2 (CR2) (CD21) is a glycoprotein of approximately 140kDa which recognises mainly C3bi and C3dg/C3d (Reynes et al 1985, Lambris 1988). CR2 is not found on PMN, monocytes or macrophages but is found on lymphocytes, follicular dendritic cells and thymocytes and it has also been implicated in B cell activation (Lambris 1988). CR2 binds to C3 at the C3d site which is similar in sequence to a stretch of amino-acids in the Epstein - Barr virus (EBV) and is thus thought to be the EBV receptor on B lymphocytes (Frade et al 1985).

Complement receptor 3 (CR3) binds the C3 fragment C3bi in the presence of Mg++ and Ca++ ions (Fearon 1980), this receptor, which is a high affinity/low density receptor (Gordon et al 1987), is found on PMN, monocytes and macrophages as well as on follicular dendritic cells and natural killer cells. A second binding site found on CR3 interacts with the β-glucan cell wall component of zymosan and lipopolysaccharide (LPS)(Wright and Jong 1986, Ross et al 1987). Recent work has shown that yeasts opsonised with C3bi bind avidly to CR3 on PMN, but ingestion and the oxidative burst of phagocytosis are mediated by the binding of the yeast glucans to the secondary binding site of CR3 (Cain et al 1987). CR3 is made up of a non-covalently associated 165kDa α chain and a 95 kDa β chain (Ross et al 1987). The β chain of CR3 is identical to the β chains of two other receptors, the integrins LFA-1 and p150/95 (Sanches-Madrid et al 1983, Lanier et al 1985).

Complement receptor 4 (CR4) (p150/95) is found on PMN, neutrophils and macrophages and binds C3bi and to a lesser degree C3dg and C3d in the presence of calcium ions (Vik and Fearon 1985). The physiological role of CR4 has not been established, but it appears to be similar to CR3 (which has an identical β chain) and it appears to be the major C3 receptor on tissue macrophages and may be involved in clearance of C3b opsonised particles or immune complexes(Myones et al 1988); CR4 was identified because it binds to C3dg, and yet it is distinct from the C3d/dg receptor (CR2) found on lymphocytes (Frade et al 1985). The presence of a fifth receptor (CR5) was suggested in studies (Micklem and Sim 1985) in which C3dg was found to bind to platelets in the absence of divalent cations.

Studies using opsonised sheep erythrocytes have shown that phagocytosis can be triggered directly by high concentrations of IgG opsonins bound to cells. Small amounts of bound IgG can trigger phagocytosis by activating the classical pathway and depositing C3 opsonins on the target surface. However, erythrocytes opsonised

with C3 in the absence of IgG do not promote phagocytosis and it appears that C3 fragments mediate attachment to the neutrophil surface, while immunoglobulins promote ingestion (Ehlenberger and Nussenzweig 1977, Newman and Johnson 1979). In contrast to the findings with erythrocytes, phagocytes bind to C3 opsonised micro-organisms in the absence of immunoglobulin (Roos et al 1981). Using the yeast cell wall preparation - zymosan (Kemp and Turner 1986) have shown that PMN can bind and ingest zymosan which has been opsonised with C3 plus IgG or with C3 alone. However ingestion was not observed when the zymosan was opsonised with IgG alone in the fluid phase and this was probably due to poor attachment of the PMN to the IgG opsonised particles in solution. One possible mechanism for the triggering of phagocytosis in the absence of IgG involves the monocyte β- glucan receptor (Czop 1978 (a)& (b). This receptor may promote phagocytosis of micro-organisms and yeasts which have glucan rich cell walls but not sheep erythrocytes where these monosaccharides are masked by sialic acid.

1.3 Opsonisation by immunoglobulins

1.3.1 IgG opsonins

IgG is the major heat stable factor in human serum responsible for antigen-specific opsonisation. It is particularly important in defence against infection with most encapsulated bacteria and some virulent strains of nonencapsulated bacteria. For example, opsonisation by IgG is required for ingestion of *Diplococcus pneumoniae* (Johnston et al 1969), groups A, B and C meningococci (Roberts 1967) and *Pseudomonas aeruginosa* (Young and Armstrong 1972). IgG binding to target cells is independent of divalent cations and occurs at 4^{o}C as well as 37^{o}C (Pillemer et al 1954). Immunoglobulins may bind to distinct carbohydrate or protein structures on the surface of target cells.

Human IgG comprises four distinct subclasses(IgG1, IgG2, IgG3 and IgG4) which are known to differ in their involvement in specific antibody responses and in their ability to express different effector functions. IgG1 is quantitatively the most important and accounts for 60-65% of IgG, IgG2 accounts for 20-25% while IgG3 and IgG4 account for 5-10% and 3-6% respectively(Jefferis and Kumararatne 1990). The subclasses IgG1 and IgG3 are the major opsonic proteins and both are able to interact with FcγRII and FcγRIII on neutrophils. In the case of the high affinity FcγRI receptor on mononuclear cells, IgG1 and IgG3 again account for most of the opsonic population but IgG4 has also been shown to have some activity.

1.3.2 IgA opsonins

Although earlier work had suggested that IgA had no opsonic role, there is now evidence to the contrary (Fanger et al 1983). IgA has been shown to enhance opsonisation induced by sub-optimal doses of IgG (Fanger et al 1983) and IgA bound to erythrocyte surfaces can activate the alternative complement pathway (Hiemstra et al 1987). Yeaman and Kerr (1987) found that monomeric or dimeric IgA, isolated from the sera of patients with alcoholic cirrhosis, simulated the binding of C3b to mannan surfaces. IgA opsonisation of erythrocytes, yeasts, bacteria or inert particles (Gorter et al 1987, Yeaman and Kerr 1987) can stimulate phagocytosis by PMN and monocytes possessing the Fcα R receptor (Albrechtsen et al 1988, Monteiro et al 1990) (see **Figure 1.1 (a)**).

IgA deficiency is the most common immunodeficiency, affecting 1 in 700 Caucasians. There are at least 3 variants of IgA deficiency; affecting both subclasses, one subclass or one subclass and an IgG subclass. Oxelius et al (1981) claimed that around 20% of individuals with IgA deficiency also had an IgG2 deficiency and they were thus particularly susceptible to bacterial infections.

1.4 Opsonisation by complement component C3

1.4.1 Activation and control of complement

The term "complement" is now known to embrace at least 20 different plasma proteins which interact to generate a range of biologically active molecules. There are two major activation pathways which converge at the quantitatively dominant component **C3.** These are: i) the classical pathway (CP)
ii) the alternative pathway (AP)

Both complement pathways produce C3 convertase enzymes which cleave C3 into C3a and C3b fragments. Subsequently C3b may become bound to nearby surfaces and act as an opsonin or it may act as a focus for the generation of C5 convertase, thereby contributing to the lytic membrane attack sequence(MAC) (see **Figure 1.2**).

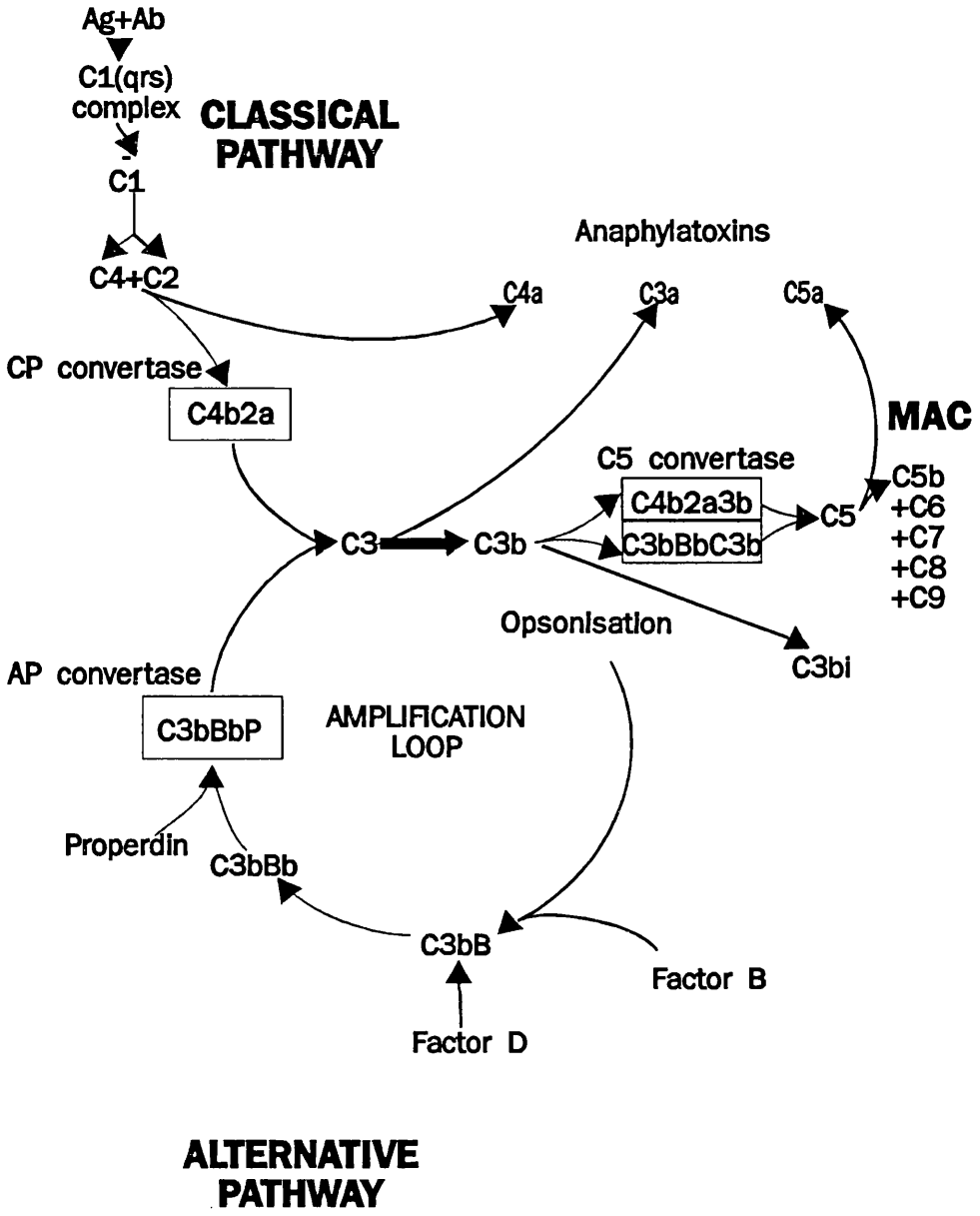


Figure 1.2 Activation of the alternative and classical complement pathways . (Adapted from Turner 1983.)

1.4.1.1 Activation of the classical pathway (CP)

Activation of the CP occurs after C1q binds to IgG or IgM in immune complexes and aggregates or to polyanions such as DNA, RNA, and lipopolysaccharides (Law and Reid 1988). C1q consists of 18 polypeptide chains (6 each of chains A, B and C); each chain has 81 N-terminal amino acids with the collagen-like repeat sequence (Gly Xaa Yaa) whereas the C terminal region comprises 136 amino acids without this repeat. The collagen-like sequences form into triple helices stabilised by disulphide bridging between the A and B chains, while the C chains form disulphide bridges with

an adjacent triple helix. The C-terminal sequences form a globular region which binds to immunoglobulins (Reid 1983). Thus in the C1q molecule there are 6 collagenous "stalks" terminating in 6 globular "heads" (Porter and Reid 1978). (see **Figure 1.3**)

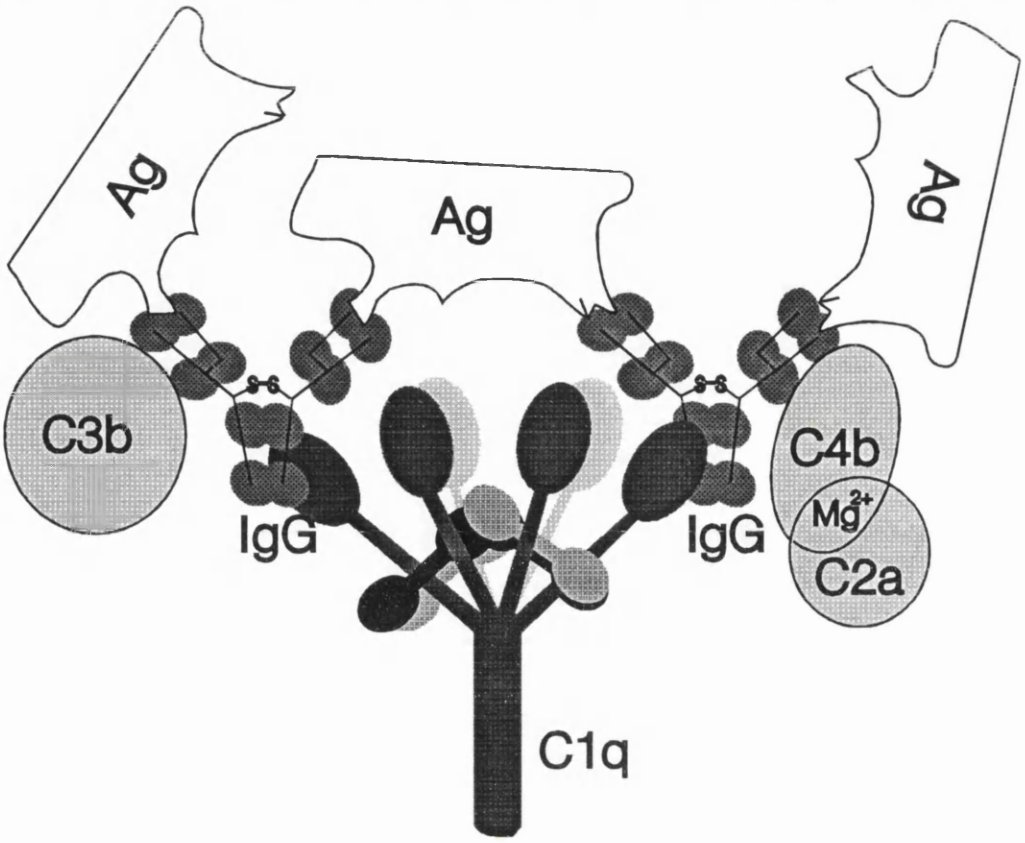


Figure 1.3 Interactions between the C1 complex, specific antibody bound to antigen and the complement components C2, C3 and C4. (Adapted from Law & Reid 1988).

The enzymic activity of C1 is due to the calcium - dependent activation of the two C1r and two C1s molecules which are bound to the C1q in the fluid phase. The C1-inhibitor (C1INH) controls the activation of C1 in two ways :(Ziccardi 1985) (i) In serum C1INH interacts reversibly with the C1 complex preventing spontaneous activation of the pro-enzymic C1r.(ii) After C1q is activated and bound to antibodies, C1INH removes the activated C1r and C1s from the C1 complex, preventing these from interacting with any other C1q molecules (Sim and Reboul 1981). When C1q binds to immunoglobulins and other efficient activators of the classical pathway, the inhibitory effect of C1INH is overcome and C1r and C1s are activated. The globular "heads" of the C1q bind only weakly to native IgG, but bind avidly to the CH2 domain of the IgG heavy chain and to the CH3 domain of the IgM heavy chain in immune complexes or other aggregates of immunoglobulins (Whaley 1985). C1q binds to different IgG subclasses with differing avidity, for example it binds strongly to IgG 1 and 3 but less to IgG2 and not at all to IgG4. C1q is released from the C1INH inhibition when 2 or more C-terminal " heads" interact with a suitable

immune complex causing a conformational change in the C1q and allowing the auto-activation of the pro-enzymic C1r. Activated C1r, in turn, activates the pro-enzyme C1s (Dodds et al 1978) due to the close association of the C1r and C1s on the collagen-like arms of the C1q .

The first substrate of the C1 esterase is the 3-chain C4 which is split by the C1s in the alpha chain to yield a C4a anaphylatoxin and the major fragment C4b (Schreiber and Müller-Eberhard 1974). Native C4 does not have enzymic activity but the splitting of C4 to yield C4b reveals a reactive acyl group which can bind covalently to adjacent amide or hydroxyl groups (Campbell et al 1981). C4b can react with C2 in the presence of magnesium ions, and when this occurs close to an activated C1s moiety the C2 can be split by the C1 esterase to yield the non-catalytic C2b (30 kDa) and catalytic C2a (70kDa) fragments (Nagasawa and Stroud 1977). The C4b2a complex is the **classical pathway C3 convertase enzyme** and cleavage of C3 by this enzyme yields C3a and C3b fragments. Formation of the CP C3 convertase is controlled in part by C4 binding-protein (C4bp) which binds to C4 and prevents binding to C2 (Sanches-Madrid et al 1983).

1.4.1.2 Complement component C3

The major pivotal protein of both complement pathways,**C3**, is a 2 chain structure comprising a short βchain (75kDa) linked to a longer α chain (115kDa) by a single disulphide bond (see **Figure 1.4(a)&(b)**).

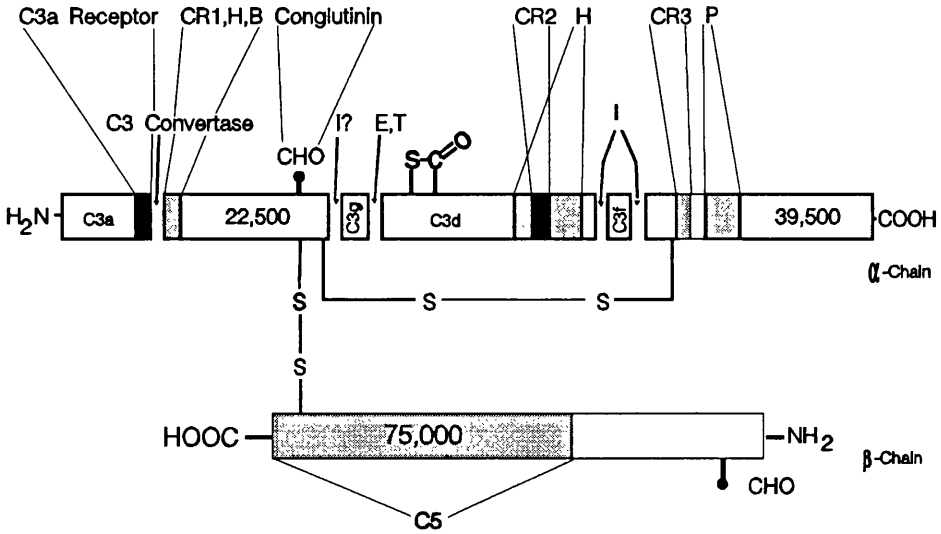


Figure 1.4(a) Schematic representation of the C3 molecule showing the postulated binding sites of CR1, CR2, CR3, C3a receptor, Factor B (B), Factor H (H), properdin (P), C5 and conglutinin. The sites of cleavage by C3 convertase, Factor I (I), elastase (E) and trypsin (T) are indicated together with the C3 fragments produced. (Adapted from Lambris 1988.)

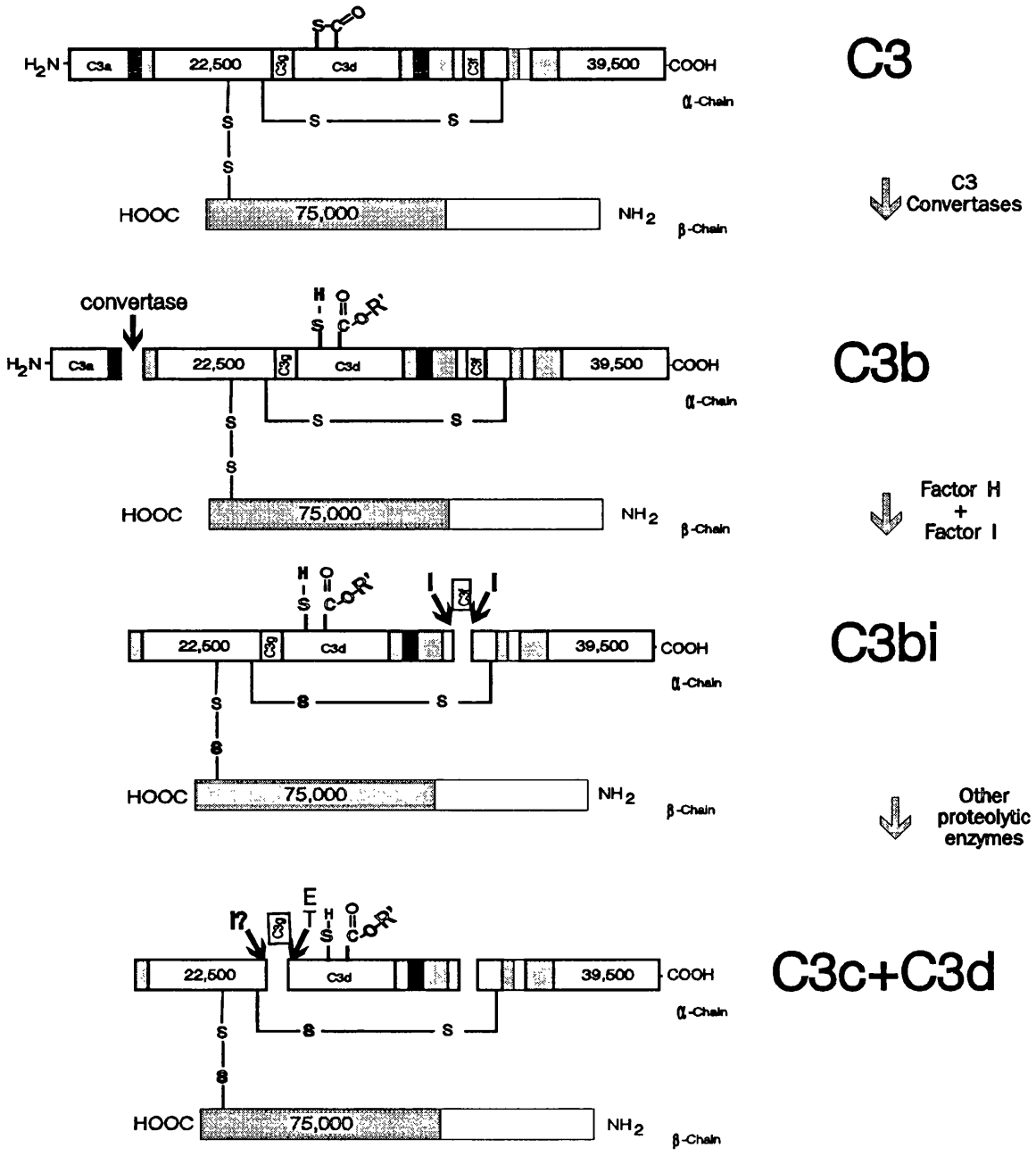


Figure 1.4(b) Schematic diagram showing the stages of C3 processing and the fragments produced. Note:R' represents the side chain of an accessible protein or carbohydrate, I is Factor I, E is elastase and T is trypsin.

The central role of C3 is demonstrated by the large number of complement proteins which interact with C3. These include the complement receptors CR1, CR2 and CR3, the alternative pathway proteins properdin and Factors H and B and the classical pathway component C5. Activation of C3 is controlled by proteolytic enzymes such as the C3 convertases, Factor I and elastase or tyrpsin-like enzymes. The fragments produced on proteolysis of C3 are shown in **Figure 1.4 (b)**. C3 convertase enzymes catalyse the conversion of C3 to C3b and C3a. C3a is an anaphylatoxin and (like C4a)

promotes increased vascular permeability and smooth muscle contraction. The release of the C3a results in a conformational change in the C3, revealing a previously buried internal thiolester group (Janatova et al 1980, Pangburn et al 1980, Tack et al 1980, Sim et al 1981). The exposed thiolester is extremely reactive to nucleophilic attack and will bind covalently to neighbouring amino or hydroxy groups forming amide or ester links respectively (Law and Levine 1977, Law et al 1979, Gadd and Reid 1981) (see **Figure 1.5**). **This conformational change activating the C3 molecule is the basis of C3 mediated opsonisation since activated C3 readily binds to the surfaces of adjacent micro-organisms, opsonising them for subsequent phagocytosis.**

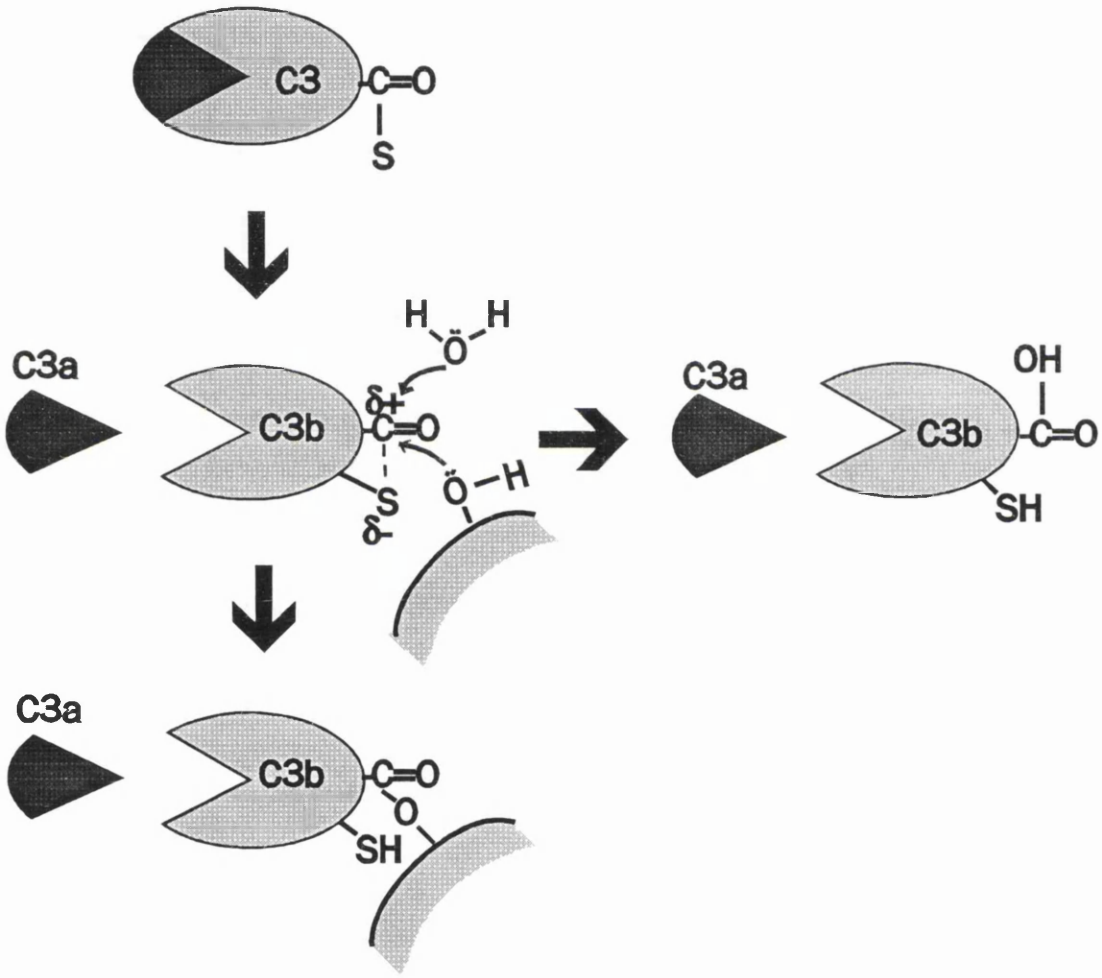


Figure 1.5 Alternative fates of nascent C3b. Depending on the nature of the nearby ÖH group the molecule is either inactivated by water or bonds covalently to a hydroxyl group (adapted from Sim et al 1981).

Activation and binding of C3b must be tightly controlled to prevent binding to host cells. The exposed thiolester group is extreme reactive to nucleophilic attack and if it does not bind to a surface within 50 milliseconds it will be hydrolysed by water (Law and Reid 1988) and will then be unable to bind to neighbouring host cells. In addition, sialic acid residues on mammalian cell membranes make these relatively refractive to C3 mediated opsonisation.

1.4.1.3 Activation of the alternative pathway (AP)

Activation of the alternative pathway (AP) of complement involves six serum proteins, C3, properdin and Factors B, D, H and I (Fearon and Austen 1977, Müller-Eberhard and Schreiber 1980, Schreiber et al 1978). The AP convertase enzyme (C3bBb) can be activated by the divalent cation magnesium, even in the absence of calcium. Takada et al (1987) showed that magnesium ions cause conformational changes in C3 and Factor B which facilitates Factor B binding to cationic sites on C3b and the binding of C3 to anionic sites on Factor B.

Activation of the AP does not require the binding of specific antibodies to the target cell surface, but relies on the molecular structure of the target cell itself.

AP activation can be divided into four phases - (Pangburn et al 1981).(see **Figure 1.6**).

(i) Initiation,
(ii) C3b deposition,
(iii) Discrimination
(iv) Amplification

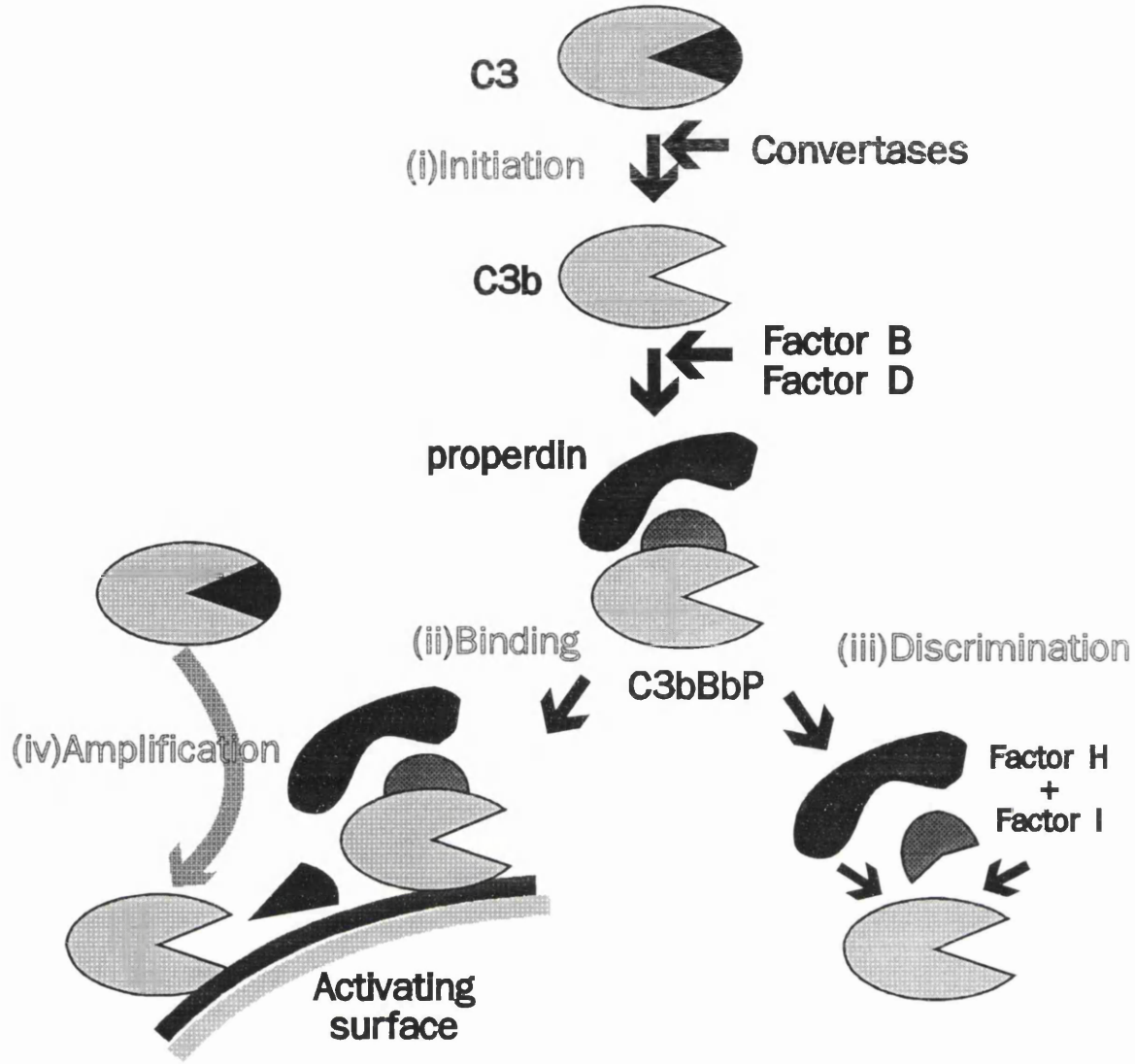


Figure 1.6 Alternative fates of the alternative pathway C3bBbP convertase, either binding to activating surfaces or hydrolysed by Factors H+I in the fluid phase. (Adapted from Turner 1983).

(i)Initiation

The AP can be initiated by a low level "tickover " of activation, possibly caused by a serum protease, small nucleophile or water gaining access to the internal thiolester group of the C3 (Fearon and Austen 1975, Schreiber et al 1978, Müller-Eberhard and Schreiber 1980). In the fluid phase this activated C3 is converted to $C3(H_2O)$,

which resembles C3b except that C3a is not necessarily lost from this molecule. $C3(H_2O)$ can bind to factor B in the presence of Factor D and magnesium ions forming the **fluid-phase C3 convertase** (Pangburn et al 1981, Isenman et al 1981, von Zabern et al 1981) .

(ii) Deposition

The $C3(H_2O)$ or C3b molecules produced by the fluid-phase convertase are metastable and can bind covalently to amino or hydroxy groups on nearby surfaces(Law et al 1977, 1979).

(iii) Discrimination

In the fluid phase or on surfaces which do not support AP activation, the alternative pathway convertase enzymes are readily inactivated by Factors H and I (Pangburn et al 1977, Schreiber et al 1978), preventing further activation of the alternative pathway. Factor I is a serine protease which regulates the AP and CP C3 convertases using Factor H or C4-binding protein as cofactor. Factor I acts by splitting the α'chains of C3b or C4b, producing inactive C3bi or C4bi forms which are unable to form convertases with Factor B and C2 respectively (Pangburn et al 1977, Law et al 1979). C3bi can then be broken down into C3c and C3dg by Factor I and trypsin-like enzymes (see **Figure 1.4 (b)**).

(iv) Amplification

The cell surface is important in the activation of the alternative pathway. On non-activating surfaces the control proteins (Factors H and I) prevent binding of activated C3bBb. Most mammalian cells are non-activating and there is good evidence that the presence of sialic acid is a critical factor preventing activation. Activating surfaces appear to provide "protected sites", where the affinity of C3b for Factor H is decreased,(Fearon and Austen 1977, Fearon 1978, Kazatchkine et al 1979, Pangburn et al 1978, Schreiber et al 1978) permitting positive feedback and amplification of the AP (Fearon and Austen 1977, Pangburn and Müller-Eberhard 1978). The stabilising protein, properdin binds to C3bBb in the fluid phase and especially on activating surfaces and increases the half-life of the convertase from 2.5 minutes to over 15 minutes (Medicus et al 1976). Properdin binding is not necessary for initiation of AP activation, but it amplifies activation by preventing spontaneous decay of the AP convertase (Schreiber at al 1978).

Haemolytically active C3 fragments may become bound to host cells following random activation in the fluid phase despite the sialic acid layer. Control proteins such as CR1 (Fearon 1979), decay accelerating factor (DAF)(Nicholson-Weller et al 1982) and membrane cofactor protein (MCP)(Seya et al 1986) act on these C3 molecules, dissociating any Factor B bound to C3b and forming haemolytically inactive C3bi.

Although binding of antibody to antigen is not required for AP activation, complexed IgG is a potent AP activator because of IgG-C3 interactions (Gadd and Reid 1981). When C3 is activated in the fluid phase in the presence of IgG opsonised micro-organisms, 20% of the C3b formed binds to the IgG. It is thought that the binding of the C3 to IgG stabilises the C3 convertase and protects this from attack by Factors H and I (Fries et al 1984). C3 binds to the $F(ab')_2$ rather than to the Fc portion of IgG (Gadd and Reid 1981). Thus C3 and C1q bind to IgG at distinct epitopes. The FcγRII and FcγRIII receptors on PMN have low affinity for opsonised monomeric IgG, however the IgG-C3b hybrid molecule binds to both the FcγR and CR1 receptors and particles opsonised with this hybrid molecule can be phagocytosed even in the presence of serum concentrations of IgG which would normally block the Fcγ binding (Kurlander and Batker 1982, Malbran et al 1987). In addition, whereas C3b - CR1 interactions do not initiate phagocytosis in resting PMN or monocytes, this is stimulated by the efficient simultaneous CR1 and FcγR engagement by the C3b-IgG hybrid.

1.4.1.4 C5 and the membrane attack complex

C5 is the first molecule of the membrane attack complex (MAC) and is a homologue of C3 and C4 (although it does not contain the internal thiolester group characteristic of these proteins). C5 is cleaved by the C3bBbC3b AP convertase or the C4b2a3b CP convertase to release C5a and C5b. C5a is a highly potent anaphylatoxin, whereas the larger C5b becomes surface bound and undergoes a conformational change which initiates the assembly of the MAC. A recent model of the interaction of the complement components forming the CP C5 convertase proposes that C4 is covalently bound to the cell surface and that C3b is then covalently bound to the C4b through an acceptor site on the C4b α'chain (Law and Reid 1988).

The 5 plasma glycoproteins C5b, C6, C7, C8, C9 interact as intact proteins, producing the membrane attack complex (MAC) which is responsible for lesions which allow the diffusion of intracellular components out of the cell and ultimately lead to cell death. The MAC comprises C5b-8 associated with between 1 and 18 molecules of C9 dependent on the availability of monomeric C9.

1.4.1.5 Activation of the classical pathway of complement by antibody independent routes

Antibody independent activation of the classical pathway is well documented although the physiological importance of such mechanisms remains to be elucidated. Protamine and C-reactive protein (CRP) are the best known of such activators.

CRP is an acute phase protein(made up of non-covalently associated polypeptides forming a pentameric structure - a "pentraxin"). CRP was so named because it binds to the C-polysaccharide of pneumococci in the presence of calcium ions. CRP also binds to phosphorylcholine in the lipid of cell membranes (especially after the fluid bilayer has been damaged) and to LPS and polycations such as poly-L-hisidine and poly-L-lysine. One domain of the CRP molecule has a sequence which closely resembles the CH2 of IgG and it is thought that C1q binds to this region in a manner reminiscent of its binding to IgG, thereby activating the classical pathway and promoting deposition of C3b and C4b opsonins on cell surfaces.

1.4.2 Polymorphisms of complement proteins

The complement proteins are grouped on the human genome in gene clusters or supergene families and there appear to have been extensive exon shuffling and gene rearrangements resulting in the observed distribution. The genes encoding human C4, Factor B and C2 are found in the MHC Class III region on chromosome 6 (Carrol et al 1984, Porter 1984). The different allotypes of these proteins are inherited as complotypes in strong linkage disequilibrium with HLA haplotypes of the flanking MHC classes I and II.

In man the C4 gene is duplicated and is found as two closely linked isotypes C4A and C4B straddling the 21 hydroxylase locus and within 400kb of the HLA-DR locus on chromosome 6 (Dunham et al 1987). C4 is the most polymorphic complement protein and more than 35 different structural allotypes have been identified to date, with the majority of the changes localised to the C4d region. The protein products of the C4A and B isotypes have different, complementary functions; the C4b fragment of C4B binds readily to carbohydrate surfaces (e.g. to IgG-coated erythrocytes) whereas the C4b fragment of C4A proteins bind to amino groups on surfaces(e.g.. on immune complexes). Thus the haemolytic activity of C4B is much greater than that of C4A (Law et al 1984, Isenman and Young 1984, Awdeh and Alper 1980), while C4A readily forms the classical pathway convertase catalysing C3b binding to immune

complexes. Cross-over between the isotypes occurs frequently and this has yielded the great variety of allotypes. There are frequent silent "null"-alleles of each of the C4 loci in all populations tested (Hauptmann et al 1988). These null alleles lead to the half-null state with homozygous or heterozygous deficiency of C4A or C4B proteins in sera. For example, in Caucasians only 56.8% of the population have four functional C4 genes, 34.5 % of the population have three genes, 7.9 % have two genes and 0.8% have only one functional gene. Porter (1983) suggested that the different C4 isotypes represented a selective advantage in the opsonisation of microorganisms and solubilisation of immune complexes and that these functions were complementary.

Four structural C2 alleles and one null allele have been described (Alper 1976, Segurado and Arnaiz-Villena 1989 (a)). Homozygous C2 deficiency is the most common of the primary complement deficiencies (0.01% of the population) and has been associated with systemic lupus erythematosus (SLE) (Perlmutter and Colten 1989) and other diseases such as chronic vasculitis (Friend et al 1975) and linear scleroderma (Hulsmans et al 1986), although apparently healthy individuals with C2 deficiency have been described. C2 polymorphisms can be identified using haemolytic overlays (Alper 1976) or using isoelectric focusing (IEF) and silver staining (Segurado and Arnaiz-Villena 1989 (a)).

There are two common Factor B polymorphisms which can be identified on high voltage electrophoresis, namely the Bf*S (slow) form and the Bf*F (fast) form (Segurado and Arnaiz-Villena 1989 (b)). In addition there are two minor forms Bf*S1 and Bf*F1 and at least three other rare polymorphisms.There are no known clinical associations with any of these polymorphic states at the present time.

The C3 gene is found on chromosome 19 in man. There are two common C3 polymorphisms (C3*F (fast) and C3*S (slow)) which are inherited as autosomal codominant genes with an allelic frequency of 0.77 - 0.8 for the C3*S in Caucasian populations. 16 other common variants have been found and shown to exhibit altered calcium binding. The allelic frequency of C3*F is raised in patients with mesangiocapillary glomerulonephritis and partial lipodystrophy, and these individuals also have high levels of C3 nephritic factor(C3NeF).

1.4.3 Complement deficiencies

Complement protein deficiencies can be divided into deficiencies of early CP components, AP components, components of the membrane attack complex, control proteins and complement receptors (reviewed by Perlmutter and Colten 1989). Complement deficiencies may not necessarily cause disease, but predispose to disease states. In many cases individuals with proven deficiencies are apparently healthy and this may be because of the network of overlapping functions in the complement system which are able to compensate for the defect. The disease most commonly associated with complement deficiencies is the immune complex disease systemic lupus erythematosis (SLE). This is characterised by focal lesions, fibroid necrosis of connective tissue and disseminating lesions, particularly in the skin. SLE in association with complement deficiencies differs from classic SLE as there is an increased prevalence of discoid lesions, a low incidence of renal disease, low titres of anti-DNA antibodies and little evidence of immunoglobulin or complement in the skin lesions (Agnello 1978)

Early CP complements are involved in immune complex clearance and homozygous deficiencies in C1q, r or s, C2, C3 and C4 lead to immune complex disease (Atkinson 1989). The only disease reported in individuals with C1q or C1r deficiency which is definitely inherited is SLE (Perlmutter and Colten 1989). C2 deficiency is the most prevalent homozygous complement defect, the most common disease associated with this defect is SLE but glomerulonephritis and inflammatory bowel disease have also been described(Slade et al 1978). Patients with C2 deficiency appear to be susceptible to infections such as recurrent septicaemias (Newman et al 1978) and the bacterium most often cultured is *Streptococcus pneumoniae.*C4 is encoded at the two loci C4A and C4B in the MHC Class III on chromosome 6. There are 4 active C4 genes and although homozygous deficiencies of C4 are rare, heterozygous deficiencies are relatively common (see **Section 1.4.2**). The serum levels of C4 reflect the number of active genes, although the wide range of C4 concentrations in normal serum makes it difficult to ascribe heterozygosity by measuring serum C4 levels. The C4A null allele is associated with immune complex disease and the homozygous C4A null allele has been found in 11.7% of Caucasians with SLE compared with an incidence of SLE of 0.9% in the normal Caucasian population. There is also a slightly raised incidence of insulin dependent diabetes in individuals with C4B null alleles (6.9% compared with 2.7% in the normal population) (Hauptmann et al 1988). The C4b fragment of C4B binds readily to carbohydrate surfaces and so the haemolytic activity of C4B is much greater than that of C4A (Law et al 1984). Thus individuals who are

homozygous for the C4B null alleles are probably more susceptible to bacterial infections than normal individuals (Hauptmann et al 1988).

Deposition of C3 on activating surfaces is amplified by the alternative pathway of complement and consequently deficiencies of Factor B or Factor D, the control proteins Factors H and I, or the stabilising protein properdin are very likely to affect the amounts of the C3 opsonin binding to micro-organisms. C3 deficiencies are relatively rare, this is probably because affected individuals do not survive the overwhelming bacterial infections with encapsulated organisms associated with C3 deficiency (Alper et al 1972). Homozygous deficiencies of Factor B have not been described, probably for the same reason as the low frequency of C3 deficiencies. Deficiencies of Factor D have been described recently and these may be associated with a high incidence of upper respiratory tract infections(Leigh et al 1986). The stabilising protein properdin has the important role in opsonisation of stabilising the alternative pathway convertase. The normal serum concentration of properdin is 19-25μg/ml. The group led by Dr A.Sjöholm (Lund) has identified three states of properdin deficiency which are termed Variants 1-3. Variant 1 deficiency is defined as having less than 0.1mg/l, Variant 2 deficient sera have between 1 and 2mg/l while in Variant 3 deficiency the properdin is dysfunctional although the serum levels are at 19mg/l. Properdin deficiencies were first identified in three males, one of whom died of *Neisseria meningitidis* group C infections (Sjöholm et al 1982). A detailed family history revealed three previous cases of similar infections with fatal outcome. Subsequently Braconier et al (1983) found that sera from two apparently healthy adults with properdin deficiency did not opsonise *Streptococcus pneumonii* serotype 23F efficiently but normal opsonic function was restored on addition of small amounts of properdin. The properdin gene has been localised to Xp11.23-Xp21.1 and therefore the properdin deficiency is sex linked (Goundis et al 1989, Goonewardena et al 1988). Although properdin deficiencies appear to be associated with meningococcal sepsis, it also appears that properdin deficient individuals may be healthy, possibly because of acquired, protective, specific anti-meningococcal IgG or IgM antibodies in these individuals (Sjöholm et al 1988).

Terminal complement component deficiencies are frequently associated with repeated neisserial infections, e.g. *N.meningitidis* and *N.gonorrhoea,* since lysis of these organisms is apparently required for effective killing (Perlmutter and Colten 1989).

Deficiencies in control proteins lead to reductions in the levels of complement components since activation can proceed unchecked. The most common deficiency of a control protein is C1 esterase inhibitor (C1INH) deficiency. C1INH deficiency causes hereditary angio-oedema (HAE) which is characterised by episodic bouts of circumscribed non-inflammatory oedema involving subcutaneous tissue and mucus membranes which ranges from a swollen ear to death from respiratory failure. Factor I deficiencies have been linked with frequent episodes of meningitis; the omission of this control protein leads to rapid turnover and wasteful consumption of C3 and C3 levels are about 30% of normal in individuals deficient in Factor I (Perlmutter and Colten 1989). Factor H deficiencies (Thompson and Winterborn 1981, Nielsen et al 1989) lead to reduction in C3, Factor B, properdin, C5 and C6-9 levels and therefore to a reduction in haemolytic complement activity.

Deficiencies of the complement receptor CR3 and the related LFA-1 and p150/95 molecules have been described (Ross et al 1985(b), Springer et al 1985) in association with bacterial infections, and this has led to the suggestion that these molecules can function as phagocytic receptors for bacteria.

1.5 The yeast opsonisation defect

In 1968 Miller and colleagues described a familial defect in immunity in an infant who presented with the triad of erythroderma, failure to thrive (FTT) and diarrhoea. The child appeared to be particularly susceptible to infections and the family history revealed paternal and maternal relatives with severe atopic eczema and dermatitis respectively. Plasma from this individual had a defect in immune function and failed to opsonise yeasts for phagocytosis by neutrophils from a normal donor. In 1970 Miller and Nilsson proposed that the cause of the yeast opsonisation defect was a functional inadequacy of the complement component C5 and in 1972 Miller and Koblenzer proposed that this C5 deficiency was the underlying cause of Leiner's disease - a non-specific erythematous or eczematous eruption of extensive distribution occasionally seen in infants. Although Miller and Koblenzer (1972) had suggested that the yeast opsonisation defect was caused by a functional deficiency of C5, subsequent work demonstrated that C5 deficient serum could sustain normal yeast opsonisation (Yamamura and Valdimarsson 1978, Rosenfield et al 1976). It is now thought (Hurwitz 1981) that Leiner's disease is a purely clinical entity, diagnostically apparent as generalised seborrheic dermatitis, intractable severe diarrhoea, marked wasting and dystrophy and recurrent local and systemic infections, and that familial Leiner's disease with C5 dysfunction is a more dangerous variant of this.

Using a microscopic assay to determine the average number of yeast particles taken up by a polymorph (the so-called Y/P index), Soothill and Harvey (1976) found a low index when they studied the sera of 11 out of 43 infants and children who had presented with frequent unexplained infections. More surprisingly, they also found that serum from approximately 5% of a population of healthy adults showed similar defective function and this incidence was confirmed in independent studies by Kerr et al (1983). The frequency of the defect was also found to be about 6% in a population of healthy school children (Levinsky et al 1978). Subsequently, an increased incidence of defective opsonisation was found in atopic individuals (Turner et al 1978, Siccardi et al 1980) and in children with chronic diarrhoea (Candy et al 1980). The latter group found that 23% of patients with protracted diarrhoea of undetermined cause had defective yeast opsonisation, whereas in those patients with "toddler diarrhoea" the incidence of poor opsonisation was only 4%, similar to the incidence in the healthy population. When four patients with defective yeast opsonisation and toddler diarrhoea were treated with plasma infusions the opsonic function improved in every case and in 3 out of 4 the chronic diarrhoea was also controlled.

Soothill and Harvey (1977) showed that the serum of a C3 deficient individual did not opsonise bakers yeast whereas serum from a C5 deficient patient was still functionally normal. In addition these workers reported normal function in sera from patients with agammaglobulinaemia and found that purified IgG and IgM failed to correct the defect; they also found that the lysis of sheep red cells (classical pathway) and rabbit red cells (alternative pathway) were normal in defective sera. Therefore they proposed that a "factor", necessary for the opsonisation of yeast particles was missing from the sera with defective opsonisation. Larcher et al (1981) established that the putative factor was produced in the liver since the serum of patients with fulminant hepatic failure (FHF) showed defective opsonisation which normalised when the patients recovered.

In 1982 Richardson et al reported that the incidence of poor yeast opsonisation in the newborn was 9.9%, almost double the frequency observed in adults. Subsequently in a follow - up investigation at one year of age Richardson et al (1983) studied the incidence of serious infection and atopy in 26 infants with the deficiency, compared with a control group matched for age, sex, parental atopy, parental smoking and social class. In 18 of the 26 individuals the defect persisted at one year and the incidence of atopy and infection (particularly otitis media) was significantly higher in the group with the serum defect than in the matched controls.

The first assays of opsonisation measured the phagocytosis of opsonised particles by PMN cells as a measure of the opsonic potential of a particular serum (Wright and Douglas 1903). Miller et al (1968) and Soothill and Harvey (1976, 1977) used heat killed bakers' yeast particles in their studies and expressed the results as numbers of yeast particles ingested per polymorph. Other techniques for the measurement of opsonisation have included the uridine uptake method of Yamamura and Valdimarsson (1977) and the Coulter counter assay of Levinsky et al (1978). Both of these assays measured phagocytosis of yeast or micro-organism particles by detecting those particles which remained external to the the phagocytes after the incubation period. In another modification Kerr et al (1983) developed an assay measuring fluorescence from ingested FITC-treated yeasts. Crystal violet was added to the buffer and this quenched background fluorescence from non-phagocytosed yeasts.

Bakers' yeast was originally selected for the microscopic assays because it was believed that antibody mediated opsonic mechanisms would not be involved to any significant extent. The assumption that the assay was indeed evaluating C3 opsonic mechanisms remained unestablished until Turner et al (1981) developed an assay in which trypsin was used to cleave C3c from the C3b moieties covalently bound on the yeast surface. The levels of C3c released were measured in radial immunodiffusion assays and were shown to correlate well with the direct phagocytic assay (Levinsky et al 1978) and with a neutrophil iodide uptake assay (Roberton et al 1981). These experiments established that the sera designated as poorly opsonic bound reduced amounts of C3 moieties to the yeast particles, suggesting that this was probably responsible for the reduced phagocytosis. Subsequently, Turner et al (1985 (a)) showed that there were two patterns of deposition of C3 on the yeast surface; rapid or slow and these were readily differentiated in a 30 minute assay incubation. Since suboptimal C3 deposition would be expected to occur in association with primary C3 and properdin deficiencies (Sjöholm et al 1982), in neonates with immature complement systems (Richardson et al 1982), and in patients with liver disease (Larcher et al 1981), it is important to eliminate these possibilities before concluding that a serum has the common opsonic defect.

In an attempt to characterise the molecular basis of the defect Turner et al (1985 (a)) measured the concentrations of complement components C3, C4, properdin, Factor B and Factor H in six individuals with poor yeast opsonisation and found that these were all normal. The total haemolytic complement function and Factor H function were also normal for these sera. Studies using MgEGTA suggested that both the alternative

and classical pathways contributed to the deposition of C3b on the yeast surfaces. Nevertheless a role for IgG could not be excluded since this is thought to influence AP activation (Schenkein and Ruddy 1981). A significant observation was that small amounts of normal serum mixed into poorly opsonic sera corrected the defect in vitro (Turner et al 1985a) and such "functional correction assays" using the C3c elution technique generated data which parallelled the results previously obtained with the phagocytic assay (Soothill and Harvey 1976). In a linked investigation, Turner et al (1985 b) also described the partial purification of a correcting factor of apparent molecular weight 70 000-80 000, eluting between IgG and albumin on S300 SF gel filtration and eluting immediately after IgG on anion exchange chromatography. This partially purified material had no functional properdin activity, although in attempts to evaluate the wider significance of poor opsonisation of baker's yeast, it was shown to bind to zymosan at 4^{o}C, to be insensitive to heating to 50^{o}C but functionally inactivated by heating to 56^{o}C.

In an attempt to define the relevance of the defect to potentially pathogenic organisms Turner et al (1986) measured both C3b deposition and chemiluminescence using the yeasts *S.cerevisiae* and *C.albicans* and the bacteria *S.aureus* and *E.coli*. Sera which showed poor opsonic function with *S.cerevisiae* were also significantly less effective at opsonising these other organisms compared to normal sera and thus it was concluded that sera exhibiting poor yeast opsonisation had a general opsonic defect. It was suggested that sera with defective C3 deposition had a relative rather than absolute defect, since reduced amounts of opsonin were usually bound rather than none at all.

An important aspect of all the studies described above is the fact that the assays have always used **diluted serum** (ranging from 5-20% depending on the assay). The demonstration of poor opsonic function is critically dependent on this and the interpretation of the clinical associations described needs to take this into account. This point is taken up again in **Section 6.5.**

1.6 Aims

The major aim of the studies described here was to establish the **molecular basis of the common opsonic defect**. In approaching this question it was necessary to take into account the (unpublished) scepticism expressed in some quarters regarding the findings of Turner et al (1985 (a)&(b)). In the absence of any molecular characterisation of the putative factor the explanation of the defect was thought most likely to reside in some abnormality of the known complement components. Therefore it was judged necessary to review the problem again without pre-conceptions using, wherever possible, improved reagents and technology.

The following possibilities were considered:

1) Suboptimal complement activation arising from limiting amounts of one or more complement component. For example serum levels of a single component less than mean minus 2SD (calculated from the healthy population) or between mean minus 1SD and mean minus 2SD for more than one component, might give rise to such an effect.

2) The presence of a functionally abnormal allelic variant of a known component.

3) The presence of an as yet uncharacterised component or co-factor with a major role in the regulation of C3b deposition under the assay conditions used.

The possibilities (1) and (2) were both addressed in **Chapter 3**. Possibility (3) is the subject of investigations described in **Chapters 4, 5** and **6**.

CHAPTER 2

MATERIALS AND GENERAL METHODS

MATERIALS

METHODS

2.1 Chemicals and general buffers (most of the chemicals were purchased from BDH and were AnalaR grade or equivalent)

Chemical	Supplier	Code
Acetic acid	BDH	10001
Acrylamide	BDH	44313
Agarose	ICN	95 201 4
Ammonium persulphate	Sigma	A 9164
Bovine serum albumin	Sigma	A 4503
Biotin-aminocaproate-N-hydroxysuccinimide ester	Sigma	B 2643
Bromophenol blue	BDH	20015
Calcium chloride	Sigma	C 3881
Citric acid	BDH	10081
Diethyl barbituric acid	BDH	10415
DMSO	Sigma	D 5879
Di-sodium carbonate	BDH	10240
Dithiothreitol (DTT)	Sigma	D 9779
EDTA	Sigma	E 5134
EGTA	Sigma	E 4378
Formaldehyde	BDH	10113
Glutaraldehyde	Sigma	G 6257
Glycine	Sigma	G 2879
Hydrogen peroxide	Sigma	H 1009
Magnesium chloride	Sigma	M 0250
Mannan	Sigma	M 3640
Methanol	BDH	10158
NN' methylenebisacrylamide	BDH	44300
OPD	Sigma	P 1526
PAGE blue 83	BDH	44246
Potassium chloride	BDH	10198
Potassium dihydrogen phosphate	BDH	10203
Silver nitrate	BDH	10233
Sodium acetate	BDH	10236
Sodium azide	BDH	10369
Sodium barbitone	BDH	10365
Sodium carbonate	BDH	10240
Sodium chloride	BDH	10241

Chemical	Supplier	Code
Sodium dodecyl sulphate (SDS)	Sigma	L 4509
Sodium hydrogen carbonate	BDH	10247
Sodium dihydrogen orthophosphate	BDH	10245
di- Sodium hydrogen orthophosphate	BDH	10249
Sucrose	BDH	10274
TEMED	Sigma	T 8133
Tris	BDH	10315
Tween 20	BDH	66368

General buffers

CFD	Diethyl barbituric acid	4.400 mM	pH 7.3
(complement	Sodium barbitone	0.897 mM	
fixation diluent)	Sodium chloride	145.000 mM	
	Magnesium chloride	0.826 mM	
	Calcium chloride	0.252 mM	
Carbonate buffer	Di-sodium carbonate	15.000 mM	pH 9.6
for ELISA coating	Sodium hydrogen carbonate	35.000 mM	
Carbonate buffer	Sodium hydrogen carbonate	100.000 mM	pH 8.3
for biotinylation			
Citrate/Phosphate	Citric acid	50.000 mM	pH 5.2
Buffer for ELISA	Sodium hydrogen phosphate	100.000 mM	
OPD (ELISA substrate)	o-phenylene diamine (Sigma)	10 mg	pH 5.2
	Citrate/phosphate buffer	20 ml	
	30% Hydrogen peroxide	10 μl	
PBS	Sodium chloride	140.000 mM	pH 7.3
Phosphate buffered	Potassium chloride	2.700 mM	
saline)	Di-sodium hydrogen phosphate	8.000 mM	
	Potassium di-hydrogen phosphate	1.500 mM	
PBS-Tween	PBS (as above) with	0.05%(v/v)	pH 7.3
(PBS-T)	Tween 20		
VBS	Diethyl barbituric acid	4.400 mM	pH 7.2
Veronal buffered	Sodium barbitone	1.800 mM	
saline	Sodium chloride	145.000 mM	
VBS^{++}	VBS with		pH 7.2
	Magnesium chloride	5.000 mM	
	Calcium chloride	5.000 mM	

2.2 Serum samples

The following serum samples were used:

1) Sera from 179 apparently healthy adult blood donors attending the West End blood donor centre on 12 and 13/4/88 and 17 and 18/10/88. These sera were numbered **A 1 - 179.**

2) Sera from 11 healthy adult laboratory staff members working in the Department of Immunology, Institute of Child Health. 5 of these sera were numbered **H 1-5**, the other 6, which were used regularly, were coded as **LB1**, **LB2**, **LB3** and **HB1**, **HB2** and **HB3**. The LB (low binding) sera were named in this way because levels of C3 opsonins bound to zymosan after incubating in these sera in the functional opsonic assay were below the normal range. In contrast the HB (high binding) sera bound normal amounts of C3 opsonin in this assay.

3) Sera from 400 patients attending Immunology and Dermatology clinics at the Institute of Child Health between 1987 and 1988. These were numbered **I 1 - 400.**

4) Sera from 59 apparently healthy infants aged approximately 4.5 months undergoing a formula feed trial. These were numbered **N 1 - 59.**

5) Sera from 19 hospitalised children aged between 3 months and thirteen years undergoing "cold" surgery. These were numbered **C1 -19.**

Preparation of serum samples

Blood obtained from patients or volunteers was allowed to clot for 1-2 hours at room temperature in preservative-free glass tubes. The clots were rimmed and cellular material was separated from the serum by centrifugation at 1000g for 15 minutes in a refrigerated MSE centrifuge. Sera were aliquoted into 0.5ml 'Eppendorf' tubes and stored at -70°C until use. A pooled serum stock was prepared by mixing 1ml serum aliquots from 100 of the 179 healthy adult blood donors. This pooled stock was re-aliquoted and stored at -70°C until use. Aliquots of sera were used once only after thawing.

2.3 Antisera

The antisera used are listed in **Table 2.1.**

Biotinylation of antisera

The method of Guesdon et al (1979) was followed. Antibodies were diluted and made up to 1 mg/ml in 0.1M Carbonate ($NaHCO_3$) buffer pH 8.3. Biotin -aminocaproate N-hydroxysuccinimide ester (Sigma UK Ltd) at a concentration of 1mg/ml in DMSO (Sigma UK Ltd) was added to a final concentration of 0.01M and the antibody/biotin mixture incubated for two hours at room temperature. Unbound biotin was removed by overnight dialysis against PBS .

Table 2.1 A listing of the antibodies used in subsequent experiments

POLYCLONAL ANTIBODIES	CODE	SUPPLIER
Sheep anti-human Factor B	AHP 039 H	Serotec
Sheep anti-human Factor B HRPO	AHP 039 P	Serotec
Sheep anti-human Factor H	60-209-1	ICN
Sheep anti-human Factor I	AHP 036 H	Serotec
Sheep anti-human FactorI HRPO	AHP 036 P	Serotec
Sheep anti-human C3	R-109	Scipac
Sheep anti-human C3c	AHP 031 H	Serotec
Sheep anti-human C3c HRPO	AHP 031 P	Serotec
Sheep anti-human C4	AHP 032 H	Serotec
Sheep anti-human C4 HRPO	AHP 032 P	Serotec
Sheep anti-human C1q	AHP 033 H	Serotec
Sheep anti-human C1q HRPO	AHP 033 P	Serotec
Sheep anti-human transferrin HRPO	AHP 081 P	Serotec
Sheep anti-mouse IgG HRPO	A 6782	Sigma
Goat anti-human properdin	032-03	ATAB
Goat anti-human IgA (alpha chain)	I-0884	Sigma
Goat anti-human IgA (alpha chain) HRPO	A-7032	Sigma
Goat anti-human IgG (gamma chain)	I-3382	Sigma
Goat anti-human IgG (gamma chain) HRPO	A-6029	Sigma
Goat anti-human IgM (mu chain)	I-0759	Sigma
Goat anti-human IgM (mu chain) HRPO	A-6907	Sigma
Goat anti-rabbit IgG HRPO	A 4914	Sigma
Donkey anti-rabbit Immunoglobulin ^{125}I	IM 134	Amersham
ANTIBODIES NOT AVAILABLE COMMERCIALLY	CODE	GIFTS FROM
Mouse anti-human properdin	HYB 3-3	Dr C. Koch Copenhagen
Mouse anti-human Factor H	Ox 24	Dr R.B.Sim Oxford
Rat anti-human C3g	Clone 9	Professor P.J.Lachmann Cambridge
Rabbit anti-human mannose-binding protein		Dr S.Thiel Oxford

2.4 Chromatography

2.4.1 Gel filtration

Serum proteins were separated according to their apparent molecular radii using a Sephadex S300 SF gel filtration column (Pharmacia) of dimensions (85x2.6cm) equilibrated in PBS (Oxoid) containing 10mM EDTA. The column was run at 12 ml per hour using a P3 pump (Pharmacia), the absorbance of the material flowing through the column was continuously measured using a UV-1 flow-through cell (Pharmacia) and fractions were collected into separate tubes in a Frac-300 fraction collector (Pharmacia). Absorbance plots were recorded using the REC 482 Chart recorder system (Pharmacia).

2.4.2 Ion exchange

Serum proteins were separated according to charge using anion exchange chromatography on an FPLC system (Pharmacia) which consisted of a GP-250 gradient controller running two P-500 pumps and set up with the HR 5/5 Mono Q anion exchange column, a 500μl loading loop and F-7 manual valve system, a UV-1 flow-through cell and a Frac-100 fraction collector. Plots of absorbance at 280nm were recorded on an REC-482 chart recorder.

2.4.3 Chromatofocusing

Serum proteins were separated according to charge using the FPLC system described above with the exception that the strongly cationic Mono-Q anion exchange column was replaced by a weak cation Mono-P chromatofocusing column. The use of Polybuffer 4-6 (Pharmacia) allows separation of proteins on the basis of pI in this system.

2.4.4 Affinity chromatography

Antibodies were purified from ascitic fluid and coupled to Sepharose 4B columns using the following procedures:

2.4.4.1 Preparation of antisera

Silicone dioxide treatment of ascites (Neoh et al 1986)

This treatment removes the lipid which could clog up the protein-A column used to purify the antibodies.

1) 3ml of ascites were centrifuged for 5 minutes at 13000g in an MSE microfuge to remove particulate matter.
2) An equal volume of VBS was added followed by 90mg of silicone dioxide and the suspension was incubated at room temperature for 30 minutes.
3) The silicone dioxide / lipid mixture was precipitated by centrifuging at 6500 g for 20 minutes at 4°C.

Protein A purification of antibody (Pharmacia Separation News Vol 13.5)

1) The lipid - depleted ascites fluid was diluted with an equal volume of loading buffer (1.5 M glycine/ 3 M NaCl pH 8.9) and applied to a protein A column (bed volume 8ml) . The column was washed with loading buffer until the absorbance at 280nm was less than 0.02 absorbance units.
2) Antibodies were eluted from this column in 0.1M citric acid at the following pH: -
mouse IgG 1 molecules were eluted at pH 6,
mouse IgG 2a molecules were eluted at pH 5
mouse IgG 2b and IgG 3 molecules were eluted at pH 4.
3)The protein eluted from this column was quantified by measuring the absorbance at 280nm and calculating the concentration according to the formula :

$$\text{Protein concentration} = \frac{\text{absorbance at 280 nm}}{\text{extinction coefficient}} \times 10 \text{ mg/ml}$$

using the extinction coefficient (E_{280}) of 13.0 for mouse immunoglobulin.

4) The column was washed in 0.1M citric acid pH 3 to regenerate the column and to remove any residual antibodies still bound.

2.4.4.2 Coupling of antibodies to Sepharose 4B (Pharmacia)

1) Mouse immunoglobulin (30 mg) was dialysed overnight against "coupling buffer" 0.1M $NaHCO_3$ /0.5M NaCl pH 8.3.

2) 1g of cyanogen-bromide activated Sepharose resin (Pharmacia UK Ltd) was washed with 200 ml of 0.001 M HCl on a scintered glass filter, this re-hydrates and swells the beads and the acid pH prevents activation of the cyanogen bromide residues.

3) The antibody solution was mixed with the activated Sepharose for 2 hours at room temperature in coupling buffer and then the suspension was centrifuged at 1000 g for 10 minutes to collect the Sepharose. During this incubation period the cyanogen bromide residues are activated by the high pH of the coupling buffer and this promotes antibody coupling to the resin.

4) The amount of antibody binding to the Sepharose was calculated by measuring the absorbance at 280nm of the unbound material and following the calculation described above (see **Section 2.4.4.1**).

5) The active sites on the resin which had not reacted with antibody were inactivated by incubation for two hours at room temperature in 0.2M glycine/ 0.1M $NaHCO_3$ / 0.5M NaCl .

6) The antibody coupled Sepharose gel was transferred to a column and washed with a Sodium acetate "washing" buffer of 0.1M NaAc / 0.5M NaCl pH 4.5. The absorbance of the washes was measured till these fell below 0.02 OD_{280} units.

7) The column was then washed 3 times using alternate cycles of washing and coupling buffers (pH 4.5 and 8.3 respectively). This alternate low and high pH washing removes unbound antibody and blocks any remaining unbound residues.

2.5 Electrophoresis and blotting

2.5.1 SDS - PAGE (Laemmli 1970)

10% polyacrylamide gels were prepared using the following stock solutions:

1) 30% acrylamide with 0.8% NN'methylene bisacrylamide
2) 0.75M Tris 0.01M EDTA pH8.8
3) 1M Tris pH 6.8
4) 10% w/v sodium dodecyl sulphate (SDS)
5) 1% ammonium persulphate
6) TEMED N, N, N^1, N^1,-tetramethylethylene diamine

0.75mm Thick SDS -PAGE slab gels containing 10% acrylamide solution with 0.1% SDS in Tris EDTA buffer were cast in the Biorad "Protean II" apparatus. Stacking gels, containing 5.2% acryamide and 0.1% SDS in Tris buffer, were cast above the main gels. The 10% gels were polymerised with ammonium persulphate (APS) (final concentration 0.1%) and TEMED (final concentration 0.1%) while the stacking gels contained 0.1% APS and 0.5% TEMED.

Sample preparation: Protein samples containing approximately 25mg protein in 50μl were boiled for 5 minutes with 25μl of a "sample buffer" of 6% w/v SDS,40% w/v sucrose, 0.02 % w/v PAGE blue 83 and 0.01 M Tris pH8. For vigorous reduction of the samples, dithiothreitol was added to the "sample buffer" to a final concentration of 40mM. Molecular weight markers (Sigma MW-SDS-70L) were included on each gel. These contained α-lactalbumin (14.2 kDa), trypsin inhibitor (20.1 kDa), trypsinogen (24 kDa), carbonic anhydrase (29 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), egg albumin (45 kDa) and bovine serum albumin (66 kDa). Human serum albumin (68 kDa)(Sigma A 3782) was used as an additional marker.

Electrophoresis: The gels were run in the "Biorad protean II" apparatus using a Tris-glycine buffer of 0.192M glycine/0.025M Tris/0.1% SDS pH 8.3. Following electrophoresis the gels were stained or blotted.

2.5.2 Staining

Coomassie staining: Gels were stained overnight in a solution of 0.025% Coomassie brilliant blue R (Sigma UK Ltd) in 50% methanol/ 5% glacial acetic acid and were destained in 7.5% glacial acetic acid/ 5% methanol.

Silver staining: (Morrisey et al 1981) Coomassie stained gels were fixed in 50% methanol 10% glacial acetic acid for 30 minutes, in 5% methanol/ 7% acetic acid for 30 minutes and in 10% glutaraldehyde for 30 minutes. The gel was washed overnight in deionised water and reduced with 5μg/ml of dithiothreitol (DTT) for 30 minutes and treated with 0.1% silver nitrate for a further 30 minutes. The gel was washed rapidly in distilled water and rinsed twice in a developer of 0·0185% formaldehyde in 3% Na_2CO_3 until all the coomassie blue stained protein bands changed colour to brown. The reaction was terminated by the addition of 2.3M citric acid until the pH returned to neutral. The gel was rinsed in distilled water and finally in 0.03% sodium carbonate solution to prevent bleaching.

2.5.3 **Immunoblotting** (in the Bio-rad tank blotting system)

The SDS-PAGE gel, membranes, filter papers and scotchbrite pads were soaked in 0.192M glycine/ 0.025M Tris pH 8.3 with 20% methanol. A "sandwich" was made from the cathode side of the cassette consisting of the scotchbrite pads, 2-3 layers of Whatmann 3MM filter paper, the gel, a "Hybond C Extra" (Amersham) membrane, 2 - 3 more layers of filter paper and pads. The cassette was closed and submerged in the tank with running buffer pre-cooled to 4^{o}C. The gel was blotted at 0.35 Amps for two hours with stirring. Following the blotting step the membrane was marked to indicate the position of the wells and blocked with 3% skimmed milk (Marvel) in PBS/ 0.02% sodium azide for a minimum of 1 hour. The antibodies were diluted in the skimmed milk/PBS/azide solution and washes were also carried out in this. Primary antibodies were diluted to approximately 1/1000 , the secondary antibody used was anti -species immunoglobulin, at 1 x 10^5 cpm/ml. The blot was washed for 6 x 5 minute periods after incubation with the antibodies. Following the final wash the blot was enclosed in "Saran wrap" and autoradiographed at -80 ^{o}C between fast intensifying screens with Cronex "Fast" film for 2 days - 1 week depending on the intensity of the signal.

2.6 ELISA procedures for measuring total levels of serum complement proteins

Principle

"Sandwich" type ELISA procedures were used for the detection of these proteins.The capture antibodies were polyclonal antibodies raised in sheep against the specific human complement proteins. The unbound sites were blocked with bovine serum albumin in PBS-Tween buffer. The detector antibodies were either horseradish - peroxidase labelled polyclonal sheep antibodies (see **Section 2.3**), or, where possible, biotinylated monoclonal antibodies which were detected with the streptavidin - peroxidase system. Colour was developed with an o-phenylene diamine substrate in a phosphate-citrate buffer with hydrogen peroxide. The plates were read at 492nm on a Titertek Multiscan Plus plate reader (Flow UK Ltd) and analysed using the Titersoft programme (Flow UK Ltd).

Methods

The methods used in the ELISA assays are shown in **Flow diagram 2.1** and the reagents are shown in **Table 2.2**. The antibody codes are given in **Section 2.3**.

Flow diagram 2.1 General principles of "sandwich type" ELISA procedures used for detection of serum complement components

CAPTURE	Antibody coating onto Dynatech IMMULON plates (overnight at $4^{o}C$)	
WASH	4 x in PBS-Tween	
BLOCK	0.3% BSA in PBS-Tween (2 hours at room temp.)	
WASH	1x PBS-Tween	
SERA	Sera diluted in PBS-Tween (2 hours at room temp.)	
WASH	4x PBS-Tween	
DETECTION ANTIBODY	**HRPO labelled**	**Biotinylated**
INCUBATION	Antibody diluted in PBS-Tween (1 hour at room temp.)	
WASH	4 x in PBS Tween	
STREPTAVIDIN PEROXIDASE	N.A.	Dilute in PBS-Tween (1 hour at room temp.)
WASH	N.A.	4 x PBS-Tween
SUBSTRATE	OPD in phosphate/citrate H_2O_2 (15-30 minutes in dark at room temp.)	
STOP	4N H_2SO_4	
COLOUR	Absorbance 492nm	

Table 2.2 Specific methods for sandwich type ELISA procedures used to measure serum concentrations of complement components

	Factor B	properdin	Factor H	Factor I	C4	C3
Capture Antibody	1/500 AHP 039H	1/1000 ATAB 032-03	1/2000 ICN 60-209-1	1/1000 AHP 036 H	1/2000 AHP 032 H	1/2000 AHP 031 H
Washes	5 x PBS-Tween					
Block	0.3% BSA in PBS-Tween					
Washes	1 x PBS-Tween					
Standard curve doubling dilutions starting from	1% of pooled serum standard	10% of pooled serum standard	1% of pooled serum standard	20% of pooled serum standard	1% of pooled serum standard	1% of pooled serum standard
Dilutions of test serum	0.1%	1%	0.1%	2%	0.1%	0.1%
Washes	5 x PBS-Tween					
Detector antibody	1/4000 HRPO AHP 039 P	1/4000 biotinylated HYB 3-3	1/2000 biotinylated OX 24	1/2000 HRPO AHP 036 P	1/4000 HRPO AHP 032 P	1/4000 HRPO AHP 031 P
Washes	5 x PBS-Tween					
Streptavidin peroxidase	N.A.	1/4000	1/4000	N.A	N.A.	N.A.
Washes	N.A.	5 x PBS-Tween	5 x PBS-Tween	N.A	N.A.	N.A.
Substrate	OPD in Phosphate/Citrate with H_2O_2					

Note: N.A. Stands for not applicable.

2.7 Classical and alternative pathway erythrocyte lysis assays

Principle

The assays most commonly used to measure the activity of the CP and AP in sera measure lysis of erythrocytes stabilised in agar gels (Lachmann and Hobart 1978, Truedsson et al 1981). For assays of total haemolytic complement (CH50), sheep erythrocytes are treated with anti-species immunoglobulin to produce the antibody/antigen immune complex which activates the CP. The AP is not activated by sheep erythrocytes due to the presence of sialic acid residues on the erythrocyte surfaces. AP assays commonly use rabbit or guinea-pig erythrocytes. The AP can be activated by agar and MgEGTA buffers and guinea-pig erythrocytes immobilised in the agar are lysed by "bystander lysis " in this system.

2.7.1 Total haemolytic complement

Antibody-bound sheep erythrocytes were generated as follows: 1ml of SRBC (Tissue culture services) was washed in CFD buffer and resuspended in 10ml CFD. 50 μl of rabbit anti sheep antiserum (Wellcome) were added and the suspension was incubated at 4^{o}C with mixing for 45 minutes. The cells and attached antibody were washed twice in CFD to remove unbound antibody and the final pellet was resuspended in 1 ml CFD.150 μl of the cell suspension were mixed with 12 ml of 1.5% Noble agar (Oxoid UK Ltd) in CFD at 45^{o}C and the resulting mixture was poured onto a level 10 x 10cm lantern slide. Wells of 1mm diameter were punched in the agar on the lantern slide and 20μl volumes of the standard serum dilutions and the test sera were added to these. The standard serum pool was diluted in CFD from neat to 1/32 in doubling dilutions. Test sera were diluted 1/2 in CFD. The slides were incubated at 37^{o}C overnight and the zones of haemolysis were measured using a calibrated loupe.Results were calculated by plotting the $\log_{10}$ of the dilutions of the standard serum against the diameters of the haemolysed rings in order to construct a standard curve. The CH50 of each test serum was calculated by comparing the diameter of the zone of haemolysis with the standard curve.

2.7.2 Alternative pathway lysis plate assays

10% guinea pig erythrocytes (2.5 x10^{9} cells/ml) (Tissue culture services) were washed three times in PBS, pelleting the cells between washes by centrifugation for 10 minutes at 1000g. The final pellet was resuspended in 10ml PBS containing 7mM $MgCl_2$ and 10mM EGTA and was mixed with ICN agarose which had been melted

and cooled to 56°C .The cells, suspended in 1.2% agarose were poured onto a 10 x 10 cm lantern slide. The slide was left to set and stored at 4°C until use.3mm diameter wells were cut and 7 μl serum samples were loaded into these. A standard serum pool was diluted in PBS from neat to 1/32 and 7μl aliquots of each dilution were loaded. The slides were incubated overnight at 4°C followed by 1 hour at 37°C. The diameter of the zones of haemolysis produced by the sera were measured and the activity of the alternative pathway of each serum was calculated by comparison with the standard curve drawn of the $\log_{10}$ of the dilutions of the standard serum pool.

2.8 The Functional opsonic (C3c elution) assay

Turner et al (1981) established a direct assay for measuring C3 derived opsonins on zymosan surfaces - the "C3c elution assay". This measured C3c fragments released by trypsin treatment from C3b/C3bi fragments bound to zymosan. This assay correlated with the Coulter counter assay of Levinsky et al (1978)(r_S = 0.87 $P<0.001$) which in turn had been shown to correlate with the slide opsonisation test (Miller et al 1968) (r_S = 0·87 $P<0.001$).(see **Flow diagram 2.2**).

Flow diagram 2.2. The stages of the C3c elution assay.

16.67% serum in VBS^{++} mixed with approximately $2x10^6$ zymosan particles

↓

Incubate 30 minutes 37°C

↓

Stop reaction with 0.1M EDTA in VBS

↓

Wash three times with 0.01M EDTA in VBS

↓

Add diluted trypsin (2% solution in 0.01M EDTA in VBS)

↓

Load zymosan and trypsin into wells of RID plates

↓

Measure precipitin rings

Method

10 μl of a zymosan suspension (approximately $2x10^8$ particles per ml in PBS /0.02% sodium azide) were incubated in glass bacteriological tubes for 30 minutes at 37°C with 50 μl of human serum diluted to 16.67% in 240 μl of VBS^{++}. The deposition of C3 fragments on the particles was terminated by the addition of 1ml ice-cold VBS/ 100mM EDTA, pH 7.4. The zymosan pellets were washed three times with 1ml volumes of PBS / 10mM EDTA. After washing, the particles were partially dried and incubated at 37°C for 15 minutes with 10μl trypsin diluted to 2% in VBS/ 10 mM EDTA. The total contents of each tube were then added to the well of a single radial diffusion plate (RID) containing sheep anti-human C3c antiserum (Scipac; Sittingbourne, Kent) at a final dilution of 1/2000. After diffusion overnight at room temperature in a humid atmosphere, C3c fragments were quantitatively measured with reference to a C3 standard. This standard was set up by serially diluting a 20% stock of a serum pool in PBS/ 10 mM EDTA and adding this to sequential wells in the RID plates. It was possible to calculate a binding coefficient for each sample by including in every assay a serum known to give high levels of C3b binding to zymosan (HB1) and a serum known to give low levels of binding(LB1).

$$\text{Binding Coefficient (BC)} = 1 - \frac{HB1_{C3\%} - Test_{C3\%}}{HB1_{C3\%} - LB1_{C3\%}} \times 100\%$$

Comment

Variations in the binding coefficients (BC) calculated using similar formulae to the above have been used extensively in this thesis. In all cases the HB1 and LB1 refer to the same two healthy adult laboratory workers (see **Section 2.2**). In all tests where this calculation was used the LB1 serum gave values at, or just above background whereas the HB1 serum values were found to be amongst the highest values. The use of the binding coefficient calculation also facilitated the statistical comparisons of data from widely different assays, including some (see **Chapter 5**) where there were values high within the normal range which would have exceeded the upper limit of a standard curve used for calibration. Note that the calculation ascribes negative values to those binding coefficients which are less than that of serum LB1. The Clinical Immunology Laboratory at ICH used the C3c elution assay routinely and ascribed a threshold value of 15% Binding Coefficient. Sera with values below this threshold were regarded as having poor opsonic function.

CHAPTER 3

INVESTIGATION OF FUNCTIONAL COMPLEMENT ACTIVATION AND LEVELS OF SELECTED COMPLEMENT COMPONENTS IN SERA OF DIFFERING OPSONIC POTENTIAL

3.1 Introduction

In 1976 Soothill and Harvey tested 4 sera from patients with poor opsonic function and compared the opsonisation indices (measured by a phagocytic assay) with classical and alternative pathway function (measured by haemolytic assays). Haemolytic complement activity was found to be within the normal ranges in these sera and this was confirmed by Kerr et al (1983) who found that the CH50 and C3 levels in sera with poor opsonic function were within the normal ranges. In 1977 Soothill and Harvey measured complement activation to inulin in sera which were poorly opsonic in the phagocytic assay and suggested that the opsonic defect lay in the alternative pathway.

In the present study it was important to establish whether there was any evidence that the opsonisation defect was due to low levels of a known complement component or to poor functioning of the AP or CP. A selected group of sera of known opsonic potential were assayed for alternative and classical pathway activity using the erythrocyte lysis assays, whilst the concentrations of known complement components were measured by ELISA assays.

3.2 Classical and alternative pathway erythrocyte lysis assays

Protocol

A small number of sera were tested using the CH50 and AP plate lysis assays and the results were compared with those obtained using the C3c elution assays.

Results

Two sera with poor functional yeast opsonisation (i.e C3c elution levels of 0% compared to a normal range of 15-140%) had levels of alternative pathway lysis activity within the normal range. One of the sera exhibited normal CH50 lysis whereas the other serum gave a value just below this range. In contrast serum H2, with yeast opsonisation within the normal range, showed a CH50 value below the normal range. The levels of C3 moieties bound to the zymosan surface were not correlated with the levels of CP and AP function measured by the erythrocyte lysis assays(See **Table 3.1**).

Table 3.1 C3c elution functional opsonisation assay and measurements of total complement activity (CH50) and alternative pathway activity in 7 selected sera.

NAME	C3c ELUTION	CH 50 *	AP LYSIS
LB1	0.00%	92%	90%
I15	0.00%	68%	110%
HB1	100.00%	116%	130%
HB2	90.48%	116%	130%
H2	76.19%	48%	90%
H4	104.76%	ND	130%
H5	123.81%	132%	110%

Normal ranges:

C3c elution :15 - 140 % (established at Institute of Child Health)
CH50 :70 - 130% "
AP Lysis :60 - 140% (established at Hammersmith Hospital)

* Assay kindly performed by Mr N. Seymour

Comment

These results confirm previous findings that there is no evidence of any profound abnormality in either classical or alternative pathway activity in sera with poor functional opsonisation.

3.3 ELISA assays

Introduction

The alternative pathway amplifies deposition of C3 on the surface of micro-organisms and there are many reported cases of deficiencies in AP components reducing levels of C3 opsonisation. Low levels of C3 (Kerr et al 1983), Factor D (Leijh et al 1986), properdin (Sjöholm 1982), Factor I (Perlmutter and Colten 1989) and Factor H (Thompson and Winterborn 1981) have all been shown to affect levels of C3 opsonisation.Therefore, it was necessary to establish that levels of these components were not low or low within the normal range in the sera with poor opsonic function. Sandwich ELISA assays were set up to measure concentrations of complement components in the 77 randomly selected sera from the population of healthy blood donors. These sera had been tested in the functional opsonic assay and 5 sera (6.5%) had poor opsonic function. The alternative pathway (AP) components C3, Factor B, properdin, Factor H and Factor I as well as the classical pathway component C4, were measured and the values obtained were compared with the C3c elution results.

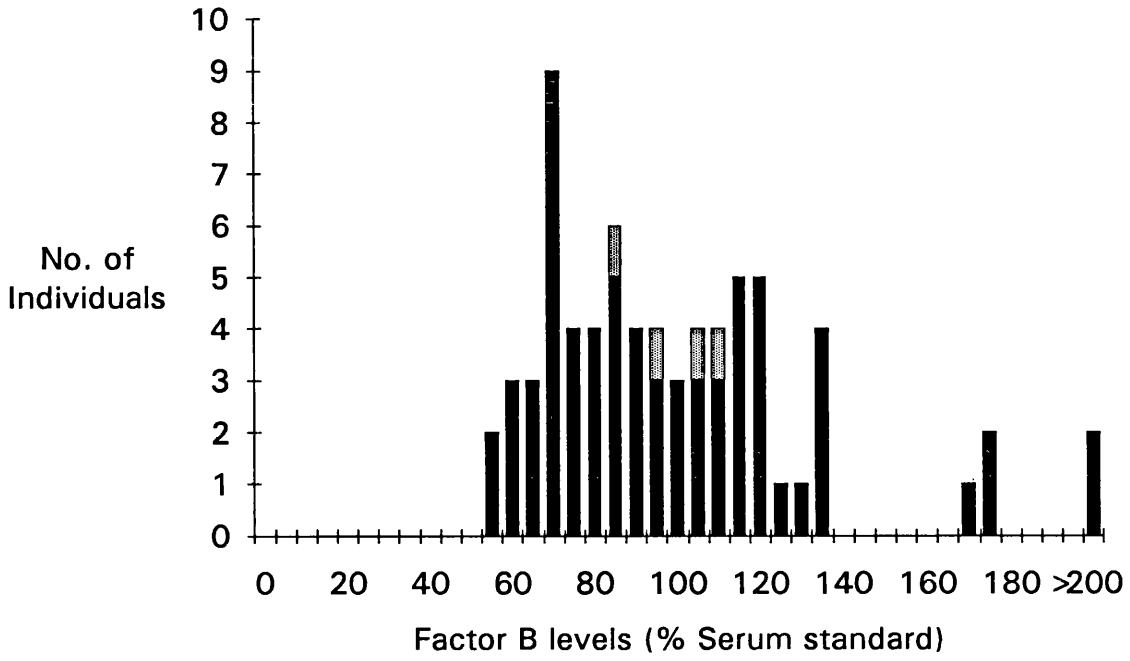
Methods

The methods used are described in **Section 2.6**. 73 out of the 77 sera were assayed for levels of properdin and Factors B, H and I. C3 and C4 assays were performed on 71 out of the 77 sera by Mr Seymour. 4 out of the 5 poorly opsonic sera were tested in these ELISA assays. Representative standard curves for each of these assays are illustrated in **Appendix 1**. Results were expressed as percentages of the pooled human standard (for properdin and Factors B, H and I) and as international units (for C3 and C4). Each serum was assayed in duplicate in all assays. The results from each well were compared with the duplicate well and only the results within 5% of the duplicate were used. A standard serum pool prepared from 10 healthy adult ICH staff members was included in each assay. The intra-assay variation of this pool had been established previously for all these assays and only the assays where the values of this pool were within the range : mean $\pm$ 1.5 x (intra-assay standard deviation), were used. These are standard conditions applied to the ELISA procedures used in the clinical Immunology laboratories, ICH.

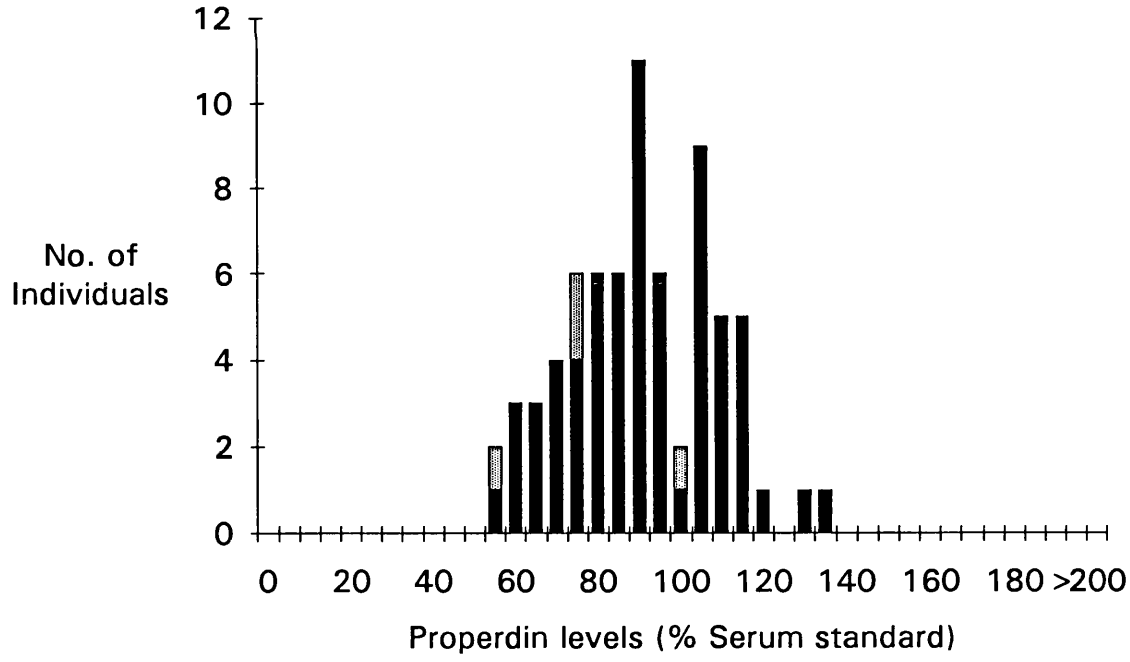
Results

Detailed results on the individuals tested can be found in **Appendix 2**. The distribution profiles for the 6 proteins in the population studied are illustrated in **Figure 3.1 (a)-(f)**.

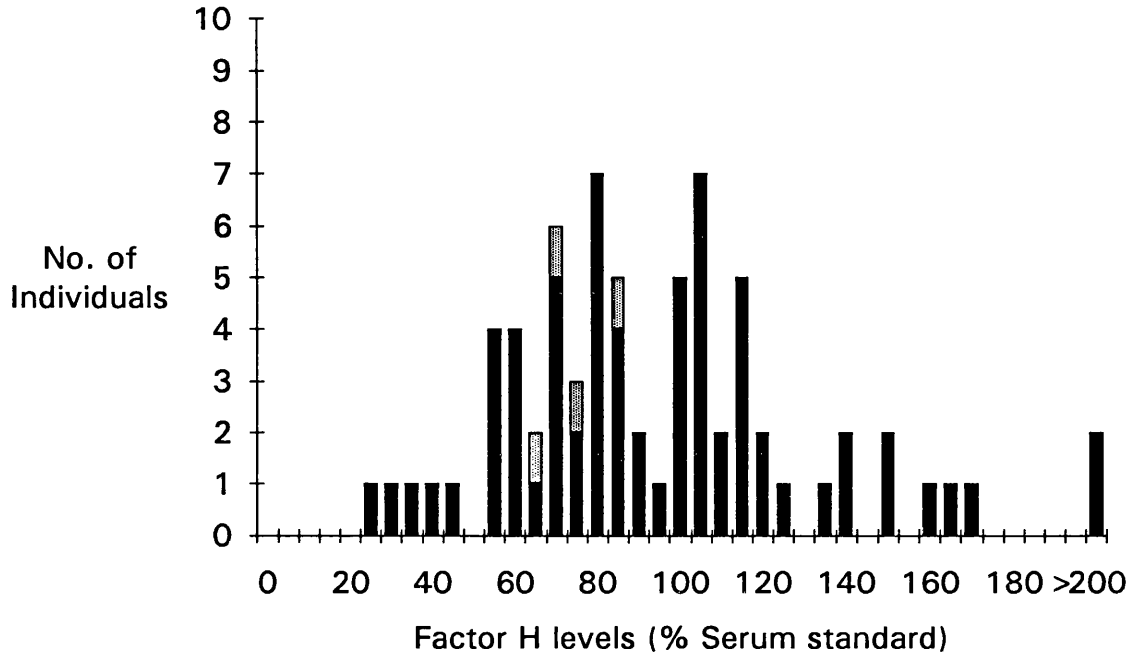
3.1(a)


No. of Individuals
0
1
2
3
4
5
6
7
8
9
10
0 20 40 60 80 100 120 140 160 180 >200
Factor B levels (% Serum standard)

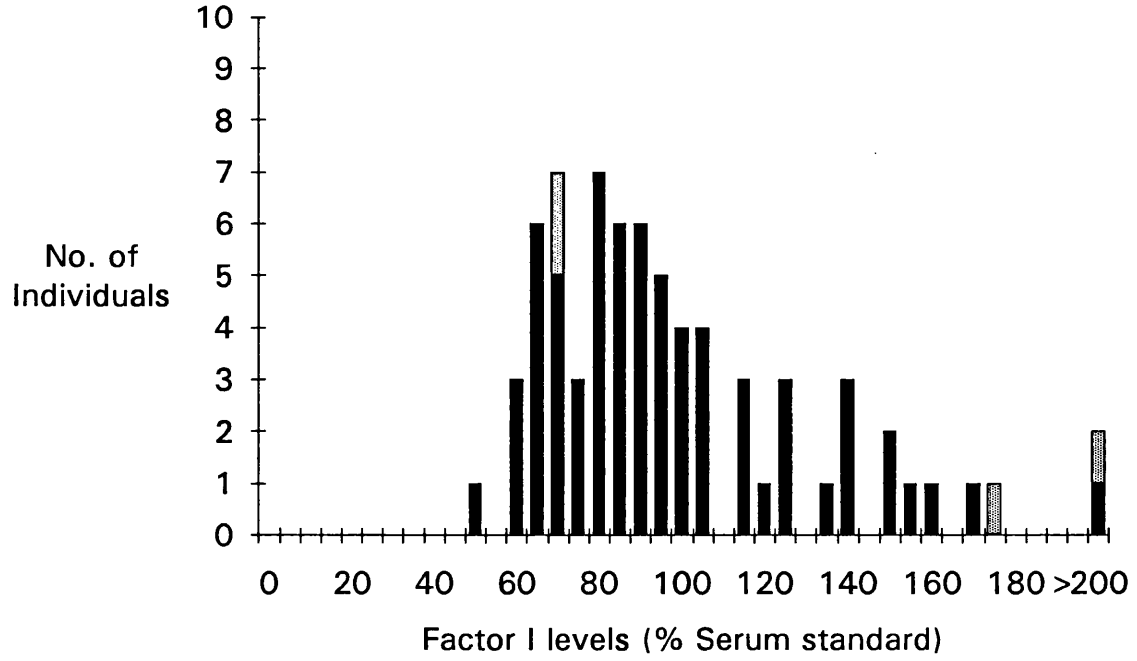
3.1(b)


No. of Individuals
0
2
4
6
8
10
12
0 20 40 60 80 100 120 140 160 180 >200
Properdin levels (% Serum standard)

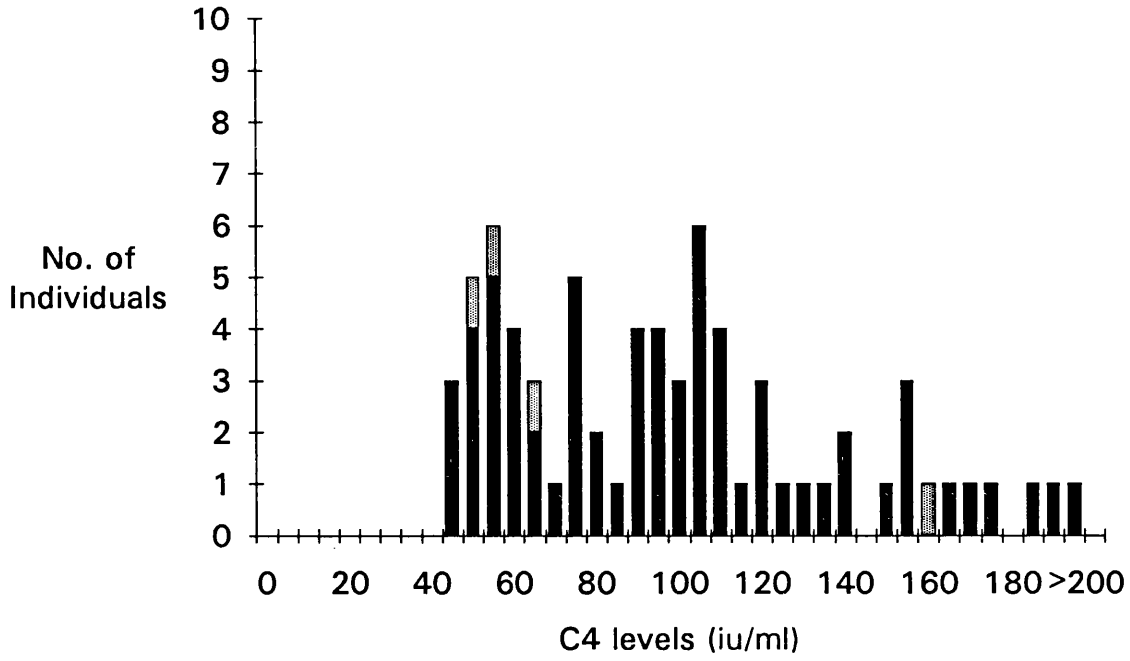
3.1(c)


No. of Individuals
10
9
8
7
6
5
4
3
2
1
0
0 20 40 60 80 100 120 140 160 180 >200
Factor H levels (% Serum standard)

3.1(d)


No. of Individuals
10
9
8
7
6
5
4
3
2
1
0
0 20 40 60 80 100 120 140 160 180 >200
Factor I levels (% Serum standard)

3.1(e)



3.1(f)

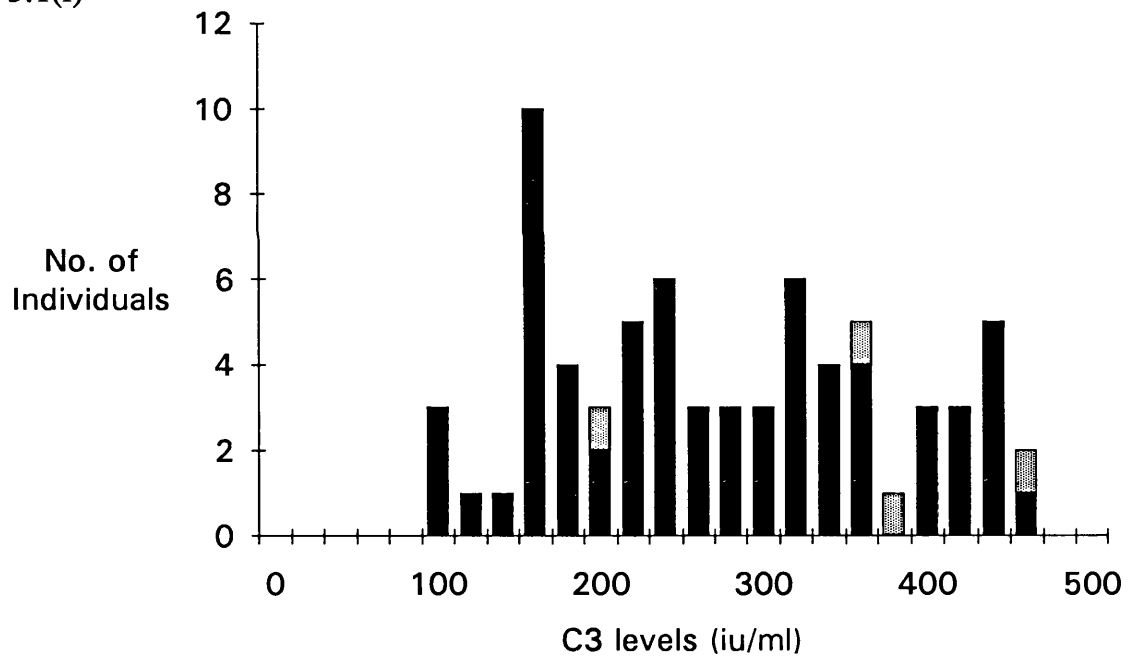


Figure 3.1 (a) - (f) Total levels of components Factor B, properdin, Factor H and Factor I (expressed as % of the values in the standard serum pool) in 73 sera from healthy adult blood donors and levels of C4 and C3 in 71 sera (expressed as iu/ml). The shaded boxes represent the sera previously shown to have defective opsonisation as measured by the functional C3c elution assay.

Comment

These experiments suggest that the yeast opsonisation defect is unlikely to be caused by deficiencies in activity of the alternative or classical complement pathways or by low concentrations of critical complement components. There was no evidence to support the view that two different components were low in individuals with the defect, since the four sera with poor opsonic function would have been expected to group at or near the lowest levels of the distribution profiles if this was the case (see **Figures 3.1 (a)-(f)**). On the contrary, with the exception of properdin and C4, in each assay 2 or more of the poorly opsonic sera had levels above the median value. No quantitative assays were carried out for Factor D. However, it is an essential component of the alternative pathway and the normal functional activity observed in AP lytic assays (in this study as well as in previous reports) suggest that the levels of this protein were not limiting in the sera studied.

3.4 Allotyping

Introduction

Different allelic forms of many complement components have been described. The yeast opsonisation defect could theoretically be linked to the presence of an allelic form with impaired functional activity. Candidate proteins for such allelic variation would include C4, C2, C3, Factor B and Factor D. A further possibility would be the total absence of all allelic variants from one of the duplicated C4 loci. For example, it is known that the C4b fragments generated by individuals homozygous for C4B null alleles bind poorly to carbohydrate surfaces because only the C4A loci are translated to protein and C4b of the C4A isotype does not bind to carbohydrates as avidly as the C4b of the C4B isotype.

Methods

7 selected sera from individuals with the opsonic defect were sent to Dr Susan Cross (Department of Genetics, University of Oxford) for C3 and Factor B allotyping; these included I 10, I 11 and I 12, three members of the same family.

Results

All the sera tested had common C3 allotypes and there was no evidence of any association between the yeast opsonisation defect and a dysfunctional C3 allotype. However, two siblings I 10 and I 12 as well as serum LB1 had the relatively rare FS0.7 Factor B allotype.

Table 3.2 Factor B and C3 allotyping in 7 selected sera from individuals with defective yeast opsonisation.

Serum sample	Factor B	C3
I 10	FS0.7	SS
I 11	SS	SS
I 12	FS0.7	SS
I 16	SS	SS
I 17	FS	FS
I 18	SS	SS
LB1	FS0.7	SS

Note: The F allotype represents the faster migrating band on SDS-PAGE while the S allotype represents the slower moving band.

3.5 Discussion

The limited studies reported in this chapter provide no evidence for the view that poor yeast opsonisation arises from a dysfunction of either an alternative or classical pathway complement component. Moreover, assays measuring serum concentrations of the components properdin, Factor B, Factor H and Factor I produced no evidence that the defect was due to very low levels of any of these components or of levels which were low within the normal range (see **Appendix 2** for individual protein profiles). These observations confirm and extend the previously published studies of these aspects (Soothill and Harvey 1976, Turner et al 1985 (a)).

The allotyping studies were, of necessity, incomplete. For example, polymorphisms of components such as properdin, Factor H and Factor I are either undescribed or only partially described and therefore could not be studied. Using seven selected sera no evidence was found of any association with polymorphic variants of the two major complement components C3 and Factor B (despite the presence of a rare allele of Factor B in three individuals). Subsequently a collaborative link was established to study C4 allotypes in isolated DNA from 5 individuals with the defect, but this was abandoned when the nature of the defect was established.

In the light of the above findings the following investigations centred on the deposition of complement moieties on zymosan surfaces.

CHAPTER 4
THE USE OF ZYMOSAN AS A BINDING SUBSTRATE IN STUDIES OF SERA OF DIFFERING OPSONIC POTENTIAL

4.1 Introduction

In a series of publications Turner et al (1981,1985(a)&(b)) described the use of trypsin to release C3c fragments from surface bound C3b/C3bi moieties on zymosan previously incubated with serum - the so called "C3c elution assay". This procedure was shown to correlate well with other assays of opsonisation such as the microscopic slide opsonisation test used by Soothill and Harvey (1976) and the Coulter Counter assay of Levinsky et al 1978). Using this assay Turner et al (1981) showed that sera with normal opsonic activity bound C3b moieties to the zymosan much more rapidly than sera with poor opsonic function and studies using MgEGTA buffers suggested that both CP and AP contributed to the deposition of C3b. A modification of this assay was also described (Turner et al 1985 (a)&(b)) which permitted correction of the opsonic defect by both intact serum and chromatographic fractions. These studies suggested that there was probably a single defect associated with the absence of a critical factor. The putative factor was shown to bind avidly to zymosan at 4°C and to lose its correcting activity when heated to 56°C (but not 50°C). Gel filtration studies indicated a molecule eluting between IgG and albumin (~ 70-80 kDa).

In the studies described in this Chapter, the previous findings were refined and extended using improved assay procedures.

4.2 C3c elution assay

Protocol

A detailed description of the method is given in **Section 2.7.**

4.2.1 The use of different assay buffers in the C3c elution assay

Introduction

In the C3c elution assay, (Turner et al1985a) sera were tested at 16.67% concentration in buffers(such as VBS++ or CFD) containing magnesium and calcium ions and capable of supporting both alternative and classical pathway complement activation. Calcium ions are required for assembly of C1 and therefore buffers such as MgEGTA which contain magnesium ions but not calcium ions permit activation of the alternative but not the classical pathway while the chelator EDTA prevents activation of either pathway. Asghar et al (1987) showed that the alternative pathway convertase(C3bBbP) could be formed in 2mM $MgCl_2$,10mM EGTA, but not in 0·2mM $MgCl_2$,10mM EGTA. These buffers were used in an attempt to

establish whether differences between sera with normal and poor opsonic function could be attributed to differences in alternative pathway function.

Experiment

The C3c elution assay protocol was followed with the exception that the zymosan and sera were incubated in either PBS containing 0.8mM $MgCl_2$ and 0.25mM $CaCl_2$ (the same concentrations as in the CFD buffer), PBS containing 2mM $MgCl_2$ and 10mM EGTA, PBS containing 0.2mM $MgCl_2$ and 10mM EGTA or PBS containing 40mM EDTA.

Results

The sera incubated in PBS containing 0.8mM $MgCl_2$ and 0.2mM $CaCl_2$ bound much more C3 from HB1 than from LB1 and accordingly these were set at 100 and 0 (BC%) respectively. Incubation of the HB1 and LB1 sera in EDTA or 0.2 mM Mg^{++} and 10mM EGTA prevented any binding of C3 to the zymosan. Incubation in 2mM Mg^{++} and 10mM EGTA showed very little binding, with slightly more C3 bound from HB1 than LB1 . (See **Figure 4.1).**

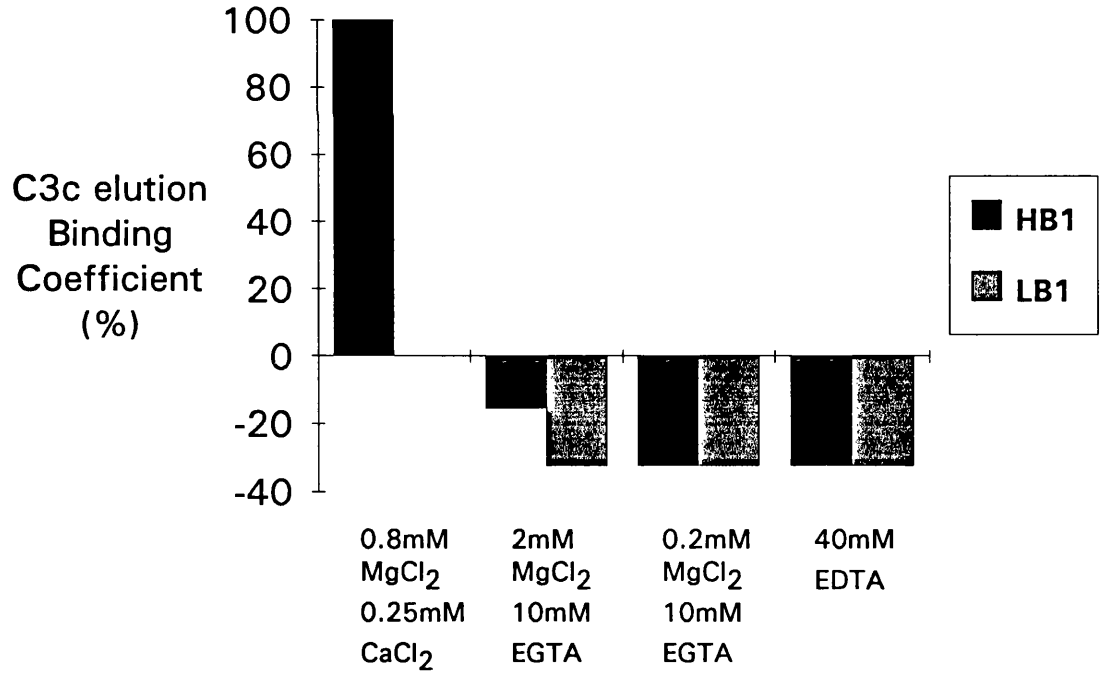


Figure 4.1 C3c elution assay in buffers supporting CP and AP ($MgCl_2$ and $CaCl_2$), AP only (MgEGTA) or not supporting complement activation (EDTA).

Comment

In Mg EGTA buffers the differences previously seen with buffers containing magnesium and calcium were no longer evident and the discrimination between the HB1 and LB1 sera was removed. These findings suggest that the differences between the HB1 and LB1 binding coefficient values which were observed in PBS^{++}, (as well as in CFD and VBS $^{++}$ which are not shown here) involved the classical rather than the alternative complement pathway. There was even greater discrimination between the amounts of C3 bound to zymosan from sera with either normal or poor opsonic function in the buffer VBS^{++} (containing 5mM MgCl2 and 5mM CaCl2) and therefore VBS^{++} was used in subsequent C3c elution assays.

4.2.2 Assays of sera obtained from a population of apparently healthy adults

Protocol

A random selection of 77 serum samples from 179 blood donors (described in **Section 2.2**) were tested by Ms L.A. Alterman of the Clinical Immunology lab at ICH using the C3c elution assay. The binding coefficients of the sera were calculated by comparison with results from the HB1 and LB1 sera included in each assay.

Results and Conclusion

The plot of number of individuals, against the binding coefficient values (see **Figure 4.2**) shows a skewed distribution. The mean, standard deviation and median values for this population were 80.66 ± 42.51 and 80.95 respectively. Five individuals of the 77 (6.5%) showed evidence of poor C3b opsonisation with binding coefficients of less than 15%, these sera were well separated from the lowest values of the rest of the population (normal range 15-140%). These sera are highlighted with grey shading in **Figure 4.2**. These findings concur with the previously reported data (Soothill and Harvey 1976, Kerr et al 1983) that the incidence of poor yeast opsonisation is between 5 and 8% in the normal adult population.

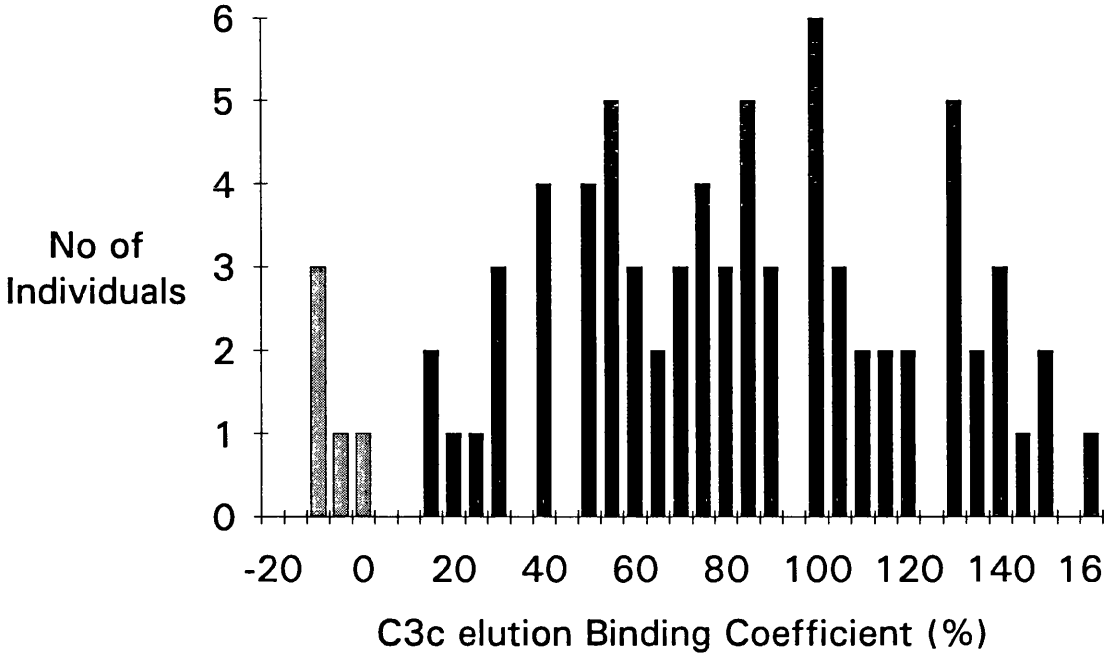


Figure 4.2 Measurement of C3b binding coefficients using the C3c elution assay to study serum samples from 77 healthy adults. The bars highlighted in grey represent sera with binding coefficients below the 15 % threshold.

4.2.3 Assays of sera obtained from a population of hospitalised children.

Introduction

Serum samples from 335 patients attending for immunology or allergy consultations at Great Ormond Street Hospital (GOSH) between January 1987 and August 1989 were also analysed using the C3c elution assay. The test was requested by the consultants in these two clinics as part of the general laboratory investigations and were carried out by Mr N. Seymour of the Clinical Immunology Laboratory at ICH.

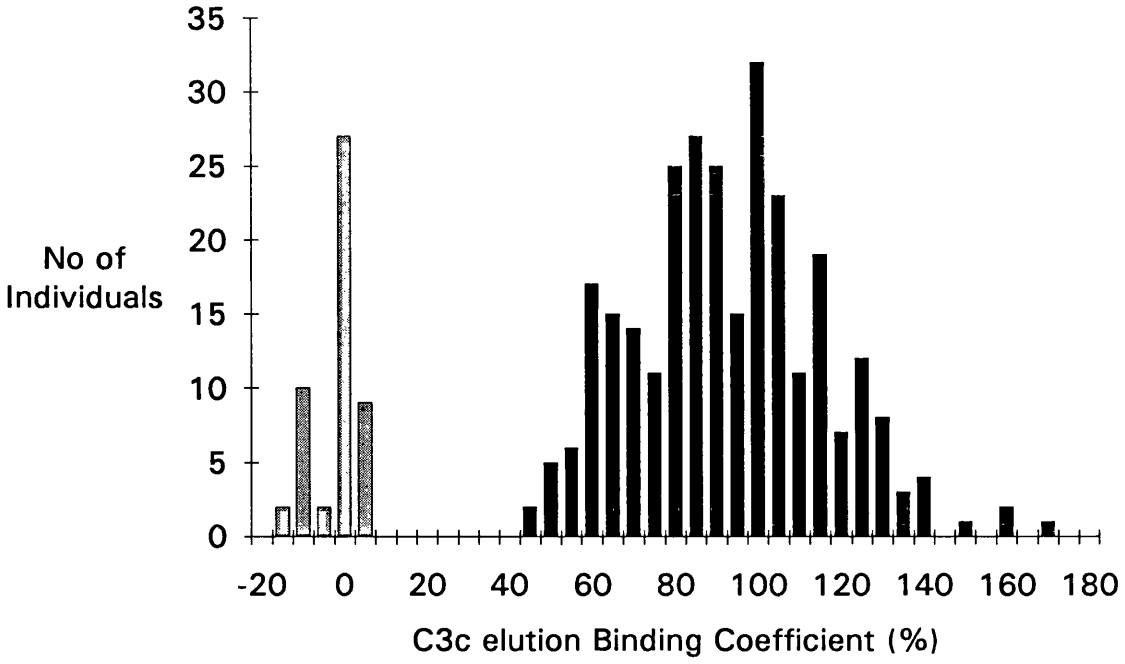


Figure 4.3 Measurement of C3b binding coefficients using the C3c elution assay in sera obtained from 335 children attending Great Ormond Street Hospital. The sera with binding coefficients below the 15 % threshold are again highlighted in grey.

Results

The distribution of the sera in this assay appears bimodal, with extremely good discrimination between the two peaks (see **Figure 4.3.**). In this population the mean, standard deviation and median values were 80.60± 39.87 and 89.47 respectively. These values are very similar to the values for the normal adult population (quoted in **Section 4.2.2**). However, 50 out of the 335 patient sera tested (ie 14.93%) had binding coefficient values below 15% as opposed to the 6.5% in the normal population tested.

Comment

14.9% of these patients showed poor yeast opsonisation, which is more than double the 6.5% found in the normal healthy adult population and this confirms the previous findings (Soothill and Harvey 1976, Turner et al 1978, Candy et al 1980) that the incidence of poor yeast opsonisation in populations of hospitalised children (presenting with immunological complaints or allergy) is higher than in the normal adult population. The Chi -squared test applied to these populations with a threshold at 15% binding coefficient, showed that the possibility that the two samples came from the same population was 7.6% at 1 degree of freedom.

4.3 C3c elution Correction assays

Introduction

Correction assays are frequently used in studies of complement protein deficiencies. Small amounts of normal sera or of proteins, suspected to be absent from or dysfunctional in the immune deficient serum, are added in a dose response fashion and the functional activity of the mixture is measured. Here correction assays have been used in order to investigate whether or not defective opsonic function might be due to the absence of a previously unrecognised essential factor or to low levels of a known complement component.

4.3.1 Studies using whole sera

Protocol

The correction assays were carried out essentially as described for the direct assays. 50μl volumes of LB1 serum were mixed with increasing volumes (5-25μl) of HB1 serum or LB2 serum and VBS^{++} was added to give a final volume of 300μl. Thus the concentration of the LB1 serum was 16.67% whereas the concentrations of the correcting sera ranged from 1.67 to 6.66%.

Results

The addition of 4.91% and 6.55% of HB1 to serum LB1 used at 16.67% raised the binding coefficient to 100% , whereas 6.55% HB1 incubated without the LB1 serum gave a binding coefficient of only 5%. In contrast 6.55% LB2 serum mixed with the 16.67% LB1 gave only a 10% binding coefficient (see **Figure 4.4.**)

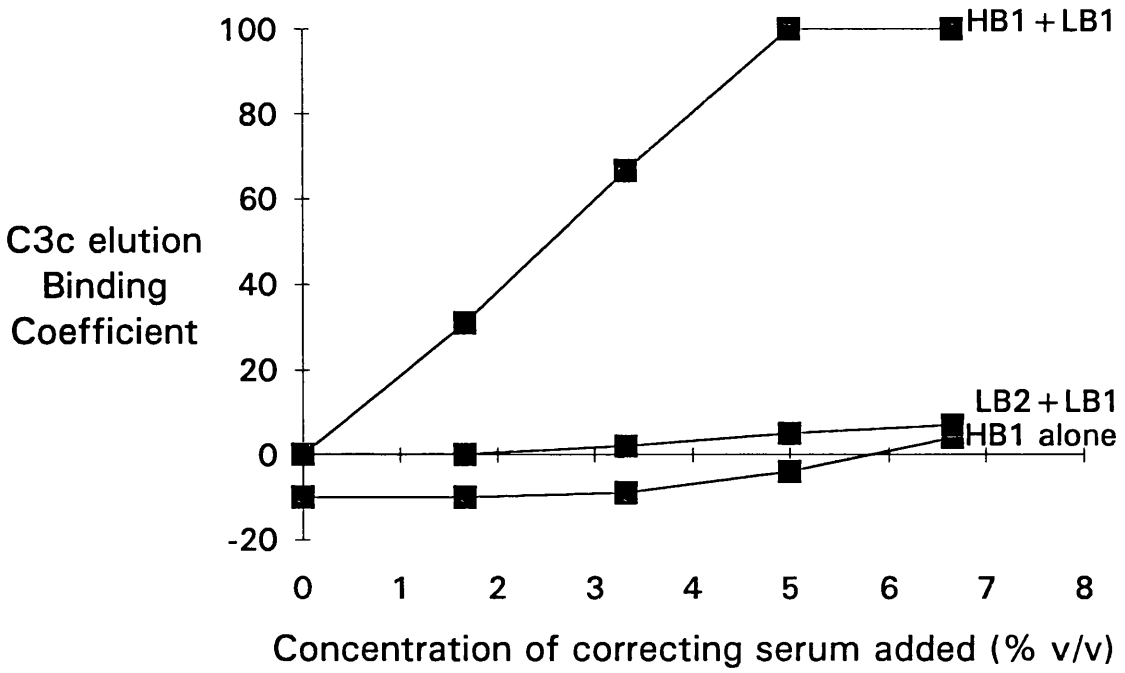


Figure 4.4 Attempted correction of the defect in poorly opsonic serum LB1 using increasing volumes of HB1 serum (normal function) or of LB2 serum (poor opsonic function).

Comment

When low concentrations of HB1 serum were mixed with LB1 serum, the amount of C3 binding to the zymosan surface was far greater than the C3 bound from either serum used alone at these concentrations. Thus it appeared that the HB1 serum contained factor(s) which promoted the binding of the C3 from the LB1 serum, thus "correcting" the defect. Titration of LB2 serum into the LB1 serum did not show this correcting effect, which suggested that the LB1 and LB2 sera probably shared a common defect and also that the correcting effect was not simply due to an increase in C3 concentration in the assay. These finding confirm the earlier observations of Turner et al (1985a).

4.3.2 Studies using KSCN treated serum

Introduction

C3 and C4 molecules contain reactive thiolester groups which can be transiently activated. When these are in the active state in the presence of carbohydrate surfaces, the molecules bind covalently to these via ester links (Law and Levine 1977). Reagents such as KSCN or NH_4OH which inactivate the thiolester in the fluid phase thus prevent subsequent binding to such surfaces (Whaley 1985).

Experiment

HB2 serum samples were dialysed at 4^{o}C for 4 hours against either a solution of 0.5M KSCN or PBS. Following this treatment the samples were dialysed overnight against two changes of PBS. In each dialysis step the ratio of buffer to serum was 200:1. The effect of these treatments was measured in the C3c elution and correction assays. In the correction assays the treated sera and serum LB1 were diluted to 5% and 16.67% respectively in CFD.

Results

HB2 serum treated with KSCN did not bind C3 to the zymosan surface in direct assays (see **Figure 4.5 panel (b)**). However KSCN treatment did not abrogate the ability of serum HB2 to **correct the opsonic defect** (see **Figure 4.5 panel (a)**) and HB2 serum treated with KSCN retained more correction activity than when treated with PBS. Thus it appeared that neither C3 nor C4 moieties could be responsible for the correcting activity of the HB2 serum.

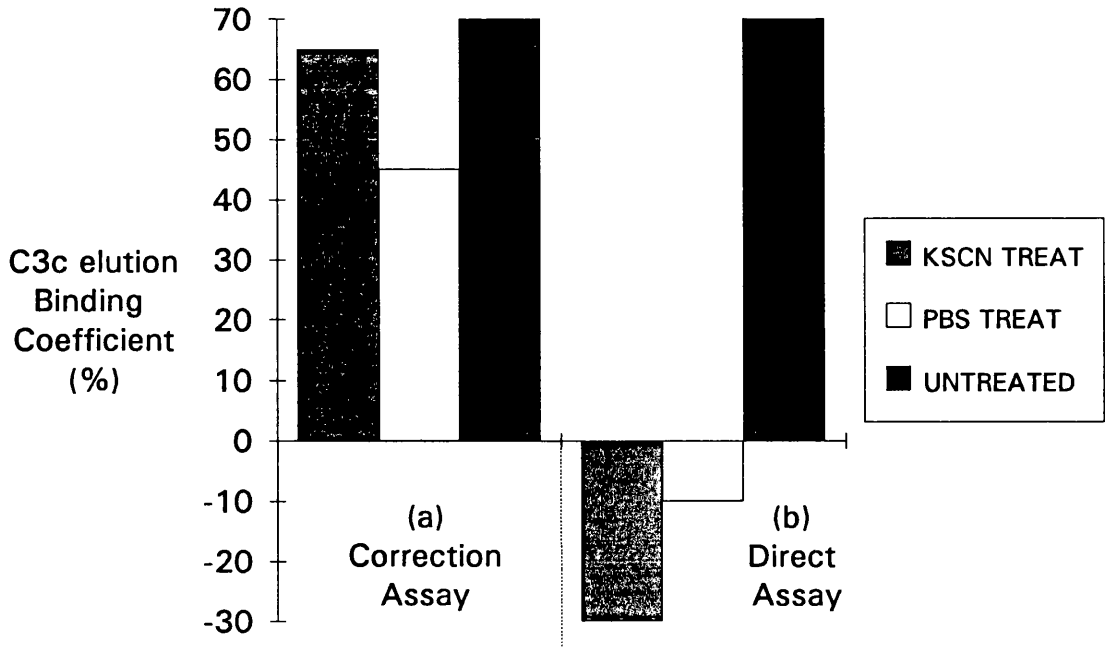


Figure 4.5 Correction of poor opsonic function in serum LB1 by addition of KSCN treated normal serum.

Comment

In this experiment the ability of the C3 and C4 to bind to the zymosan surface was destroyed and yet the potential of the HB2 serum to correct the opsonisation defect in the LB1 serum was unimpaired. This strongly suggested that the correcting factor was not C3 or C4. The use of a C3 deficient serum (See **Section 4.3.5**) confirmed this finding.

4.3.3 Studies using sera from laboratory animals

Protocol

Sera from laboratory animals were used in correction assays in order to investigate whether an animal equivalent of the human correcting factor could cross species barriers and correct the opsonisation defect in human sera. Rabbit and pig sera obtained from healthy laboratory animals undergoing no treatment, were aliquoted and stored at -70^{o} until use. The sera were thawed and assayed both in C3c elution assays and in correction assays with LB1 serum. In the correction assays the sera were titrated into serum LB1 diluted to 16.67% in CFD.

Results

Animal C3 moieties were not detected on the surface of the zymosan using the anti-human C3c antibody (Scipac) although such fragments were presumably bound. However, when these animal sera were mixed with LB1 serum the deficiency was corrected in the human serum and human C3 became bound to the zymosan surface. (See **Figure 4.6**).

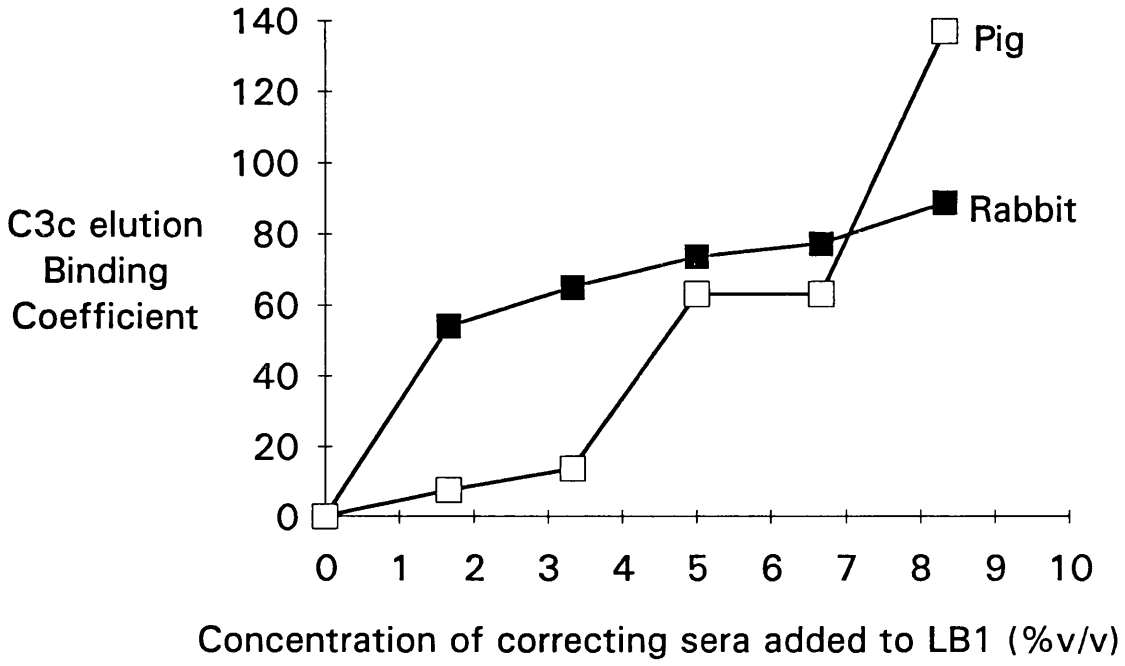


Figure 4.6 Dose dependent correction of poor opsonic function in human serum LB1 by addition of serum obtained from pig and rabbit

Conclusion

These results suggested that a factor or protein of analogous function was present in the mammalian sera tested and that this was functionally able to cross species barriers, correcting human serum with poor opsonic function by promoting the binding of the **human C3** in the LB1 serum onto the zymosan surface.

4.3.4 Studies using heat-treated sera

Introduction

Many of the complement components are thermolabile (Whaley 1985) and their enzymic activities can be abrogated by maintaining the serum at defined temperatures. In particular Factor B is sensitive to heating at 50°C whereas the initiating antibody (IgG) of the classical pathway activation is resistant even to heating at 56°C.

Experiment

HB2 and rabbit sera were held for 30 minutes at 4°C, 50°C or 56°C. The treated sera were then mixed with LB1 serum and assayed in the standard correction assay.

Results

The treatment of the sera at 4°C and 50°C did not affect the correcting potential of the human or rabbit sera (see **Figure 4.7**), and the binding coefficient values of these were consistent with the values for untreated sera (data not shown). In contrast the 56°C treatment completely abrogated the correcting potential of the sera.

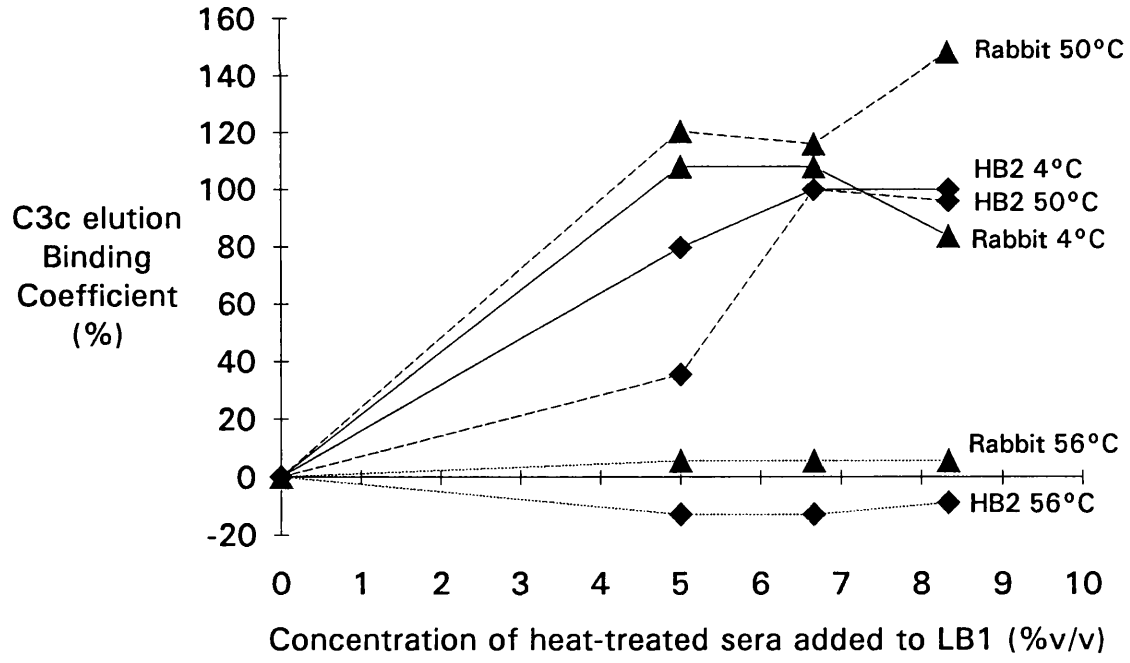


Figure 4.7 Correction of poor opsonic function in serum LB1 using various heat treated sera.

Conclusion

The heat treatment studies suggested that the correcting factor was not Factor B, which is sensitive to 50°C treatment, nor IgG, which is resistant to 56°C. Once again the animal and human sera appeared to behave in similar fashion suggesting that the rabbit serum contained an analogue of the human factor.

4.3.5 Studies using human sera lacking various complement components

Protocol

In a further attempt to establish the identity of the factor responsible for the correction of the defect, sera deficient in C3, properdin or Factor D, were assayed for their ability to correct the opsonic dysfunction in LB1 serum. The C3 deficient serum was kindly provided by Dr Abol Farhoudi (Tehran) and the C3 deficiency was independently confirmed by Dr R. A. Thompson (Birmingham). The properdin deficient serum was kindly provided by Dr A. G. Sjöholm (Lund) from a family with a history of infections with *Neisseria meningitidis* and the properdin deficiency had been established by ELISA and confirmed by alternative pathway erythrocyte lysis assays. The normal mean human properdin concentration in serum is 19-25mg/l whereas this serum had less than 0.1mg/l of properdin. Serum from a Factor D deficient individual was kindly provided by Professor M. Daha (Leiden). Factor D ELISA assays and functional Factor D titration assays (Leijh et al 1986) had shown that this serum contained no Factor D.

Results

Using C3c elution correction assays sera lacking the components C3 and Factor D were able to correct the opsonisation defect in the LB1 serum, whereas the properdin deficient serum did not correct the defect (see **Figure 4.8**). In direct C3c elution assays there was little C3 bound to the zymosan from any of the deficient sera (data not shown).

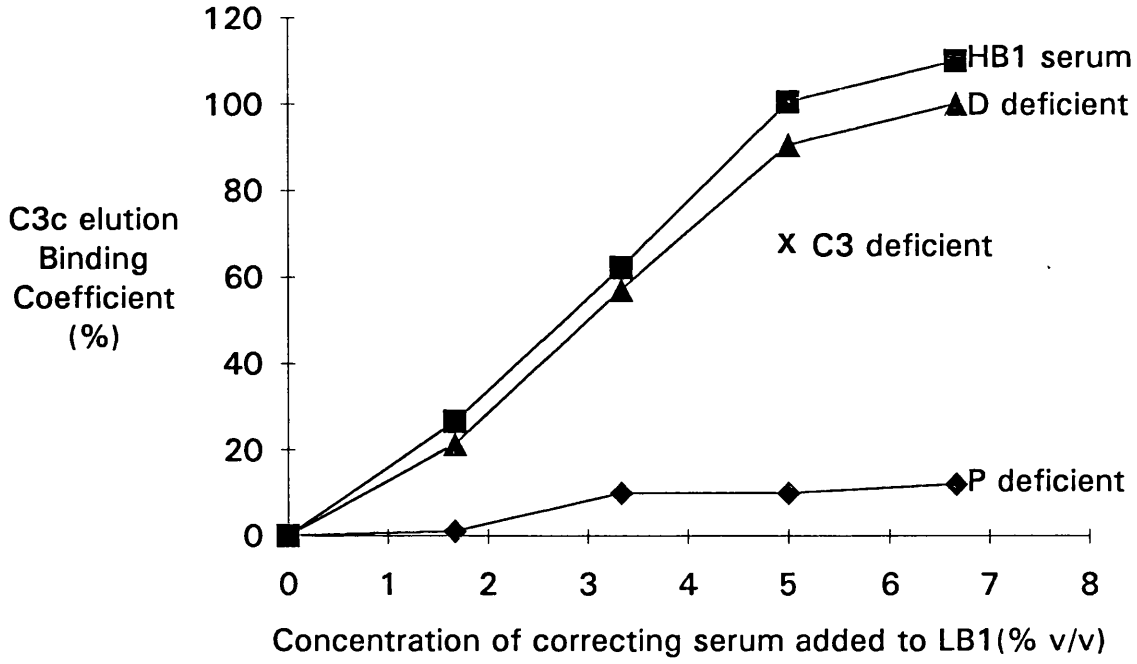


Figure 4.8 Attempted correction of defect in opsonic serum LB1 using various human sera. The volume of C3 deficient serum available was sufficient for only one measurement at 5%.

Conclusion

These experiments suggested that the correcting moiety was neither C3 nor Factor D. However the results with the properdin deficient serum suggested either that properdin might be the factor involved or, if not, that there was a co-existing molecular defect in this serum.

4.3.6 Studies using purified complement components

Introduction

In view of the results reported in **Section 4.3.5** it was necessary to ascertain whether properdin was the factor missing from poorly opsonic sera. Accordingly a properdin rich preparation was made using an affinity column and this material was added to LB1 in a correction assay. The alternative pathway control proteins Factor H and Factor I were similarly purified and mixed with the LB1 serum in an attempt to correct the opsonisation defect.

Experiment

Properdin was purified from pooled human serum by affinity chromatography (see **Chapter 2)** using the monoclonal antibody HYB 3-3 kindly supplied by Dr C.Koch (Copenhagen). Affinity purified Factors H and I were prepared using anti-Factor H (MRC Ox 23) and anti-Factor I (MRC Ox 21) respectively, these reagents were kindly supplied by Dr. R.B. Sim (Oxford). The eluted fractions were dialysed into PBS and were diluted to give concentrations of the protein equal to or greater than those found in normal human sera. The mean concentrations of properdin, Factor H and Factor I in normal human serum were taken to be 19-25μg/ml, 133 μg/ml and 30-50μg/ml respectively (Rother and Rother 1986).15μl of serum with normal opsonic function would thus contain approximately 0.375μg of properdin, 2μg of Factor H and 0.6μg of Factor I. The amounts of the purified components added were 0.375 and 3.75μg of properdin,2μg and 6.99μg of Factor H, and 0.6μg and 3.72μg of Factor I. These purified proteins were mixed with the LB1 serum in standard correction assays.

Results

None of the three complement components properdin, Factor H or Factor I, tested at the above concentrations, was able to correct the opsonic deficiency in LB1 serum (data not shown), whereas intact serum HB1, included in the same assay, gave full correction.

Conclusion

Although there was no evidence to support the view that the control proteins Factor H and Factor I might be involved (immunochemical levels appeared to be normal in poorly opsonic sera) (see **Table 3.4**) the addition of affinity purified protein was undertaken to test the possibility that one of the proteins might be functionally aberrant. In fact, as expected, neither preparation was active. More surprisingly, the purified properdin also failed to correct the opsonic deficiency of LB1 serum. This observation was apparently at variance with the failure of the properdin deficient serum to correct the defect **(Figure 4.8)** and this discrepancy is discussed further in **Chapters 5** and **6.**

4.3.7 Studies using IgG fractions

Introduction

Staphylococcal Protein A is known to bind to Caucasian IgG1, IgG2 and IgG4 and therefore it is possible to remove most of the IgG from a serum sample and to measure the correcting potentials of the IgG depleted and IgG enriched fractions.

Experiment

1ml of HB1 serum was run through a 3.8ml Protein A column (Pharmacia UK Ltd) in glycine /NaCl (see **Chapter 2**). This column had the capacity to bind 85μg of IgG, and presumably would not be overloaded by the IgG in the human serum (approximately 30μg). The flow-through fractions and fractions eluted with 0.1M citric acid pH3 were dialysed against water, lyophilised and resuspended in 1ml of PBS (the volume of the original serum). A control was prepared by diluting HB1 serum with the Protein A running buffer; this was then dialysed and treated in an identical fashion to the fractions.

Results

The fractions were assayed by the Ouchterlony test using a sheep anti-human IgG antiserum (Unipath UK Ltd) in the central wells (data not shown). The flow through material did not appear to contain IgG whereas the eluted material had approximately the same concentration of IgG as in the control serum which had not been passed down the column. All the fractions were assayed for their correcting potential when mixed with the LB1 serum (see **Figure 4.9**). In this correction assay IgG enriched fractions were less active than the fractions depleted of IgG, suggesting that IgG had no significant role in the correction process.

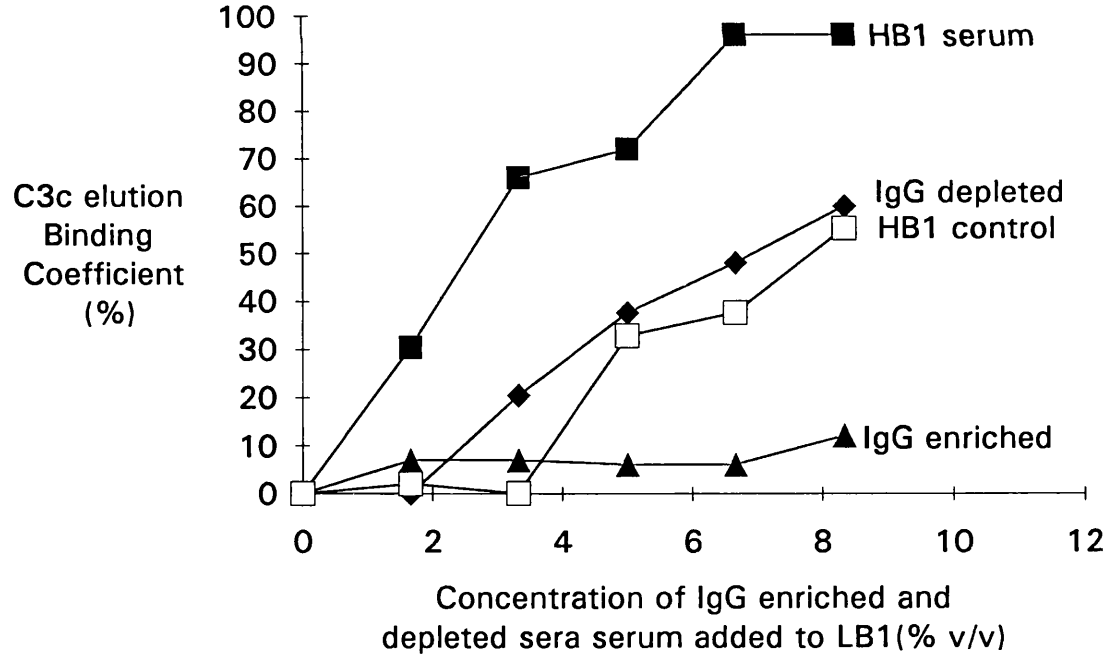


Figure 4.9 Attempted correction of the opsonic defect in serum LB1 using IgG - depleted and IgG - enriched sera and appropriate controls.

Conclusion

The above results were again suggestive that IgG was not itself the correcting factor, but they were not conclusive since it was still possible that a minor IgG fraction of potent opsonic potential, but below the detection limit of the Ouchterlony assay, had passed through the column.

4.4 ELISA procedures for measuring complement components bound to zymosan

Introduction

Newman and Mikus (1985) established that C3b on zymosan surfaces is rapidly converted to C3bi. Bound C3b is more resistant to trypsinisation than C3bi, so it was important to establish that the differences in the amounts of C3c eluted from zymosan opsonised with normal and poorly opsonic sera were not due to different ratios of C3b to C3bi bound. DiScipio (1981) found approximate stoichiometry of 1:1:1 for properdin, Factor B and C5 binding to C3b on zymosan. Therefore assays were set up to measure complement components bound to the zymosan particle. These assays resembled the C3c elution assay but differed in that the complement moieties adhering to the zymosan surface were measured directly rather than following release by trypsinisation. The technique for measuring C3 binding to zymosan had originally been developed by Ms L.A.Alterman of the Clinical Immunology Laboratory, ICH and sera were evaluated in parallel using both the C3c elution assay and the ELISA technique. The best correlation between the assays was observed when sera were tested at 5% concentration in the ELISA procedure (Spearman-rank correlation 0.491, $p < 0.001$).

Method

Sera with normal opsonic function from HB1 and 5 laboratory staff, and sera with poor opsonic function from LB1 and 2 apparently healthy laboratory staff as well as from 3 GOSH patients (see **Section 2.2**) were assayed in the C3c elution assay and in the ELISA procedure. The latter was identical to the C3c elution assay except that the trypsinisation step was omitted. Instead, following the incubation and washing steps, the zymosan pellets were transferred to 1.5 ml "Eppendorf" tubes pre-coated for one hour at room temperature with a solution of 1mg/ml bovine serum albumin (Cohn Fraction V Sigma UK Ltd) in PBS. The particles were resuspended in 100 μl PBS and 100 μl of PBS containing the various antisera were added. The detection systems used were either horseradish - peroxidase labelled sheep anti-human C3c (Dako) at a

dilution of 1/1000 or biotinylated mouse anti-human properdin monoclonal antibody (HYB 3-3) at 1/4000. The particles were incubated in these antisera for 1 hour at room temperature and then washed 3 times with PBS. When the biotinylated antiserum was used the detection system included the extra step of a 1/4000 dilution of streptavidin peroxidase (Amersham UK Ltd) in PBS which was incubated with the particles for 1 hour at room temperature. After this incubation the particles were again washed 3 times with PBS. Colour was developed with 10μl H_2O_2 and 10 mg OPD in 20 ml phosphate /citrate buffer pH 5.2. The tubes were left in the dark for 15 minutes, spun in an "Eppendorf" centrifuge for 1 min at 1300g and the supernatants then added to the wells of a Dynatech "Immulon" microtitre plate. Absorbance values were read at OD492 on a Flow Multiscan Plus plate reader(Flow UK Ltd.). The standard sera LB1 and HB1 were included in each assay so that the results could then be expressed as binding coefficients.

Thus for C3 moieties the calculation was:

$$\text{Binding Coefficient (BC)} = 1 - \frac{HB1_{C3\%} - Test_{C3\%}}{HB1_{C3\%} - LB1_{C3\%}} \times 100\%$$

and for properdin

$$\text{Binding Coefficient (BC)} = 1 - \frac{HB1_{P\%} - Test_{P\%}}{HB1_{P\%} - LB1_{P\%}} \times 100\%$$

Results

The correlation between the zymosan binding coefficients for C3 and properdin obtained with the 10 sera are shown in **Figure 4.10**. The amounts of C3 and properdin bound to the zymosan from 10 different sera were found to correlate significantly(r_p=0.8,$p<0.001$).

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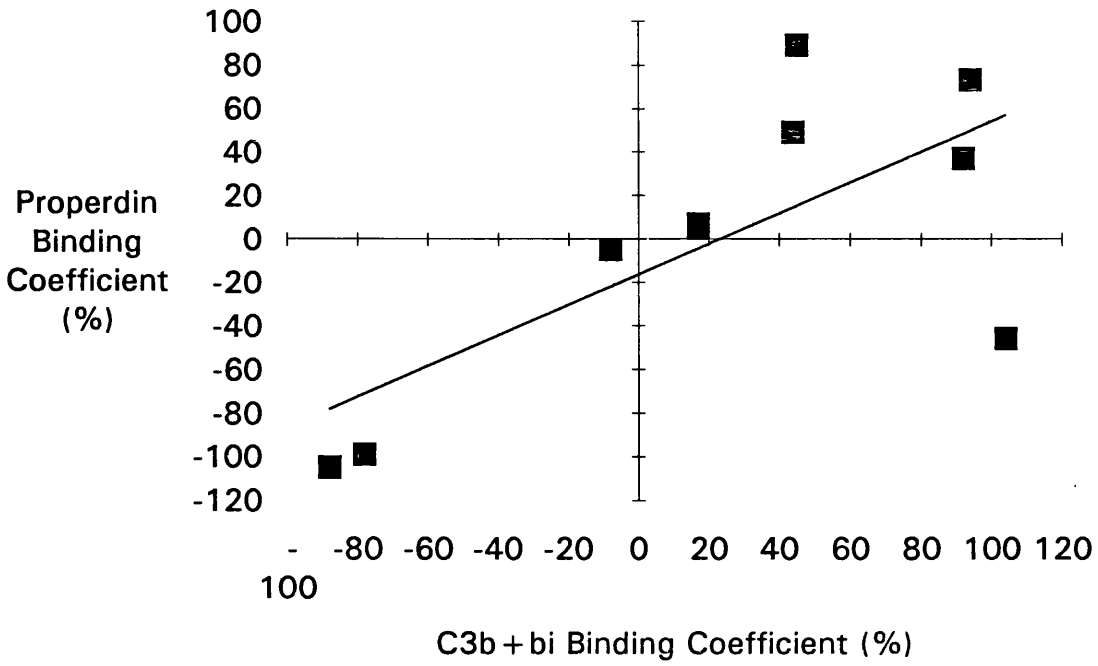


Figure 4.10 Pearson correlation of properdin and C3b+bi binding to zymosan from 10 sera, compared with binding of HB1 and LB1

Comment

The correlation between the levels of properdin and C3 bound to the zymosan surface observed here was consistent with the findings of DiScipio (1981) who showed a quantitative relationship between the levels of C3, properdin and Factor B bound to zymosan.

4.5 Discussion

The incidence of poor opsonic function in the normal adult population has been previously reported to be 5-8% (Soothill and Harvey 1976, Kerr et al 1983) and the application of the C3c elution technique to the sera of 77 healthy adults (**Section 4.2.2**) gave a similar frequency. In addition, Levinsky et al (1978) had found a similar incidence in 6% of a population of healthy school children aged 11-12 years. The studies on patients attending the immunology and allergy clinic at Great Ormond Street Hospital (**Section 4.2.3**) showed that the incidence of poor yeast opsonisation in this population (ie binding coefficients less than 15%) was 14.9% and was more

than double the incidence in the healthy adult and paediatric populations studied. When the healthy adult blood donor group and paediatric patient group were compared by the Chi square analysis, measuring the proportions of each population above and below the 15% Binding Coefficient threshold, the two samples were not significantly different (p= 0.076). Although this is superficially at variance with previously published work (Soothill and Harvey 1976, Turner et al 1978) the patient group studied here was not defined as were the earlier cohorts. In the present study the test for opsonic function was requested as part of a range of immunological investigations when the patients first presented.

The present study differed from the findings of Soothill and Harvey (1977) in assays where Mg EGTA buffers were used (**Section 4.2.1**). These authors had found that pre-incubation with inulin decreased the haemolytic complement titre of normal human sera and they therefore suggested that this demonstrated a " defect in the alternative pathway of complement". In contrast, in these studies the use of MgEGTA almost completely abrogated binding of C3 to the zymosan surface and very little discrimination was observed between sera having normal or poor opsonic function. Thus it appeared that, at the serum concentrations routinely used in these assays, the **classical pathway** plays a major role in the generation of complement components which subsequently bind to the zymosan surface.

The correction assays described in **Section 4.3.1** confirmed earlier findings of Miller et al (1968), Soothill and Harvey (1976) and Turner et al (1981 and 1985(a)&(b)), that the addition of a small amount of normal serum corrected the opsonic defect and could raise the (%) Binding Coefficient to within the normal range. In subsequent correction assays normal sera were treated in order to selectively remove or inactivate known complement components and sera genetically deficient in selected components were also studied.

The ability of normal human serum to opsonise yeast or zymosan is markedly dependent on the concentration of C3 in the serum (Kerr et al 1983). Therefore, it was important to establish that the C3 bound to the zymosan in the correction assays came from the LB1 rather than from the correcting sera used. This question was addressed in **Sections 4.3.2, 4.3.3 and 4.3.5** :

In **Section 4.3.2** the thiolester groups of the C3 and C4 were inactivated by incubating serum in KSCN. Following such treatment no C3b become bound to yeast in the standard assay, but the sera continued to function normally in correction assays. Similarly, when animal sera were tested in standard assays using the anti-

human C3c antiserum, there was no detectable cross-reacting C3 binding to zymosan (**Section 4.3.3**), yet these sera were able to promote the binding of LB1 C3 to the zymosan in correction assays. As expected, assays with a C3 deficient serum (**Section 4.3.5**) showed no C3 binding to zymosan in the C3c elution assay, but still this was able to correct LB1 serum. Furthermore, the C3 in serum LB1 was functionally normal (see **Table 3.1**) and known to be present in concentrations within the normal range (data not shown). Thus it seems very unlikely that the common opsonisation defect described here is due to an abnormality of the C3. In all these assays it appears that the addition of a small amount of a factor present in normal sera was able to correct the functional defect in LB1 and other similar sera.

Several conclusions were drawn from the correction studies using the C3c elution correction assay (reported in **Sections 4.3.4 to 4.3.7**) .

(i) The correcting moiety was heat stable at 50^{o}C, but not at 56^{o}C, suggesting that it was neither heat labile Factor B nor thermostable IgG.

(ii) IgG depleted serum was still able to correct the defect.

(iii) Affinity purified preparations of properdin, Factor H and Factor I failed to correct the defect.

(iv) Serum naturally deficient in Factor D was able to correct the defect but another serum with proven properdin deficiency was inactive.

The properdin findings in (iii) and (iv) were superficially at variance and focused attention on this AP control protein. However, functional and total properdin levels have been measured in several sera with the opsonic defect (see **Figure 3.1(b)**) and found to be normal, and SDS-PAGE, IEF and immunoblotting experiments (data not shown) failed to show any qualitative difference between the properdin present in LB1 and HB1 and HB3 sera. Further studies with properdin deficient sera are described in **Chapters 5** and **6**.

The above results suggest that the factor which promotes the binding of C3 to the zymosan surface is labile at 56^{o}C and is present in other mammals as well as in man. This factor is not C3, nor IgG, Factor B, C4, Factor H, Factor I, nor Factor D. It appeared that deficiencies in this factor affected the initiation of the classical complement pathway but that this factor was not IgG.

The C3c elution assays provided a useful body of data, but these assays were difficult to use in purification studies (See **Chapter 5 and 6**) because of the overnight incubation step necessary before the active fractions could be identified. In addition the zymosan particle was not ideal for the study of the binding of complement components because of the presence of yeast proteins in the zymosan wall which prevented the surface identification of bound complement components by iodination and SDS-PAGE studies (data not shown). Moreover, it was technically difficult to remove unbound complement components and ELISA reagents from the zymosan surface during the centrifugation and washing steps of the ELISA based studies and there was a risk that the particulate material would be lost.

As a result of these difficulties experiments were initiated using purified components of the zymosan cell wall as the complement activating surface. The major constituents of the yeast cell wall are β-glucans and α-D mannans and binding studies undertaken with these preparations are described in **Chapter 5.**

CHAPTER 5

INVESTIGATIONS OF SERA OF DIFFERING OPSONIC POTENTIAL USING MANNAN COATED ELISA PLATES

5.1 Introduction

Zymosan consists almost entirely of the two carbohydrate polymers β-D glucans and α -D mannans (Phaff 1963, Bacon 1969). The β-D glucans are made up of β-glucosyl residues linked 1,3 and 1,6 and branched 1,3 (Czop 1986) while the α -D mannans are made up of α -D mannose units linked 1,6 and branched 1,2 (Bacon 1969)(see **Figure 5.1**).

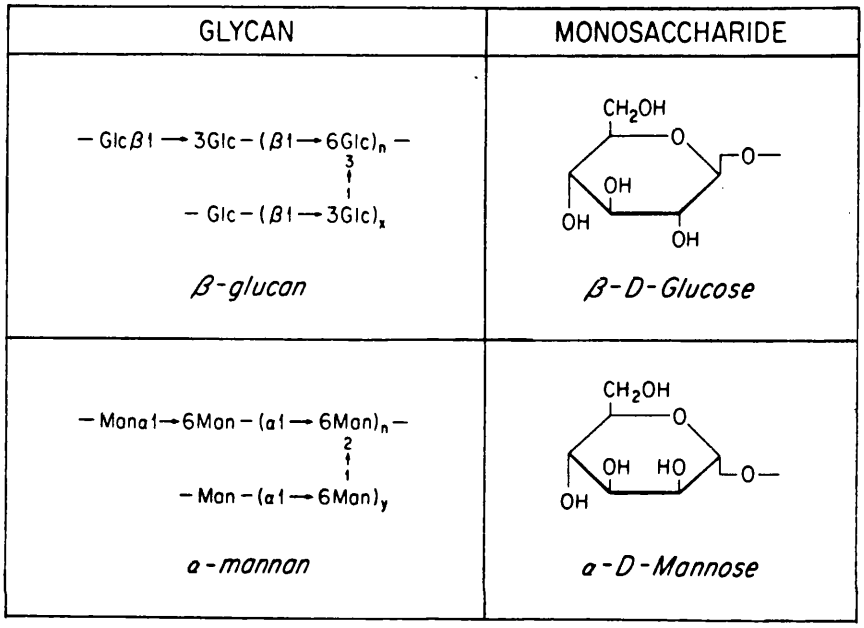


Figure 5.1 Structures of yeast cell wall components α-D mannan and β-D glucans. (Adapted from Czop 1986)

Alkali treatment of zymosan removes mannans and produces particles containing only β-glucans (Bacon 1969). Using the C3c elution assay there was a poor correlation between the amount of C3c bound to yeast zymosan and the amount bound to yeast glucan particles from selected sera (data not shown). This suggested that the mannan component of zymosan might be responsible for the differences in the levels of C3 binding from different sera. Yeaman and Kerr (1987) had shown that mannan was able to bind to the surface of microtitre plates and accordingly this technique was modified in the present study to measure the binding of complement moieties and immunoglobulins to mannan coated surfaces. The mannan used was α - D mannan, prepared commercially by fractional precipitation with cetyltrimethylammonium-bromide (the Cetavlon method).

5.2 Standard assay of complement components binding to mannan

Introduction

The C3c elution assay (see **Chapter 4**) was modified to measure complement components bound to mannan.

Method

The wells of Immulon Dynatech micro-ELISA plates were filled with 100μl volumes of mannan (Sigma, Poole, UK) at 0.5 mg/ml in carbonate/bicarbonate "coating" buffer pH 9.6. After incubation overnight at 4^{o}C, the mannan coated plates were washed three times with PBS-Tween, once with PBS (without Tween-20) and once with VBS. Duplicate serum samples were diluted to 5% in VBS $^{++}$ in Micronic tubes (Flow Laboratories) and 100μl aliquots were loaded into the wells of the mannan-coated microtitre plates using a multichannel pipette. The plates were incubated at 37^{o}C for 30 minutes and then washed four times with PBS-T. Bound ligands were detected by incubation at room temperature for 1 hour with the following indicator antibodies diluted in PBS-T:

- horseradish peroxidase labelled polyclonal sheep anti- human C3c, C4, Factor B and transferrin (Serotec UK Ltd) The anti-C3c and anti-C4 reagents were used at dilutions of 1/10 000, and the anti-Factor B and anti-transferrin antibodies were used at 1/2000.
- biotinylated anti-properdin antibody clone HYB 3-3,(a gift from Dr Claus Koch) used at a dilution of 1/4000.
- anti-C3bi. This rat monoclonal anti-C3g clone 9 (kindly provided by Professor P. J. Lachmann) was used at 1/1000.

After the incubation with the indicator antibodies the plates were washed four times with PBS-T. The plates which had been incubated with the anti-properdin and anti-C3g monoclonal antibodies were then incubated for a further hour with streptavidin-peroxidase (Amersham UK Ltd) at 1/4000 and peroxidase labelled sheep anti-mouse IgG (Sigma, Poole, UK) diluted to 1/500 in PBS-T. Following this incubation step the plates were washed four times with PBS-T. Colour was developed following incubation for 15-30 minutes in the dark at room temperature with 100 μl per well of a solution of o-phenylene diamine in phosphate-citrate buffer pH 5.3 containing H_2O_2. The colour reaction was stopped by the addition of 100 μl per well of 4N H_2SO_4 and absorbances were measured at 492 nm using a Titertek Multiskan ELISA plate reader and the Titersoft Program (Flow UK Ltd).

A serum known to give high levels of C3b binding to zymosan (HB1) (see **Chapter 2**) and a serum known to give low binding in the same system (LB1) were included in every assay and used to calculate a binding coefficient for each test system.

$$\text{Binding Coefficient (BC)} = 1 - \frac{HB1_{OD492} - Test_{OD492}}{HB1_{OD492} - LB1_{OD492}} \times 100\%$$

5.2.1 The use of different concentrations of serum in the mannan-binding assay

Introduction

In the C3c elution assay (**Chapter 4**) sera were diluted to 16.67% v/v since this was found to give good discrimination between the amounts of C3 bound to the zymosan using sera of differing opsonic potential and the C3c released was readily measured by RID after trypsinisation. However, when zymosan bound C3 was assayed by ELISA (**Section 4.4**), better discrimination was obtained using 5% serum. Sera were therefore diluted to different concentrations in VBS^{++} in the mannan- binding assay to find the optimal concentration giving the greatest discrimination between sera differing in their opsonic potential.

Method

The standard assay for C3bi binding to mannan was performed in duplicate with the exception that HB1 and LB1 sera were titrated into VBS^{++}, yielding final serum concentrations between 1% and 17%. Absorbances were measured at OD492 and the ratios of absorbances of the HB1:LB1 sera were calculated for each concentration.

Results

The results obtained are illustrated in **Figure 5.2.** The amount of C3bi bound to mannan using serum HB1 at a concentration of 5% serum was 84% of the amount bound at 17% concentration and the curve had almost reached a plateau between these

two concentrations. In contrast, the LB1 serum diluted to 5% had only 44% of the OD492 bound at 17% and the curve between 1% and 17% serum concentrations was almost linear with no evidence of a plateau. These findings were reproduced when the C3b+bi binding to mannan was measured (data not shown). The greatest discrimination between the HB1 and LB1 sera was observed at 5% (see **Table 5.1**) and this serum concentration was, therefore, subsequently adopted for binding coefficient calculations in the mannan-binding and mannan-correction assays.

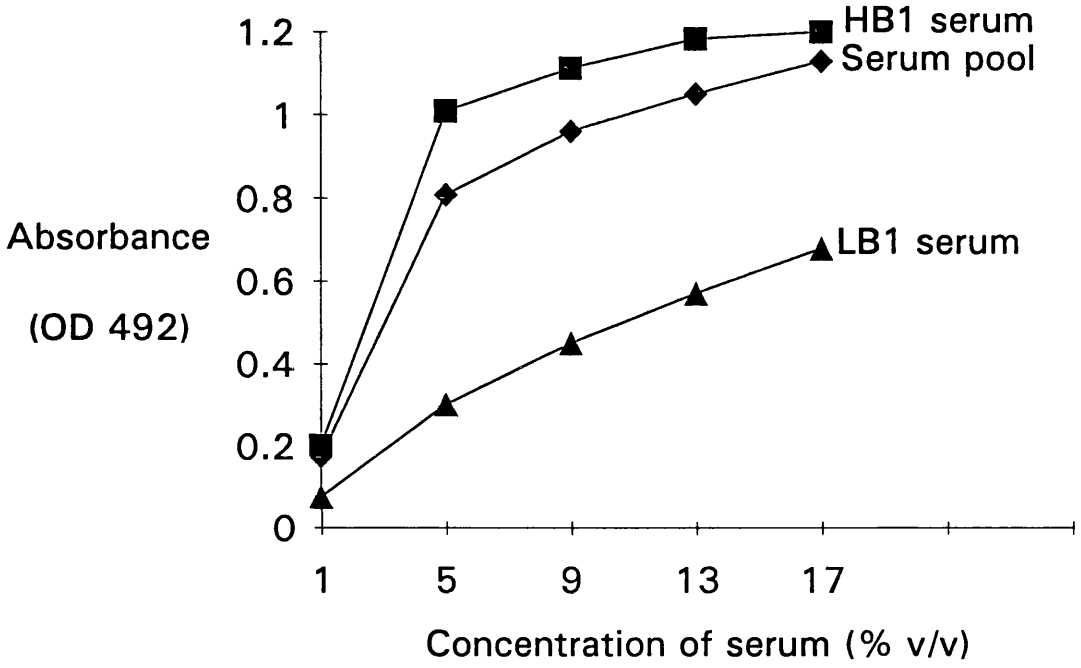


Figure 5.2 Binding of C3bi to mannan using various concentrations of a serum pool and previously studied sera with normal (HB1) and poor (LB1) opsonic function.

Table 5.1 Binding of C3bi to mannan.HB1:LB1 ratios calculated from the relative levels of C3bi binding at different serum concentrations

Serum concentration	HB1:LB1 Ratio (mean of duplicates)
1%	2.66
5%	3.33
9%	2.47
13%	2.07
17%	1.79

Comment

Discrimination between the levels of C3bi opsonins binding to mannan from HB1 and LB1 was greatest at 5% serum, with the ratio of HB1:LB1 bound of 3.33. HB1 serum concentrations above 5% appeared to saturate the mannan surface and little further binding of C3bi was detected, whereas binding of C3bi from the LB1 serum showed no evidence of saturation and so by 17% serum concentration the HB1:LB1 ratio for C3bi binding had fallen to 1.79. These findings, together with those of **Section 4.4.**, suggest that the discrimination between HB1 and LB1 is related to the function of the classical rather than the alternative pathway since AP activation is markedly reduced at serum concentrations below 20% (Ehrnst 1978).

5.2.2 The use of different buffers in the standard mannan-binding assay

Introduction

In the C3c elution assays described in **Chapter 4** there was no evidence of C3 binding to zymosan from a serum with normal opsonic potential in the presence of MgEGTA. This experiment was repeated with 40 sera using the mannan-binding assay to determine whether this more sensitive test system showed complement activation in this buffer. The MgEGTA buffer was made up in VBS and contained 7mM $MgCl_2$ and 10mM EGTA in order to increase AP complement activation (Harrison and Lachmann 1986).

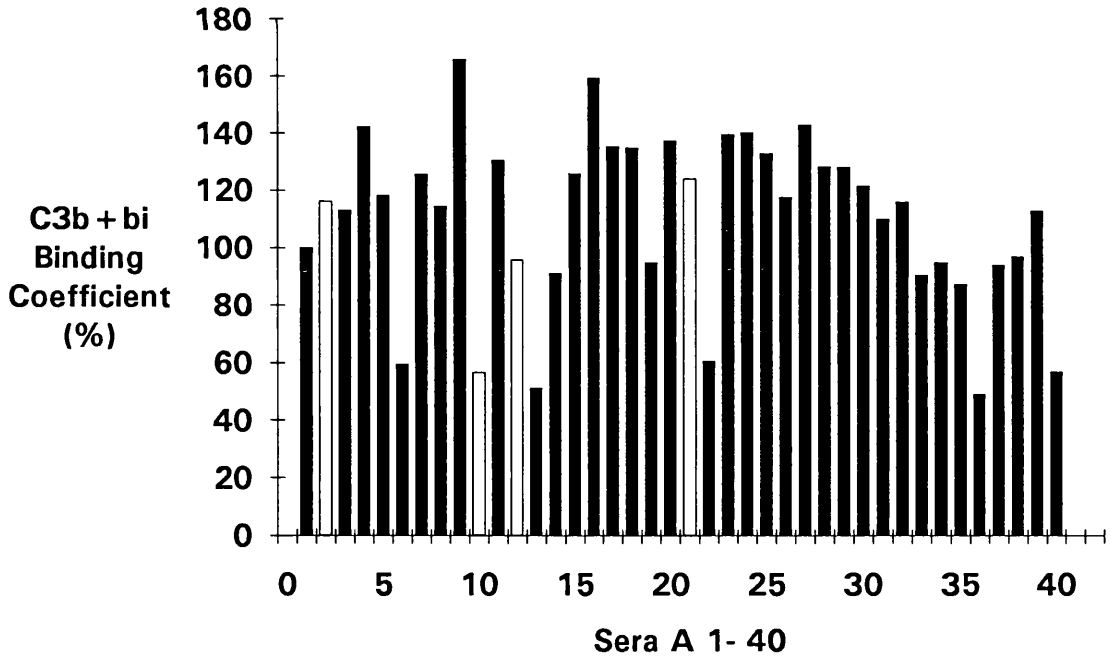
Experiment

40 sera, chosen at random from the sample of 179 blood donors (see **Chapter 2**), were analysed in duplicate at both 5% and 30% concentration in MgEGTA (7mM $MgCl_2$ and 10mM EGTA in VBS) using the mannan-binding assay. Binding of C3b+bi, C4 and Factor B were measured and the results were compared with the binding of these components in VBS^{++}.

Results

Sera diluted to a concentration of 5% in VBS containing MgEGTA showed no significant binding of any of the complement components, including C4, in mannan binding assays (data not shown). Sera diluted to 30% in MgEGTA again bound no C4, although C3b+bi and Factor B were bound in each case. There was little correlation between the patterns of binding to mannan at 5 and 30%(v/v) in MgEGTA and those observed at 5% in VBS^{++} as are shown in **Figure 5.3 (a)&(b)** and by the Spearman rank correlations in **Table 5.2.**

5.3(a)



5.3(b)

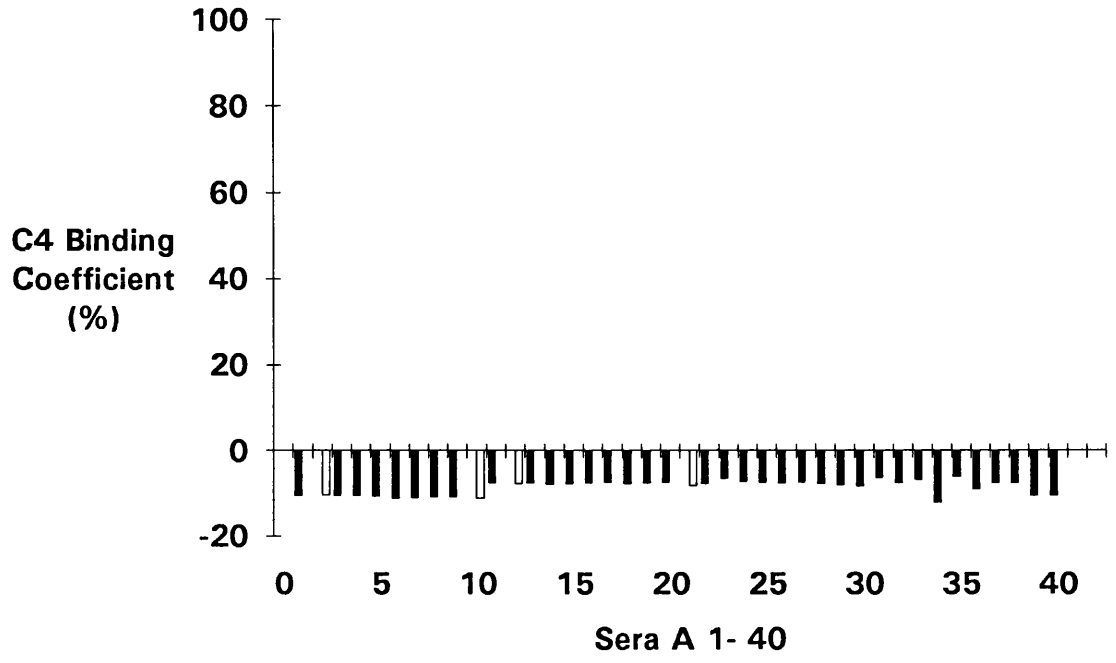


Figure 5.3 (a) and (b) Levels of C3b+bi and C4 binding to mannan from the sera of 40 healthy adult blood donors, tested at 30% concentration in Mg EGTA buffer. Means of duplicate measurements are shown.The hollow bars represent those sera with binding coefficients less than 10% in the mannan capture C3bi assay (5% sera in VBS^{++}) - see **Figure 5.4** (a)

Table 5.2 Correlations of levels of complement components bound in mannan capture assays in VBS and Mg EGTA buffers. 40 sera, selected at random from the population of healthy adult blood donors, were assayed at 5% in VBS^{++} buffer and at 5 or 30 % in Mg EGTA.

	C3 5% VBS	C4 5% VBS	Bf 5% VBS	C3 5% EGTA	C4 5% EGTA	Bf 5% EGTA	C3 30% EGTA	C4 30% EGTA	Bf 30% EGTA	
C3 5% VBS	1	0.817	0.887	0.331	0.189	0.137	0.215	0.297	0.393	r_s
		0.000	0.000	0.039	0.237	0.394	0.180	0.064	0.014	p
		1	1							
C4 5% VBS		1	0.924	0.267	0.098	0.128	0.049	0.367	0.221	r_s
			0.000	0.096	0.542	0.425	0.760	0.022	0.169	p
			1							
Bf 5% VBS			1	0.296	0.147	0.086	0.137	0.303	0.313	r_s
				0.643	0.358	0.593	0.039	0.058	0.051	p

Discussion

Mg EGTA chelates the divalent cation Ca^{++} required for the functioning of the classical pathway convertase C4b2a (Harrison and Lachmann 1986) and complement binding to the mannan surface using 30% serum in MgEGTA probably occurred solely by activation of the AP. In this experiment there was no evidence of any binding of C4 to mannan using either 5% or 30% serum concentration in MgEGTA thus confirming that the classical pathway was not activated. When the assays were performed at 30% serum concentration, at which the alternative pathway was more likely to be activated, C3 fragments and Factor B were shown to be bound to the mannan. Significantly, in this case, the binding coefficient values of sera previously designated as poorly opsonic were within the normal range (see **Figure 5.3(a)**) and there was little correlation with results from the standard mannan-binding assay using 5% sera in VBS^{++}. These assay results, together with those of **(Section 5.2.1)**, suggest that a low serum concentration is mandatory if the opsonic defect is to be demonstrated. They also indicate that at such low concentrations the AP of complement, operating in the absence of the CP, cannot be involved in the generation of C3 moieties.

5.2.3 Assay of sera from a healthy adult population

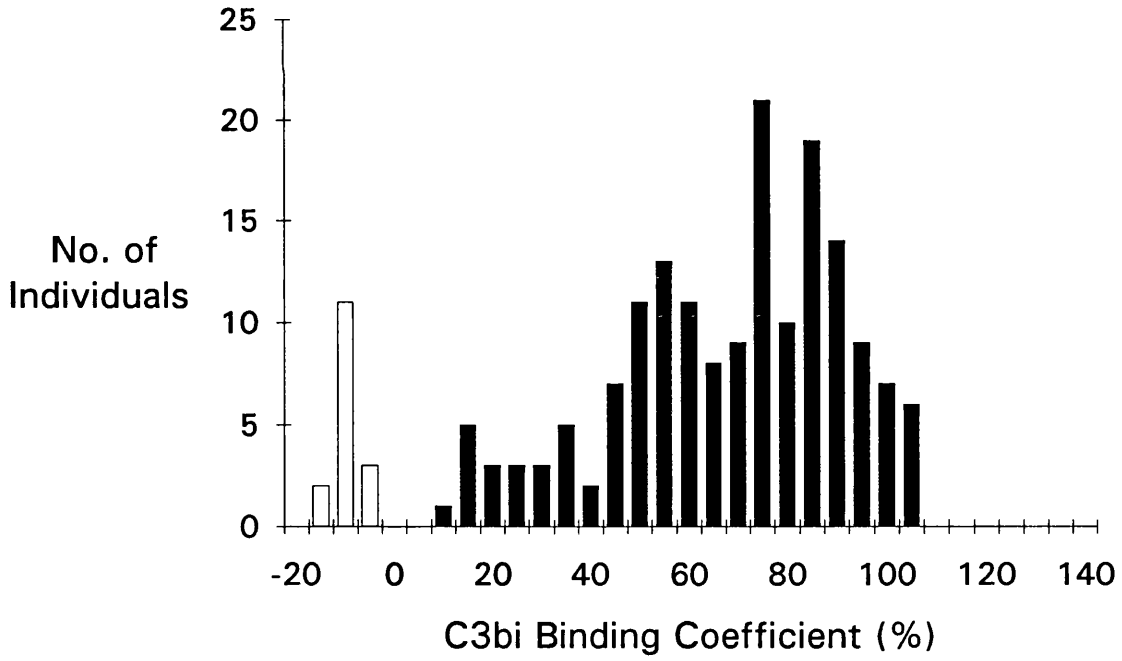
Protocol

179 serum samples were obtained from blood donors attending the West End Donor Centre (see **Chapter2**). These sera were used in the mannan-binding assay and binding coefficients of C3bi, C3b+bi, Factor B, properdin and C4 were measured.

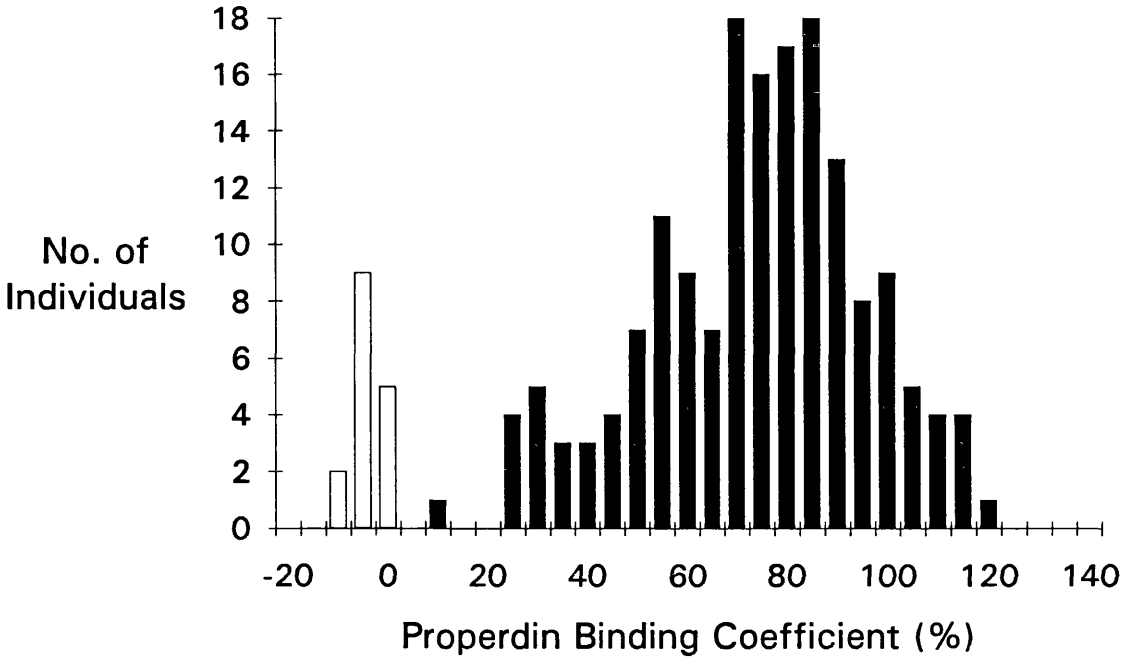
Results

The assays for the components C3b+bi(anti C3c), C3bi(anti C3g), properdin(HYB 3-3), Factor B and C4 (see **Figure 5.4 (a)-(d)**) all show similar distribution profiles where there is a large group with relatively large amounts of the components bound to the mannan and a separate smaller group binding relatively little of the component to the mannan and with binding coefficients close to or below the zero value of serum LB1. Detailed results on the individuals tested can be found in **Appendix 3.**

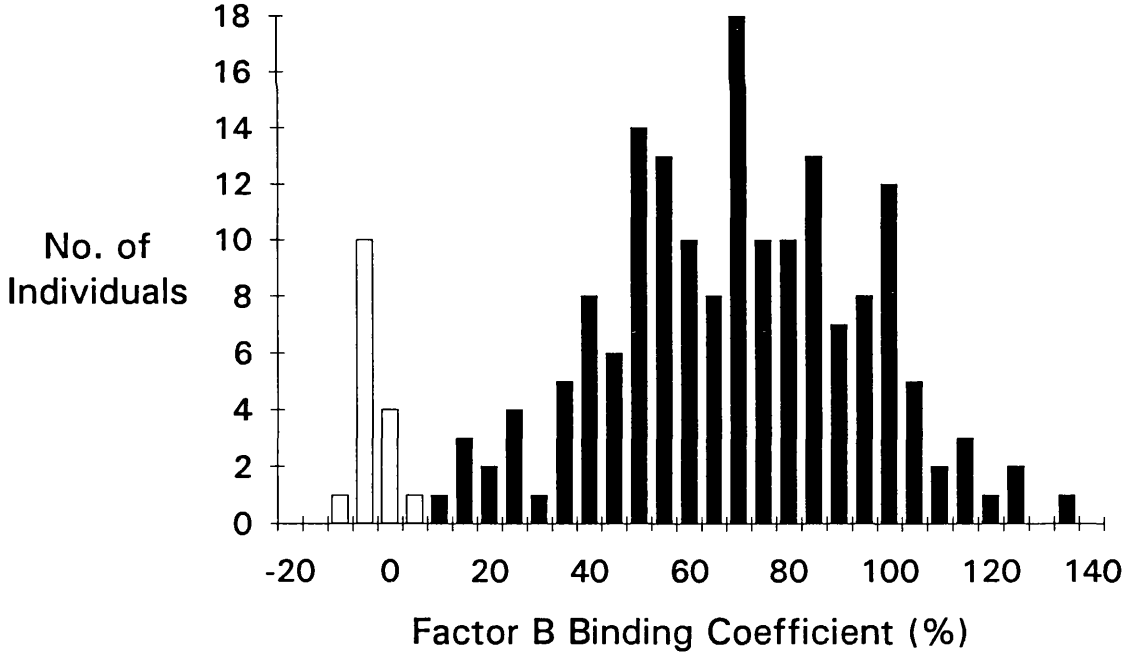
5.4(a)



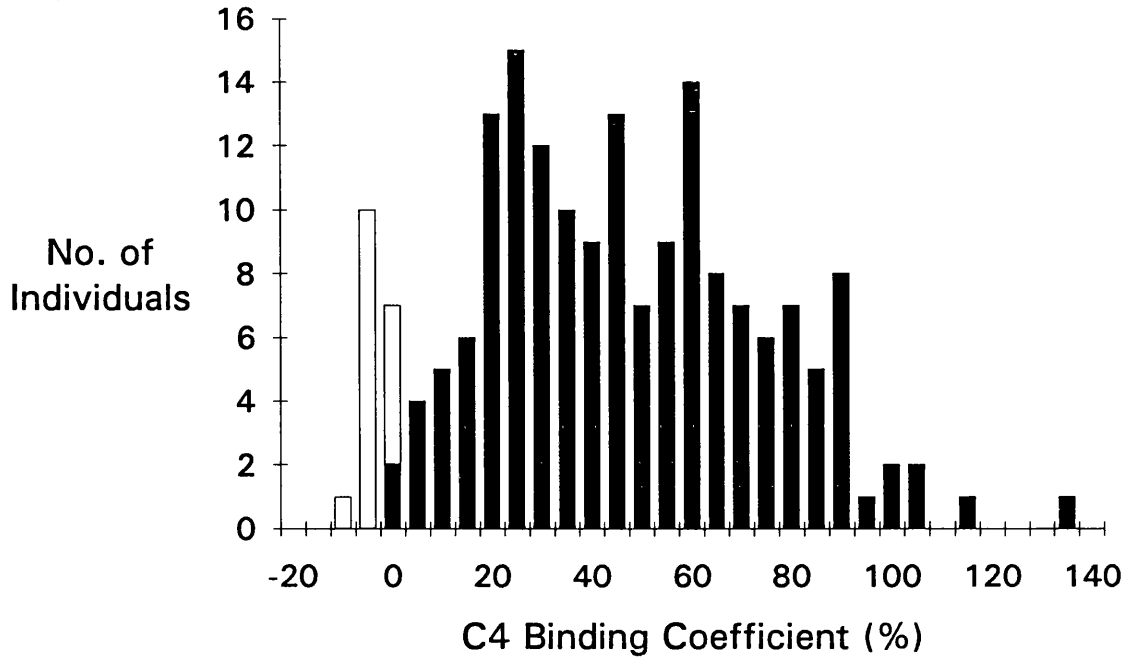
5.4(b)


No. of Individuals
18
16
14
12
10
8
6
4
2
0
-20
0
20
40
60
80
100
120
140
Properdin Binding Coefficient (%)

5.4(c)


No. of Individuals
18
16
14
12
10
8
6
4
2
0
-20
0
20
40
60
80
100
120
140
Factor B Binding Coefficient (%)

5.4(d)



Figures 5.4 (a),(b),(c),(d) Mannan capture assays for complement using sera from a population of 179 healthy adult blood donors. The open bars represent those individuals in whom a C3bi binding coefficient of less than 10% was observed (see panel (a)).

Using the anti-C4 antibody, which presumably recognised covalently bound C4b fragments, individuals with low binding coefficients were again observed but these were less clearly defined within the population profile. The normal range of binding of C3bi fragments to the mannan surface was 15-110%, and the sera with C3bi binding coefficient below 10% in this assay are represented as open forms (bars or boxes) in subsequent figures.

The assay for transferrin revealed no evidence of binding by this transport protein to the mannan coated plates nor was there any evidence of C1q binding (data not shown). In addition, in the presence of EDTA there was no detectable binding of any of the complement components to the mannan plate and if uncoated plates were used no complement components were bound (data not shown).

The mannan-binding of the complement proteins was compared by both parametric (Pearson) and non-parametric (Spearman rank) tests and these showed extremely good correlations between the different assays (see **Table 5.3**). The Pearson correlations are plotted in **Figure 5.6 (a)-(c)**. The binding levels observed also correlated well with the C3c elution assay data obtained using zymosan (see **Figure 5.5** and **Table 5.3**).

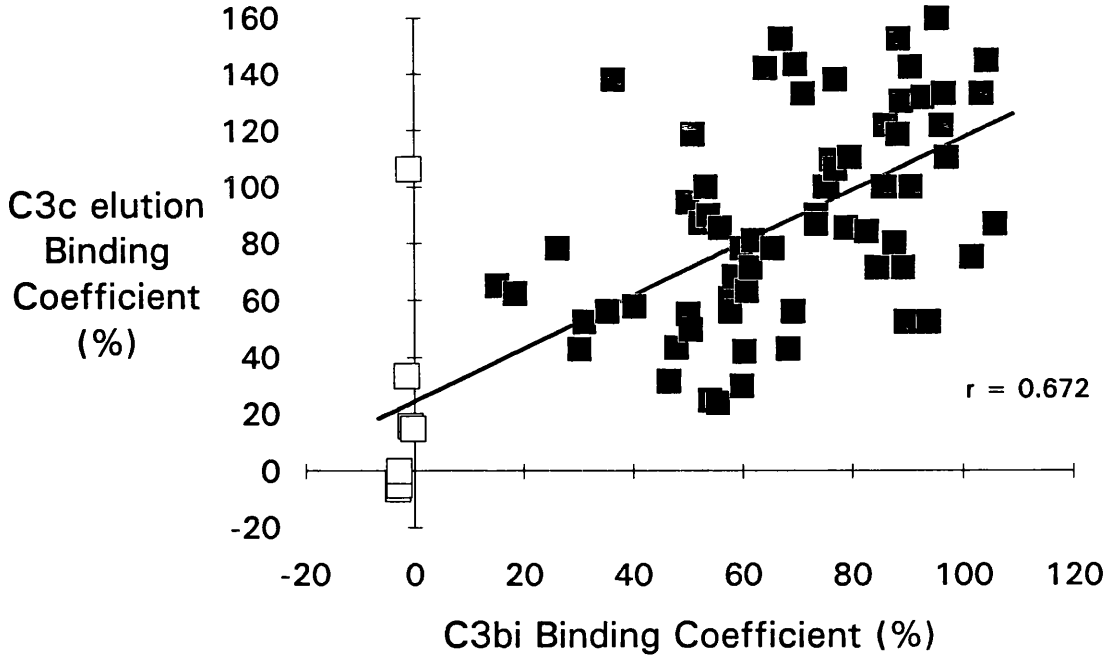
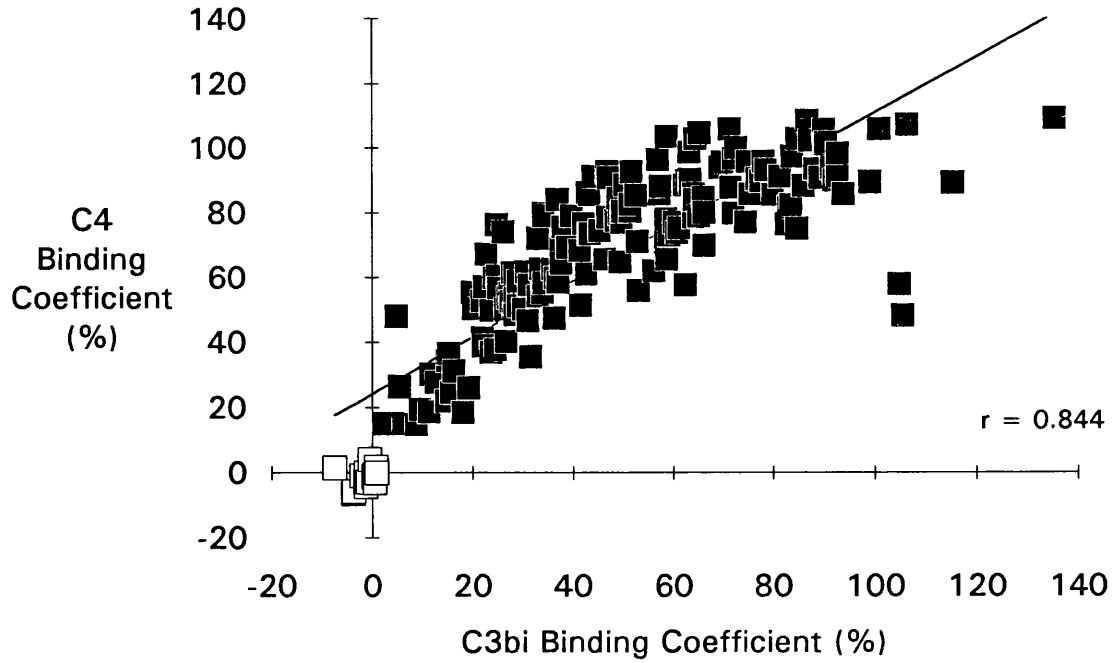
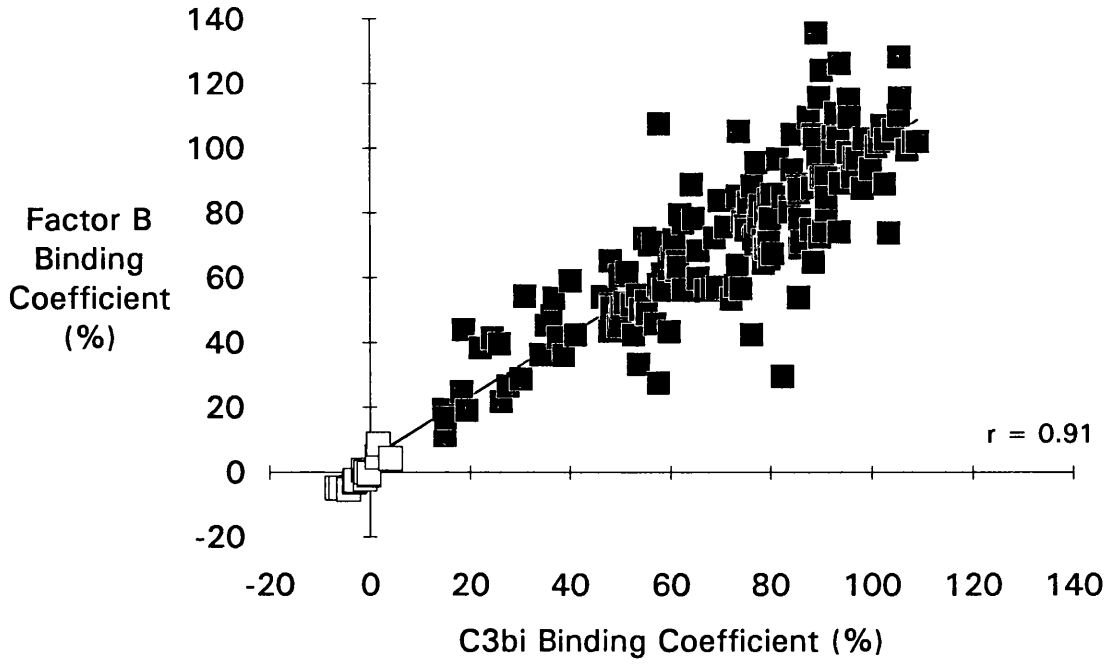


Figure 5.5 Plot of the Pearson correlation between C3bi binding to mannan and C3c eluted from zymosan in 77 sera obtained from healthy adult blood donors.

5.6 (a)



5.6 (b)



5.6 (c)

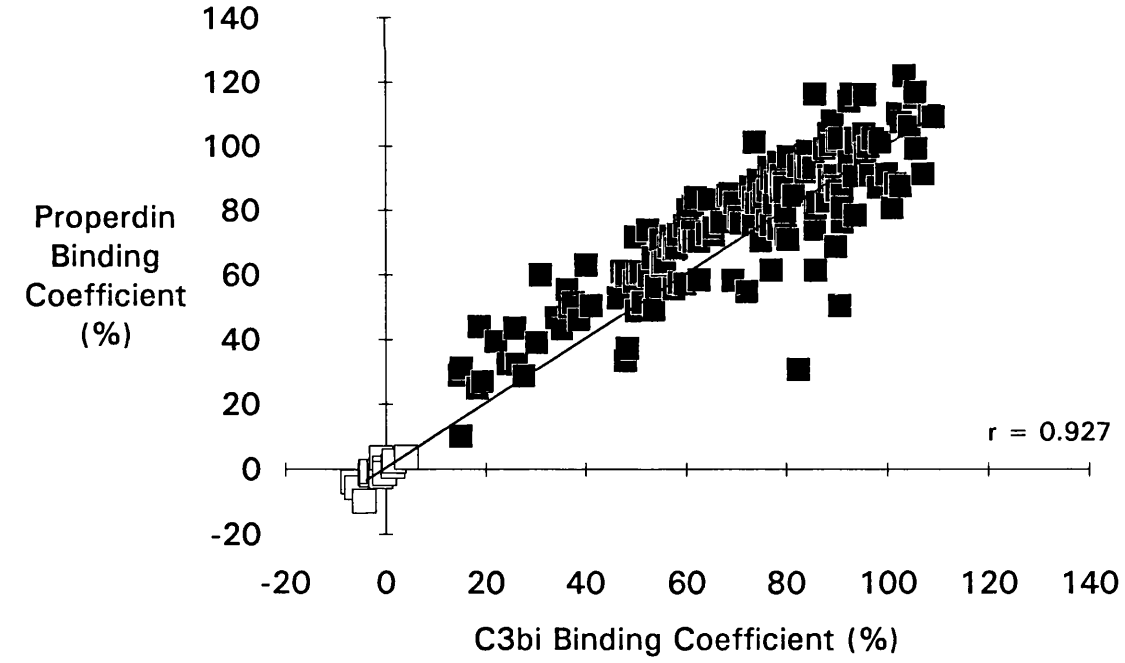


Figure 5.6 (a), (b) and (c) Pearson correlations of mannan capture complement assays in sera from 179 healthy adult blood donors.

Since the binding coefficient profiles shown in **Figures 5.4 (a) - (d)** were not normally distributed, the data was also analysed for correlations using the non-parametric Spearman rank test (see **Table 5.3**).

Table 5.3 Pearson (r_p) and Spearman rank (r_s) correlations of binding coefficients for various complement proteins measured by mannan capture assays and C3c eluted from zymosan. Comparisons between the binding coefficients observed using the mannan capture assay are also tabulated.

Correlation	n	r_p	p	r_s	p
C3c elution					
vs C3bi	**77**	**0.672**	**<0.0001**	**0.627**	**<0.0001**
vs P	**77**	**0.656**	**<0.0001**	**0.574**	**<0.0001**
vs Bf	**77**	**0.582**	**<0.0001**	**0.521**	**<0.0001**
vs C4	**77**	**0.488**	**<0.0001**	**0.495**	**<0.0001**
C3bi					
vs P	**179**	**0.927**	**<0.0001**	**0.878**	**<0.0001**
vs Bf	**179**	**0.91**	**<0.0001**	**0.882**	**<0.0001**
vs C4	**179**	**0.844**	**<0.0001**	**0.867**	**<0.0001**
C4					
vs P	**179**	**0.743**	**<0.0001**	**0.741**	**<0.0001**
vs Bf	**179**	**0.83**	**<0.0001**	**0.855**	**<0.0001**
P					
vs Bf	**179**	**0.923**	**<0.0001**	**0.890**	**<0.0001**

Comment

These results suggest that the binding of the complement proteins to the mannan coated ELISA plates is a reflection of specific complement activation by the carbohydrate surface. The levels of C3 fragments binding to zymosan in the C3c elution assay were found to correlate significantly with the C3bi mannan binding coefficients when these values were compared in the subpopulation of 77 individuals (see **Figure 5.5**). Extremely good correlations were found between the mannan binding coefficients of all the individual complement components measured when the larger population of 179 healthy blood donors was studied (see **Figure 5.6 (a) - (c)**).

5.2.4 Assays of sera from hospitalised children

Protocol

Sera from ten patients with poor yeast opsonisation attending the Immunology outpatients clinic at Great Ormond Street Hospital (GOSH) were also tested in the mannan-binding assay.

Results

The ten sera with low binding coefficients in the C3c elution assay also had low binding coefficients in the mannan-binding assays (see **Table 5.4**).

Table 5.4 Mannan capture complement assays in sera from 10 patients attending Great Ormond Street Hospital and previously defined as having poor yeast opsonisation. The ages given were at the last hospital visit, when the assays were carried out.

SERA	AGE	ASSAYS					
		zymosan BC(%)	Mannan BC(%)				
		C3c elution	C3b+bi	C3bi	C4	Factor B	properdin
I1	3yr	3	-2.9	ND	-0.6	-6.9	ND
I2	15mo	5.9	-3.6	ND	-0.9	-3.94	ND
I3	16mo	-5.6	-3.5	ND	-0.8	-6.76	ND
I4	9mo	0	0.46	-4.7	-0.2	-5.63	-2.01
I5	7yr	0	1.63	-5.9	-9.8	-8.31	-2.6
I6	8yr	0	-1.97	-7.3	-0.6	-9.15	-2.4
I7	6mo	5.3	-11.43	-3.76	-0.9	ND	-8.35
I8	2.5yr	0	13.86	0.84	1.7	ND	5.4
I9	10mo	5.3	-16.41	-5.45	-1.3	ND	-9.96
I10	9yr	-5	10.49	ND	0	ND	ND

Comment on the mannan-binding assay:

The findings in **Sections 5.2.3** and **5.2.4** confirm that the mannan- binding assay is a good replacement for the C3c elution assay since the results of the two assays correlated well and all of the sera tested which were by definition poor in the opsonisation of zymosan were also poor in the opsonisation of mannan. The microtitre-based mannan-binding assay facilitated the testing of multiple serum samples in duplicate and permitted the measurement of a range of complement moieties.

5.3 Measurement of immunoglobulins bound to mannan

Introduction

Phagocytosis of sheep erythrocytes requires both C3 fragments and IgG (Ehlenberger and Nussenzweig 1977, Newman and Johnson 1979). Similarly, there is evidence that efficient phagocytosis of zymosan and micro-organisms occurs after opsonisation with both IgG and C3 (Scribner and Fahrney 1976, Newman and Johnson 1979, Roos et al 1981). C3 probably promotes adhesion to the PMN and the immunoglobulins, C3 and lectins on the zymosan surface promote ingestion of the yeast (Kemp and Turner 1986). Hyperimmunised individuals show high levels of specific immunoglobulin (Young and Armstrong 1972) and Yeaman & Kerr (1987) have also described an anti-mannan IgA opsonin. The role of specific anti-mannan immunoglobulin is two fold, IgA and the IgG subclasses are directly opsonic, and the binding of IgM and the IgG1 and 3 subclasses also activates the classical complement pathway leading to the deposition of C3 derived opsonins.

Aim

To measure specific IgA,G and M and the IgG subclasses 1,2 and 3 bound to mannan from normal human sera and to establish whether there was any relationship with the levels of C3 derived opsonins observed previously.

Method

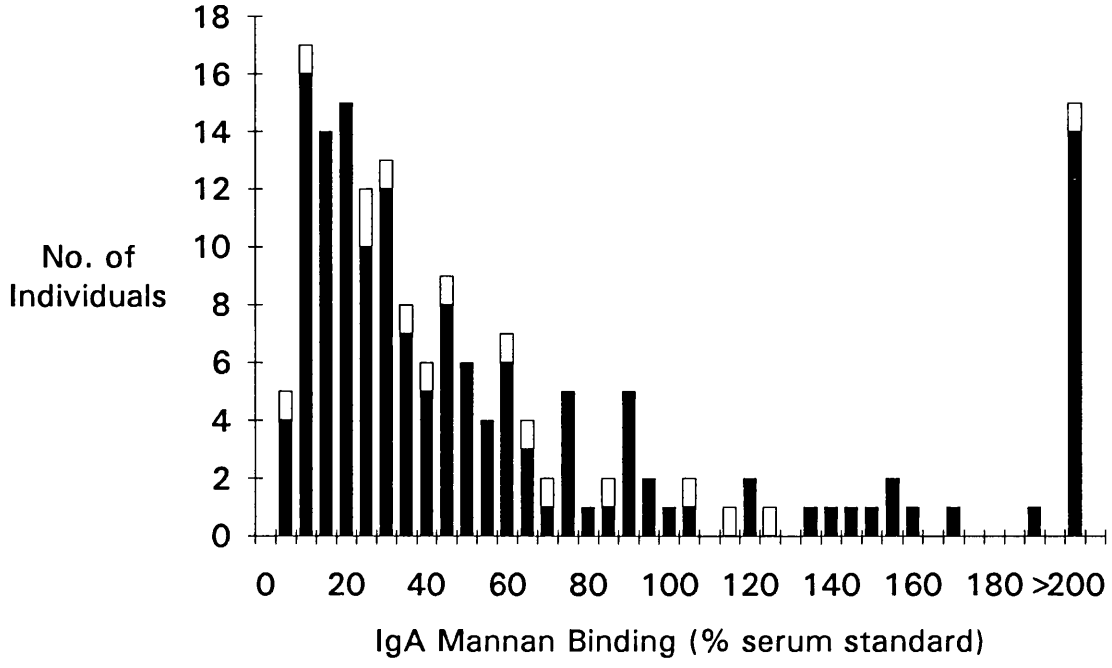
The method used was adapted from the method described by Yeaman and Kerr (1987) for measuring anti-mannan IgA. MicroELISA plates were coated with mannan as described previously. 179 sera from healthy adult blood donors were diluted to 5% using PBS-T containing 10 mM EDTA. Duplicate aliquots (100 μl) of the diluted sera were incubated in the plates for 2 hours at 37^{o}C. The plates were then washed four times with PBS-T before the addition of the appropriate indicator antibody. Peroxidase labelled goat anti-human IgA, IgG and IgM preparations (Sigma, Poole,

UK) were diluted 1/1000 in PBS-T and 100 μl volumes added to each well. After a 2 hour incubation at 37^{o}C the plates were washed four times with PBS-T and colour developed as in **Section 5.2.** Unknowns were interpolated using a logistic curve fitting programme from Titersoft (Flow UK Ltd). The amount of bound immunoglobulin was related to a standard curve constructed with doubling dilutions (0.07 - 20%) of a normal human serum pool prepared from 100 healthy adult blood donors. The binding patterns of IgG1, IgG2 and IgG3 subclass antibodies to mannan were also investigated for the 30 sera with the highest total IgG and IgM binding to mannan using biotinylated mouse monoclonal reagents purchased from Cambridge Bioscience (Cambridge, U.K.) at working dilutions of 1/500, 1/1000 and 1/250 respectively. After a further incubation with streptavidin peroxidase used at either 1/1000 (IgG2) or 1/250 (IgG1 and IgG3) the plates were developed as above and binding expressed as % of a normal human serum pool.

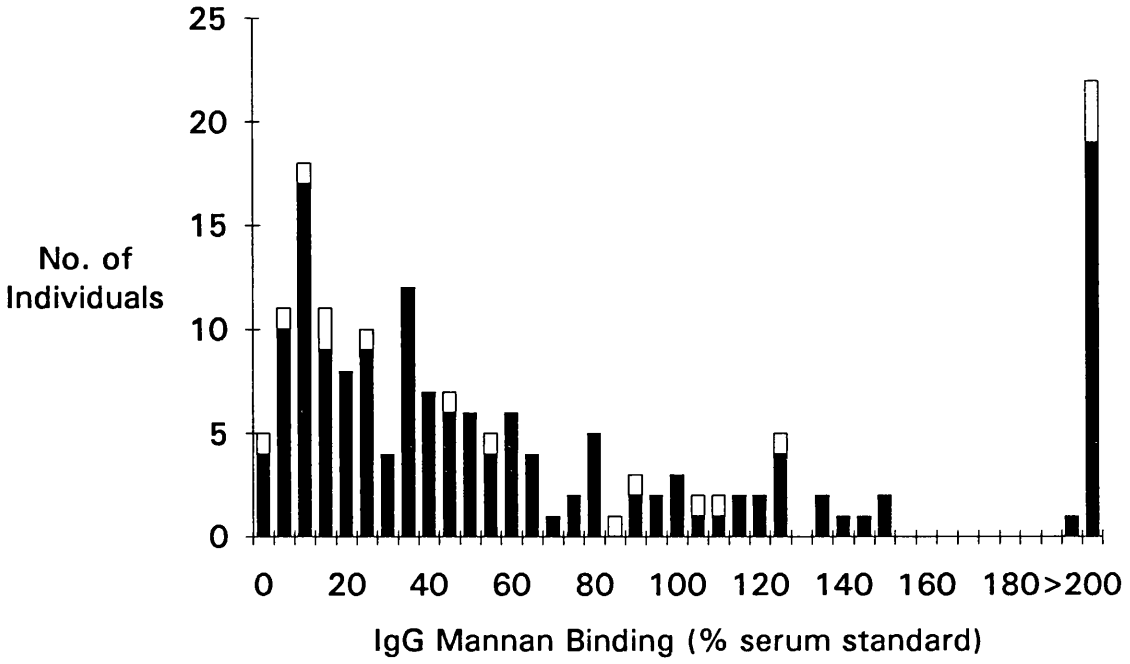
Results

The serum samples used for the study of mannan binding complement components were also investigated for specific total IgA, IgG and IgM binding in assays which were performed in the presence of EDTA in order to eliminate complement binding and possible steric hindrance. As shown in **Figure 5.7 (a)-(c)** several sera, including some with poor C3bi mannan binding activity, bound significant amounts of immunoglobulin of all three classes (>100% of pooled serum standard) but the levels of binding of the immunoglobulins did not correlate with C3bi deposition. See **Figure 5.7 (d)-(f) and Table 5.5.** Detailed results are presented in **Appendix 3.**

5.7(a)



5.7 (b)



5.7(c)

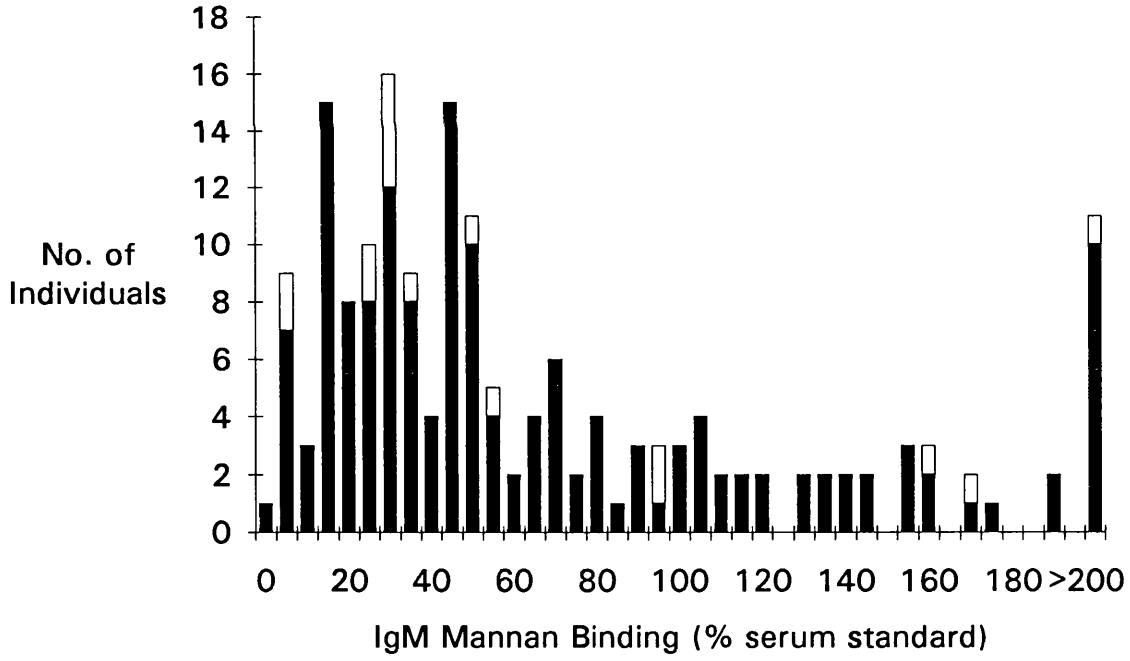
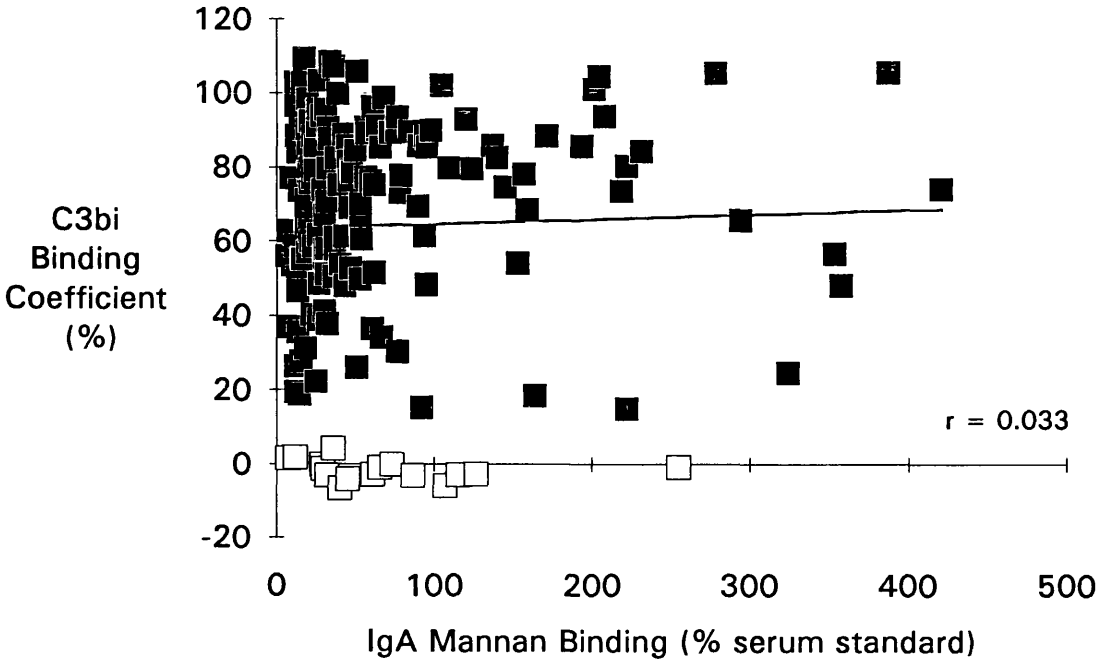
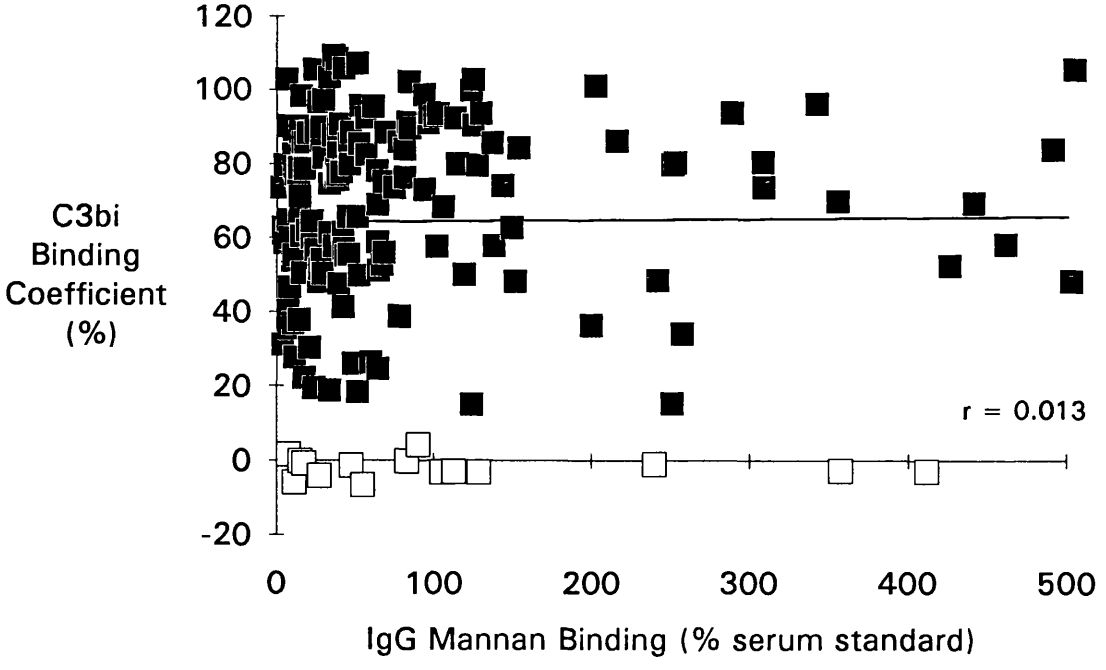


Figure 5.7 (a)-(c)Measurement of IgA,IgG,and IgM binding to mannan using sera from 179 healthy adults. Individuals with a C3bi binding coefficient of less than 10% are indicated with open bars.

5.7(d)


C3bi
Binding
Coefficient
(%)
120
100
80
60
40
20
0
-20
r = 0.033
0
100
200
300
400
500
IgA Mannan Binding (% serum standard)

5.7(e)


C3bi
Binding
Coefficient
(%)
120
100
80
60
40
20
0
-20
r = 0.013
0
100
200
300
400
500
IgG Mannan Binding (% serum standard)

5.7(f)

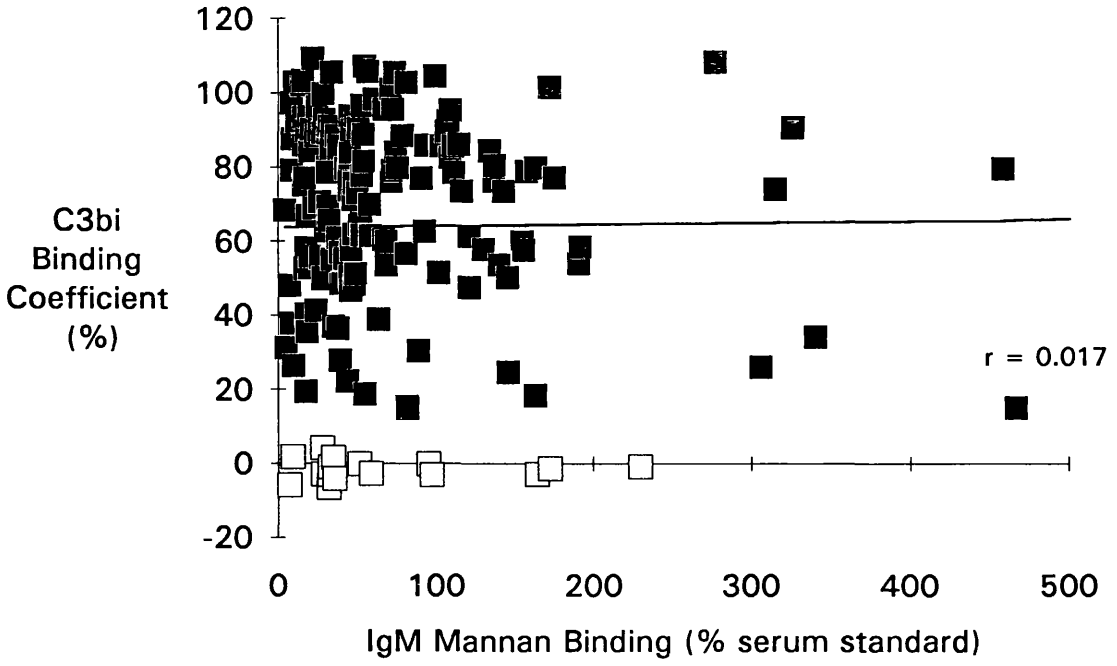


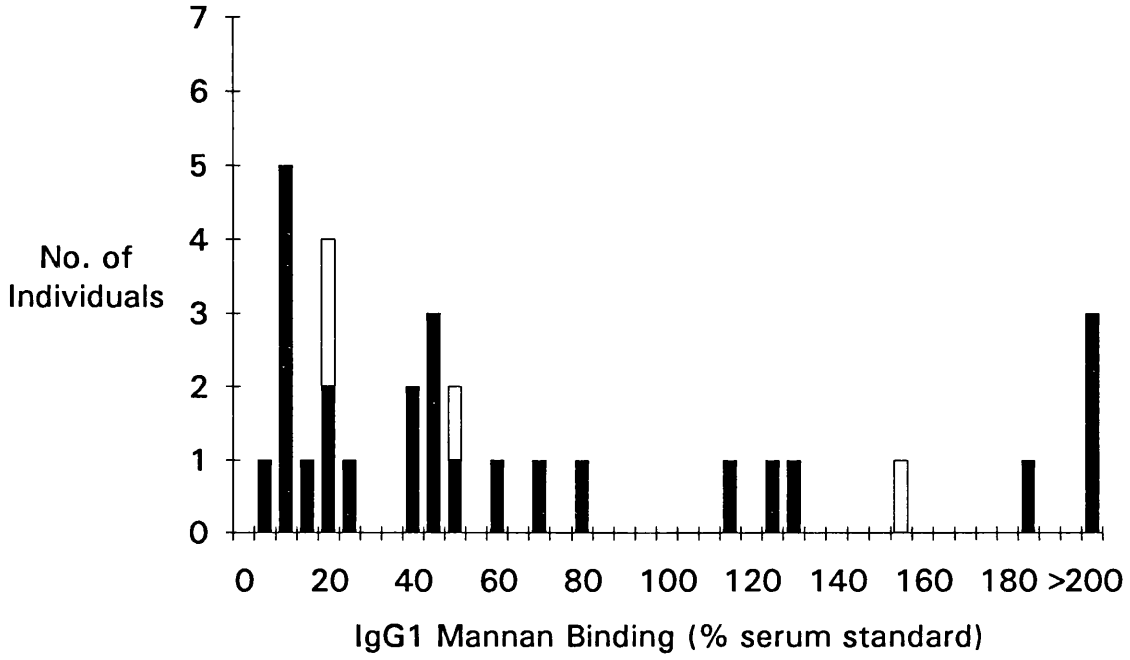
Figure 5.7 (d)-(f) Pearson correlations between results obtained using the mannan capture C3bi assay and mannan capture IgA, IgG and IgM assays. Hollow boxes identify the individuals with a C3bi binding coefficient of $<10\%$.

Table 5.5 Spearman rank correlation of mannan capture assays for complement components and mannan capture assays for IgG and IgM.

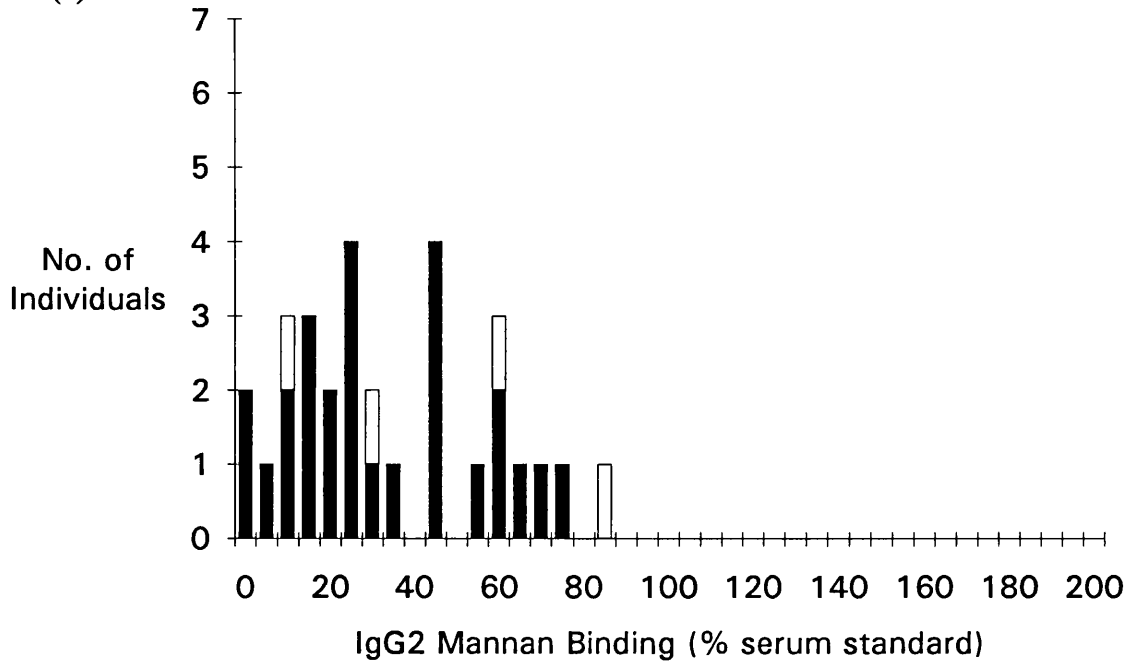
	IgG	IgM	IgA	C3bi	C4	P	Bf	
IgG	**1**	**0.06**		**0.092**	**0.104**	**0.082**	**0.082**	r_s
	(0)	**(0.44)**		**(0.230)**	**(0.174)**	**(0.288)**	**(0.287)**	**p**
IgM		**1**		**-0.02**	**0.013**	**-0.001**	**0.023**	r_s
		(0)		**(0.879)**	**(0.868)**	**(0.991)**	**(0.763)**	**p**
IgA			**1**	**N.A**	**N.A**	**N.A**	**N.A**	r_s
			(0)					**p**

Binding to mannan by antibodies of the three IgG subclasses IgG1, IgG2 and IgG3 was measured in the thirty individuals who had the highest levels of IgG and IgM binding(see **Figure 5.8 (a)-(c)**). The levels of antibodies in these subclasses were then compared with C3bi, C3b+bi, properdin, Factor B, and C4 bound to the plates (see **Figure 5.8 (d)-(f)** and **Table 5.6**). Detailed results on the individuals tested can be found in **Appendix 4.** In this subpopulation there was again a highly significant correlation between all of the non-immunoglobulin proteins studied but no correlation in binding to mannan between any subclass and the complement proteins.

5.8 (a)



5.8(b)



5.8(c)

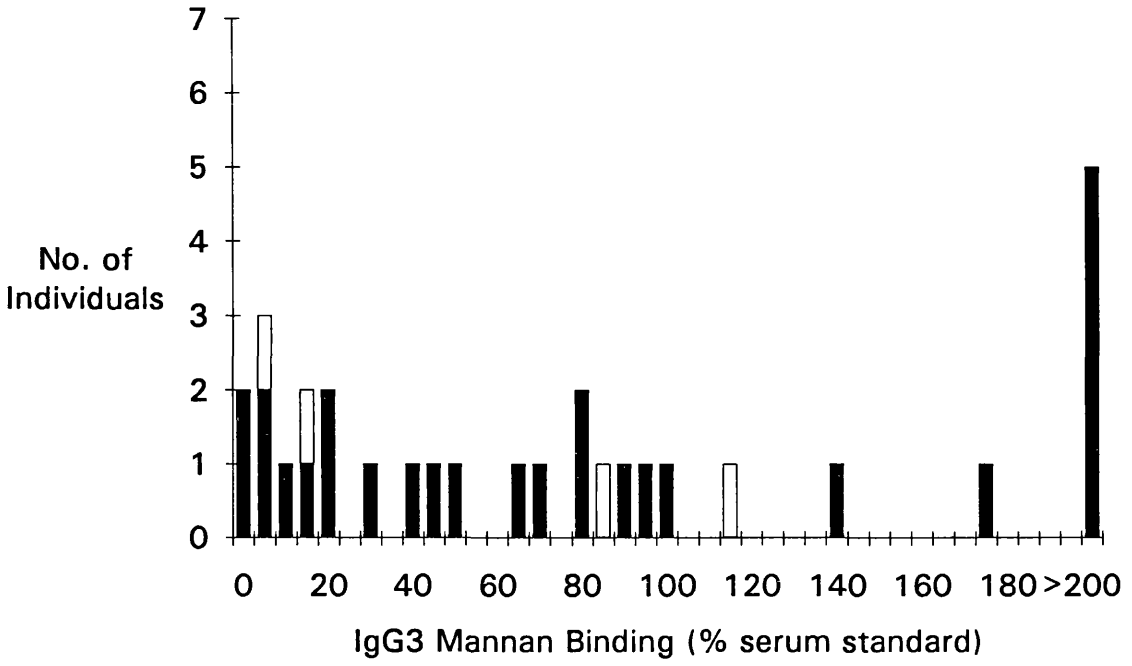
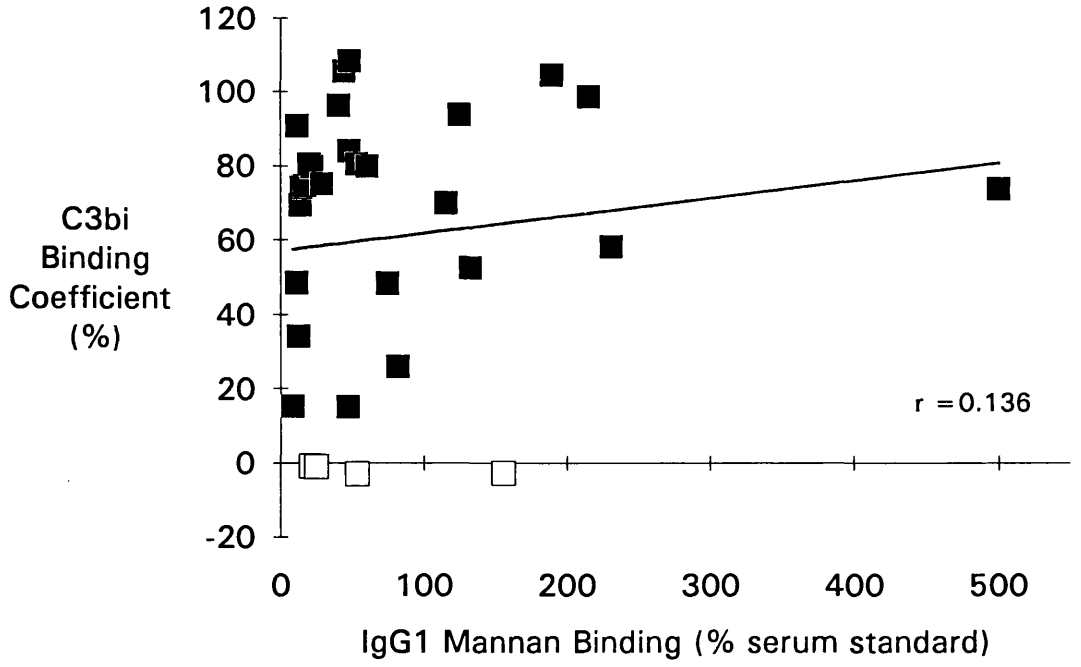
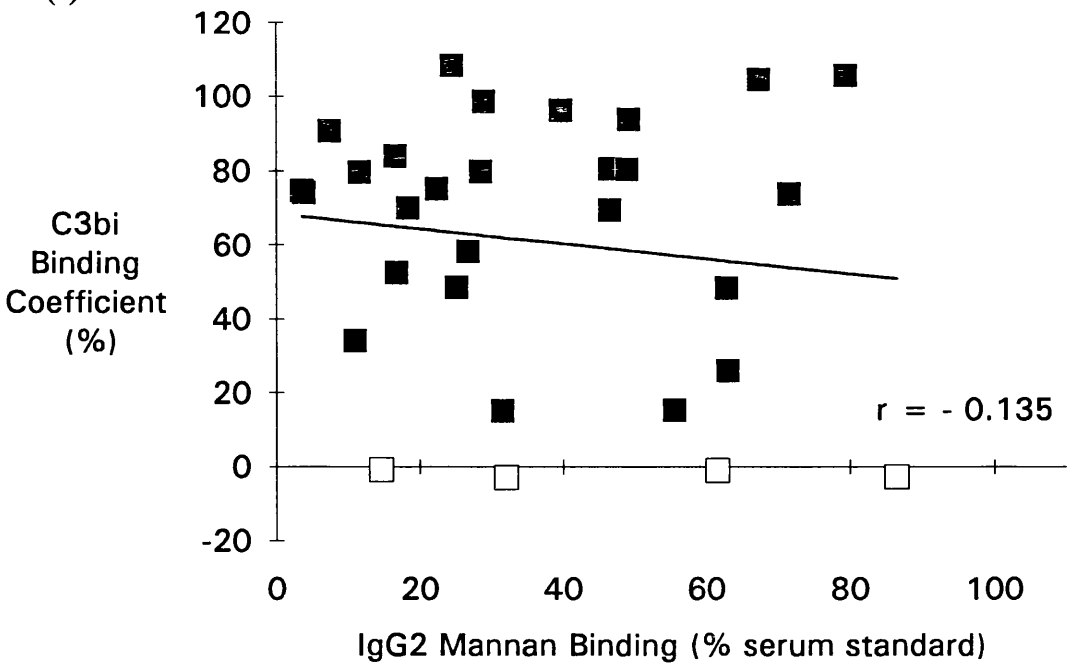


Figure 5.8(a) - (c) IgG subclasses 1,2 and 3 binding to mannan from the 30 sera with the highest levels of IgG or IgM mannan binding

5.8(d)



5.8(e)



5.8(f)

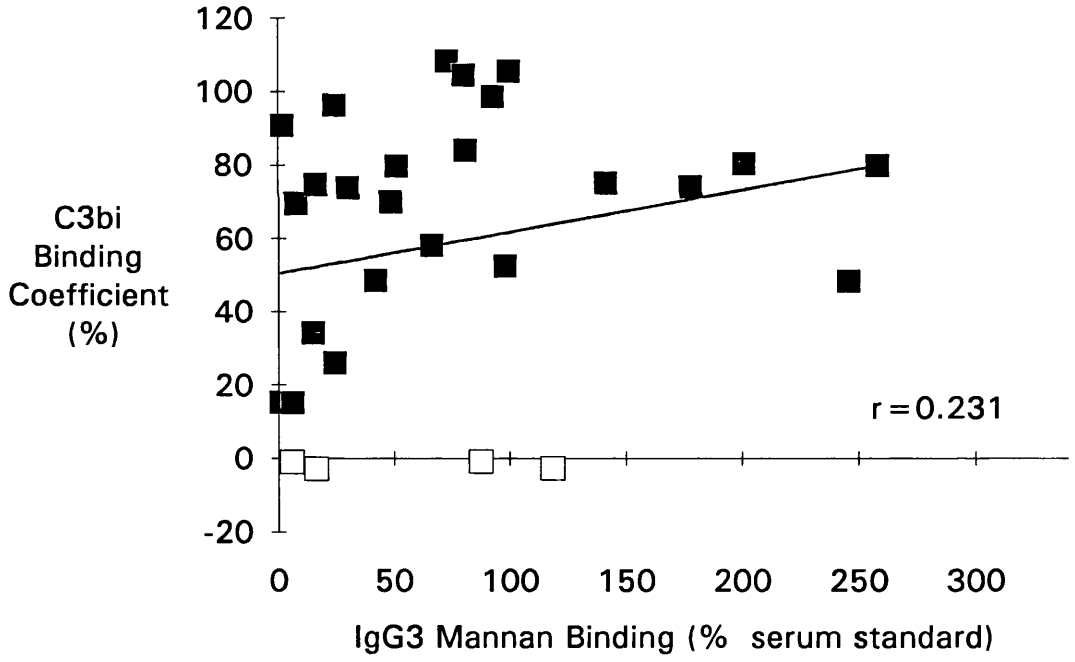


Figure 5.8 (d)-(f) Pearson correlations of results obtained using the mannan capture C3bi assay and mannan capture IgG subclass assays from the 30 sera with the highest IgG or IgM mannan binding.

Table 5.6 Spearman rank correlations of mannan capture complement assays with mannan capture Ig subclass assays.

	IgG1	IgG2	IgG3	C3bi	C4	P	Bf	
IgG1	1	0.42	0.369	0.112	0.155	0.340	0.264	r_s
	(0)	(0.021)	(0.053)	(0.555)	(0.413)	(0.066)	(0.159)	p
IgG2		1	0.057	-0.070	-0.098	0.016	0.061	r_s
		(0)	(0.771)	(0.714)	(0.606)	(0.931)	(0.747)	p
IgG3			1	0.309	0.272	0.067	0.268	r_s
			(0)	(0.110)	(0.162)	(0.351)	(0.168)	p

5.4 Mannan-binding correction assays

These assays resembled the mannan-binding assays described in **Section 5.2** with the exception that small amounts of normal sera, treated sera or fractions from chromatographic separations were added to the LB1 serum incubated at 5% in VBS^{++} on mannan-coated microELISA plates.

5.4.1 Correction using whole sera

Introduction

The aims of these experiments were to establish whether low concentrations of sera with normal opsonic potential could correct the opsonic defect in LB1 and other poorly opsonic sera, and whether the poorly opsonic sera could cross-correct.

Experiment

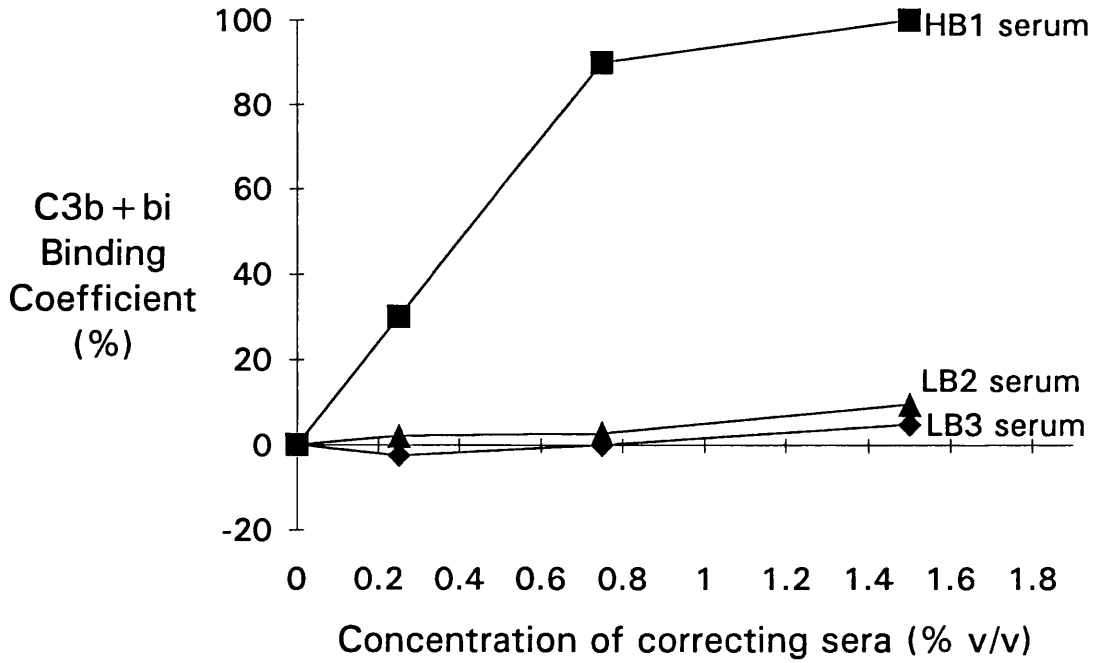
The LB1 serum at 5% concentration in VBS^{++} was mixed with increasing volumes of the normal opsonic serum HB1 and with the poorly opsonic sera LB1, LB2 and LB3 to give final concentrations of these sera ranging from 0.25 to 1.5%.100 μl aliquots of these mixtures were added in duplicate to the wells of mannan coated microtitre trays and the standard correction assay procedure was then followed. The plates were assayed for the binding of C3b+C3bi(anti-C3c), C3bi(anti-C3g), C4, properdin and Factor B (see **Figure 5.9**). In a second assay the 16 sera from the healthy adult population found to have low binding coefficients in the mannan-binding assay (see **Section 5.2.3**) were set up in a chequerboard array so that each serum was

tested for it's ability to correct or to be corrected by all the other sera. Each serum was diluted to 5% in VBS^{++} and all the other sera in turn were diluted to 1.5% and incubated with this serum. The LB1 and HB1 sera were included to provide the negative and positive controls respectively. The binding of C3bi, C4 and properdin was measured in this assay (see **Table 5.7**).

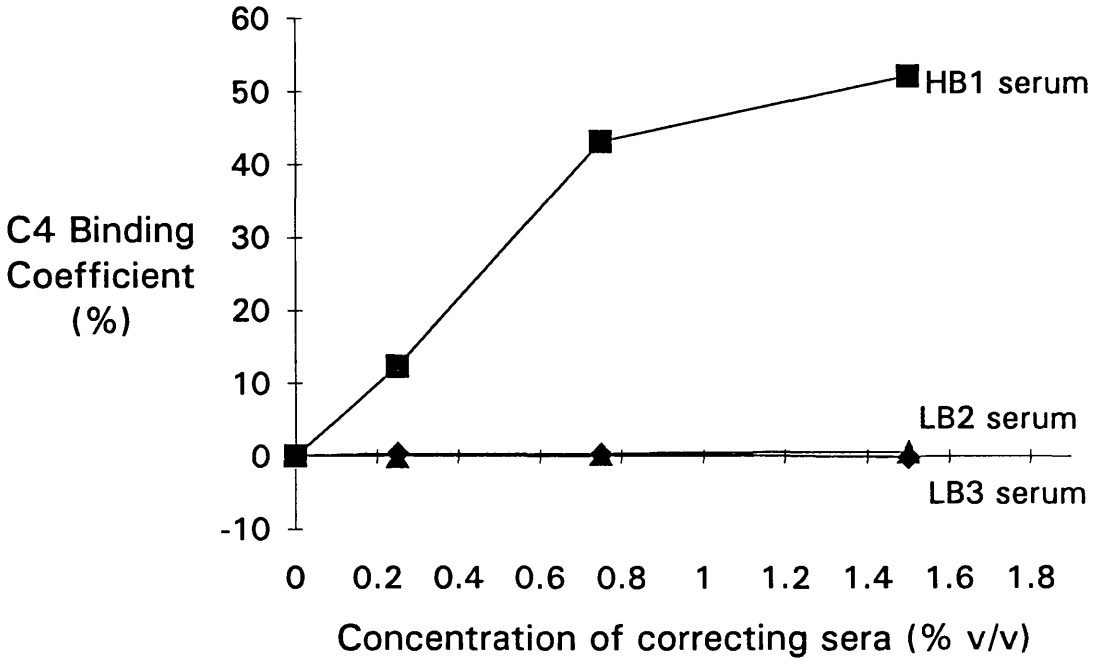
Results

Sera showing poor binding of complement components to mannan in the mannan ELISA assays could be corrected by the addition of small amounts of normal sera (see **Figure 5.9(a)-(e)**). This parallels the results obtained using the C3c elution assays. However, the titration of one serum with low binding coefficient into another similar serum (LB1) did not improve the binding coefficients (see **Figure 5.9(a)-(e)** and **Table 5.7**). There was some variation in response evident in the correction assays (**Table 5.7**), for example, serum "E" was not fully corrected by HB1 serum, serum "P" was partially corrected by a few poorly opsonic sera and serum "m" partially corrected other sera.

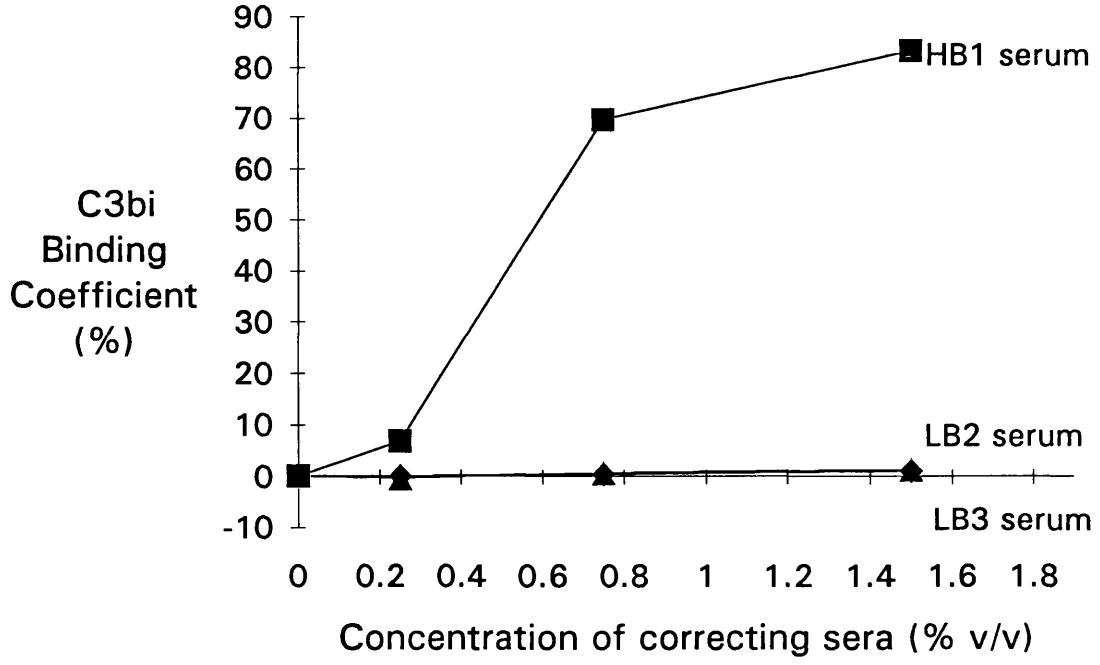
5.9(a)



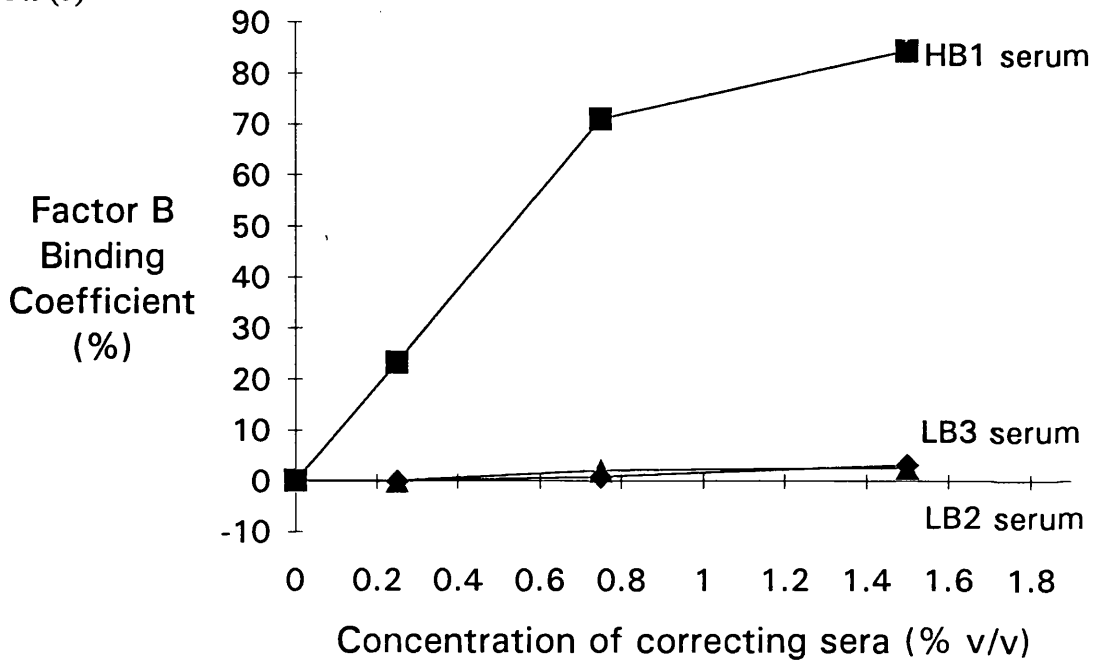
5.9(b)


C4 Binding Coefficient (%)
60
50
40
30
20
10
0
-10
HB1 serum
LB2 serum
LB3 serum
0 0.2 0.4 0.6 0.8 1 1.2 1.4 1.6 1.8
Concentration of correcting sera (% v/v)

5.9(c)


C3bi Binding Coefficient (%)
90
80
70
60
50
40
30
20
10
0
-10
HB1 serum
LB2 serum
LB3 serum
0 0.2 0.4 0.6 0.8 1 1.2 1.4 1.6 1.8
Concentration of correcting sera (% v/v)

5.9(d)



5.9(e)

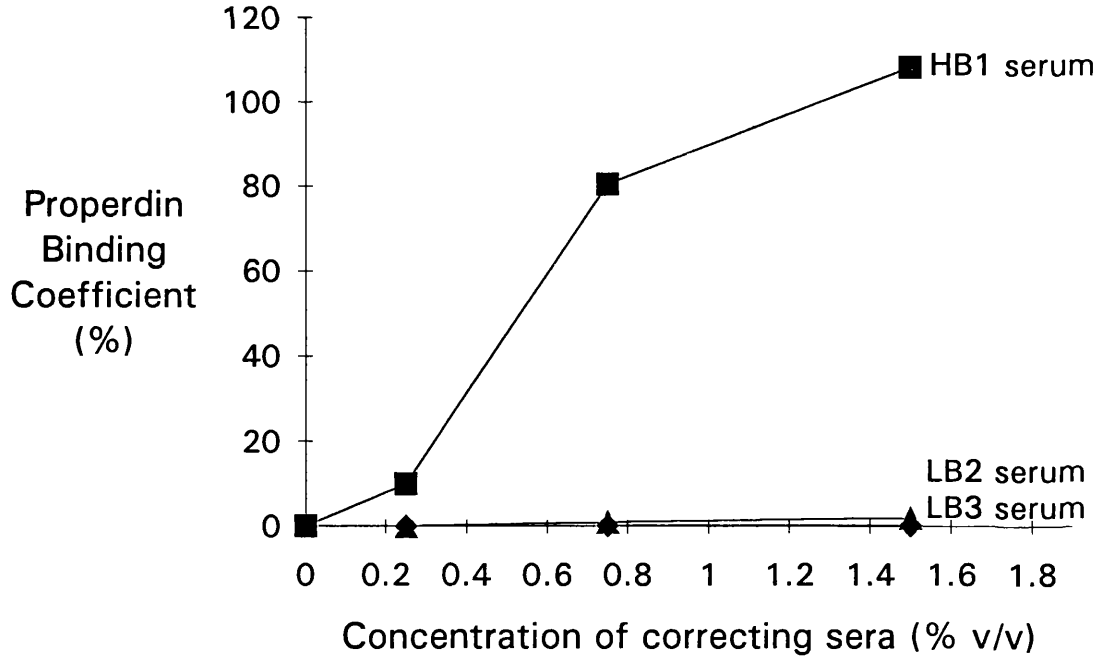


Figure 5.9 (a) - (e) Use of mannan capture assays for investigation of functional correction of opsonic defect. Various concentrations of a serum with normal opsonic function (HB1) and of sera with poor functional activity (LB2, LB3) were added to a serum (LB1) with established opsonic dysfunction. Binding coefficients for (a) C3b+bi (b) C4 (c) C3bi (d) Factor B and (e) properdin were then determined in the usual manner.

Table 5.7 Chequerboard mannan capture correction assays using HB1 and 16 poorly opsonic sera (measuring properdin binding). The columns (A - Q) represent the sera to be corrected (at 5% serum concentration) and the rows (a - q) represent the sera used for the attempted correction (at 1.5% concentration).Thus serum A is HB1 at 5% while serum a is HB1 at 1.5%. Sera B - Q are16 sera from the blood donor group found to exhibit low levels of complement binding to mannan; in addition, none of these 16 sera was able to correct the functional defect in LB1 serum. Similar results were obtained on measuring C3bi and C4 mannan binding moieties (data not shown).

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
a	\	+	+	+	*	+	+	+	+	+	+	+	+	+	+	+	+
b	+	\															
c	+		\													*	
d	+			\													
e	+				\												
f	+					\										*	
g	+						\									*	
h	+							\								*	
i	+								\							*	
j	+									\							
k	+										\						
l	+											\					
m	+	*			*	*							\				
n	+													\			
o	+														\		
p	+															\	
q	+																\

Code:

+	x > 50 BC(%)
	x < 10 BC(%)
*	25 BC(%) > x > 10 BC(%)

Comment

These assays provide strong evidence that there is a common defect in LB1 and the 16 other sera with opsonic dysfunction which resulted in their inability to cross-correct each other. In contrast, HB1 and another normal sera were able to correct the low binding coefficient of LB1 and the other functionally deficient sera.

5.4.2 Correction using NH_4OH treated sera

Introduction

NH_4OH treatment of serum inactivates the thiolester group in the binding-site of C3 and C4 thus preventing the formation of covalent ester links between these and carbohydrate surfaces. Sera similarly inactivated with KSCN were shown to retain their correcting potential (See **Chapter 4**) even though the C3c elution assay showed no C3 binding to zymosan from these sera.

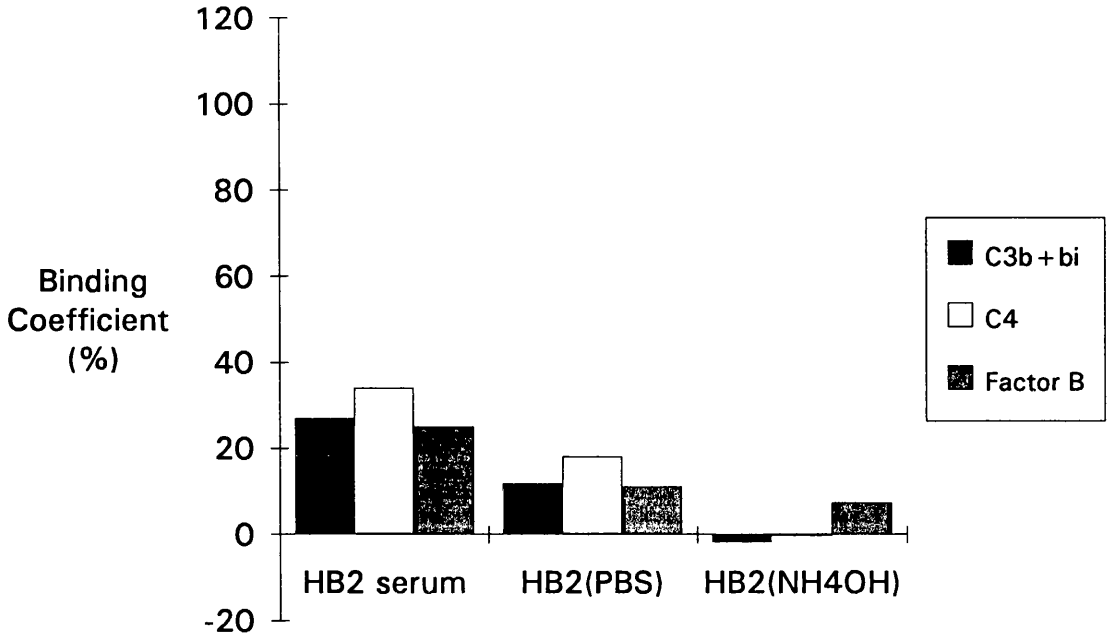
Experiment

HB2 serum was treated with NH_4OH by incubating 19 volumes of the serum with 1 volume of 2.5M NH_4OH at 37°C for 30 - 45 min. At the end of this incubation period the serum was returned to neutral pH by the addition of 1M HCl. A control was established by the addition of 1 volume of PBS to 19 volumes of HB2 serum and this was incubated as above and then a volume of PBS equal to the volume of HCl was added. The sera were diluted during the NH_4OH treatment and therefore sera were used at 2.5% rather than 5% concentration in the mannan-binding and mannan-correction assays.

Results

In the mannan-binding assays shown in **Figure 5.10 (a)**, the incubation of HB2 for 45 minutes at 37°C with PBS reduced the amounts of C3, C4 and Factor B bound to the mannan by approximately 50% when compared with the untreated serum. However, when the serum was incubated under similar conditions with NH_4OH, the levels of C3 and C4 bound reduced to approximately 0% binding coefficient. Some residual Factor B binding was observed, it is unclear whether this was artifactual or represented activated Factor B binding to mannan. In the correction assays **(Figure 5.10 b)** the binding coefficient values of the HB2 serum incubated at 37°C were again lower than the control, but both the PBS and the NH_4OH treated HB2 serum corrected the LB1 serum significantly.

5.10(a)



5.10(b)

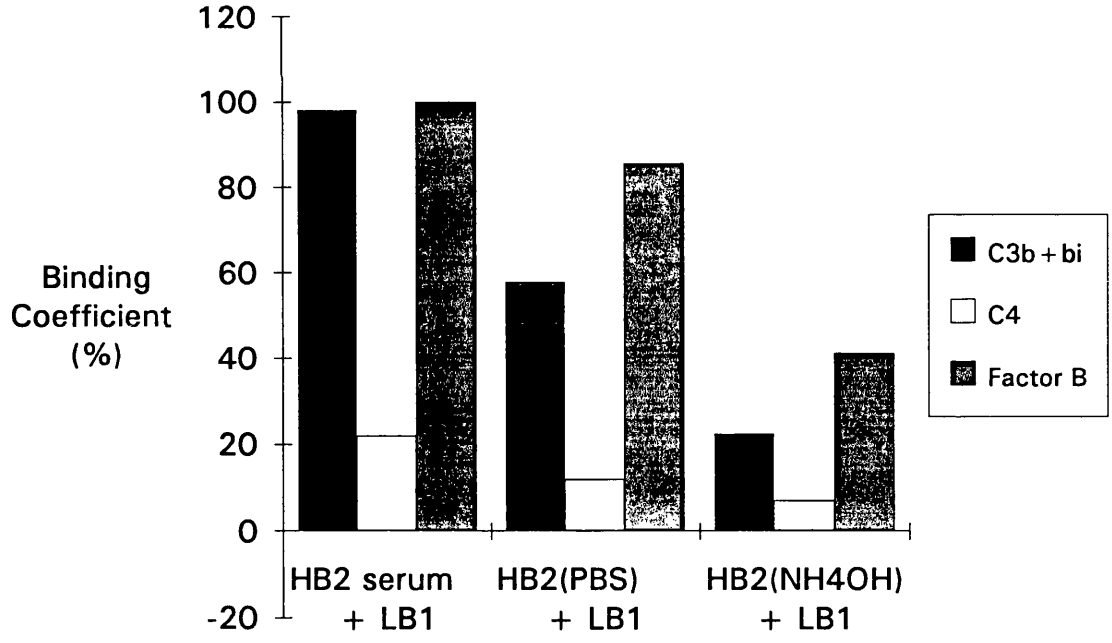


Figure 5.10 (a) and (b) Direct (a), and correction (b) mannan capture assays using a poorly opsonic serum (LB1) and an NH_4OH treated serum.

Note: HB2 serum was used at a final concentration of 2.5% in the direct and correction assays.

Comment

NH_4OH treatment of serum reduced the amounts of the C3 and C4 components binding to the mannan surface in mannan- binding assays to undetectable levels. However this treatment did not prevent the HB2 serum from successfully correcting the opsonic defect in the LB1 serum and this suggests (as was found in **Section 4.3.2**) that the correcting factor is probably not C3 or C4. The standard binding and correction assays showed that the incubation of the HB2 serum with PBS reduced specific binding of complement moieties to mannan. This probably reflected a generalised activation of complement during this incubation, possibly by alternative pathway tickover. Consequently C3 may have bound to the walls of the "Eppendorf" tubes or been consumed in the formation of the fluid phase $C3(H_2O)$ and subsequently inactivated by Factors H and I.

5.4.3 Studies using animal sera

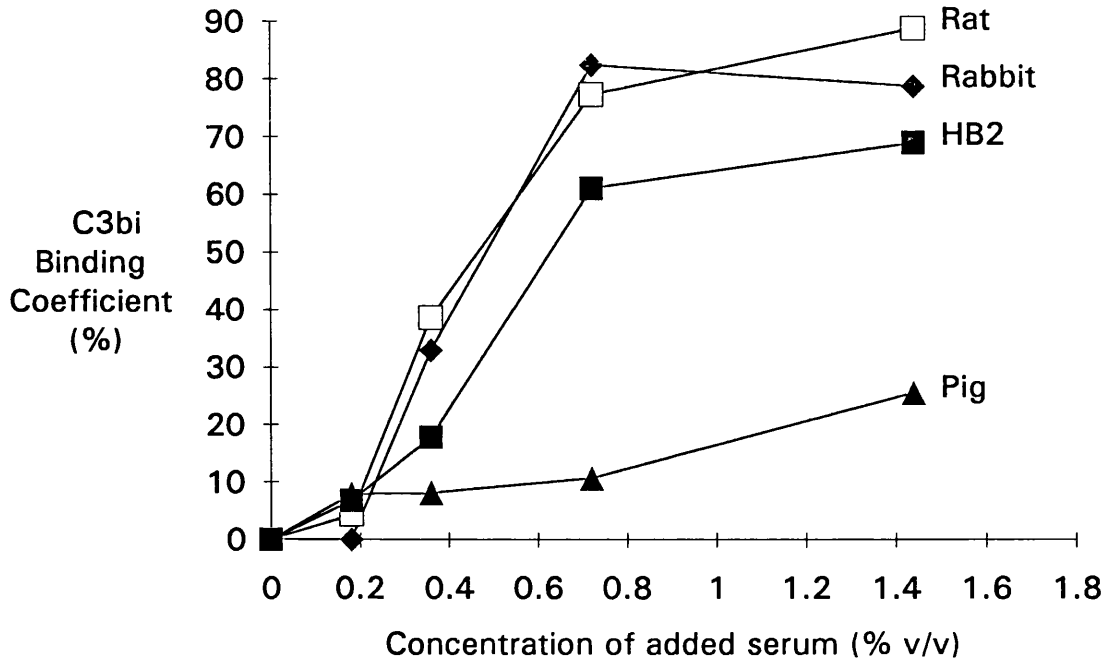
Protocol

Sera from eutherian mammals (pig, rabbit, and rat) and from marsupials (echidna, possum, quokka and tamar) were studied using the mannan-binding correction assay to determine the existence of animal factors with the ability to correct the human opsonic defect.

Results

Complement components binding to mannan from the animal sera tested were not detected using antisera specific for human complement proteins. However (in agreement with the findings obtained in the zymosan system) all of the animal sera tested were able to correct the defect in LB1 serum to some extent (see **Figure 5.11 (a)** and **5.11 (b)**.

5.11(a)



5.11(b)

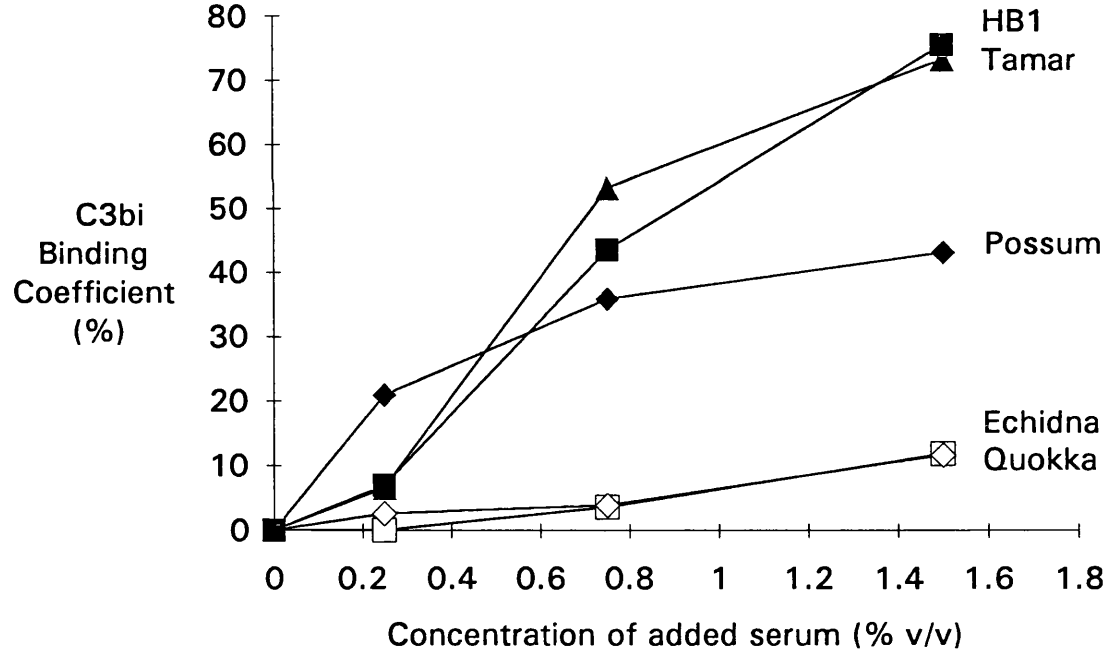


Figure 5.11 Mannan C3bi correction assays. Various sera from eutherian mammals (panel a) or marsupials (panel b) were added at different concentrations to serum LB1 and subsequent C3bi deposition was measured.

Comment

Marsupials have evolved separately since ancestral forms diverged from the eutherian mammals in the Mesozoic period (60-80 million years ago). The sera of the marsupials tested contained factor(s) which corrected a human serum with poor opsonic function, suggesting that this factor was present in the ancestral mammalian/marsupial line prior to speciation.

5.4.4 Studies with sera lacking known components

Protocol

The sera used in this experiment were the Factor D deficient serum (from Professor M. Daha, Leiden) used previously and three properdin deficient sera provided by Dr. A Sjöholm (Lund). These properdin deficient sera were described as: Variant 1 containing less than 0.1 μg/ml properdin (Söderström et al 1987)(this was the same serum as used in the experiments described in **Chapter 4**), Variant 2 containing between 1 and 2μg/ml properdin (Sjöholm et al 1988) and Variant 3 containing 19μg/ml of properdin (normal levels) but known to be dysfunctional (Dr A.Sjöholm personal communication). These sera were mixed with the LB1 serum in a mannan-binding correction assay and were also assayed in the direct mannan- binding assay. Levels of bound C4, C3b+bi, C3bi, properdin and Factor B were determined as previously described.

Results

In correction assays, (**see Figure 5.12 (a)**) the Factor D deficient serum and the properdin deficient serum Variant 3 were able to correct the opsonisation defect which suggested that neither Factor D nor properdin was the factor absent in the LB1 serum. However, the properdin deficient sera variants 1 and 2 did not correct the LB1 serum and there was no increase in the binding of the complement components to mannan. In the correction assays there a was good correlation between the binding coefficients of all the complement components (C3b+bi, C3bi, C4, properdin and Factor B) bound to the mannan surface (data not shown). In the mannan-binding assay (see **Figure 5.12 (b)**) there was no binding of any of the complement components tested from the properdin deficient sera Variants 1 and 2. In assays using Factor D deficient serum and properdin Variant 3, the binding of the complement components did not correlate- the C4 and C3 moieties bound, but with properdin deficient (V3) serum no properdin or Factor B were bound and using the Factor D deficient serum no Factor B was bound to the mannan surface.

5.12 (a)

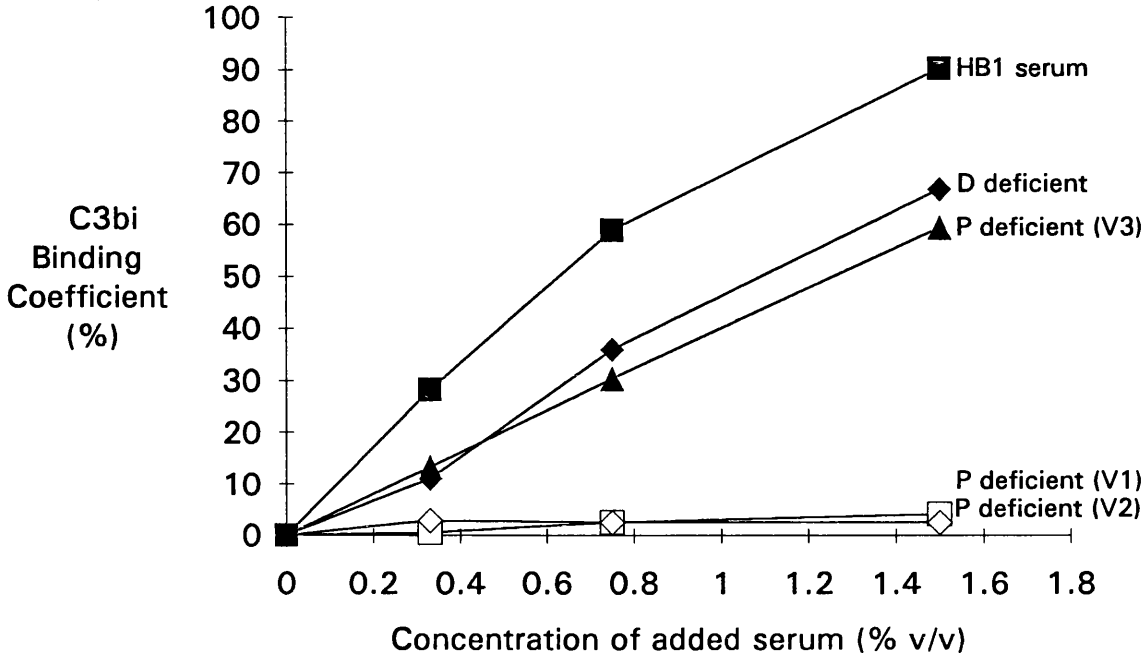


Figure 5.12(a) Mannan capture C3bi assay of attempted correction of poorly opsonic serum (LB1) with Factor D deficient serum and three properdin deficient sera (Variants 1-3).

5.12(b)

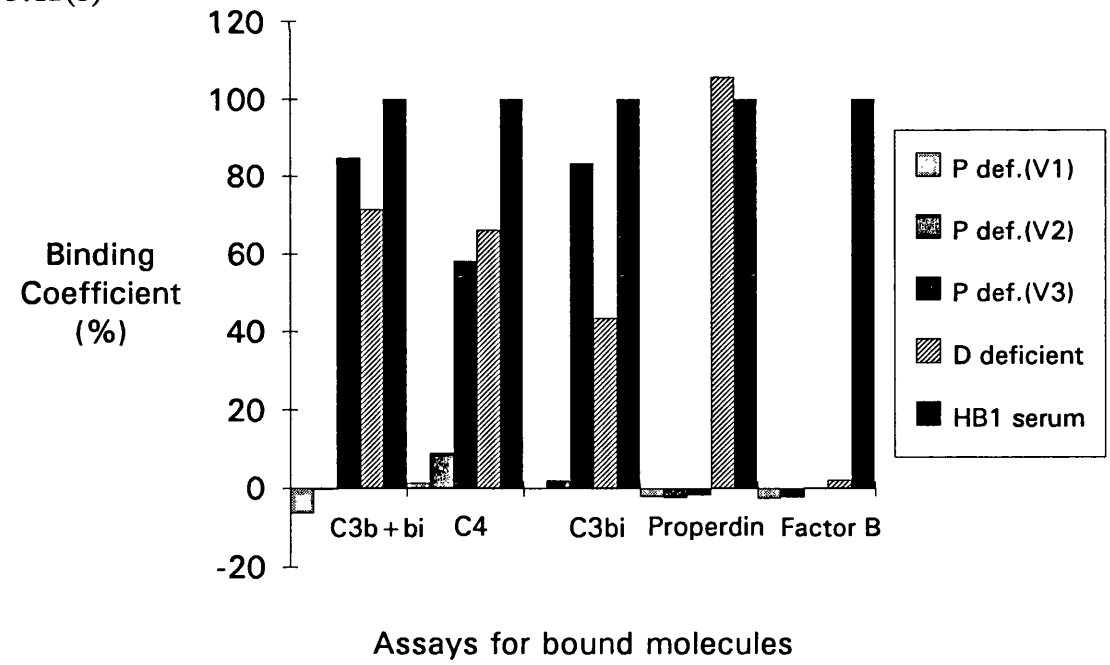


Figure 5.12(b) Mannan capture assays measuring various complement moieties bound from control serum (HB1) or sera deficient in properdin (V 1-3) and Factor D.

Comment

The results presented in this section again suggest that the absence of Factor D from a serum does not influence the ability of that serum to correct the common opsonic defect. Therefore it is unlikely that a Factor D abnormality underlies the defect. However the D deficient serum tested by the mannan-binding assay gave different results from those obtained using the C3c elution assay (see **Section 4.3.5**). Although there was little material detected following C3c elution, the levels of C4, C3bi, C3b+bi and properdin bound to mannan were within the normal ranges,and only Factor B showed reduced levels of binding.

As expected, the mannan binding assay results confirm that Factor D deficiencies influenced the AP rather than the CP and the differences observed between the C3c elution and mannan binding assays probably reflects a greater role for the AP in the C3c elution assay which uses 16.67% serum. Results with the three properdin deficient sera were more difficult to evaluate. The assays with Variants 1 and 2 suggested that properdin might be implicated in the opsonic defect and yet the results obtained with the Variant 3 serum contradicted this. Therefore it seemed that, either Variant 3 was not a properdin deficient serum, or else that there might be a coexisting defect in Variants 1 and 2. In the direct mannan- binding assay the Variant 3 serum failed to deposit properdin or Factor B on the mannan surface and when this serum was tested in an AP lysis assay (see **Chapter3**), the levels of alternative pathway activity were well below the normal range. Both these assays suggested that properdin was dysfunctional or absent in the Variant 3 serum. The use of an anti-properdin ELISA (see **Chapter 3**) confirmed that the concentration of properdin was approximately 19μg/ml in this serum,and it was concluded that the protein was indeed dysfunctional.

The apparent discrepancies between these three properdin deficient sera were eventually resolved in **Chapter 6**.

5.4.5 Studies with human sera depleted of known components

Introduction

In addition to the studies in **5.4.4** with sera naturally deficient in properdin, it was possible to prepare sera immunochemically depleted of properdin and to test these in the mannan-binding correction assay.

Experiment

Serum HB1 equilibrated in PBS containing 10mM EDTA was depleted of properdin by passing it through a column containing the anti-properdin monoclonal antibody HYB3-3 immobilised on a Sepharose matrix (see **Section 2.4.4**). The flow-through material from the anti-properdin affinity column was readjusted to the volume of the original serum and was tested in an anti-properdin ELISA. The concentration of properdin in the flow-through material was found to be less than 0.1 μg/ml (the detection limit of the ELISA) and thus had a similar concentration to the Variant 1 properdin deficient serum. In addition, two other affinity resins were prepared using polyclonal anti IgG and anti albumin antibodies (from Scipac UK Ltd) respectively and serum HB1 equilibrated in PBS/10mM EDTA was also passed through these. The flow-through materials from these affinity columns were not assayed for concentrations of IgG or albumin as these columns were being used as controls for the affinity purification technique and not necessarily to deplete the serum of IgG and albumin (which would have been difficult as the concentrations of these in serum are much greater than that of properdin). A separate aliquot of HB1 serum was diluted to 1/10 with PBS EDTA buffer and this and the flow-through materials from the affinity columns were diluted similarly and tested for their activities in the mannan-binding correction assay and the direct mannan-binding assays. *Note*: sera were diluted during chromatography and therefore the concentration of the sera used in the direct mannan-binding assay (**Figure 5.13(b)**) was 2.5% rather than 5% .

Results

In the mannan-binding correction assay the untreated HB1 control serum and the preparations which had been passed through the affinity columns corrected the LB1 serum equally well and the binding levels of all the complement components assayed were significantly correlated with each other. This is evident in the C3bi binding assay (**Figure 5.13(a)**). However, in the case of the direct mannan-binding assay, although there were high levels of C4, C3b+bi and C3bi binding to the mannan surface from all the preparations, there was no detectable properdin or Factor B binding from the properdin depleted serum (see **Figure 5.13b**).

5.13(a)

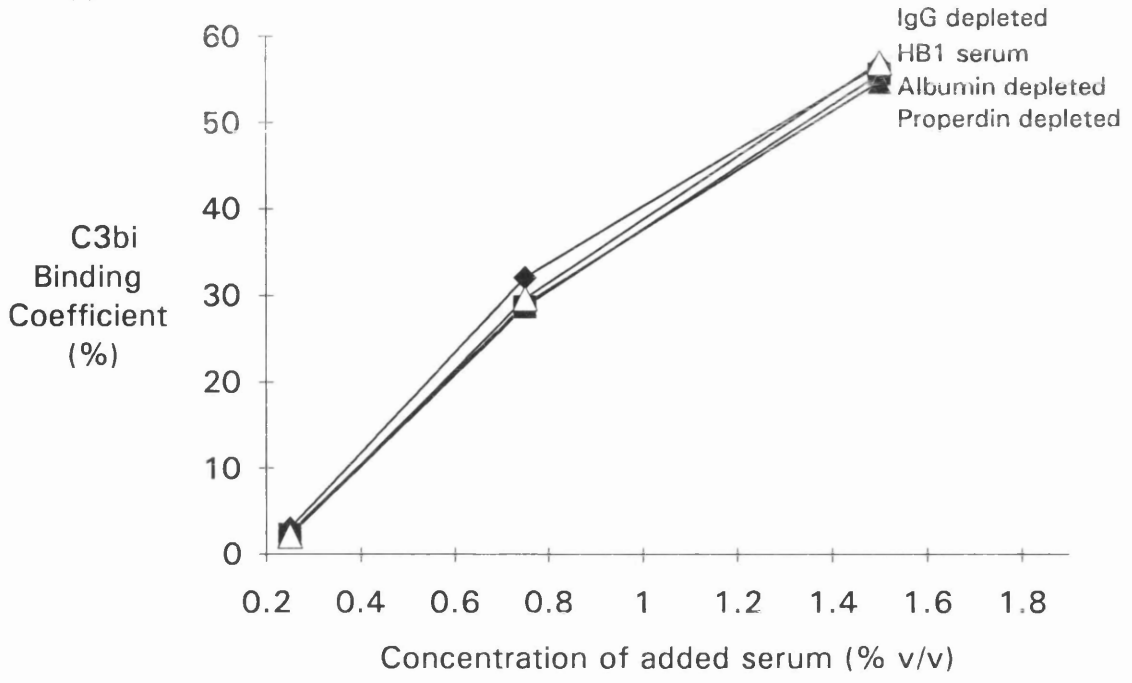


Figure 5.13(a) Mannan capture C3bi correction assays. Increasing concentrations of serum HB1(untreated) or of the same serum following affinity depletion of IgG, albumin or properdin, were added to serum LB1. Similar results were obtained for binding of the other complement components tested (data not shown).

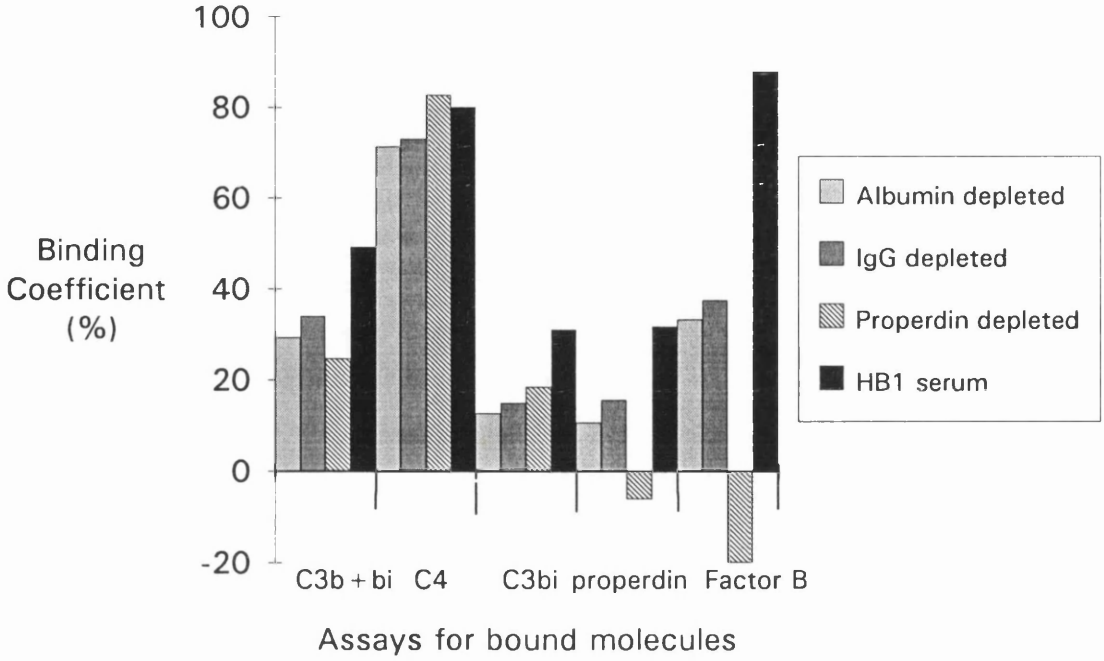


Figure 5.13(b) Mannan capture complement assays measuring C3b+bi, C4, C3bi, properdin and Factor B from a normal serum depleted of albumin, properdin or IgG. Note that these preparations were tested at 2.5% serum rather than 5%.

Comment

Affinity depletion of properdin from serum HB1 yielded a serum with immunochemical properdin levels of less than 0.1μg/ml in the ELISA assay described in **Chapter 2.** This preparation reacted in the mannan-binding assay and mannan-binding correction assays in the same manner as the Variant 3 properdin deficient serum assayed in **Section 5.4.4** i.e. in the correction assays this serum was able to correct LB1 serum raising binding coefficient levels into the normal range. In the direct mannan-binding assay the properdin depleted serum bound normal levels of C4 and C3 fragments but neither properdin nor Factor B could be detected. These studies provide further support for the view that a properdin deficiency is unlikely to be the basis of the common opsonic defect. In addition, further assays of properdin concentration and function were carried out on selected poorly opsonic sera using the techniques described in **Chapter 2.** There were no differences in the quantitative levels of properdin between this group and the general population, nor were there any differences in AP function (using rabbit erythrocyte lysis assays), immunochemical properties (using SDS-PAGE and Immunoblotting) and pI (using Isoelectric focusing of affinity purified properdin) (data not shown).

When considered together, the experiments outlined in **Sections 5.2.2, 5.4.4** and **5.4.5** suggest that the factor which is deficient or dysfunctional in the poorly opsonic sera may be a classical CP component. Activation of the classical pathway is usually preceded by formation of an antibody-antigen complex which then reacts with C1q. It would therefore appear reasonable that the concentration of mannan-specific complement-activating antibodies in sera would have a significant effect on the activation of the CP at the mannan surface and sera such as LB1 might be expected to have had low levels of such antibodies. However, in these experiments no C1q appeared to bind to mannan and there was no correlation found between the levels of the immunoglobulins IgA, IgG, IgM or the subclasses IgG1,2,3 bound to mannan and the levels of the complement components bound. This suggests that, although the CP was implicated in the processes leading to opsonisation, the levels of specific anti-mannan immunoglobulins in a serum were not critical in determining the observed binding pattern.

5.4.6 Studies using fractionated sera

Introduction

Turner et al (1985b) showed that specific fractions of HB1 serum purified by anion exchange chromatography corrected serum LB1 in the C3c elution assay. In the present study, HB1 and HB2 sera were fractionated by gel filtration, anion exchange and chromatofocusing techniques(see **Chapter 2**) separating on the basis of effective molecular radius, charge and pI respectively. The fractions obtained were tested for their ability to correct LB1 serum in the mannan correction assay, and specific complement components were assayed using appropriate ELISA procedures.

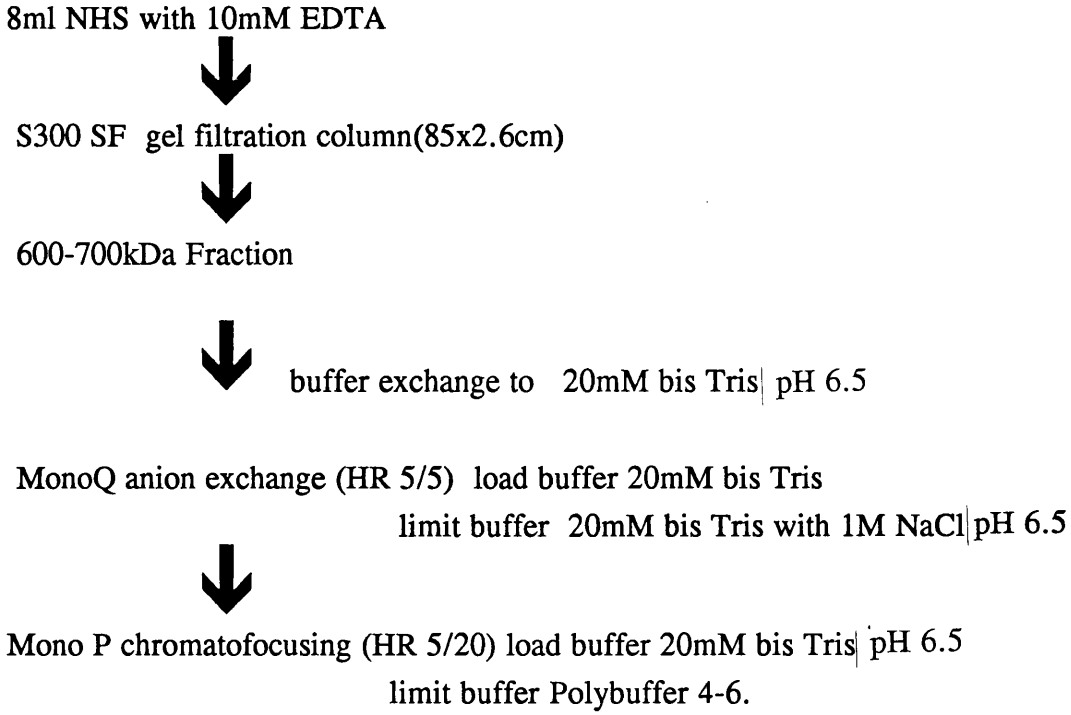
Aim

To purify the correcting moiety in order to establish its identity and to distinguish it from other candidate molecules.

Methods

The steps used in the purification of the correcting protein are shown in **Flow-diagram 5.1.**

Flow diagram 5.1 Fractionation of normal human serum (NHS) to isolate the correcting protein.

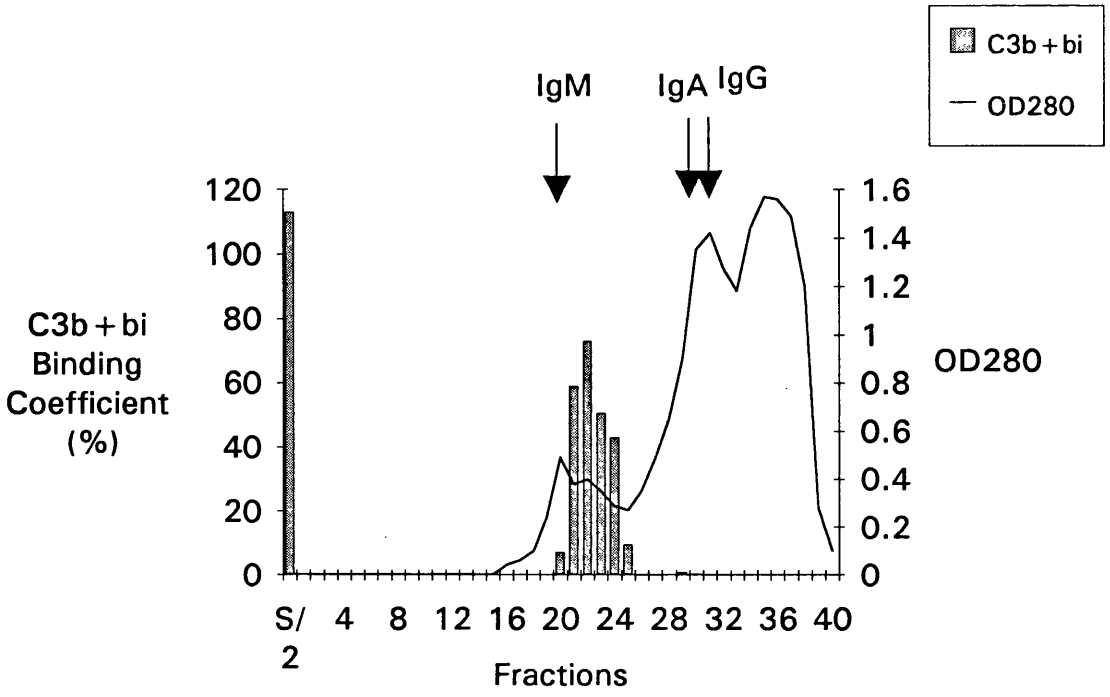


8ml of normal human serum HB1 were mixed with 0.5M EDTA to give a final concentration of 0.01M EDTA. This was run at 12ml/hr through a S300-SF gel filtration column of dimensions 2.6x85 cm in PBS- 10mM EDTA running buffer. The absorption of the eluate was measured at 280nm and fractions were collected over 30 minute periods. These fractions were then tested for their ability to correct the opsonic defect in LB1 serum.Those fractions showing correcting activity were dialysed and concentrated in 20mM bis-Tris pH 6.5 (conductivity 0.4μSi/cm^2) using an Amicon stirred cell,(Amicon UK Ltd). 500μl volumes were loaded onto the Mono Q HR 5/5 anion exchange column (Pharmacia UK Ltd) equilibrated in the bis-Tris buffer with a limit buffer of 20 mM bis-Tris pH 6.5 containing 1M NaCl. This chromatography run consisted of a stepped gradient with a rapid rise to 0.3 M NaCl to remove contaminating proteins and a gradual increase to a final concentration of 1M NaCl to purify the correcting protein. The absorbance of the eluted fractions was measured at OD280 and the fractions were again tested in the correction assay and in assays for various complement components. Correcting fractions from the Mono Q column were again concentrated in the Amicon cell and returned to the 20mM bis Tris buffer. This was loaded onto a Mono P chromatofocusing column and run at 0.2 ml per minute with a start buffer of bis-Tris and a limit buffer of Polybuffer 4-6 (Pharmacia UK Ltd). The fractions were tested for correcting activity and the concentrations of complement components were measured in the fractions using the assays described in **Chapter 2**. Total levels of IgA, IgG and IgM were measured in the column fractions using techniques established by Dr J. G. Shields at ICH and described in detail in " Lymphokines and Interferons. A practical approach". Editors Clemens,Morris and Gearing (IRL press). Briefly these assays consisted of an antibody capture phase of affinity purified goat anti- alpha, gamma or mu chain and horseradish peroxidase labelled detector antibodies which were also specific for the heavy chain of the immunoglobulin classes. These antibodies were obtained from Sigma UK Ltd and are catalogued in **Table 2.1**. Immunoglobulin concentrations were calculated with reference to a secondary standard prepared by Dr Shields.

Results

Figure 5.14 panel (a) shows the absorbance at OD280 of the S300 gel filtration chromatography of 8ml of HB1 and the positions of the correcting fractions and positions of the marker immunoglobulins IgA, IgG, IgM (see **panel (a)**). The positions of the complement components C3, C4 and Factor B are shown in **panel(b)**). The correcting moieties were found between fractions 45 and 50 with the maximum concentration in fraction 47 i.e. approximate molecular weight 600-700K. This region was distinct from the fractions containing high concentrations of the other

5.14(a)



5.14(b)

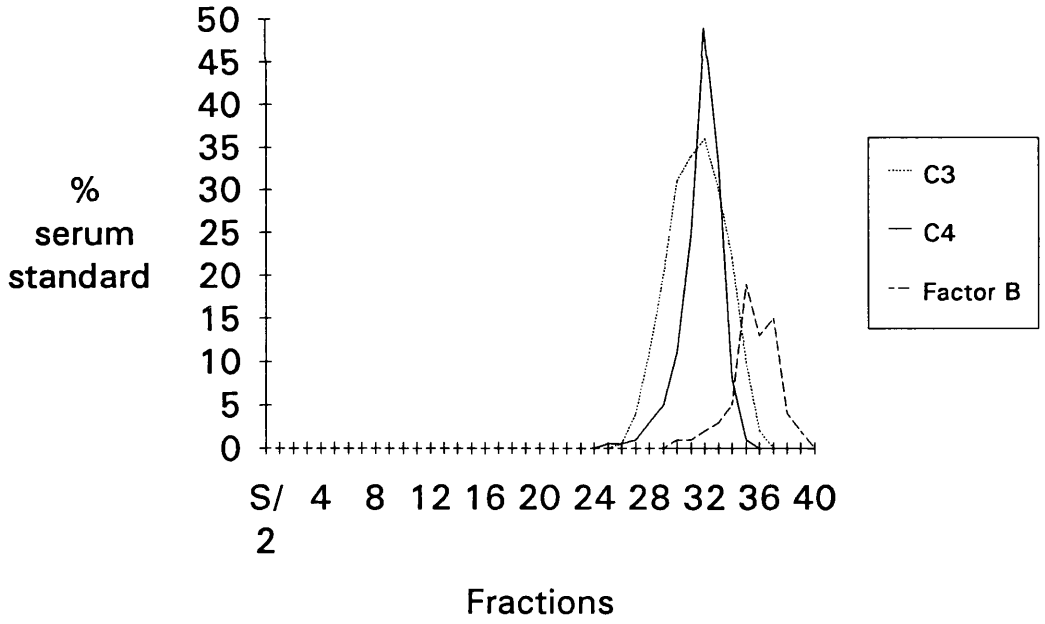
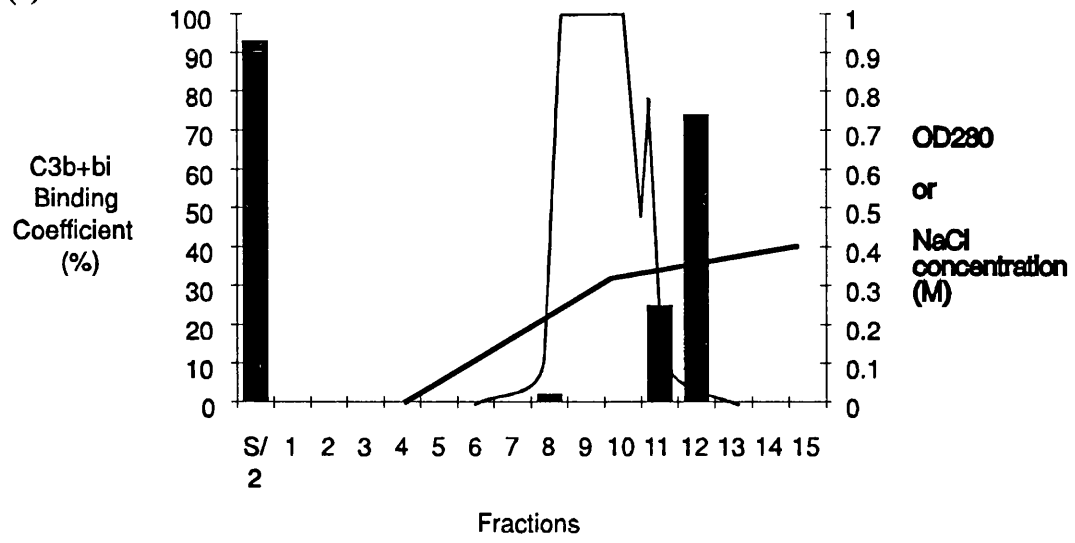


Figure 5.14(a) Fractionation of 8ml of normal serum(HB1) by S-300 SF gel filtration (column dimensions 2.6x85cm). The line represents the absorbance of the fractions at OD280 and the bars represent the mannan capture C3b+bi correction assay of LB1 corrected with the column fractions.S/2 is the starting material diluted 1/2.
(b) Elution positions of the complement components C3, C4 and Factor B in HB1 serum fractionated by S300-SF gel filtration (column dimensions and flow rate as for panel (a)).

5.15(a)



5.15(b)

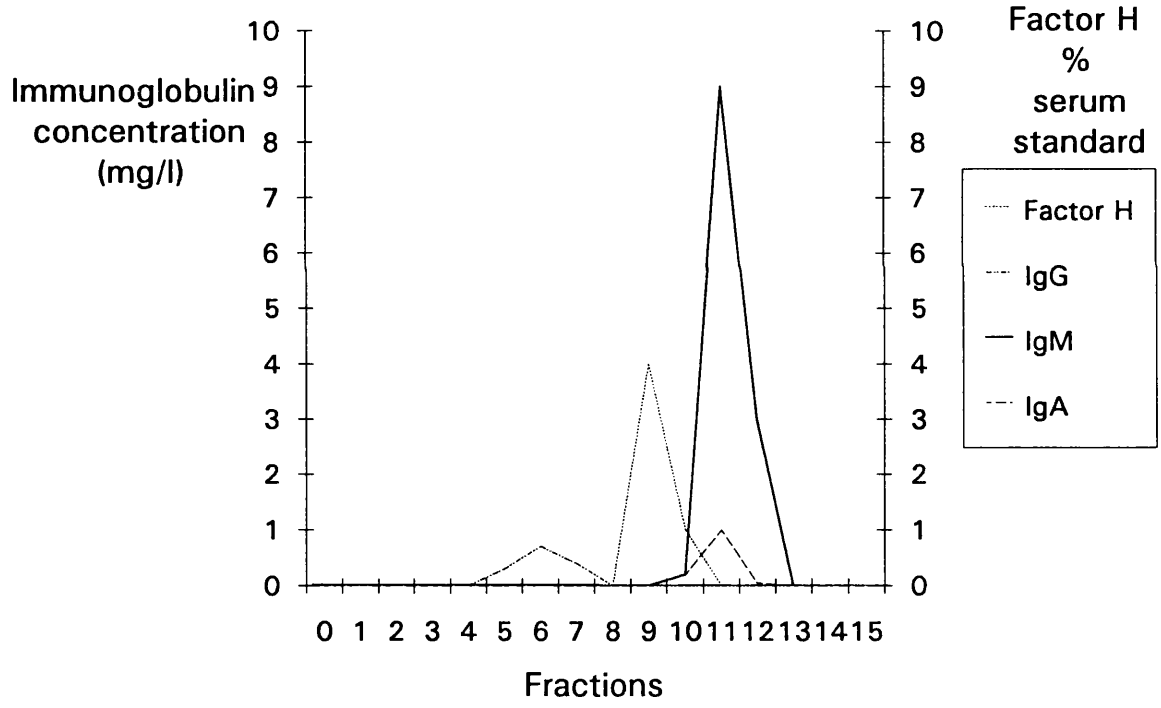


Figure 5.15(a) Anion exchange fractionation of pooled material (fractions 20-25) from the S-300SF gel filtration separation (see **Figure 5.14a**) and mannan capture C3bi+bi correction assay of LB1 serum corrected with column fractions (solid bars). The recorded OD280 is shown as a thin line whereas the gradient of increasing ionic strength is indicated by the thick line. (b) Elution positions of complement component Factor H and immunoglobulins IgG,IgA and IgM.

Results (Contd.)

molecules tested(see panels **(b)**). Small amounts of properdin, Factor H, IgA and IgM were found in this region, but these were largely removed during the subsequent ion-exchange chromatography (see **Figure 5.15**).

The trace in **Figure 5.15 panel (a)** shows the absorbance at OD280 following MonoQ anion exchange chromatography of the fractions 20-25 from the S300 gel filtration and the results of assays for correcting potential. The panel below shows the elution positions of IgA, IgG, IgM and Factor H . No detectable levels of C3, C4, Factor B, properdin or Factor I were found in fractions from this chromatography run. Mono-P chromatofocusing of Mono-Q Fraction 12 showed that the correcting fraction had a pI of approximately 4.5. This result is not shown here because by this stage of the purification the levels of protein were at the limits of detection.

Conclusion

Material able to correct the opsonic dysfunction in serum LB1 appeared to have a molecular weight of 600-700kDa, a pI of approximately 4.5 and eluted from an anion exchange column at a conductivity of $20\mu Si/cm^2$ (approximately 350mM NaCl). The peak of correcting activity was distinct from the positions of greatest concentration of all the complement components tested.

5.5 Discussion

The use of mannan coated ELISA plates to detect anti-mannan immunoglobulins was first described by Yeaman and Kerr (1987). In the investigations reported here plates coated with this glycan were found to be equally suitable for the measurement of surface-bound complement components. Mannan is a complex carbohydrate consisting of mannose units linked alpha 1-3 and alpha 1-6. However, the cetyl-trimethylammonium bromide (Cetavlon) preparation of mannan from *S. cerevisiae* does not remove all the protein from the mannan preparation (Sigma UK Ltd personal communication) and this was confirmed by the observation that the ELISA "coating buffer" absorbed at OD280 (data not shown). It seems probable that plate coating is facilitated by the presence of this peptide material in the mannan preparations.

The levels of C3 derived opsonins bound to mannan using sera of healthy adults and hospitalised children were shown to correlate well with the results of the C3c elution assay and all of the sera with poor opsonic function in the elution assay also gave low values in the mannan-binding assay. Levels of C3bi, C3b+bi, C4, properdin and

Factor B binding to mannan were shown to correlate extremely well with each other. This led to the hypothesis that the binding of all these components was linked. Furthermore, using mannan binding assays, the optimal concentration for discrimination between sera with either normal or poor opsonic function, the use of MgEGTA and the correlation of the binding of C4 with the other components all suggested that, using dilute serum: **the deposition of C3 derived opsonins on mannan surfaces involved the classical pathway.**

The correction assays confirmed earlier work which had shown that sera with normal opsonic activity were able to correct the defect, raising binding coefficient values to within the normal range. In contrast, when 16 sera with poor opsonisation were tested, none of these was able to correct the other sera to any significant extent, suggesting that all shared a common defect. Similar assays were also used to show that sera with known Factor D and properdin (Variant 3) immunodeficiencies, and sera which had been treated to remove or inactivate the complement components C3, Factor B, Factor D or properdin, were all able to correct the dysfunction. The inability of two properdin deficient sera (Variants 1 and 2) to correct the opsonic defect could possibly be explained by the presence of a coexisting defect in both of these sera. The studies reported in **Chapters 3, 4** and **5** suggest that sera with the common opsonic defect are not deficient in any known alternative pathway component. Indeed, much of the experimental work which has been presented in this chapter and **Chapter 4** appears to suggest that the activation of the classical pathway precedes the deposition of C3 derived opsonins on the mannan or zymosan surface. Nevertheless, assays for mannan - specific IgA, IgG and IgM antibodies and IgG subclasses 1,2 and 3 revealed no evidence of a correlation between the levels of the mannan binding immunoglobulins and the deposition of complement moieties from the same sera. The other possible explanation for these observations is that the classical pathway of complement is activated by an antibody independent mechanism in the experiments described. Established examples of such mechanisms include the lysis of retroviruses following C1q binding in the absence of antibody (Cooper et al 1976), the Ca^{++} dependent binding of the acute phase C-reactive protein (CRP)(5 subunits of 21 kDa) to pneumococci (Kaplan and Volanakis 1974), the Ca^{++} dependent binding of serum amyloid protein (SAP) (5 or 10 subunits of 23.5k Da) to agarose (Pepys & Dash 1977), the mouse Ra-reactive factor (RaRF) (320 kDa), which binds to *Salmonella* rough core polysaccharides (Ra chemotype) in Ca^{++} containing buffers, activating C4 and C2 in the absence of C1 (Ji et al 1988) and rat serum mannose-binding protein (Ikeda et al 1987)(650 kDa) which binds to mannan and N-acetyl glucosamine in the presence of Ca^{++}.

Partial purification of material able to functionally correct the opsonic deficiency indicated a protein of 600-700 kDa with a pI of approximately 4.5, eluting from the mono-Q anion exchanger at a conductivity of $20\mu Si/cm^2$. A literature survey, searching for a mammalian protein of molecular weight 600-700kDa able to bind to mannan and activate the classical pathway of complement, revealed that the rat serum mannose-binding protein (MBP) (Ikeda et al 1987) had these characteristics. This molecule was thus a putative rodent analogue of the human "opsonic correction factor". Further studies on the relationship between the opsonisation defect and MBP are reported in **Chapter 6.**

CHAPTER 6

THE ASSOCIATION BETWEEN MANNOSE BINDING PROTEIN (MBP) AND OPSONIC FUNCTION

6.1 Introduction

The survival of glycoproteins in the circulation is dependent on the terminal sugar residues of the oligosaccharide moiety, so that glycoproteins with sialic acid residues have half-lives of days, whereas those terminating with other sugars only survive for hours (Achord et al 1977(a), Schlesinger et al 1976, Stahl et al 1976 (a)&(b)). Glycoproteins terminating in mannose or N-acetyl glucosamine are rapidly removed from the circulation by the liver (Stockert et al 1976, Winkelhake et al 1976, Schlesinger et al 1976, Stahl et al 1976 (a)&(b), Achord et al 1977(a)), the receptors for these carbohydrates are situated on the endothelial cells and Kupffer cells of the hepatic sinusoidal cells (Achord et al 1977 (b), Hoyle & Hill 1988).

Initial attempts to isolate such receptors from the livers of rats (Mizuno et al 1981, Townsend &Stahl 1981, Wild et al 1983) or rabbits (Kawasaki et al 1978, Kozutsumi et al 1980) using mannan-Sepharose affinity chromatography, isolated a different oligomeric mannose-binding protein produced by hepatic cells of the parenchyma and excreted into the circulation (Maynard &Baenziger 1982, Kawasaki et al 1978, Kozutsumi et al 1980, Townsend & Stahl 1981). This lectin, called serum mannan - binding protein (MBP) (or mannose-binding protein) was shown to bind to mannose, N-acetyl glucosamine (NAG) and fucose (Townsend & Stahl 1981) in the presence of calcium ions. This calcium dependent binding could be reversed using chelators such as EDTA and therefore MBP became classified as a C-type lectin (Drickamer 1988). There are two distinct forms of the rat liver MBP , namely MBP A (which has also been termed serum MBP or S-MBP-II by Oka et al (1988)) and MBP C (which was termed liver MBP or S-MBP-I). These mannan binding proteins differed in the sizes of the polymers and monomers, in their localisation and in the ability to activate complement (Drickamer et al 1986, Ikeda et al 1987). MBP-C comprises 6 monomers of 32kDa held together by disulphide bridges forming a 200kDa protein while MBP-A has approximately 20 identical monomers of 31kDa which polymerise to form a 650kDa protein. MBP 's isolated from rat, rabbit and human serum have recently been shown to activate the classical pathway of complement with an absolute requirement for C4 (Ikeda et al 1987). In these assays sheep erythrocytes were coated with mannan using chromium chloride and these were then sensitised with the various MBP preparations. Guinea-pig serum, which had been passed through a mannan-Sepharose 4B column at $4^{o}C$ to remove anti - mannan antibodies and endogenous guinea pig MBP, was incubated with the MBP sensitised erythrocytes. Binding of

guinea pig complement to MBP on mannan coated erythrocytes activates the classical pathway and lyses the cells by passive haemolysis. The requirement for C4 was established when a C4 deficient serum failed to lyse the sensitised erythrocyte targets. A human mannose binding protein of unknown function had been described by Summerfield and Taylor in 1986 and the complement activating properties of rat MBP suggested that the human lectin was a strong candidate for the role of the correcting factor in the various assays described in **Chapters 4** and **5**. Accordingly a polyclonal rabbit antibody, raised against highly purified MBP, was acquired and used to measure MBP concentrations in the investigations described in this chapter.

6.2 Assays for MBP

6.2.1 Preparation of anti-MBP antiserum

This was kindly provided by Dr S. Thiel (MRC Immunochemistry unit, Oxford),but since the specificity of this reagent is critical for the interpretation of the results presented here, the preparation is described in detail:

The antiserum to human MBP was raised in a rabbit using MBP prepared from 6 litres of outdated human plasma. Citrated plasma was made 20mM with respect to calcium, allowed to clot for one hour at 37^{o}C and left overnight at 4^{o}C. The serum was dialysed extensively against a 50mM TrisHCl/1M NaCl/20mM $CaCl_2$ pH 7.8 starting buffer and applied to a 130ml mannan- Sepharose 4B column prepared in the same manner as the affinity columns described in **Chapter 2**. The column was washed with 2 volumes of starting buffer and the bound protein was displaced with EDTA (50mM TrisHCl/1M NaCl/10mM EDTA pH 7.8), re-chromatographed on a 13 ml mannan-Sepharose column and eluted with mannose (50mM TrisHCl/1M NaCl/20mM $CaCl_2$/50mM mannose pH 7.8) and then successively fractionated by Superose 6 - gel filtration (50 mM TrisHCl/1M NaCl/1mM EDTA/ 0.05%w/v Tween 20 pH 8) and Mono Q (HR 5/5) anion exchange chromatography (50 mM TrisHCl/100mM NaCl pH 8 eluted with a NaCl gradient). Contaminating IgM was removed using an anti-human IgM affinity column. N- terminal amino-acid sequencing confirmed the purity of the MBP preparation which was then used to raise the rabbit antiserum. IgG was isolated from the antiserum by Na_2SO_4 precipitation and passage of the redissolved precipitate through a DE52 ion exchange column. The antibodies in this preparation were shown to be specific for MBP by probing on an immunoblot of whole serum. Further tests established that there was no cross-reactivity with C1q.

6.2.2 Enzyme Immuno-Assays for MBP (see **Flow Diagram 6.1**)

MBP was measured by two ELISA techniques with different capture layers (see **Flow diagram 6.1**). The mannan-capture assay was asymmetric, with a capture phase of mannan coated ELISA plates and a detector of polyclonal anti-MBP. Test sera were incubated in the wells in an imidazole buffer containing calcium (described by Kawasaki et al 1983), using the mannan-binding capacity of MBP to attach MBP from the samples onto the solid phase for subsequent detection. In contrast, the antibody capture assay was symmetric with the same polyclonal antibody used as capture and detector. In this case the detector antibody was biotinylated by the method of Guesdon et al (1979). Although the same antibody was used for both capture and detection in this assay, the antibody was polyclonal and therefore capable of recognising different epitopes on the MBP surface. This arrangement reduces the possibility of the capture and detector antibodies competing for the same sites which could sterically hinder detector antibody binding.

Flow diagram 6.1 A brief outline of the ELISA procedures used to measure human MBP. These techniques are explained in greater detail in the accompanying text.

Stages	**Mannan capture**	**Antibody capture**
Coat 4°C overnight in carbonate buffer	Mannan (0.5mg/ml) (Sigma M-3640)	1/1000 Rabbit anti-human MBP
Washes	3x PBS-Tween, 1x PBS and 1x imidazole	4x PBS-Tween
Block (1hr RT)	NA NA	4% normal goat serum in carbonate buffer
Washes	4x PBS-Tween	
Sera	Test and standard sera diluted in imidazole buffer / 50mM calcium (2hrs 37°C)	Sera diluted in PBS-Tween / 10mM EDTA (1hr 37°C)
Washes	4x PBS-Tween	
Conjugated detector antibody	1/500 Rabbit anti-human MBP in PBS-Tween (2hrs 37°C)	1/1000 Biotinylated rabbit anti-human MBP in PBS-Tween (1hr 37°C)
Washes	4x PBS-Tween	
Amplification	1/500 Peroxidase labelled sheep anti-rabbit IgG in PBS-Tween (1hr 37°C)	1/1000 Streptavidin peroxidase in PBS-Tween (1hr 37°C)
Washes	4x PBS-Tween	
Colour development	OPD substrate with H_2O_2 in citrate/phosphate buffer. Reaction stopped with H_2SO_4 Absorbance read at OD_{492}	

(i) Measurement of MBP by mannan capture assay

Dynatech Immulon MicroELISA plates were coated overnight at 4°C with mannan M 3640 (Sigma UK Ltd) diluted to 0.5mg/ml in carbonate/bicarbonate buffer pH 9.6. The coated plates were washed three times with PBS- Tween (PBS-T) (PBS pH 7.3 (Oxoid UK Ltd) with 0.05% Tween 20), once with PBS without Tween, and once with imidazole buffer. Sera or column fractions were diluted to 5% in imidazole buffer (40 mM imidazole/HCl pH 7.8 with 1.25M NaCl) containing 50 mM $CaCl_2$ and 100μl aliquots were added in duplicate to the wells of the mannan coated plates which were then incubated at 37°C for two hours. The plates were washed and then rabbit anti-human MBP (diluted to 1/500 in PBS-T) was added to all wells and incubated at 37°C for two hours.After further washes the plates were incubated at 37°C for 1hr with horseradish peroxidase sheep anti-rabbit IgG conjugate (Serotec UK Ltd) at 1/500 in PBS-T. The plates washed and a solution consisting of 10μg o-phenylene-diamine in 20ml citrate-phosphate buffer pH 5.2 (10.4g citric acid plus 14.4g Na_2HPO_4/litre) containing 10μl 30% H_2O_2 was added to all wells and incubated at room temperature in the dark for 15-30 minutes. The colour reaction was stopped by adding 100μl of 4N H_2SO_4 to each well and the plates were read at 492nm using a Titertek Multiskan Plate Reader (Flow UK Ltd). An MBP binding coefficient was then calculated as described previously for the binding of complement components using the HB1 and LB1 sera. In addition, the serum pool (described in **Chapter 2**), previously calibrated against a purified preparation of MBP (by Dr S.Thiel MRC Immunochemistry unit Oxford), was serially diluted from 20% to 0.156% in imidazole buffer to provide a gravimetric standard curve(see **Appendix 1**), and the MBP concentration in the test sera was determined by reference to this secondary standard. The detection limit of the assay was 2.5μg/litre.

(ii) Measurement of MBP by antibody capture assays

MBP was also assayed using an antibody capture sandwich ELISA in which the rabbit anti-MBP serum was used as both the capture and detector antibody. The capture antibody was diluted 1/1000 in carbonate/bicarbonate pH 9.6 and incubated on the plates overnight at 4°C, the detector antibody was diluted 1/1000 in PBS-Tween and incubated with the plates for 1 hour at 37°C. This antibody was biotinylated by the method of Guesdon et al (1979) and the assay developed with streptavidin peroxidase at 1/1000 in PBS-Tween for 1 hour at 37°C. Sera or column fractions were diluted in PBS-Tween containing 10mM EDTA and the unknowns were assayed by reference to the secondary standard serum pool described above(see **Appendix 1**).

6.3 Confirmation that isolated human MBP is able to correct the common opsonic defect

MBP is known to bind to mannan-Sepharose columns in the presence of calcium and this process can be reversed on addition of the chelator EDTA (Mizuno et al 1981, Kawasaki et al 1978, Wild et al 1983). To establish whether MBP was the active correcting moiety, the correcting fractions from the gel filtration chromatography (600-700kDa molecular weight) were dialysed against an imidazole buffer containing 50mM calcium and loaded onto a mannan-Sepharose column. Bound material was eluted with 5mM EDTA and this eluate was applied to a Mono Q anion exchange column and eluted with an NaCl gradient. Fractions were tested in the mannan-binding correction assays and in the mannan-capture and antibody-capture assays for MBP. The correcting fractions from the final purification step (Mono Q anion exchange chromatography), were electrophoresed on an SDS-PAGE gel, immunoblotted and probed with the anti-MBP antibody.

6.3.1 Purification of material able to correct the common opsonic defect

Introduction

The purification steps used in **Chapter 5** were extended in an attempt to establish whether or not the active fractions contained MBP.

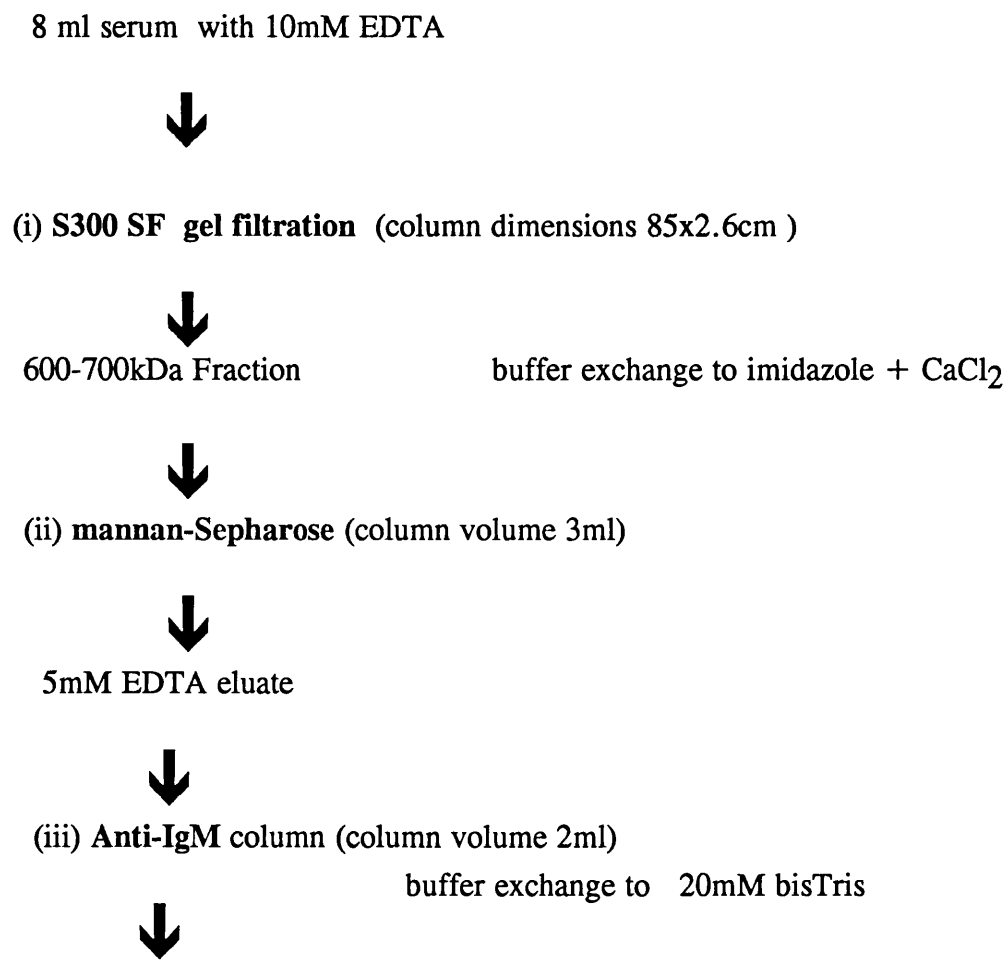
Fractions from the active region of the S300 gel-filtration separation were pooled, dialysed against imidazole buffer containing calcium and loaded onto a mannan-Sepharose column. Thus, if the correcting fractions contained MBP, this would bind to the mannan-Sepharose in the presence of calcium. Material bound to the mannan-Sepharose column was eluted with imidazole buffer containing 5mM EDTA and passed through an anti-IgM affinity column to remove this immunoglobulin (studies reported in **Chapter 5** had shown that the active fractions from Mono Q contained significant quantities of IgM). Flow through material was dialysed against bis-Tris, and loaded onto the FPLC Mono Q anion exchange column,(as described in **Section 5.4.6**) and fractions were eluted with a gradient of NaCl. As these experiments were carried out before the antiserum was received from Dr S.Thiel, activity was measured in mannan capture correction assays (see **Chapter 5**) and samples from all the fractions were stored at -70^{o}C for subsequent measurement of MBP concentration.

Methods

Four procedures were used sequentially in an order to isolate material with correcting activity from 8ml of HB1 serum, 8ml of HB2 serum and 50ml of a human serum pool. The same procedures were also carried out on 8ml volumes of LB1 and LB3 sera (see **Flow Diagram 6.2**) .

These procedures were:
i) Sephacryl S300 Gel filtration
ii) Mannan-Sepharose 4B affinity chromatography
iii) Anti- IgM Sepharose 4B affinity chromatography
iv) Mono Q anion exchange chromatography

Flow diagram 6.2 Isolation of correcting factor from normal human sera.



(i) **Sephacryl S300 gel filtration:**

500 mM disodium EDTA, pH 8, was added to 8ml of serum to give a final concentration of 10 mM EDTA and the serum was fractionated by gel filtration on a column of Sephacryl S300 (85 x 2.6cm) equilibrated in PBS/10mM EDTA buffer, pH 7.3.

(ii) **Mannan-Sepharose affinity chromatography:**

Correcting fractions eluted from the Sephacryl S300 column in the region corresponding to a molecular weight of 600-700 kDa were dialysed extensively against 40 mM imidazole/HCl pH 7.8 containing 1.25M NaCl. 1M $CaCl_2$ was added to give a final concentration of 50 mM calcium and the fractions were loaded (at room temperature) onto a 3ml mannan-Sepharose affinity column which had been equilibrated with imidazole buffer containing 50 mM calcium (MBB). The column was then washed with this buffer until the OD_{280} was less than 0.02 absorbance units. The bound fraction was eluted with imidazole buffer containing 5mM EDTA.

(iii) **Anti-IgM Sepharose affinity chromatography:**

The 5mM EDTA eluate from the mannan-Sepharose affinity column was passed through a Sepharose coupled anti- IgM affinity column and the breakthrough material, depleted of IgM, was used in the final purification steps.

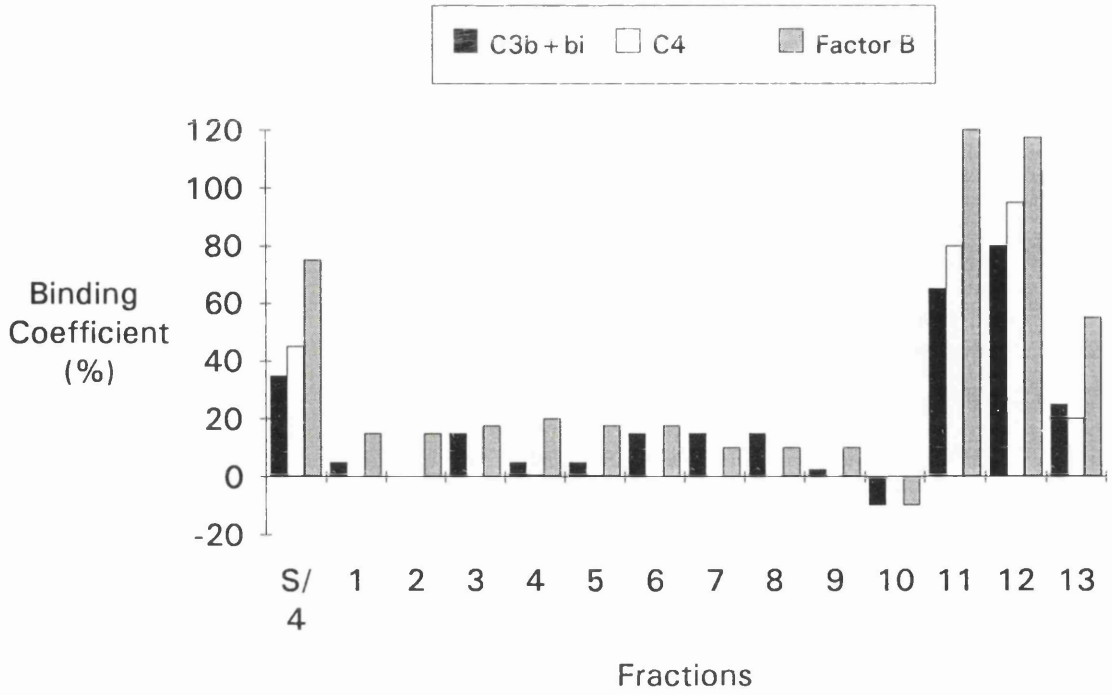
(iv) **Mono Q anion exchange chromatography:**

The flow-through material from the anti-IgM affinity chromatography step was concentrated and dialysed against 20 mM bis Tris pH 6.5 using an Amicon stirred cell (Amicon UK Ltd). The dialysed sample was loaded on to a Mono Q anion exchange column linked to the FPLC system (Pharmacia UK Ltd) using a start buffer of 20 mM bis Tris pH 6.5 and protein fractions were eluted using a gradient of a limit buffer of 20mM bis Tris/ 1M NaCl.

Results

Fractions from the separation of HB2 serum on the Mono Q column were tested using mannan-capture correction assays measuring binding of C3b+bi, C4 and Factor B (see **Figure 6.1 panel (a)**). The MBP concentrations of the fractions established using the mannan capture assay are shown in panel **(b)**. In contrast, when LB1 and LB3 sera were fractionated by the four-step procedure outlined above, none of the Mono Q fractions were able to correct LB1 serum, and no MBP was found in any of these. In other experiments (results not shown) MBP concentrations were measured in correcting fractions and adjacent non-correcting fractions from HB1 and pooled serum purified using S300, mannan-Sepharose, anti-IgM and Mono Q chromatography. All the fractions which were able to correct the opsonic defect contained MBP while adjacent, non-correcting fractions did not contain detectable levels of MBP.

(a)



(b)

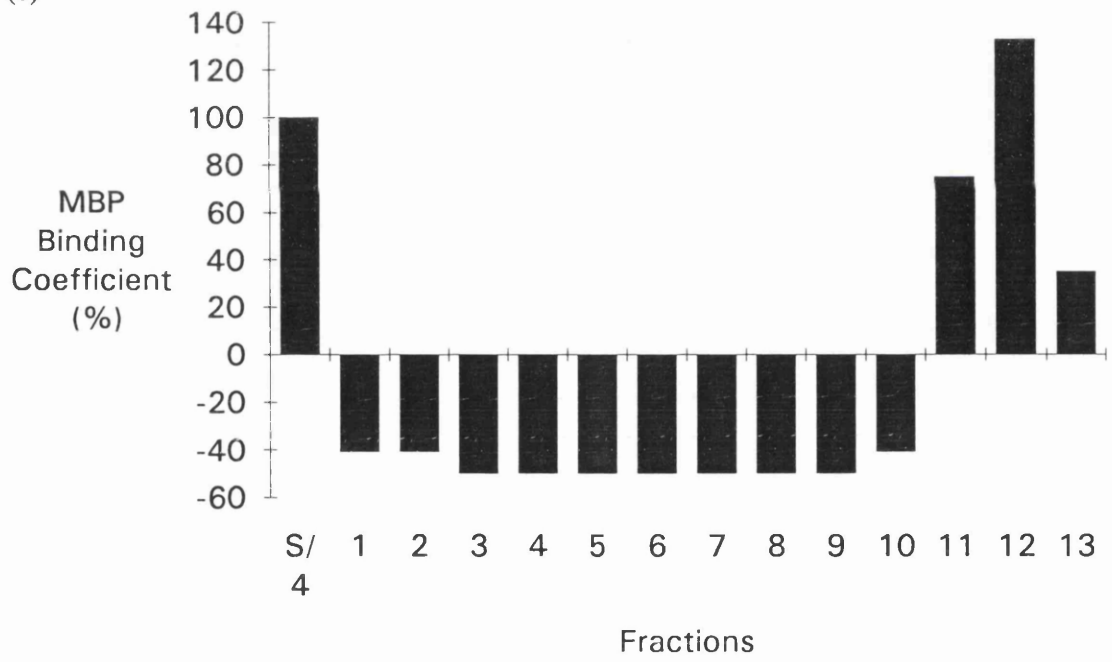


Figure 6.1 (a) Mono Q anion exchange chromatography of 8ml HB2 serum purified by the four step procedure. The fractions were added to LB1 serum and tested in mannan capture C3b+bi, C4 and Factor B assays. (b) MBP concentrations were measured using the mannan-capture ELISA and expressed as a binding coefficient.

Conclusions

These results suggest that material which bound to a mannan- Sepharose column under conditions favouring the binding of MBP was identical to the 600-700kDa protein which corrected the opsonic defect in serum LB1 (see **Chapter 5**). The MBP ELISA techniques confirmed that **all of the fractions with correcting activity contained MBP**. It was still necessary to establish that the effects observed were due to MBP and not to some co-migrating factor which could bind to mannan and activate complement. Proteins with these characteristics include anti-mannan IgM and IgG, and the pentraxin C-reactive protein (Pepys 1981, Thiel and Reid 1989). Various preparations were therefore assayed by SDS-PAGE and immunoblotting to assess their purity.

6.3.2 SDS-PAGE and Immunoblotting:

Protocol

Material prepared by the four step procedure outlined in **Flow diagram 6.2** was vigorously reduced by boiling in the presence of 40 mM DTT and duplicate samples were electrophoresed on a 10% reducing SDS-PAGE slab gel by the method of Laemmli (1970). The gel was divided for subsequent development ; half was silver stained by the method of Morrissey(1981), whereas the other half was electroblotted using the BioRad "Transblot" cell and probed with the rabbit anti-MBP antibody followed by ^{125}I-labelled anti-rabbit Ig (Amersham UK Ltd).

Results

(a) Silver stained gel (**see Figure 6.2 panel (a)**)

The four step fractionation of LB1 serum (see lanes 1 and 2) yielded two bands at approximately 30 and 60 kDa. No bands were visible at 32 or 64 kDa (the position of MBP monomers and dimers) and this suggested that there was no MBP in the Mono Q fractions 11 and 12 from LB1. In contrast, in the fractionation of HB1 and pooled serum, (lanes 3-6) there were bands visible at 32 and 64 kDa as well as at the 30 and 60 kDa (see lanes 1 and 2). In addition Fraction 12 of the human serum pool (lane 6) also contained bands of 68 kDa - which may be human serum albumin (HSA). However, the majority of the protein in the Mono Q fractions 11 and 12 of HB1 and the pooled serum (lanes 3-6) was associated with bands with molecular weights of 32 and 64 kDa.

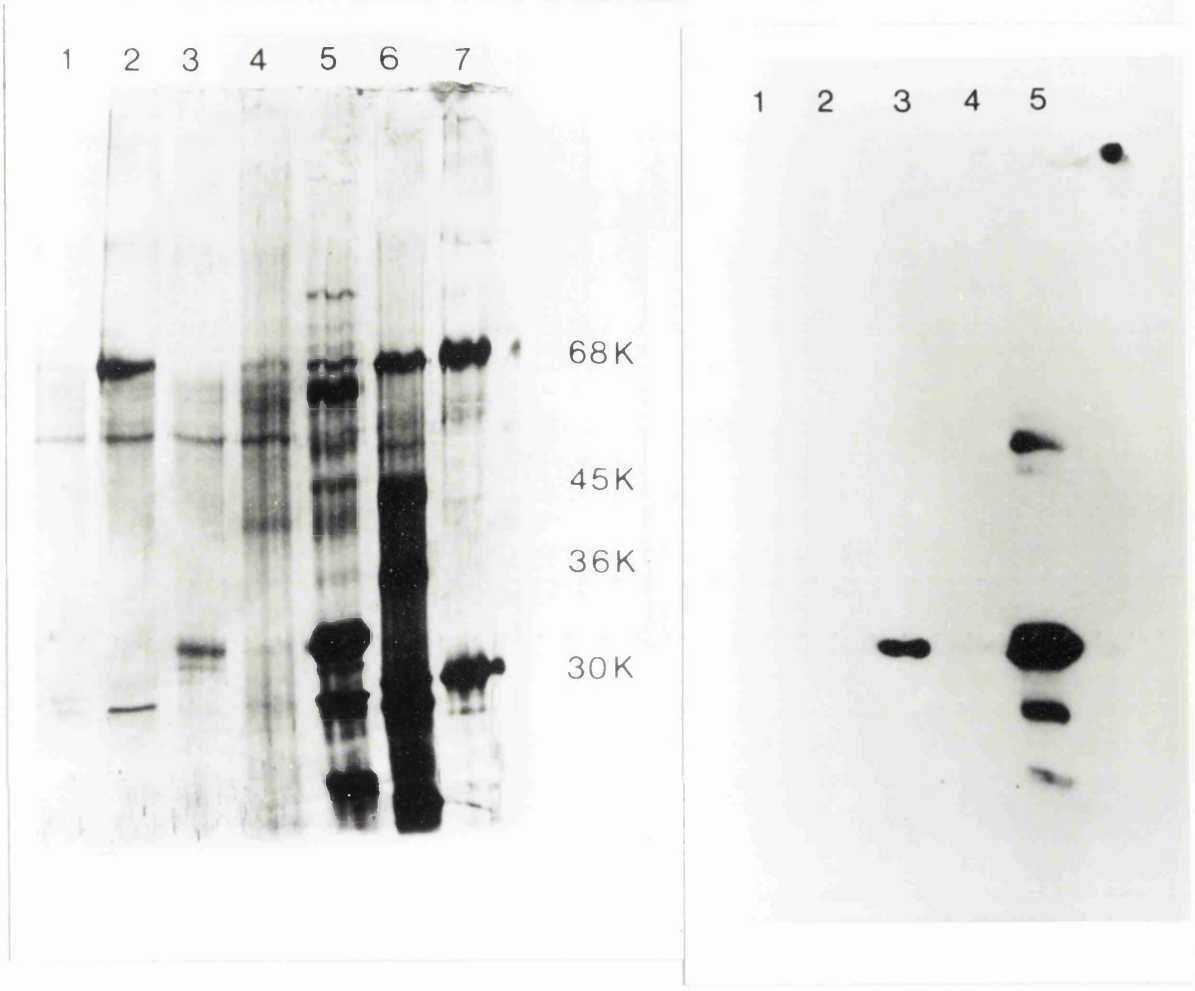


Figure 6.2

(Left panel)

SDS Page of various preparations of MBP. 10% acrylamide gel run under reducing conditions and silver stained by the method of Morrissey 1981.

Lane 1 FPLC Mono Q fraction 12 from separation of 8 ml LB1 serum

Lane 2 FPLC MonoQ fraction 11 from separation of LB1 serum

Lane 3 FPLC Mono Q fraction 12 from separation of 8 ml HB1 serum

Lane 4 FPLC MonoQ fraction 11 from separation of HB1 serum

Lane 5 FPLC Mono Q fraction 12 from separation of 50 ml pooled serum

Lane 6 & Lane 7 Molecular weight markers

(Right panel)

Immunoblot of the same gel using rabbit anti-MBP followed by ^{125}I-labelled Ig as probe.

(b) Immunoblotting (see **Figure 6.2(b)**).

This confirmed that the fractions obtained from the LB1 serum (lanes 1 and 2) contained no MBP and that HB1 and the pooled serum contained significant amounts of MBP(lanes 3-6), either of the 32 kDa monomeric or 64 kDa dimeric forms(see **Figure 6.2(b)**).The concentration of MBP in Fraction 12 was greater than in Fraction 11, which confirms the ELISA result shown in **Figure 6.1.** Subsequent sensitive ELISA assays established that the MBP concentration in serum LB1 was 4-7 μg/l whereas that of HB1 was approximately 400 μg/l. This probably explains why there was no detectable MBP in LB1 serum following the four-step fractionation procedure while, in contrast, HB1 serum gave such a strong signal in the immunoblot.

6.3.3 Correction of the opsonic defect by addition of purified MBP to serum LB1

Protocol

It was necessary to establish that MBP could correct the opsonic defect in LB1 serum in a dose- dependent fashion. An MBP enriched fraction was prepared from pooled serum by the four step purification procedure described in **Section 6.3.2.** The Mono Q fraction 12 (which had been used in the SDS-PAGE and immunblotting studies) was titrated into LB1 serum and mannan - binding correction assays were performed measuring the C3b+bi, C4 and Factor B binding coefficients.

Results

The MBP enriched preparation (which had an MBP concentration of 200μg/l) increased the amounts of C3b+C3bi, C4 and Factor B binding to mannan from the LB1 serum, in a dose-dependent manner (see **Figure 6.3**).

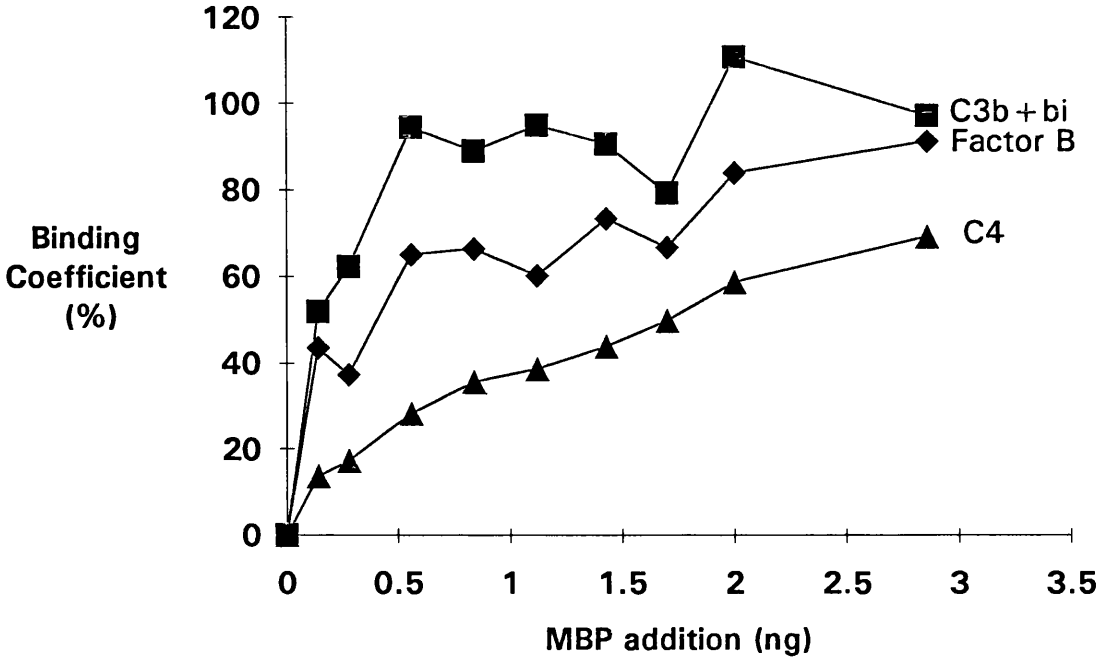


Figure 6.3 Mannan capture correction assays measuring the complement components C4, C3b+bi and Factor B following titration of an MBP enriched fraction from the four step purification of pooled human serum into LB1 serum. The opsonic defect in the LB1 serum is corrected in a dose dependent fashion.

Conclusion

All the results described in **Sections 6.3.1, 6.3.2 and 6.3.3** suggest that MBP is the protein present in normal human sera which can correct the opsonic defect in vitro. However, it was still necessary to establish that the levels of MBP were low in sera from individuals with defective opsonisation.

6.4 Measurement of MBP in whole sera

The specific anti-human MBP antiserum was used to measure the MBP concentration in whole sera from 178 healthy adult blood donors, 59 apparently healthy 4.5 month old infants, 19 cold surgery " hospital control" patients, 10 patients with the opsonic defect and 3 properdin deficient individuals.

6.4.1 Adult sera

Protocol

MBP levels were measured in 178 sera from healthy adult blood donors using the mannan-capture ELISA technique described in **Section 6.2.2** and there was sufficient sample in 102 of these sera for measurement using the antibody capture technique. MBP concentrations were also measured in the properdin deficient sera studied in **Chapters 4 and 5**. Sera LB1 and HB1 were included as controls in all the mannan and antibody capture assays and results were calculated with reference to these and expressed as binding coefficients (%). In addition, gravimetric levels of MBP concentration were calculated for the mannan-capture assay using serial dilutions of the pooled serum which had been calibrated by Dr S.Thiel (MRC Immunochemistry unit Oxford).

The MBP binding coefficients and concentrations calculated for the adult sera were compared with the binding coefficients calculated for the same sera using the mannan-capture assays for C3b+bi, C4, C3bi, properdin and Factor B (see **Chapter 5**). In addition binding coefficients determined with the C3c elution procedure were compared with MBP results in the 76 sera tested in both these assays.

Results

The levels of MBP in the adult blood donors are shown in **Figures 6.4, 6.5** and **6.6.** The sera with a mannan capture C3bi binding coefficient of less than 10 % are once again represented as open bars or boxes. It is apparent that these sera also have the lowest MBP binding coefficients and concentrations. The median MBP concentration in this healthy adult population was 92μg/l, and the range was 2.5-610μg/l. (Detailed results of MBP levels can be found in **Appendix 3**). In contrast, in the subgroup with low C3bi binding coefficients, the median value was 4.52 μg/l and the range was 1.04 - 17.75 μg/l. There was a highly significant correlation between the results obtained using the mannan capture and the antibody capture assays (see **Figure 6.7 and Table 6.1**), and no evidence of a functionally inactive MBP, unable to bind to mannan but still immunochemically recognised in the antibody capture assay. When the MBP binding coefficients obtained using the mannan capture assays are compared with the levels of C3b+bi, C3bi, C4, properdin and Factor B bound to the plates using the same sera, highly significant correlations are again observed **(Figures 6.8 (a)-(d)** and **Table 6.1.**)

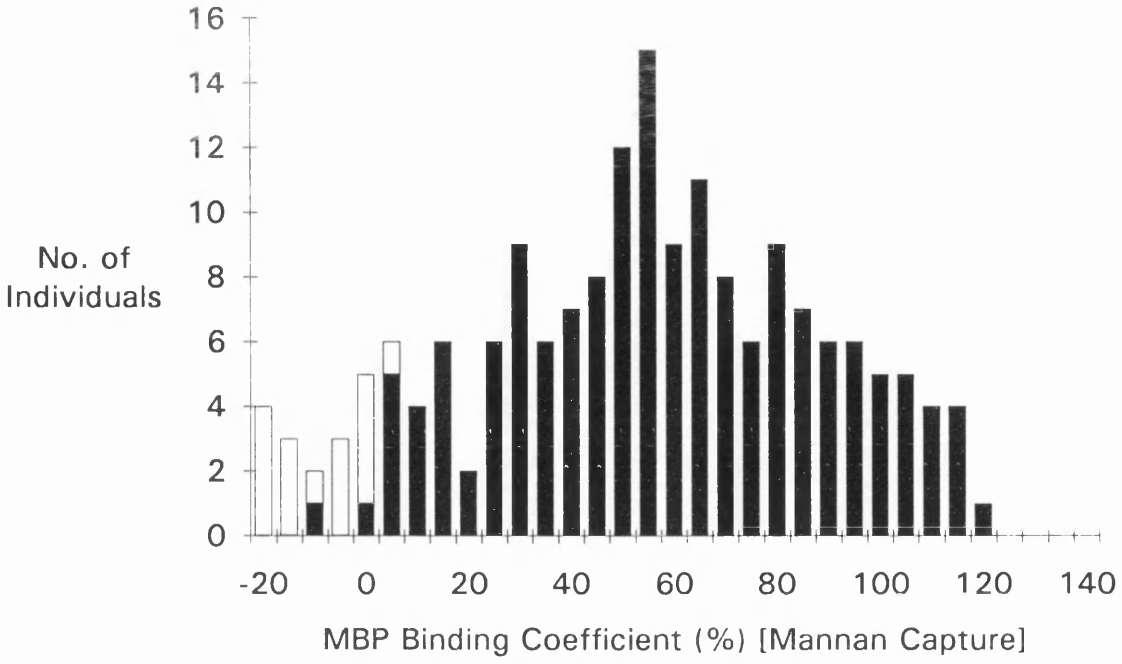


Figure 6.4 Measurement of MBP binding to mannan from 178 sera of healthy adult blood donors. The hollow bars represent sera with binding coefficients less than 10% in the C3bi mannan capture assay.

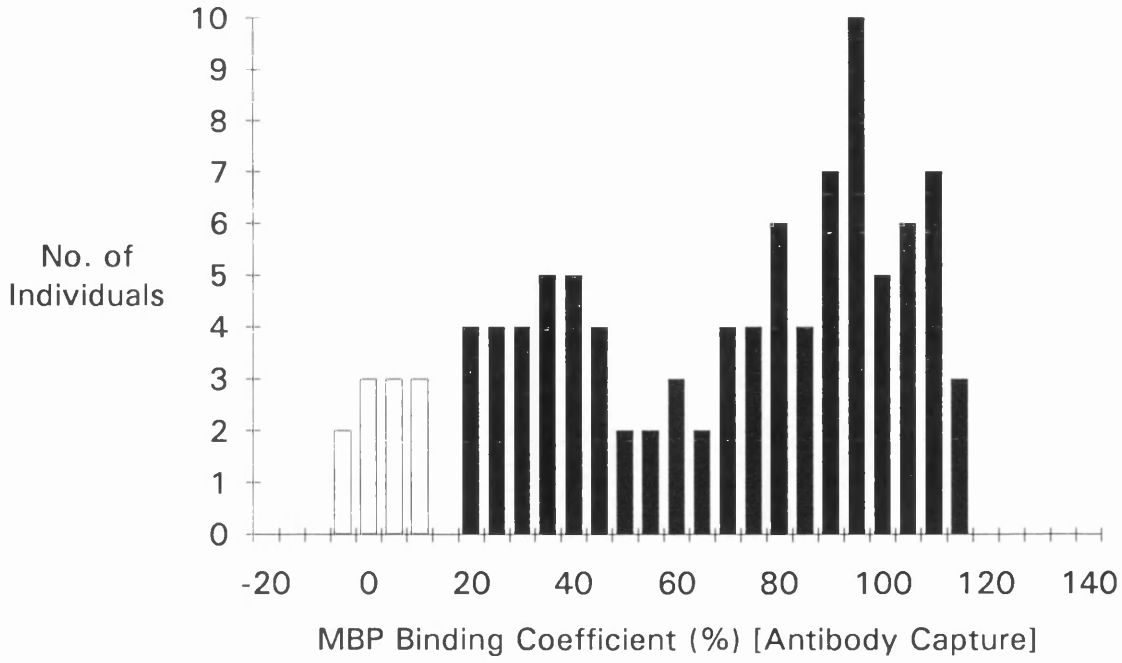


Figure 6.5 Measurement of serum MBP levels in sera from 102 healthy adult blood donors using the antibody capture MBP assay. The hollow bars represent sera with binding coefficients of less than 10% in the C3bi mannan capture assay.

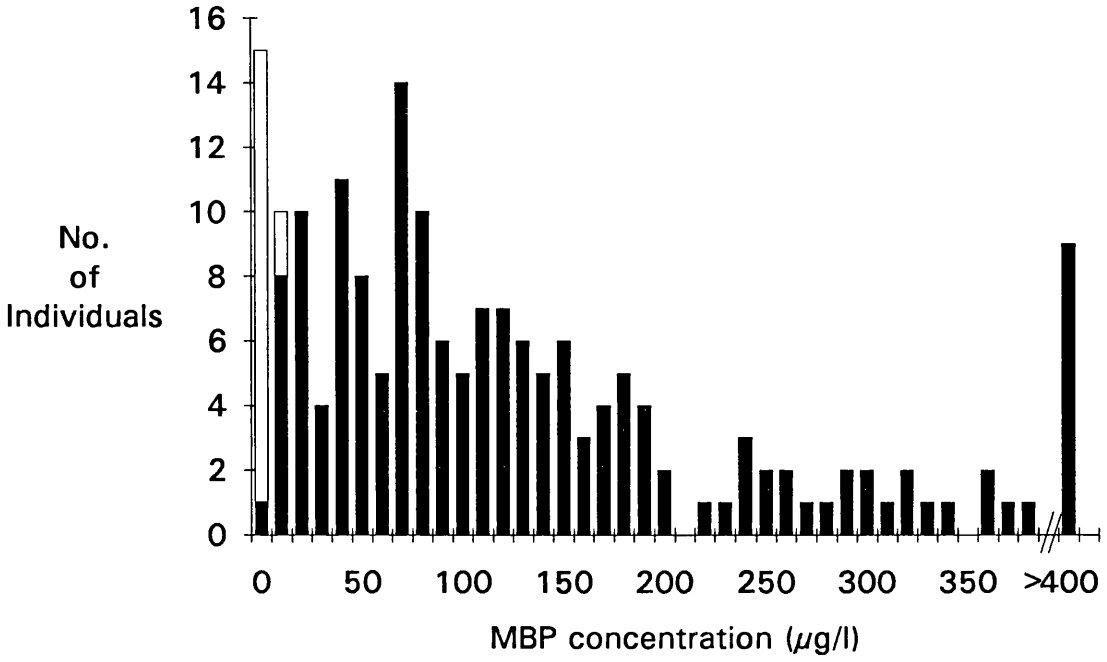


Figure 6.6 Levels of MBP in sera from 178 healthy adult blood donors. The hollow bars represent the sera with binding coefficients of less than 10% in the C3bi mannan capture assay.

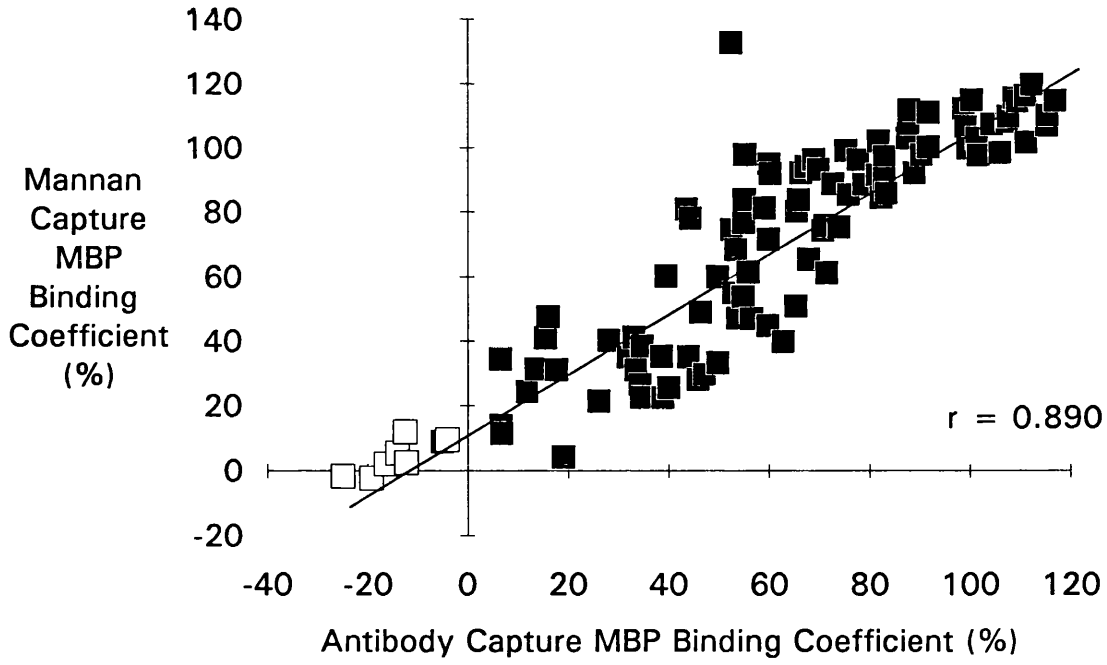
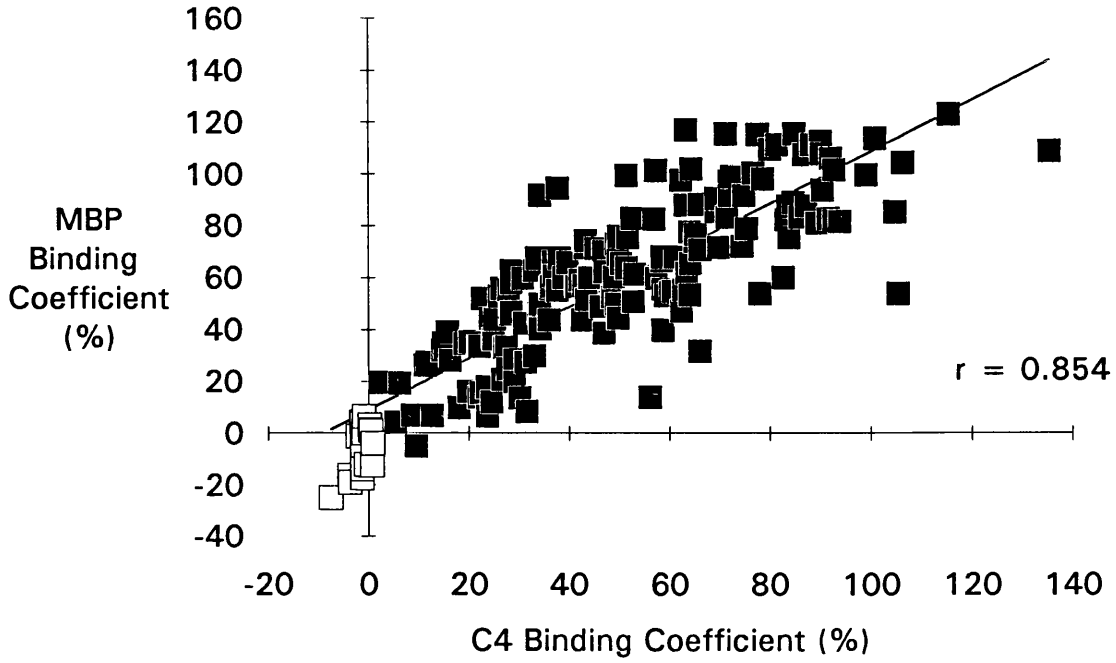
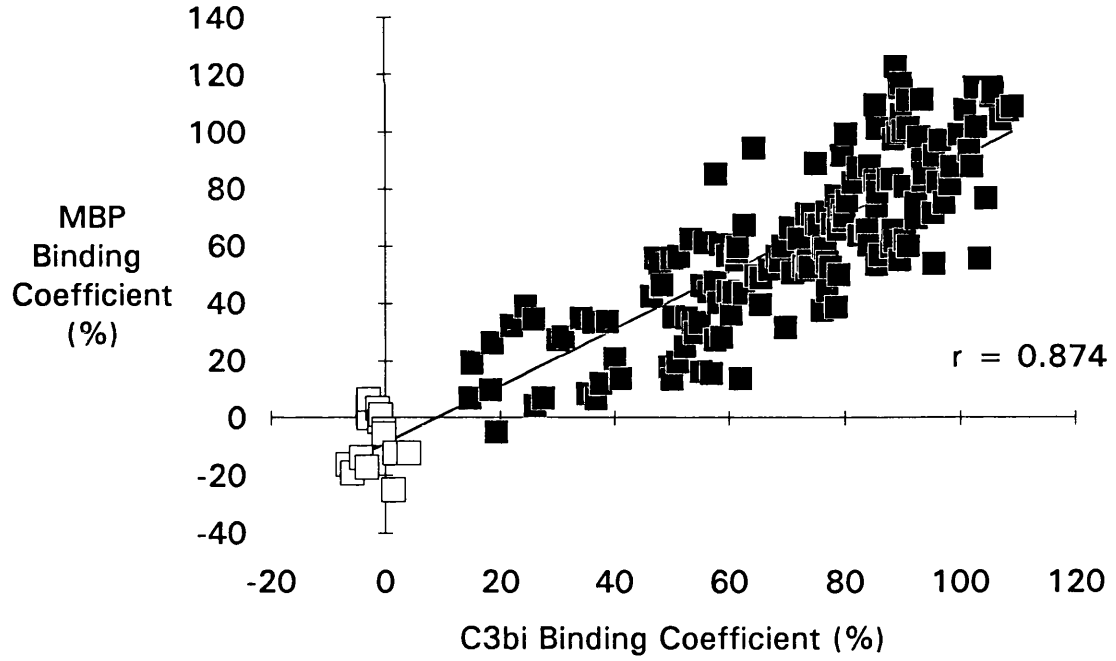


Figure 6.7 Pearson correlation between levels of MBP determined using the mannan and antibody capture assays in 102 sera from healthy adult blood donors. The hollow boxes represent those sera with binding coefficients of less than 10% in the C3bi mannan capture assay.

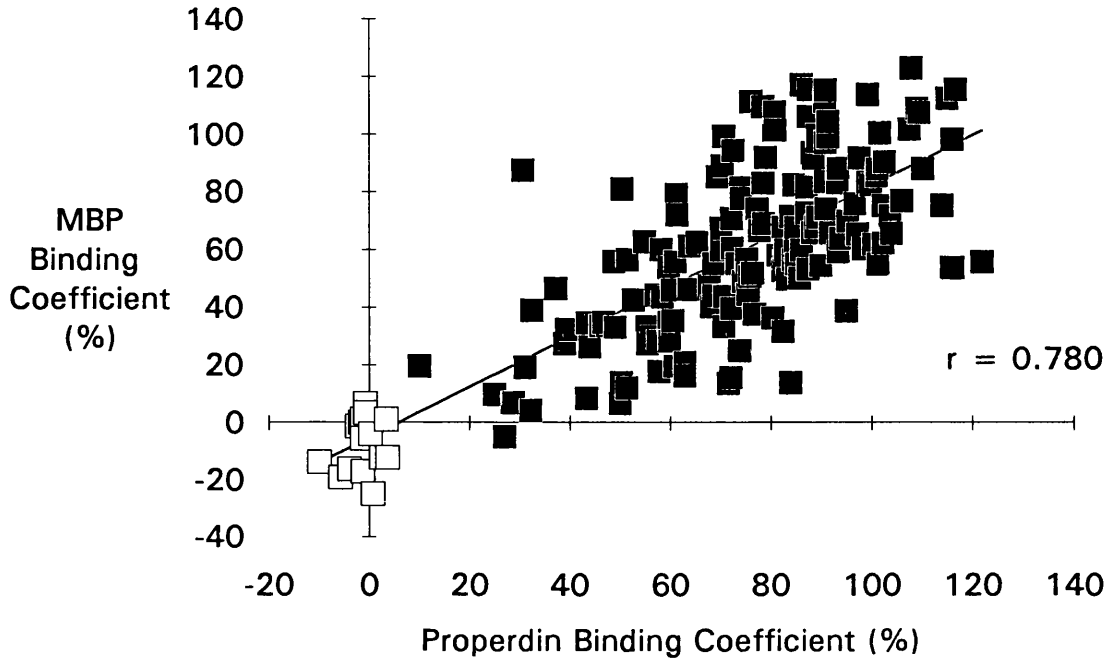
6.8 (a)


MBP Binding Coefficient (%)
160
140
120
100
80
60
40
20
0
-20
-40
-20
0
20
40
60
80
100
120
140
r = 0.854
C4 Binding Coefficient (%)

6.8 (b)


MBP Binding Coefficient (%)
140
120
100
80
60
40
20
0
-20
-40
-20
0
20
40
60
80
100
120
r = 0.874
C3bi Binding Coefficient (%)

6.8 (c)



6.8(d)

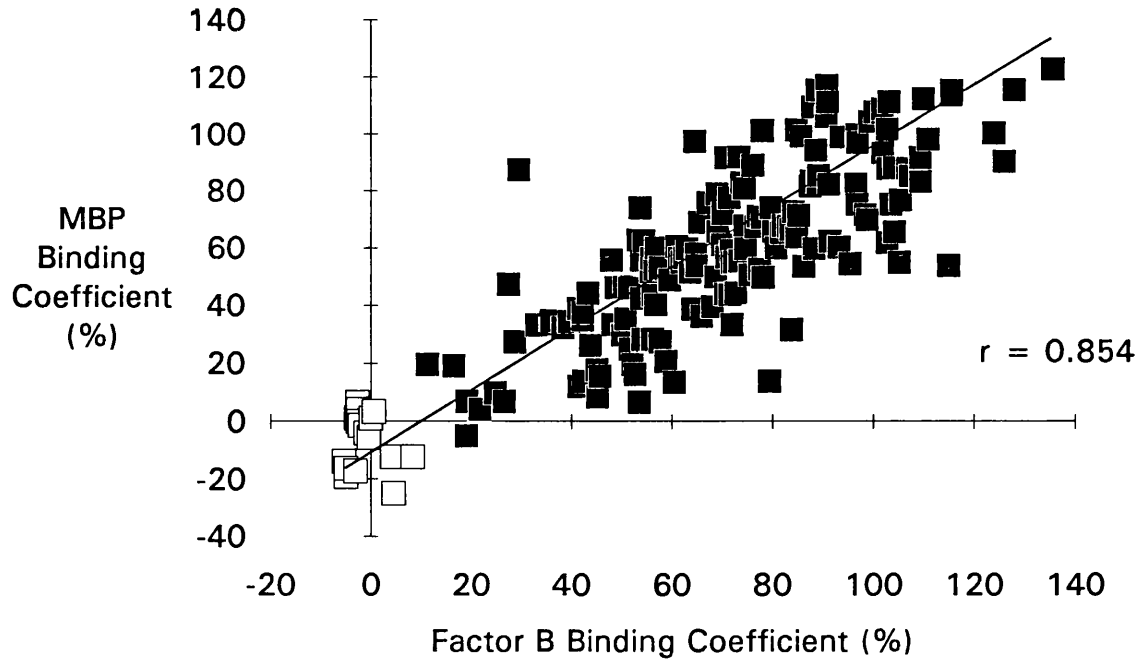


Figure 6.8 (a)-(d) Pearson correlations between MBP, C4, C3bi, properdin and Factor B binding to mannan from 178 sera of healthy adult blood donors. Open boxes represent the sera with binding coefficients of less than 10% in the mannan capture C3bi assay.

Table 6.1 Pearson and Spearman rank correlations between various assays using the population of healthy adult blood donors.

Assay	vs	n	Pearson r_p	p value	Spearman r_s	p value
*MBP mannan	**MBP antibody	102	0.890	<0.0001	0.930	< 0.0001
MBP mannan	***C3c elution	76	0.497	<0.0001	0.469	< 0.0001
MBP mannan	C3bi binding	178	0.874	<0.0001	0.874	< 0.0001
MBP mannan	C4 binding	178	0.854	<0.0001	0.858	< 0.0001
MBP mannan	properdin binding	178	0.780	<0.0001	0.736	<0.0001
MBP mannan	Factor B binding	178	0.854	<0.0001	0.836	<0.0001
MBP mannan	°IgG binding	171	0.019	N.S	0.064	N.S
MBP mannan	°IgM binding	173	0.109	N.S	0.092	N.S

Note: * MBP measured by mannan-capture assay

** MBP measured by antibody capture assay

*** Functional opsonic assay (see **Figure 4.2**)

° Immunoglobulins binding to mannan (see **Figure 5.7**)

NS Not Significant ($p > 0.5$)

Conclusion

The sera with the lowest MBP concentrations also had the lowest mannan capture C3bi values. A threshold was therefore set at an MBP concentration of 30μg/l. Serum MBP levels were highly significantly correlated with the mannan binding of the complement proteins C3, C4, properdin and Factor B. Moreover the serum levels of MBP were also significantly correlated with activity in the functional (C3c elution) opsonic assay.

6.4.2 Paediatric sera

Protocol

MBP levels were measured in the sera of 59 apparently healthy infants aged 4.5 months (N 1-59), in 10 children previously diagnosed to have the functional opsonic defect (I 1-10)(age range 6 months to 9 years) and in 19 control children undergoing "cold" surgery (C 1-19)(aged 3 months to 13 years) .

Results

The median MBP concentrations of the healthy paediatric groups (C 1-19 plus N1-59) was 143μg/l (range 2.5 - 880μg/l) and the median value for the patient group with the opsonic defect was 4.9μg/l (range 2.5-35μg/l) See **Figure 6.9 (a)** and **(b)** and **Table 6.2.**

6.9(a)

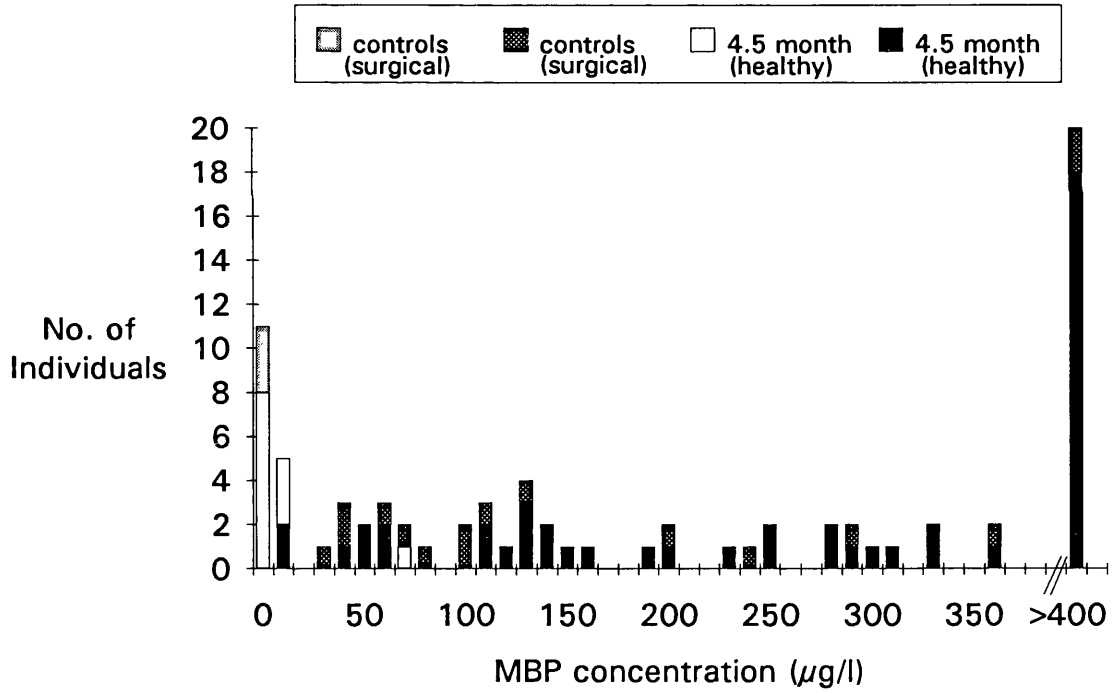


Figure 6.9(a) MBP concentrations (mannan capture assay) in 59 healthy 4.5 month old infants and in 19 control children. The sera with binding coefficient less than 10% in the mannan capture C3bi assay are represented with hollow bars for the 4.5 month old infants(N1-59) and lightly hatched bars for the surgical controls(C1-19).

6.9(b)

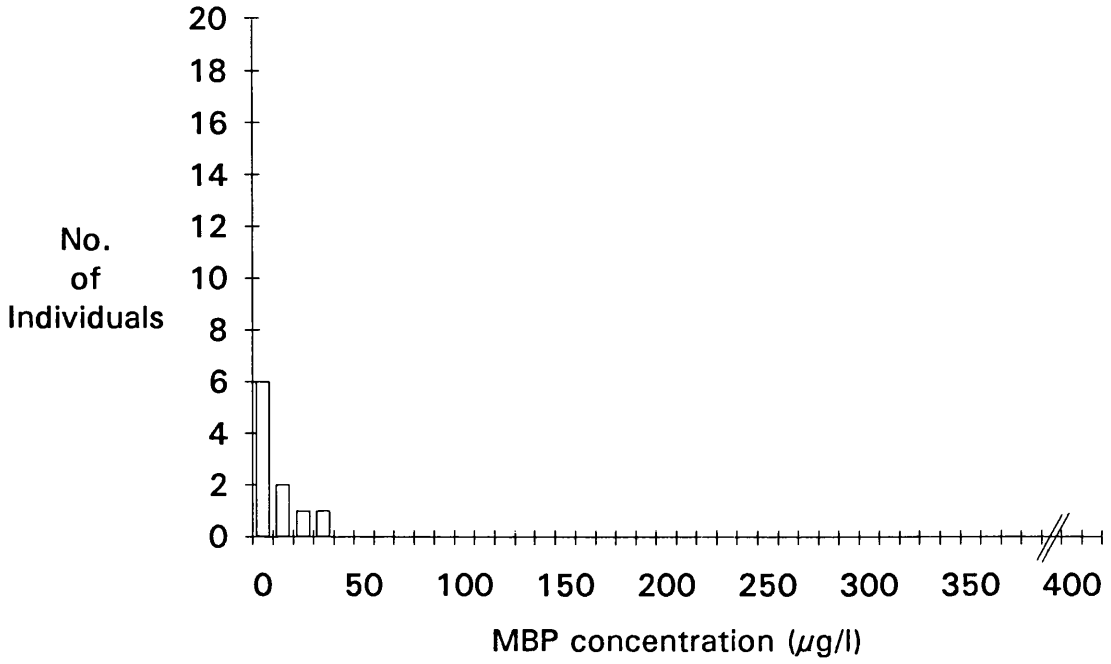


Figure 6.9 (b) MBP concentrations (mannan capture assay) in 10 GOSH patients with the functional opsonic deficiency(I1-10). All these sera are represented with hollow bars because all have binding coefficients less than 10% in the mannan capture C3bi assay.

Table 6.2 Levels of MBP in the sera of 10 GOSH patients previously shown to have the functional opsonic deficiency

Patient	Age at last hospital visit	Initial clinical presentation	C3c elution assay (BC%)	C3b+bi binding (BC%)	C4 binding (BC%)	MBP (μg/l) concentration (mannan capture)
I1	3 yr	Severe diarrhoea, FTT	0.0	-2.9	-0.6	9.4
I2	15 mo	URTI	5.9	-3.6	-0.9	4.9
I3	16 mo	RTI	-5.6	-3.5	-0.8	<2.5
I4	9 mo	URTI	0.0	0.5	-0.2	13.9
I5	7 yr	Pyrexia	0.0	1.6	9.8	17.4
I6	8 yr	URTI, migrane	0.0	-1.2	-0.6	4.5
I7	6 mo	Severe diarrhoea FTT	5.3	-11.4	-0.9	35.2
I8	2.5 yr	RTI bronchiec-tasis	0.0	13.9	1.7	23.9
I9	10 mo	Pyrexia	5.3	-16.4	-1.3	2.8
I10	9 yr	URTI FTT	-5.0	10.5	0.0	<2.5

Note: FTT Failure to thrive
URTI Upper respiratory tract infection
RTI Respiratory tract infection

(No other immunological abnormalities were found in these patients with the exception of an IgG2 deficiency in patient I3.)

Conclusion

The ten paediatric patients reported here had previously been shown to have an opsonic deficiency (see **Table 5.4**) and this is now confirmed in the various mannan binding assays. The median MBP level for this group was 4.9μg/litre and 8 of the 10 were found to have MBP concentrations below 20μg/litre.

6.4.3 Properdin deficient sera

Introduction

The three properdin deficient sera described in **Chapters 4** and **5** had generated inconsistent data in the various mannan binding assays. A possible explanation for these findings would be different levels of MBP in the three sera tested.

Method

The three properdin deficient sera were tested for MBP concentration using the mannan capture MBP ELISA procedure.

Results

The properdin deficient sera Variants 1 and 2 both had low MBP concentrations (see **Table 6.3**) which were below the threshold value of 30μg/l set for this assay (see **Section 6.4.1**). Thus these sera appeared to have co-existing deficiencies of properdin and MBP. In contrast, the concentration of MBP in the Variant 3 serum was at the top of the normal range.

Table 6.3 Levels of MBP in the 3 properdin deficient sera

Properdin deficient	Clinical presentation	Properdin concentration (mg/l)	Mannan binding assay C3bi (BC%)	Mannan binding assay properdin (BC%)	MBP concentration (μg/l)
Variant 1	40 year old healthy man	<0.1	0.00	-2.20	8.80
Variant 2	29 year old healthy man	1.0 - 2.0	1.94	-2.47	28.86
Variant 3	61 year old man with *N.meningitidis* meningitis and *E.coli* septicaemia	19.0	83.29	-1.86	658.94

Comment

The measurement of MBP in these three sera has established a rational explanation for their differing properties in the various correction assays (see **Figure 5.11(a)**)

6.5 Discussion

The observation by Ikeda et al (1987) that rat, rabbit and human MBP were able to activate guinea pig complement has now been confirmed using human MBP and autologous human complement (Super et al 1990, Ohta et al 1990). Moreover, it is now clear that MBP acts as a C1q analogue and, after interacting with C1r and C1s, is able to promote the formation of C1 esterase (Lu et al 1990, Ohta et al 1990) with the potential to cleave C2 and C4. At a serum concentration of 5% the levels of C4b and C3b complement fragments binding to mannan were found to be strongly correlated with the serum concentration of MBP (see **Table 6.1**). Furthermore, there was no correlation between these levels and the mannan binding activity of various complement fixing immunoglobulins (neither total IgG and IgM nor IgG1, IgG2 and IgG3 subclasses) (see **Figures 5.7** and **5.8** and **Table 6.1** and Super et al 1990).
It appears, therefore, that MBP is the major regulator of classical pathway activation by mannan at low serum concentrations. Presumably the findings obtained using 5% serum would also be applicable to 9%, 13% and 17% serum and would explain the observed differences between the HB1 and LB1 sera at these concentrations(see **Figure 5.2** and **Table 5.1**). Above 20% serum the differences in deposition of C3 moieties become less marked and are virtually non-existent with 30 % serum as shown by the Binding Coefficient (%) values of sera with the common opsonic defect when assayed in the presence of MgEGTA (see **Figure 5.3 (a)**).

It is well known that the alternative pathway of complement is very sensitive to dilution and the concentration of Factor D is probably the most limiting element in the pathway (Ehrnst 1978). The absence of any role for the alternative pathway in the experimental system used in these studies is not, therefore, unexpected. In contrast, the presence of detectable antibody with anti-mannan specificity and putative C1q binding activity (that is IgM, IgG1 and IgG3 antibodies) leads one to expect at least some C3b deposition as a result of antibody activation of the classical pathway. Yet this was not observed. One possible explanation is that at the dilution of serum used the IgG antibodies are spatially too far apart to be effectively bridged by C1q, however these arguments cannot be invoked in the case of IgM, which is able to activate classical pathway following binding of a single antibody molecule to the

substrate. Various aspects of this test system, particularly the nature of the mannan coat, will require much further work in order to reach a complete understanding of the mechanism involved in complement activation in this system. It is also important to stress that in calculating the Binding Coefficient, the amount of complement binding to mannan (or zymosan) from serum LB1 is set at 0%. However, it is evident (see **Figure 5.2**) that the OD492 absorbance of LB1 used at 5% is approximately 0.3 (compared with a value of 1 for HB1). Thus the amount of complement binding to mannan from LB1 and other sera with low MBP concentrations may represent the complement activated by IgM anti- mannan antibodies.

The immunoblotting experiment described here showed that MBP enriched preparations could be obtained from both pooled serum and from the serum of donor HB1 with high C3b binding. The material derived from pooled serum was subsequently shown to correct the opsonic defect in serum LB1; the complement proteins C3b+bi, Factor B and C4 becoming bound to mannan coated plates in a dose dependent fashion. In contrast, MBP was not detected in the immunoblot of material from donor LB1 with low C3b binding. However, sensitive ELISA procedures detected 5-7μg/litre of MBP in the LB1 serum. It is likely, therefore, that losses during the multistep preparation accounted for the total absence of signal from the LB1 fractions in the immunoblot whereas a strong signal was still obtained with MBP derived from donor HB1 (MBP level approximately 400μg/litre). It should be noted that donor LB1 has been used as a donor of serum for a considerable period of time and MBP assays of many such samples have consistently given values of approximately 6μg/l. It can therefore be assumed that the individuals with low serum MBP levels always remain low, even when evaluated months later. This point is of interest because other work (Ezekowitz et al 1988) has shown that MBP is an acute phase reactant (see **Chapter 7**) and it might be expected that high levels of MBP would be observed transiently in response to stress or infection. We have measured MBP levels in a patient with meningitis and have observed that the levels of MBP in serum rose from 127μg/l to 525μg/l in 5 days in the course of an acute infection. In the apparently healthy adult population there were some individuals with levels of 610μg/l and in the paediatric population there were individuals with levels as high as 880μg/l, but it is unclear whether these were undergoing an acute phase reaction at the time they were bled.

Turner et al (1985 b) reported a correcting protein of molecular weight 70-80kDa, which eluted immediately after IgG on anion exchange chromatography before any application of a salt gradient. In contrast, in the experiments described in this thesis the only fractions which corrected the opsonic defect contained MBP and these were 600-700kDa and eluted from Mono Q anion exchange chromatography at approximately 350mM NaCl. Since the results of the yeast opsonisation and mannan-binding assays correlate well, the material purified in 1985 probably contained MBP. Yet it is difficult to explain the different purification characteristics. One possible explanation for the discrepancy in molecular weight may relate to the carbohydrate-binding function of MBP. In chromatography buffers containing trace amounts of calcium and less than 0.05 M NaCl, MBP is partially retained on gel filtration columns and elutes with the albumin fraction (M. Super unpublished observation). This may explain the different results between the earlier fractionations, which were carried out in 0.05M Tris buffer using incompletely deionised water and the present studies using PBS (containing 0.14 M NaCl) and **EDTA** in Milli-Q water.

The concentration of MBP in the sera of children previously shown to have the opsonic defect was significantly lower than that of a paediatric control group and, since no other immunodeficiency was found in this group,(other than an IgG2 subclass deficiency in patient I3) it is suggested that the low MBP concentration contributes to the observed pathology. Nevertheless, it would be premature to claim that this deficiency is a sole and sufficient cause of the illnesses observed. Many aspects of the immune system remain unprobed, even in a tertiary referral centre such as GOSH and there is every possibility that other contributory factors may be relevant. For example, it is still unclear why the incidence of poor yeast opsonisation/low MBP concentration is so high in the healthy population.These concepts are discussed at greater length in **Chapter 7.**

CHAPTER 7

DISCUSSION

Mannose Binding Protein (MBP)

Mannose binding protein is a member of the family of animal lectins (Drickamer 1988). Lectins as a group have been defined as carbohydrate-binding proteins of non-immune origin that agglutinate cells and can precipitate polysaccharides and glycoproteins. It is now thought that mammalian lectins play an important role in recognition of carbohydrates associated with both cell bound and soluble proteins and that this recognition may trigger certain cellular effector functions (Thiel and Reid 1989).

Lectins have been divided by Thiel and Reid (1989) into three groups. These are:

(i) the C-type lectins which have an absolute requirement for Ca^{++} when binding to carbohydrates e.g. mannose-binding protein.
ii) the S-type lectins, which are thiol dependent e.g. β galactosidase- specific lectin
iii) the third group do not contain either the C or S- type of conserved carbohydrate-binding domain e.g. serum amyloid protein (SAP).

All C-type lectins have a carbohydrate-binding domain of approximately 130 amino-acids which contains a framework of 18 conserved amino acid residues (Drickamer 1988). These conserved amino acids are thought to stabilise the quaternary three-dimensional structure of the carbohydrate-binding domain in which 4 cysteine residues are thought to form two stabilising disulphide bridges (See **Figure 7.1)** . The precise functions of these lectins following binding to their appropriate ligands has been unclear. However, possible roles for membrane-bound lectins include the endocytosis of mannose terminated glycoproteins by the mannose receptor on macrophages (Lennartz et al 1987) elimination of desialyated erythrocytes and conjugates containing exposed galactose groups by the galactosyl receptor (Ashwell and Morell 1974), elimination of yeast particles by phagocytosis through complement receptor type 3 (Ross et al 1985(a)) and intracellular localisation of mannose-6-phosphate containing lysosomal enzymes by mannose-6-phosphate receptors. The soluble lectins may be involved in clearance mechanisms since the pentraxins SAP and CRP bind to a variety of polysaccharides on bacteria fungi and parasites (Pepys 1981).

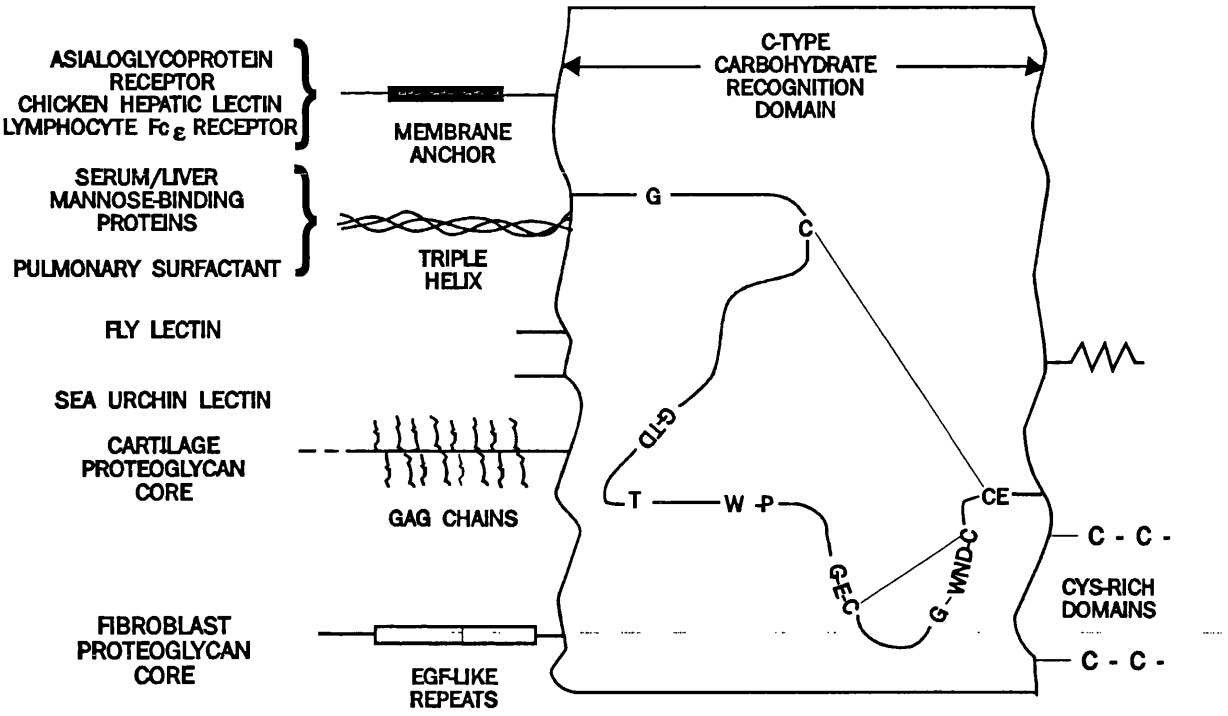


Figure 7.1 Summary of structural features of C-type animal lectins. The invariant residues found in the common carbohydrate-recognition domain of the C-type lectins are shown, flanked by schematic diagrams of the special effector domains found in individual members of the family.(Adapted from Drickamer 1988).

The asialoglycoprotein receptor was the first C-type lectin to be described. The presence of this molecule was suggested in studies measuring clearance of glycoproteins from the mammalian circulation following removal of terminal sialic acid. All C-type lectins bind to carbohydrates at and above pH 6.5 in the presence of divalent calcium. These proteins consist of a carbohydrate binding domain associated with one or more non-lectin domains which may be responsible for recognition and clearance mechanisms. The C-type lectins can be divided into membrane bound and soluble forms depending on the nature of the non-lectin domains. The membrane-bound lectins are attached by membrane anchor domains and include the asialoglycoprotein receptor found in rat (Leung et al 1985) and human liver (Halberg et al 1987) and (Bischoff & Lodish 1987) and the chicken hepatic lectin (Drickamer 1981), the lymphocyte IgE Fcε receptor (Ikuta et al 1987) and the receptor on rat Kupffer cells (Hoyle & Hill 1988). Recently reported membrane-bound lectins include the lymphocyte homing receptor (Lasky et al 1989) and the cellular adhesion factors GMP -140 (Johnston et al 1989) and ELAM -1 (Bevilacqua et al 1989).

The soluble C-type lectins include proteins with collagenous domains such as the mannose binding protein of rabbit, rat and human (Drickamer et al 1986, Ezekowitz et al 1988), pulmonary surfactant apoprotein (PSAP)(Floros et al 1986, Benson et al 1985) and conglutinin (Baartrup et al 1987, Thiel et al 1987, Young and Leon 1987). Other soluble C-type lectins lacking the collagen-like domain are cartilage proteoglycan core, and lectins in fly (Takahashi et al 1985) and sea urchin (Giga et al 1987). These have been reviewed by Drickamer (1988).

Conglutinin, pulmonary surfactant apoprotein and mannose - binding protein are C-type lectins which contain an N-terminal non-collagenous region, a collagenous "core" region and a carbohydrate-binding region containing specific recognition sites. Conglutinin, the first vertebrate lectin to be described, binds to mannose and N-acetyl glucosamine terminated glycoproteins (Young and Leon 1987). It binds particularly avidly to the glycoprotein on C3bi (Hirani et al 1986) although the physiological significance of this has not yet been established. PSAP, which is found in the lung, exhibits a calcium dependent binding to phospholipids and carbohydrates. Mannose-binding protein is a C-type lectin apparently secreted solely by the liver. In man the serum and liver forms are identical polymeric molecules based on individual polypeptide chains of approximately 32kDa (Wild et al 1983, Summerfield &Taylor 1986). The larger polymers exist as a 650kDa protein which is homologous to the 650kDa rat MBP-A and 600kDa rabbit MBP molecules (Ezekowitz et al 1988, Kozutsumi et al 1980). Thus this form of human MBP comprises approximately 18 identical 32kDa subunits. Each subunit consists of an 18-20 aa N terminal region rich in cysteine residues, a collagenous region containing 18-20 repeats of Gly- X-Y (Drickamer et al 1986) similar to non-fibrillar collagen motifs, and a C terminal globular domain which is the site of carbohydrate binding (Ezekowitz et al 1988, Drickamer et al 1986). Three monomeric chains initially interact as follows: the collagenous regions form a triple helix and the carboxy terminal regions fold up to give a globular carbohydrate recognition domain. The whole subunit structure is stabilised by three disulphide bridges in the N-terminal region and further similar covalent interactions between 3-6 different subunits stabilise the polymers.

The human MBP gene has been sequenced recently (Ezekowitz et al 1988, Taylor et al 1989) and has been localised to the long arm of chromosome 10 at position 10q11.2 - q21 (Sastry et al 1989). The genomic DNA, which is approximately 6.2 kb, consists of 4 exons and three introns homologous to the organisation of surfactant apoprotein (H-PSAP) (see **Figure 7.2**). The organisation and sequence of the human MBP gene are strikingly homologous to the genes for rat MBP-A and C. Human MBP

protein has 51% homology with rat MBP-C with 3 gaps and 48% homology with MBP-A when aligned with 7 gaps. Thus human MBP has also been termed MBP-C even though the function is more reminiscent of rat MBP-A since it is mainly found in serum and can activate complement (Ezekowitz et al 1988). Exon 1 (251 bp) contains the sequence of a 5' untranslated region (UTR), a signal sequence, the N-terminal cysteine - rich region and the collagenous region up to the glycine of the Gly-Gln-Gly "kink". Exon 2 (117 bp) encodes the sequence of the remaining 11 Gly-X-Y triplets of the collagenous region. Exon 3 (69 bp) codes for the "neck" region between the collagenous domain and the carbohydrate receptor domain (CRD) while exon 4 (3.1kb) encodes the CRD (450 bp) and 2.6 kb of 3' untranslated region (UTR).

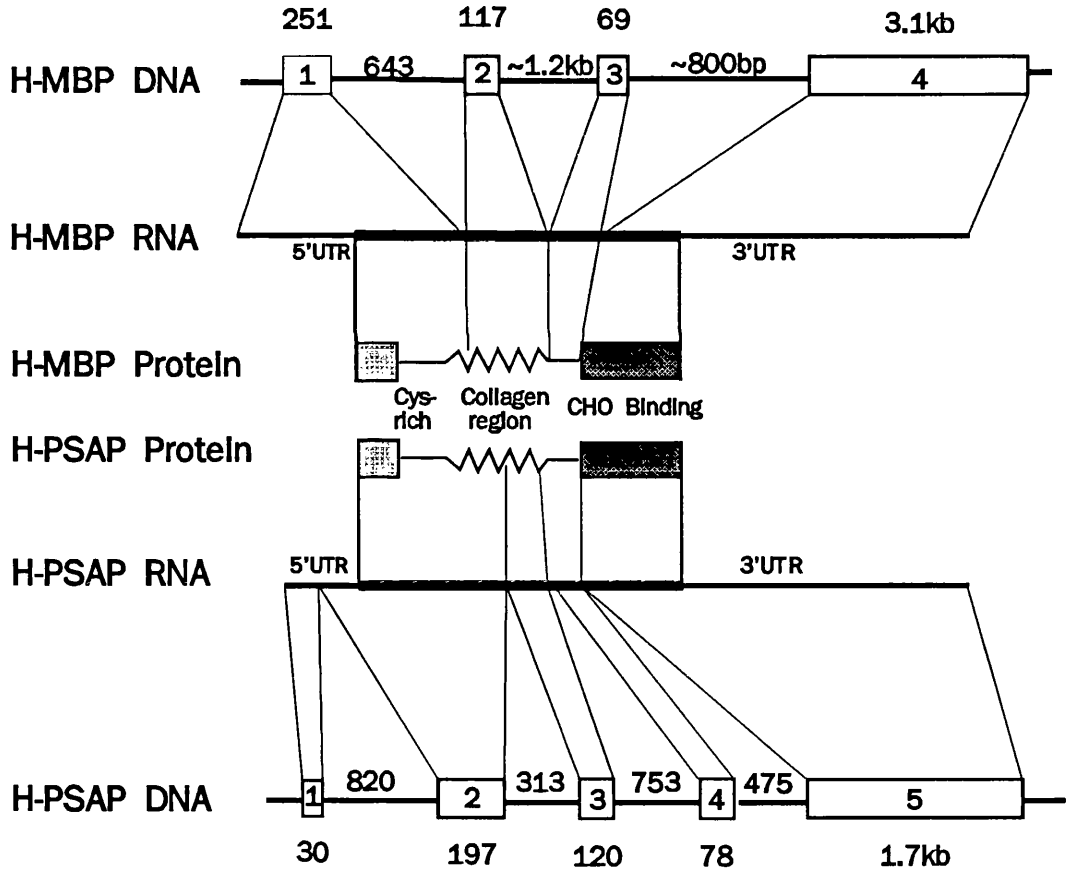


Figure 7.2 Summary of the functional regions in the human MBP-C gene, mRNA, and polypeptide and comparison with the corresponding elements for the human pulmonary surfactant apoprotein (H-PSAP).The human MBP-C shares 30% overall homology with H-PSAP as well as similar genomic and functional organisation.(Adapted from Sastry et al 1989).

The sequence of the collagenous region (from exon 1 and 2) closely resemble the sequence for rat MBP and the motif of exons coding for the collagenous region interspersed by an intron resulting in a kink in the collagen alpha helix is also found in H-PSAP (Thiel & Reid 1989) and C1q (although in C1q the sequences of exons and intervening intron are not homologous to those in MBP and H-PSAP). The 4 exons are interspersed by 3 introns of 643 bp, and approximately 1.2 kb and 800 bp respectively. Homologies between the exon arrangement of rat MBP-A and non-fibrillar collagens suggests that MBP molecules are formed by juxtaposition of exons from non- fibrillar collagens and carbohydrate-binding proteins (Drickamer and McReary 1987). Further evidence of evolution of the C-type lectins is found in the exons coding for the CRD; in the asialoglycoprotein receptor the carbohydrate-recognition domain is encoded by three exons while in the pulmonary surfactant apoprotein and human and rat MBP's these have fused with the deletion of introns to form one exon (Sastry et al 1989).

Ezekowitz et al (1988) first suggested that human MBP might function as an acute phase protein since large amounts of mRNA for MBP were noted in the liver of a victim of a road traffic accident. The 5'UTR of MBP contains TATAA and CAAT boxes at -38 bp and -79 bp from the start of the cDNA (Taylor et al 1989), and there is also a region (TAAGAAATTTCCAG) similar to the heat shock promoter of *Drosophila* at -592 bp. In *Drosophila* this region is involved in transcription initiation of the heat-shock gene products and the homologous region in MBP (and another acute-phase protein : C-reactive-protein (CRP)) may similarly be involved in stress responses (Taylor et al 1989). In addition there are three regions in the 5' untranslated region (at -245, -656 and -736) which are similar to consensus sequences of glucocorticoid - responsive elements (Taylor et al 1989). These may also be important in acute phase responses since an increased release of glucocorticoid hormones from the adrenals is a characteristic of the acute-phase response. It has also been noted that the region from -204 bp to -184 bp shares 90% homology with a region of the Serum amyloid A gene (also an acute phase-protein). Finally, the 3'UTR of MBP contains 7 AUUUA instability sequences found in many cytokines, oncogenes and growth factors and thought to be associated with rapid mRNA degradation (Sastry et al 1989).

The shapes of the final polymeric forms of H-PSAP, MBP and conglutinin resemble C1q since all of these molecules have globular "heads" linked by "connecting strands" to a central collagen-like structure (see **Figure 7.3**) (Thiel and Reid 1989).

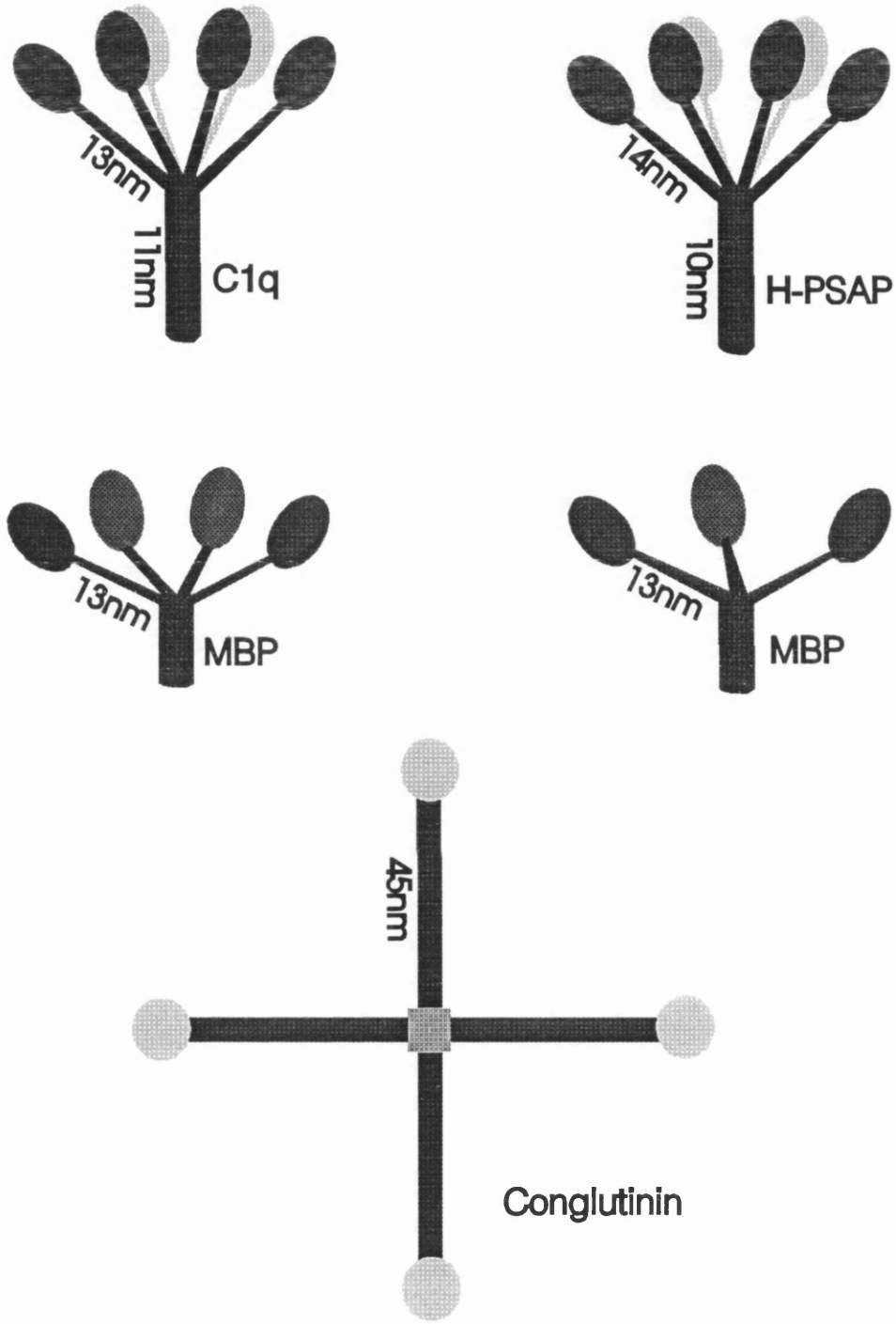


Figure 7.3 Proposed structures of C1q, H-PSAP, MBP (trimeric and tetrameric forms) and bovine conglutinin (Based on Thiel and Reid 1989).It should be noted that the conglutinin molecule is considerably larger than C1q, H-PSAP or MBP and is shown on a different scale to that used for the other molecules.

In electron microscopy the H-PSAP and C1q are virtually indistinguishable. MBP may also form similar hexameric structures, but the trimer/tetrameric forms appear to predominate in serum (Thiel and Reid 1989, Lu et al 1990). In contrast conglutinin is found only as a tetrameric structure. The collagenous regions of C1q (chains A and C), H-PSAP and human and rat MBP-A share a kink introducing disruption to the Gly-Xaa-Yaa repeat characteristic of the non-fibrillar collagens (Ezekowitz et al 1988) and this may be important in the binding of these molecules to cell-surface receptors (Thiel and Reid 1989). This kink is the site of an intron in the human and rat MBP's (Ezekowitz et al 1988, Drickamer & McReary 1987) and in the pulmonary surfactant apoprotein (White et al 1985). Preliminary evidence suggests that both H-PSAP and MBP can indeed bind to C1q receptors on lymphoid and other cell types (Malhotra et al 1990). The interaction of the collagenous regions with C1q receptors may lead to triggering of effector mechanisms such as an increase in oxidative phosphorylation,decreased IL1 synthesis, modulation of IgG synthesis and increased phagocytic and cytotoxic cell-mediated responses (Erdei and Reid 1988, Lu et al 1990). Thus it appears that binding of these collagen-like regions to a common receptor may be significant in general recognition and clearing mechanisms for the ligands of C1q, H-PSAP, MBP and conglutinin i.e. for clearance of immune complexes, glycolipids and mannose-rich organisms.

Ikeda et al (1987) measured the ability of MBP to activate complement using a passive haemolysis method which consisted of coating sheep erythrocytes with mannan using chromium chloride and then sensitising these with MBP isolated from rabbit, human and rat serum as well as the liver MBP isolated from rats. Using this system Ikeda et al showed that the serum MBP's, which were all approximately 650kDa, lysed mannan coated erythrocytes (ME) in a dose-dependent manner in the presence of guinea-pig complement. In contrast, the 200 kDa rat liver MBP did not activate complement. The addition of mannose or haptenic sugars known to be potent inhibitors of MBP binding (L-fucose,N-acetylmannosamine and N-acetylglucosamine) inhibited the MBP sensitisation of ME whereas galactose, a non-inhibitor, did not. Moreover, when these putative inhibitors were added after the sensitisation step, they did not inhibit at all. Thus MBP binding to the mannan-coated erythrocytes was mediated by the sugar binding activity of MBP. Ikeda et al (1987) showed that rabbit, human and rat serum MBP's bound to ME and activated the classical pathway in guinea-pig serum with an absolute requirement for C4. This was not due to endogenous guinea pig MBP since this had been removed at $4^{o}C$ using a mannan-Sepharose column. The absolute requirement for C4 was established with the use of

C4 deficient guinea-pig serum - this did not lyse ME sensitised with any of the MBP preparations used. These workers therefore proposed that MBP bound to cells functions like C1q in activating the classical pathway of complement. Ikeda et al did not definitely establish that MBP was acting like C1q since they did not remove the guinea-pig C1q or use C1q deficient serum. Therefore guinea-pig C1q might theoretically have become bound to the MBP-sensitised ME and then activated the CP. These points were subsequently addressed by Lu et al (1990) and by Ohta et al (1990). Moreover, Ikeda et al had used human MBP to activate guinea-pig complement, but they had not shown that human MBP was able to activate human complement. This was established in the experiments described in this thesis.

When sera from 179 healthy blood donors were incubated on mannan- coated ELISA plates at 5% concentration in Veronal-buffered saline containing 5mM $MgCl_2$ and 5mM $CaCl_2$, binding of the components C3b/bi,C3bi,properdin and Factor B correlated well with each other. These results were in agreement with DiScipio (1981) who found a stoichiometric ratio of approximately 1:1:1 for C3b,properdin and Factor B interactions with zymosan surfaces using radiolabelled complement components. He therefore proposed that binding of these components was linked, probably in the form C3bBbP -the alternative pathway convertase. In the present study there was also a very good correlation between surface bound C3b/bi and C4. The latter was presumably a measurement of covalently bound C4b fragments and the highly significant correlation with C3b/bi levels suggested that in this experimental system (using serum at 5% concentration in the presence of mannan) activation of the classical pathway and C4 cleavage is the primary regulator of C3b/bi binding to mannan or zymosan surfaces. This was confirmed by repeating selected assays measuring C4, C3b/bi and Factor B binding to mannan in the buffer, Mg EGTA, which chelates Ca^{++} and prevents assembly of the classical pathway C1 complex. Here there was no evidence (see **Figure 5.3**) of any binding of complement components when the 40 sera used were diluted to a concentration of 5%. The mannan binding patterns of the complement fixing antibodies IgG1, IgG3 and IgM (see **Figures 5.7 & 5.8**) did not correlate with C3 and C4 binding and this suggested that this experimental system was measuring an antibody independent classical pathway activation. However, highly significant correlations were found between the levels of human serum MBP bound to the mannan-coated plates and the C4, C3b/bi, properdin and Factor B subsequently bound from the 178 sera (see **Figures 6.8 (a)-(d)**). It appears that binding of MBP to mannan or zymosan surfaces precedes complement activation by the classical pathway in this experimental system.

Lu et al (1990) confirmed that the pentameric/hexameric forms of human MBP bound to zymosan particles in a calcium dependent manner could activate complement in the complete absence of C1q by interacting with $C1r_2C1s_2$ complexes via the $C1r_2$ subunit. The multistep preparation procedure used to isolate MBP and pre-incubation of zymosan and MBP preparations with the serum esterase inhibitor DFP excluded the possibility that this effect was due to any carry-over of any serine protease in the MBP preparations. Fractionation of an MBP preparation by FPLC Mono-Q anion exchange chromatography revealed that 70% consisted of trimers and tetramers (apparent molecular weights 270kDa and 360kDa respectively) while the pentameric/hexameric forms (450- 540kDa) accounted for only 10-15% of the preparation. However, classical pathway activation by MBP only occurred using the pentameric/hexameric moieties. It is possible, therefore, that activation requires multivalent binding of the CRD to the carbohydrate surface or the juxtaposition of at least 5 or 6 collagenous stalks for interaction with the $C1r_2C1s_2$ complex.This may explain the findings of Ikeda et al (1987) that the 200kDa MBP isolated from rat liver did not activate complement. Ohta et al (1990) used the passive haemolysis assay described by Ikeda et al and human serum which had been pre-adsorbed at 4°C using a mannan-Sepharose affinity column and immunoaffinity depleted of C1q and confirmed that MBP activated the classical pathway in the absence of C1q. In addition they showed that the $C1r_2C1s_2$ complex bound to MBP with high affinity (dissociation constant 1×10^{-9}) which is similar to the binding of the $C1r_2C1s_2$ to C1q (Hughes-Jones & Gorick 1982). $C1s_2$ only bound to the MBP in the presence of C1r and this binding led to activation of the C1s proenzyme with subsequent lysis only when the MBP was surface-bound. It did not occur in the fluid-phase. It appears therefore that the binding of MBP to carbohydrate surfaces and subsequent "carbohydrate-mediated" classical pathway activation parallels the binding of C1q to antibody/antigen complexes in antibody mediated activation of the classical pathway. Kawasaki et al (1989) found that the classical pathway activation by MBP acting on rough *Eschericia coli* strains K12 and B, severely reduced the colony - forming potential and thus the viability of the bacteria. MBP binding was calcium dependent and was inhibitable by mannose,N-acetylglucosamine N- acetylmannosamine, L-fucose, mannoheptulose and sedoheptulose, suggesting that MBP was recognising L-glycero-D-mannoheptose and N-acetylglucosamine of the core oligosaccharide of the *E.coli* K12 and the L-glycero-D-manno-heptose of *E.coli* B. Binding of MBP to these molecules with subsequent activation of complement may explain the avirulence of certain rough bacterial strains.

A model of the activation of complement by MBP is shown in **Figure 7.4 (a)-(c)**. This model encompasses results described in this thesis as well as the findings of Lu et al (1990) and Ohta et al (1990).

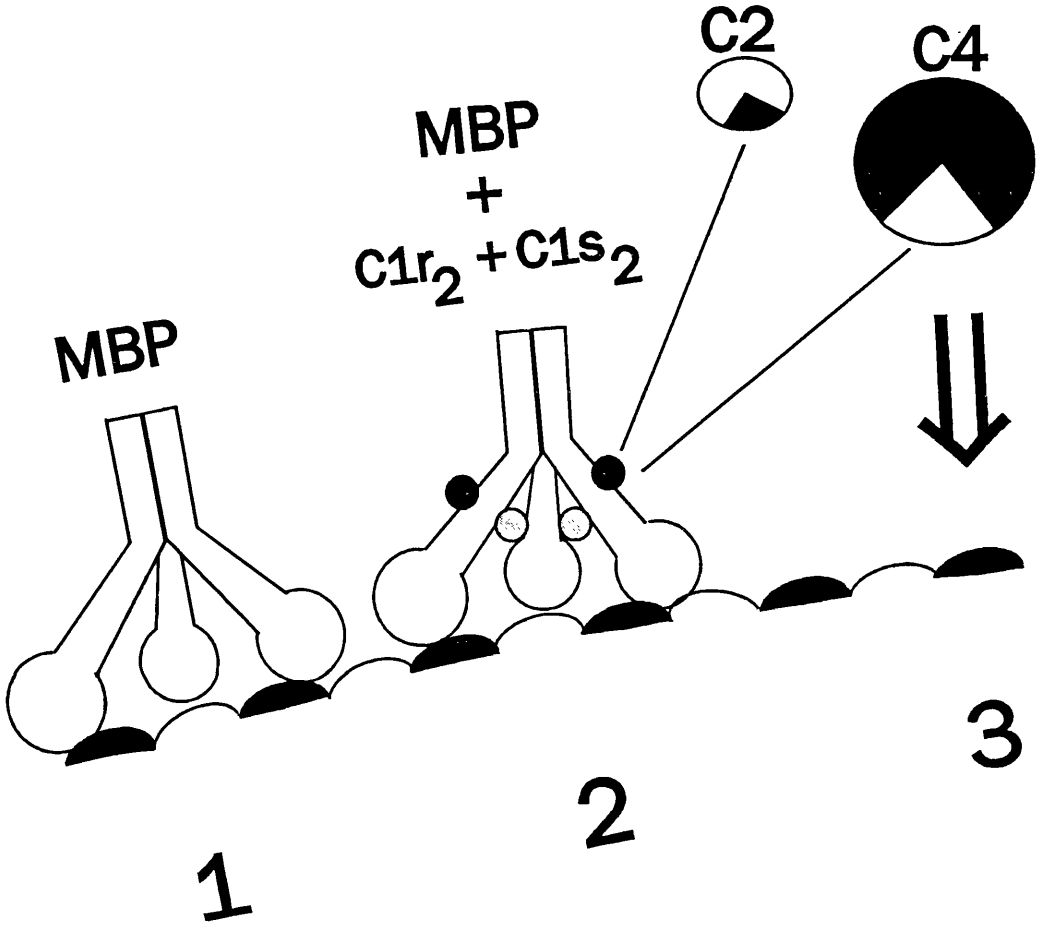


Figure 7.4 (a) Model of activation of complement by MBP:

Step 1	MBP binds to mannose rich surface through its carbohydrate recognition domains.
Step 2	Surface bound MBP interacts with $C1r_2$-$C1s_2$ to generate a C1 esterase enzyme.
Step 3	Circulating C2 and C4 are cleaved by the C1 esterase.

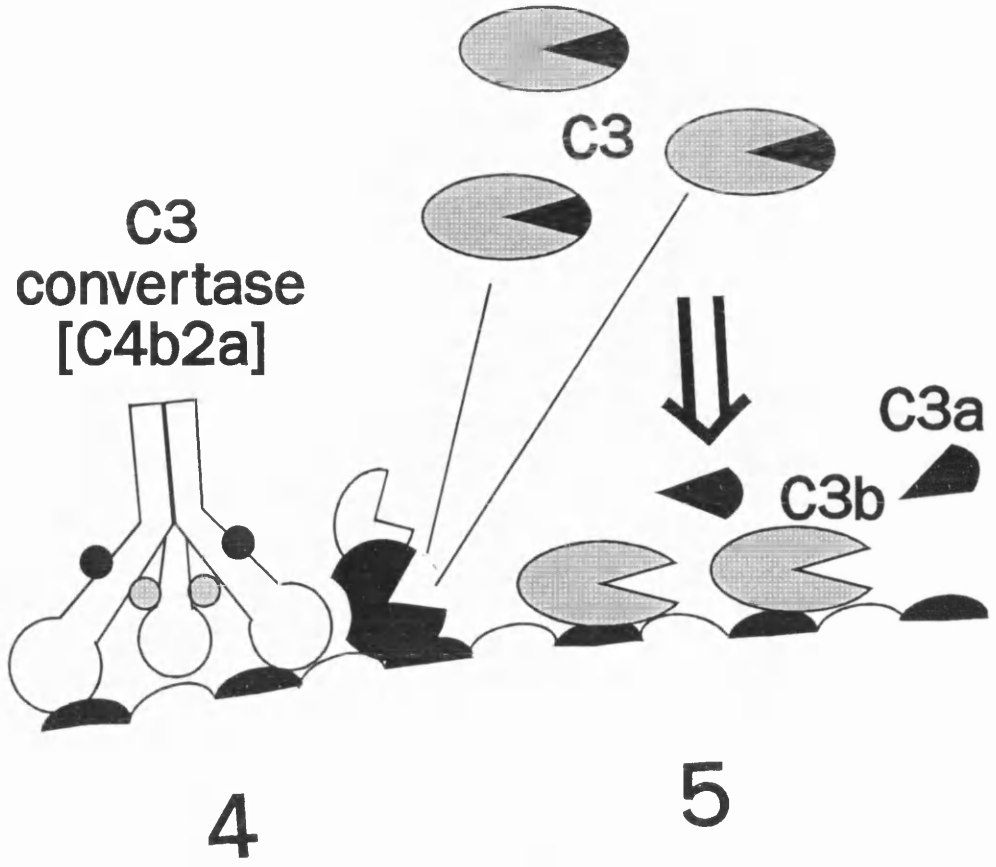


Figure 7.4 (b) Model of MBP activation of complement (continued):

Step 4	Assembly of classical pathway C3 convertase [C4b2a]
Step 5	Fluid phase C3 is cleaved into opsonic C3b and low molecular weight C3a. The C3b becomes covalently bound to the surface.

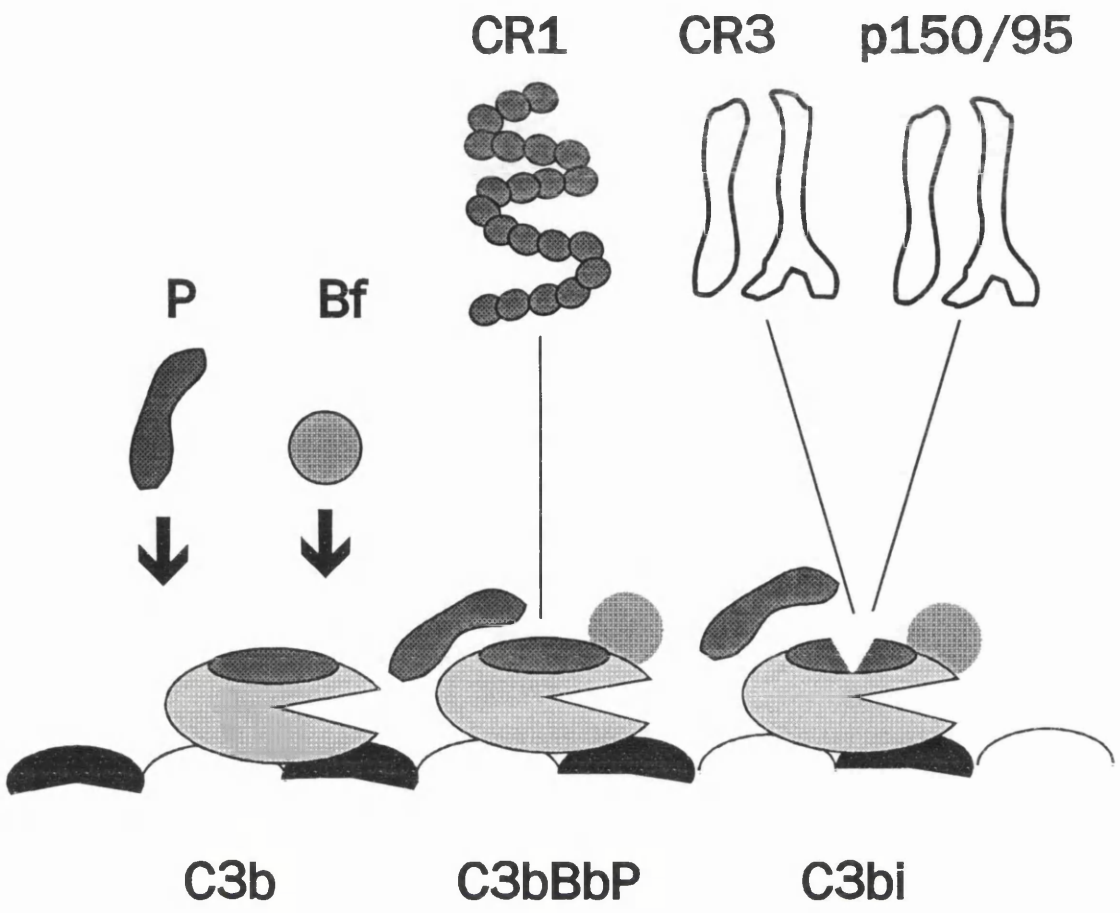


Figure 7.4 (c) Model of MBP activation of complement (continued):

Surface bound C3b binds properdin and Factor B stoichiometrically. Some of the C3b is degraded to C3bi but both C3b and C3bi are able to interact with phagocyte receptors such as CR1, CR3 and p150/95. (Adapted from Turner et al 1990 (a))

In contrast to the above findings, Schweinle et al (1989) suggested that MBP activates the alternative pathway of complement rather than the classical pathway. These workers bound MBP onto *Salmonella montevideo*, an organism with high-mannose O-polysaccharide and found that, in the presence of MBP, C3 opsonisation of the bacterium occurred via the alternative pathway and pre-sensitising of *S. montevideo* with MBP "rendered this normally serum resistant organism susceptible to complement mediated killing". This claim was based on studies using MgEGTA, various complement depleted sera and isolated complement components. Schweinle et

al measured C3 bound to *S.montevideo* (SH5770) pre- sensitised with MBP in the presence and absence MgEGTA and found that significant amounts of C3 bound to the bacteria in both instances. They also suggested that there was no difference in the amounts of C3 bound when C2 was added to a C2 deficient serum incubated with the MBP-SH5770 complexes. Addition of Factor D to a serum deficient in Factor D and C1q increased C3 binding to the MBP-SH5770 while C1q addition had no effect. Finally they used purified alternative pathway components and showed that C3 bound to the MBP-SH5770 in this system while no C1s activation was observed when the C1 zymogen was assembled from isolated C1q, C1r and C1s and then added to the MBP-SH5770 system. The difference between these results and those of other workers is difficult to reconcile as many of the experiments appear to be identical. One possible explanation for the disparate findings is that Schweinle et al pre-adsorbed the sera using the *S. montevideo* bacterium whereas Lu et al did not pre-adsorb serum and Ikeda et al, Ohta et al and Kawasaki et al pre-adsorbed using mannan-Sepharose columns. The candidate has observed that serum pre-adsorbed with zymosan at 4^{o}C did not activate classical pathway in experiments using mannan-coated ELISA plates.However, alternative pathway convertase activity was apparently unaffected when this was tested by the properdin capture/C3 detection system described by Asghar et al (1987). Thus pre-adsorption in *S.montevideo* could have selectively inactivated the classical pathway while leaving the alternative pathway unaffected. Schweinle et al suggest that MBP alters the bacterial surface, promoting amide links between the thiolester of C3b and amino groups on the bacteria It is possible that MBP opsonised SH5770 is a more suitable target for C3 alternative pathway activation than is SH5770 alone.

In addition to activating complement, MBP may fulfil various roles in host defence. There is circumstantial evidence to suggest that MBP has a role in host defence. It has already been noted that human MBP behaves as an acute phase reactant and MBP has recently been shown to inhibit *in vitro* infection of lymphoblasts by the human immunodeficiency virus (HIV), probably by binding to the high mannose glycan gp120 found in the HIV envelope (Ezekowitz et al 1989). The protein recognises mannose and N- acetyl glucosamine and the widespread occurrence of these sugars in the cell walls of pathogenic Gram negative bacteria, mycobacteria and yeasts makes such organisms putative targets for serum lectins. An organism with a cell wall rich in mannose groups could selectively concentrate MBP on its surface and this could then act as a focus for complement activation through the classical pathway. Kuhlman et al(1989) recently reported that MBP may itself be opsonic, presumably through interactions with specific C1q-like receptors on the phagocyte surface. Whether this

occurs on a significant scale in vivo remains to be established since the levels of MBP used in these in vitro assays exceeded the normal level of the protein in serum by two orders of magnitude (i.e. times 100-fold).

Since 5% of the general population have repeatedly been shown to have impaired opsonic function (Soothill and Harvey 1976, Levinsky et al 1978, Kerr et al 1983), it is clear that disease is not inevitably associated with low levels of MBP and the significance of the association should be considered. Whatever its mechanisms of action may be, MBP should probably be regarded as an accessory immune system during most of an individual's lifetime. The patients with defective yeast opsonisation have usually presented between the ages of six months and two years and this suggests that the function of MBP may be particularly important at this age.

Hammarström et al (1986) have suggested that in early infancy much of the anti-polysaccharide response may be low affinity IgG1 antibody. In most individuals isotype switching for polysaccharide antigens then occurs, resulting in a predominantly IgG2 subclass response. However, the antibody level observed in the relatively minor IgG2 subclass (generally regarded as poor in complement activation) appears to differ markedly within the population. For example, following immunisation with a 23 valent polysaccharide vaccine, Sarvas et al (1989) showed that individuals homozygous for the G2m(n) allotype had about four times more IgG2 antibody than did G2m(n) negative vaccinees. It is possible to envisage situations in which a relative deficiency of MBP may become pathologically significant in individuals in whom the IgG1 response to polysaccharides is of low affinity, in those with an isotype switch defect or in the 'low responders' having two G2m(n-) alleles. Young infants aged between 6 and 24 months would be most at risk because there is no residual maternal antibody protection and in most cases the susceptibility would be transient because of maturation in the antibody repertoire, particularly the ability to make adequate responses in the complement fixing IgG1 and IgG3 subclasses (see **Figure 7.5**).

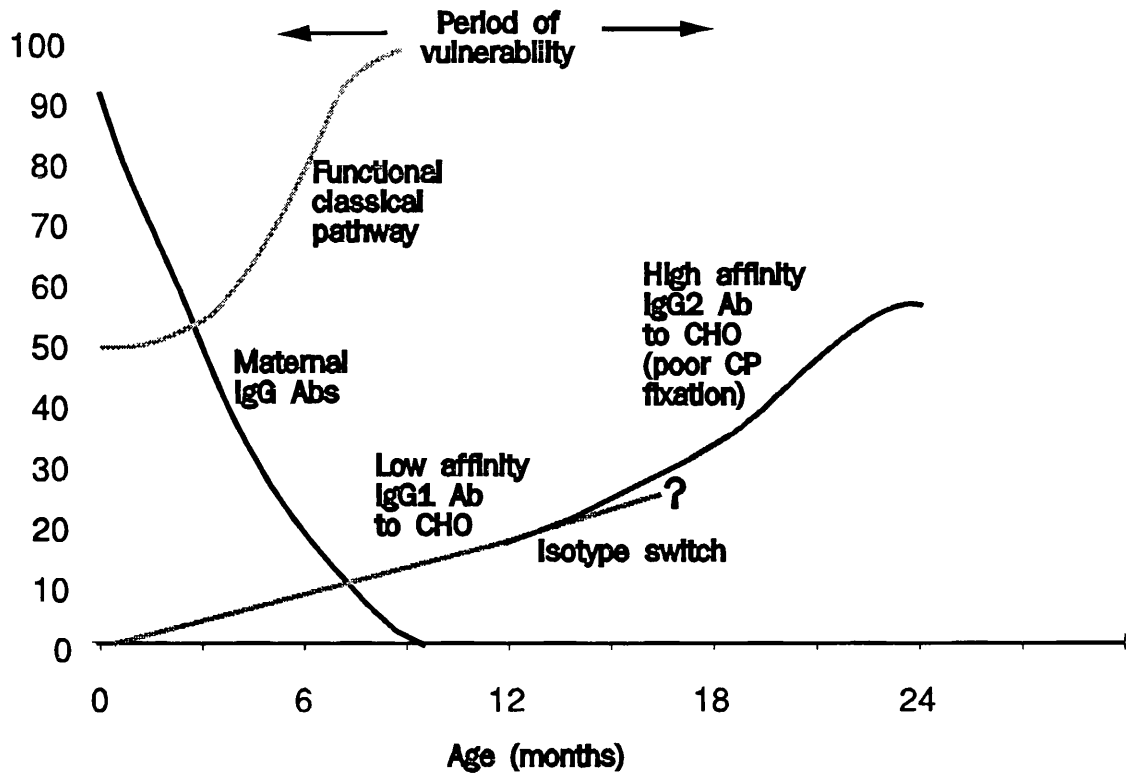


Figure 7.5 Schematic diagram illustrating the 'window of vulnerability at 6-18 months during which MBP activation of the CP might be particularly important. During this period maternal antibody has largely disappeared and the infants own repertoire of complement fixing antibodies is still extremely limited. (Adapted from Turner et al 1990(a))

Even during such a period of vulnerability it is likely that alternative pathway activation mechanisms would be adequate in the intravascular spaces.However, at **extravascular** sites, where the alternative pathway is less efficient, problems might arise in the absence of an effective MBP compensatory mechanism. Secretory IgM is known to subserve a similar compensatory role in the absence of secretory IgA.

Since low levels of MBP are apparently rather common, it is likely that coexisting defects will also occur relatively frequently. It is therefore quite plausible that a low MBP level will not in itself be sufficient for the development of pathology. Other risk factors, coexisting with the low MBP immunodeficiency, might predispose the individual to the non-specific infections and other symptoms characterising the yeast opsonisation defect (see **Figure 7.6**).

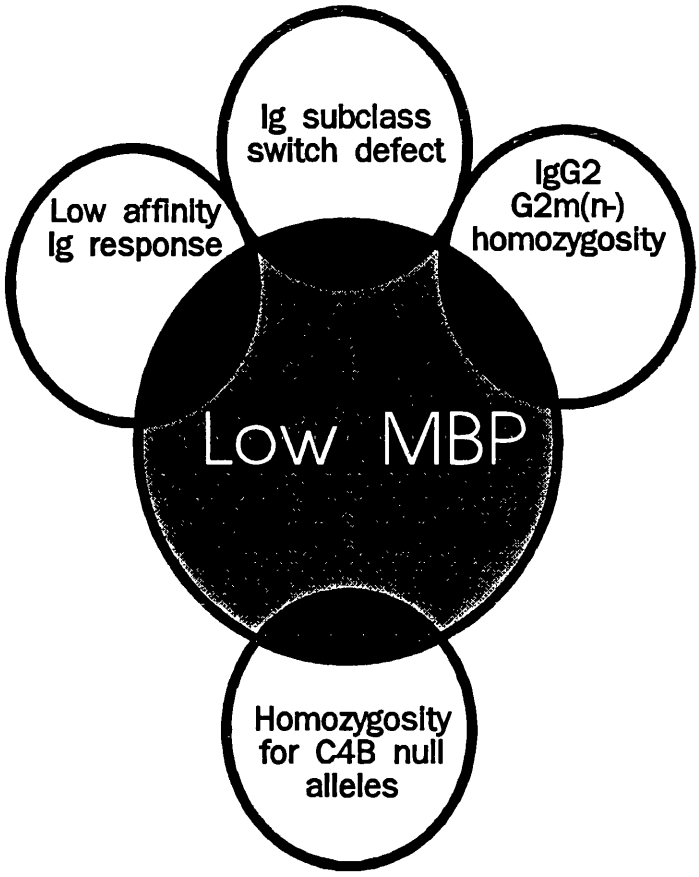


Figure 7.6 Some of the possible risk factors which may, in association with a low MBP level, precipitate immunopathology. Such co-existence may be particularly critical during the "period of vulnerability" between the ages of 6 and 24 months (see Figure 7.5) (Adapted from Turner et al 1990(b).

IgG2 is the major anti-carbohydrate immunoglobulin opsonin and promotes Fc γRII and Fc γRIII mediated phagocytosis by PMN. Levels of this opsonin could remain below a critical threshold in IgG2 subclass deficiency,or if there were a switch defect preventing switching of anti-carbohydrate response from the low affinity IgG1 to the high affinity IgG2 subclass, or if the individual were homozygous for the IgGm(n-) allotype. It is of interest that 1 out of the 10 paediatric patients with low MBP levels and clinical symptoms had an IgG2 subclass deficiency. However these studies need to be extended to establish whether there is any link between specific anti-mannan IgG2 subclass deficiencies and low MBP levels in association with clinical symptoms.

Another surprisingly frequent immunological restriction is that associated with the expression of C4 gene products. For example, approximately 8% of Caucasians lack two of the four possible functioning C4 genes (Hauptmann et al 1988) and consequently have a reduced capacity for classical pathway activation. Gene products of the C4B locus are known to be four times more active in function than the proteins encoded by the C4A locus and to interact preferentially with carbohydrate rich surfaces. Therefore individuals who have low levels of MBP and who are homozygous for C4B null alleles might be particularly susceptible to bacterial and fungal infections.

Measurements of MBP concentration in the 179 healthy blood donors showed that all the individuals with defective yeast opsonisation had a serum MBP level below 20μg/l whereas the mean MBP concentration was 176μg/l in this population. The opsonic defect is therefore associated with a relative, rather than absolute defect in MBP production. Low serum MBP concentrations could be caused by mutations in the MBP coding regions (exons) or by factors affecting control of MBP gene expression.

Consensus sequences probably involved in controlling the expression of human serum MBP have been identified in the 5' promoter regions of the gene and instability sequences have been found in the 3' untranslated region (Taylor et al 1989, Sastry et al 1989). It is possible that in individuals with persistent low levels of this acute phase protein there is an abnormality in one of these promoter regions which affects the expression of the protein.

Mutations in MBP coding exons might produce unstable MBP which would be degraded in the hepatocytes before secretion. Alternatively the mutations might produce an aberrant protein unsuitable for secretion which would then accumulate in hepatocytes as happens with α1-antitrypsin deficiency.

In an attempt to address the question of a possible abnormality in the MBP synthesised, sera from individuals with normal and abnormal opsonic function were subjected to isoelectric focusing in a pH gradient of 4-6. The gel was immunoblotted and probed with the rabbit anti-MBP antibody. Although the concentrations of MBP in the LB sera were obviously lower than in the normal sera, the pI appeared identical. This suggests that the abnormality in the MBP is not the result of a mutation in the MBP exons, but rather involves the regions controlling MBP expression.

It is still unclear why the incidence of the low MBP levels and poor yeast opsonisation is so high in the healthy population. Sastry et al (1989) have mapped the MBP gene to Chromosome 10, but at the time of writing there are no known genes in this region which could explain a selective advantage of retaining a linked defective MBP gene. Further studies are required on the genetics of this deficiency in order to establish the molecular basis of the opsonic defect at the DNA level and to explain the high incidence of the immunodeficiency in the general population.

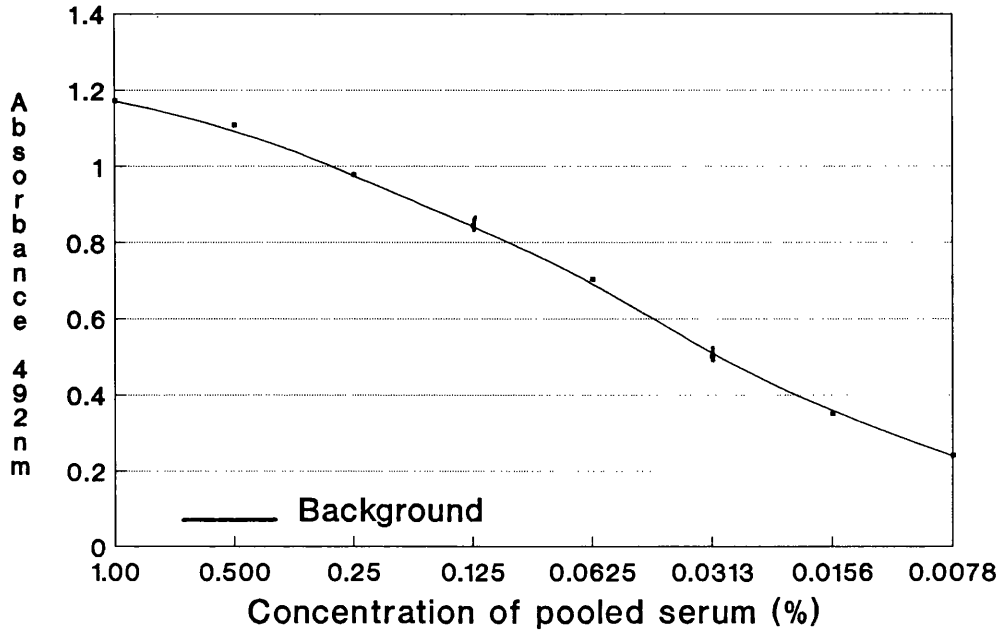
APPENDIX 1: Representative standard curves for the measurement of selected complement components in sera.

The curves represent the mean ± SD of duplicate measurements of doubling dilutions of the pooled serum standard.

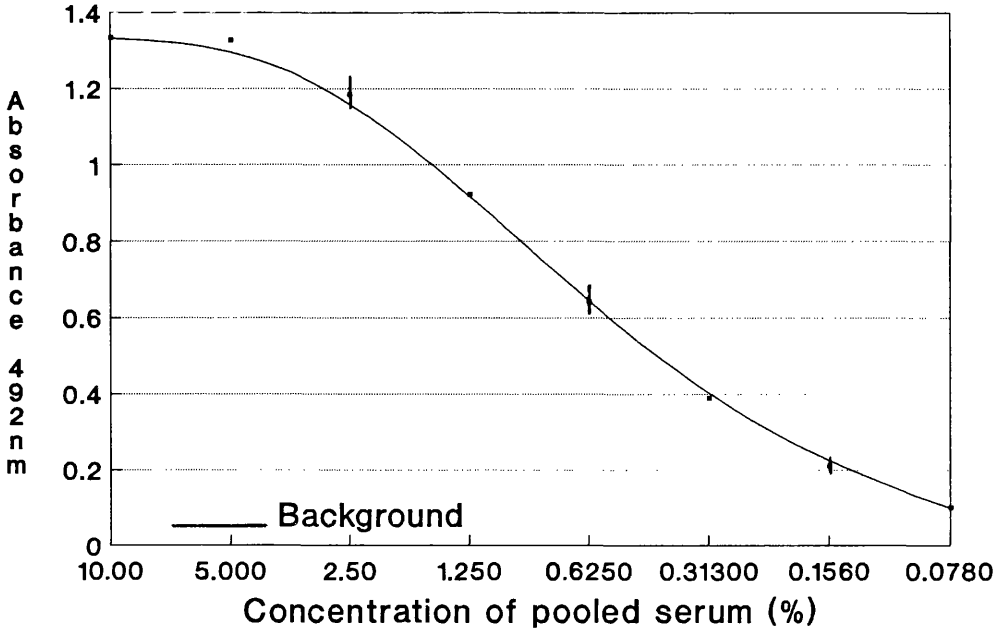
The standard curves illustrated were used to measure serum concentrations of:

1) Factor B
2) Properdin
3) Factor H
4) Factor I
5) MBP (antibody capture procedure)
6) MBP (mannan capture procedure)

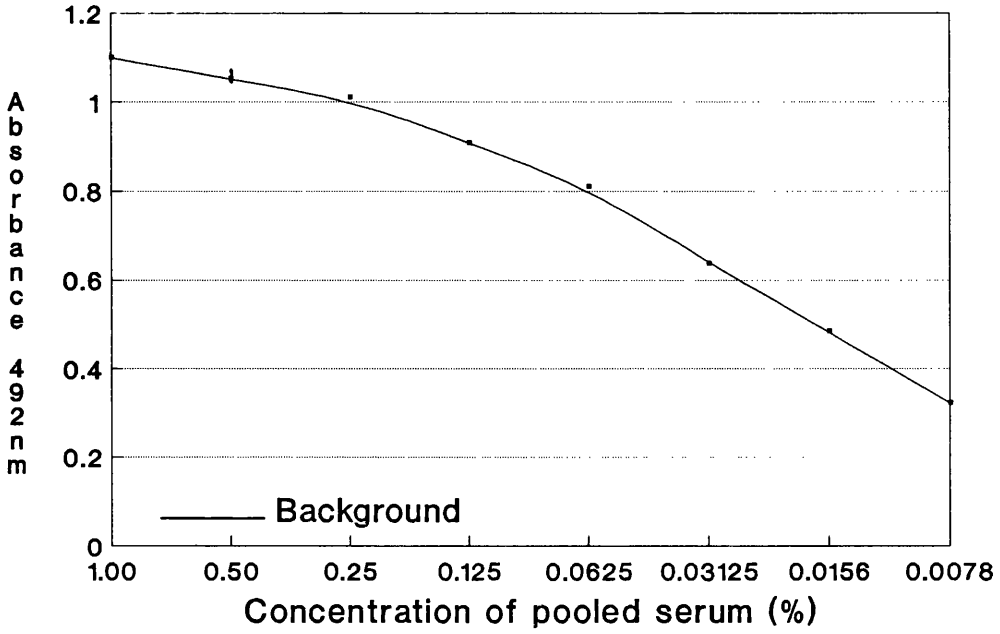
Standard curve Factor B



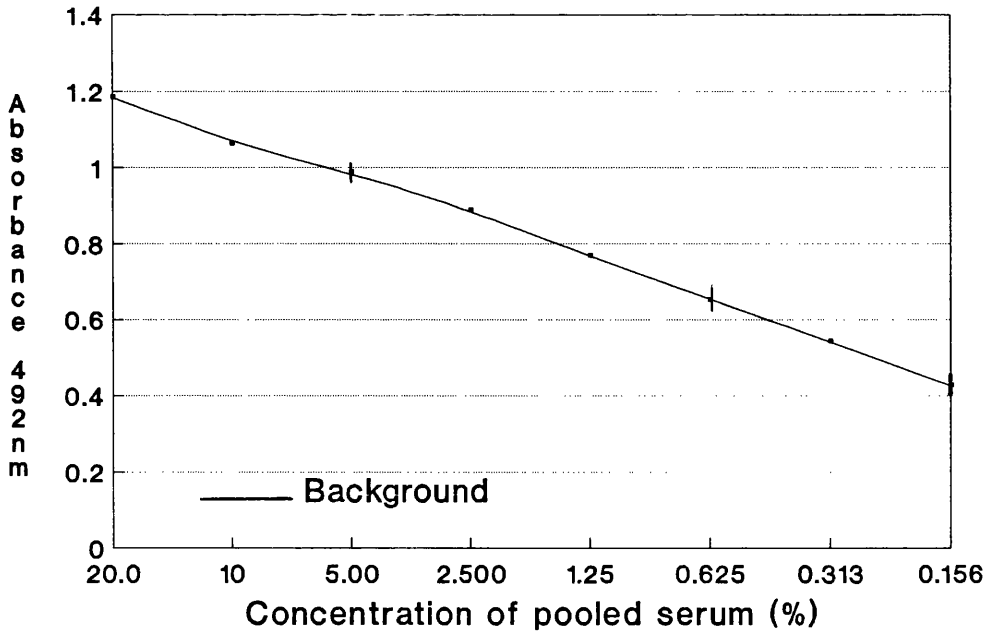
Standard curve Properdin



Standard curve Factor H

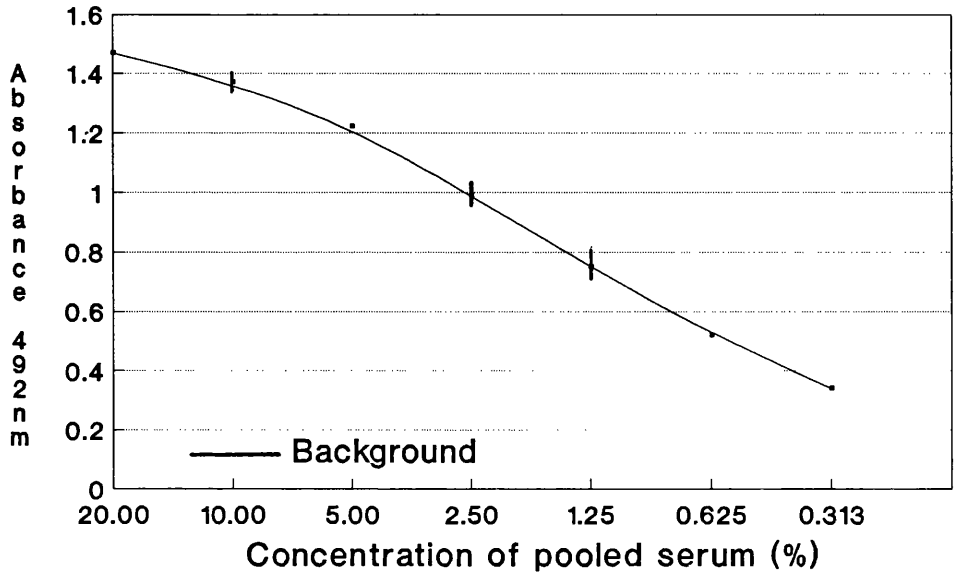


Standard curve Factor I



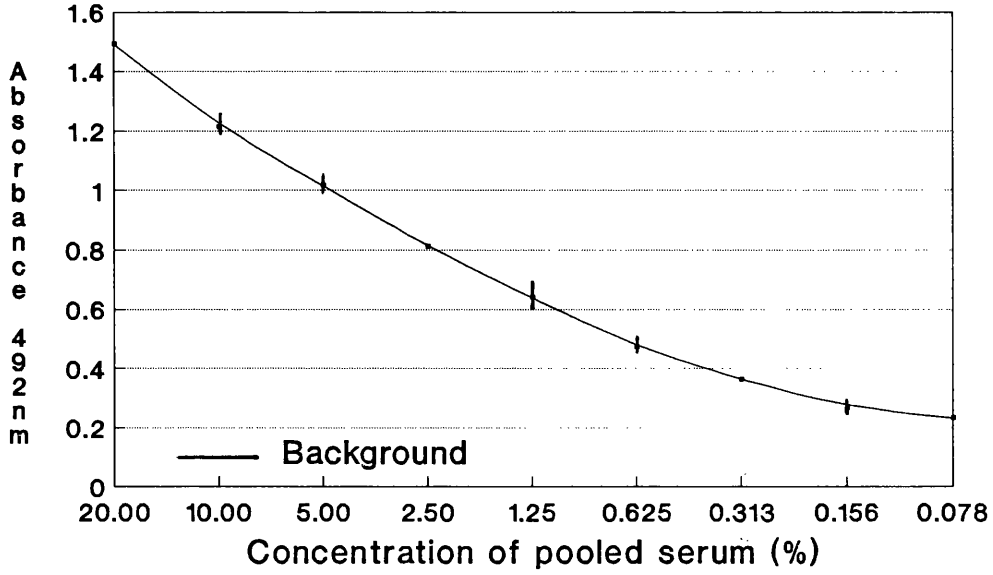
Standard curve MBP

Antibody capture assay



Standard curve MBP

Mannan capture assay



APPENDIX 2: Levels of selected complement components in 77 sera previously tested using the functional opsonic assay.

The levels shown in Columns B-G are the mean values of duplicate assays:

Column A	Code Number of serum
Column B	C3 levels (expressed as iu/ml)
Column C	C4 levels (expressed as iu/ml)
Column D	Factor B levels (expressed as % serum standard)
Column E	Properdin levels (expressed as % serum standard)
Column F	Factor H levels (expressed as % serum standard)
Column G	Factor I levels (expressed as % serum standard)
Column H	Functional opsonic activity (expressed as Binding Coefficient %)

Note: Sera from individuals with defective opsonic function (defined by the C3c elution procedure) are indicated by an asterisk.

	A	B	C	D	E	F	G	H
1	No	C3	C4	Factor B	properdin	Factor H	Factor I	YOPS
2	2	242	100	68.8	90	112	65	71.43
3	3	362	170	76	108	115.2	90	80.95
4	5	332	120	120	88	102.4	85	138.1
5	8	270	85	70.4	86	89.6	80	71.43
6	12	108	90	67.2	110	89.6	70	42.9
7	13	405	52	120	93	80	220	71.4
8	14	175	83	116	84	112	85	52.4
9	15	348	91	88	110	115.2	115	138.1
10	17	282	54	70.4	84	105.6	65	33.3
11	22	158	76	280	90	89.6	85	152.4
12	23	255	58	96	125	72	150	52.4
13	24	205	108	108	86	67.2	70	23.8
14	25	322	112	112	66	60.8	80	85.7
15	26	165	46	116	92	83.2	80	85.7
16	27	192	48	67.2	78	102.4	60	133.3
17	28	265	115	132	105	83.2	80	133.3
18	30	232	156	208	96	153.6	100	109.5
19	32	230	53	80	97	91.2	95	133.3
20	34	162	102	116	115	115.2	95	142.86
21	38	106	94	139.2	97	30.4	135	100
22	39	405	61	128	91	142.4	155	78.26
23	40	248	68	117.6	87	70.4	100	43.48
24	41	325	102	79.2	72	163.2	140	100
25	42	375	96	61.6	92	92.8	65	86.96
26	43			120.8	104	60.8	125	130.43
27	44	150	60		116	41.6	90	78.3
28	45	422	105	71.2	65	28.8	107.5	65.22
29	46	400	77	76	82	62.4	60	78.26
30	47	248	113	72.8	106	54.4	67.5	121.7

	A	B	C	D	E	F	G	H
31	48	236	106	92	91	174.4	50	121.7
32	49	186	56	135.2	85	24	117.5	100
33	50	235	72	140	113	54.4	170	86.96
34	51	178	76	112.8	82	41.6	107.5	60.87
35	52	422	120	115.2	73	124.8	125	100
36	53	163	84	170.4	139	80	125	87.5
37	54	235	155		82	67.2	87.5	25
38	55	175	75	92	108	35.2	90	75
39	56	443	110	112	76	83.2	140	56.3
40	57	262	108	124	92	139.2	120	118.8
41	58	172	56	84.8	115	73.6	95	50
42	59	188	79	89.6	71	88	70	56.3
43	60	450	95	96	64	118.4	70	106.3
44	61	460	56	70.4	90	108.8	100	118.8
45	62	430	157	88	106	107.2	105	62.5
46	63	340	130	92	78	98.2	90	56.25
47	65	316	64	84	107	64	85	106.3
48	66	125	150	104	64	107.2	105	143.75
49	*67	368	69	88	100	89.6	175	-6.25
50	69	170	110	136	110	105.6	85	42.11
51	70	330	46	108	109	144	115	110.53
52	71	470	108	80	55	78.4	65	31.58
53	72	280	62	92	119	73.6	75	57.89
54	73	190	52	108	99	100.8	80	100
55	74	333	140	64	68	56	90	131.58
56	75	172	58	73.6	80	100.8	80	68.4
57	76	363	180	58.4	72	100.8	85	94.74
58	77	170	98	73.6	93	57.6	60	142.11
59	78	360	246	96	96	73.6	80	152.6
60	79	445	96	104	95	99.2	95	15.8

	A	B	C	D	E	F	G	H
61	*80	210	57	112	78	75.2	70	-5.26
62	81	108	66	62.4	64	83.2	70	63.16
63	82	302	92	85.6	91	108.8	90	110.53
64	*83	392	160	108	75	73.6	>1000	-5.26
65	84	445	126	100	107	153.6	95	84.2
66	85	305	140	88	77	128	95	52.63
67	86	282	192	168	133	176	170	15
68	87	338	62	70.4	80	78.4	65	90
69	88	340	135	176	115	168	160	80
70	89	242	157	176	115	320	150	145
71	90	258	176	120	130	320	140	160
72	92	233	122	73.6	111	124.8	100	55
73	93			75.2	98	105.6	75	30
74	*94	472	50	96	58	67.2	70	0
75	95	348	105	104	107	116.8	90	90
76	96	206	200	59.2	85	80	75	100

APPENDIX 3: Levels of complement complement, mannose binding protein and immunoglobulins binding to mannan-coated plates from the sera of 179 healthy adult blood donors.
The levels shown in Columns C-K are the mean values of duplicate assays:

Column A	Code Number of serum
Column B	C3c elution from zymosan (functional opsonic assay) (expressed as Binding Coefficient %)
Column C	C3b+bi binding (expressed as Binding Coefficient %)
Column D	C4 binding (expressed as Binding Coefficient %)
Column E	Properdin binding (expressed as Binding Coefficient %)
Column F	C3bi binding (expressed as Binding Coefficient %)
Column G	Factor B binding (expressed as Binding Coefficient %)
Column H	MBP binding (expressed as Binding Coefficient %)
Column I	IgA binding (expressed as % of serum standard)
Column J	IgG binding (expressed as % of serum standard)
Column K	IgM binding (expressed as % of serum standard)

Note: Sera from individuals with defective opsonic function (defined as $< 10\%$ binding coefficient in the C3bi mannan capture assay) are indicated by an asterisk.

	A	B	C	D	E	F	G	H	I	J	K
1	Serum	C3c elute	C3b + bi	C4	Properdin	C3bi	Factor B	MBPm	IgA	IgG	IgM
2											
3	2	71.43	86.14	115.39	107.93	89.15	135.62	122.8	56.8	10.8	31.6
4	3	80.95	85.82	56.19	83.91	61.98	79.45	13.74	11	19.4	48
5	5	138.1	99.12	60.31	89.91	77.08	95.41	54.32	56.4	36.8	175.4
6	8	71.43	94.95	66.05	93.35	84.43	104.14	88.1	49.8	38.8	134.6
7	12	42.86	80.64	41.45	84.47	68.29	56.14	56.51	159.2	106	4.6
8	13	71.43	80.64	28.53	81.32	61.44	64.91	58.07	93.8	42	58.4
9	14	52.38	92.53	69.4	101.05	93.86	89.12	85.41	28.4	97.2	45.6
10	15	138.1	69.5	15.31	55.47	36.32	48.26	33.22	60.8	199.8	37.6
11	*17	33.3	-4.5	0.221	-0.75	-1.4	0.665	3.11	29.4	47.4	172.6
12	*20	-4.76	-11.17	-1.3	-1.34	-3.25	-3.07	-17.33	86.4	128.8	97.6
13	22	152.38	86.22	62.34	89.2	88.49	64.65	97.45	12.6	15.2	37
14	23	52.38	65.84	16.35	59.98	31.03	54.32	28.05	18.2	4	5.2
15	24	23.81	78.61	34.1	63.81	55.3	48.95	46.25	29.8	46	26.8
16	25	85.71	85.18	53.17	63.94	55.85	70.14	61.19	6.4	68.2	20.4
17	26	85.71	86.08	39.77	88.27	78.95	69.21	65.79	47.6	14.2	157.8
18	27	133.33	99.14	52.87	75.86	71.04	75.61	50.71	18.8	14.6	47.4
19	28	133.33	102.54	58.84	121.83	103.39	73.6	55.45	26.6	33.4	15.6
20	29	42.86	56.06	11.72	39	30.16	28.77	27.26	76.8	20.6	89
21	30	109.52	86	24.88	76.82	76.2	42.37	37.47	59.6	37.4	72
22	32	133.33	88.61	72	90.83	96.61	96.92	97.05	12.2	26.2	53.2
23	34	142.86	101.31	47.17	102.26	90.54	91.4	62.09	32.4	125	50.6
24	38	100	101.6	43.41	77.32	85.58	53.8	74.14	24.4	18.2	38.6
25	39	78.26	51.27	5.5	32.27	26.23	21.84	4.13	12	59.6	9.6
26	40	43.48	75.01	4.79	33.35	47.89	43.49	#N/A	43	26.4	7.2
27	41	100	83.63	28.5	65.36	53.4	54.55	62.49	10.1	10	68.8
28	42	86.96	102.69	43.52	75.32	73.49	63.78	51.63	14.6	3.2	143.2
29	43	130.43	105.85	63.53	100.38	88.8	102.81	61.79	42.4	24.8	54
30	44	78.26	91.55	46.41	74.62	65.5	59.7	48.9	18.2	51.4	32.4

	A	B	C	D	E	F	G	H	I	J	K
31	45	65.22	39.34	2.34	10.08	15.04	11.34	19.48	#N/A	124.4	466.8
32	46	78.26	92.5	24.37	57.27	59.8	43.39	44.14	#N/A	22.2	68.8
33	47	121.74	107.74	57.01	99.84	96.24	91.19	82.34	60.8	342.8	24.8
34	48	121.74	106.67	42.99	82.6	86.17	71.04	56.77	#N/A	216	115
35	49	100	105.97	57.43	80.55	85.77	78.04	101.24	90	28.6	93.8
36	50	86.96	94.89	100.89	99.19	105.67	115.46	113.61	50.8	42.8	57
37	51	60.87	91.08	31.38	55.55	57.64	57.73	27.31	30.4	36.6	46.2
38	52	100	102.42	89.22	50.48	90.57	74.51	80.86	56.4	40	326.4
39	53	87.5	83.99	27.84	73.76	52.37	51.73	24.75	#N/A	426.4	17
40	54	25	85.17	33.17	56.41	54.04	50.23	29.79	153	29	190.8
41	55	75	103.48	90.36	88.2	101.6	101.87	93.48	#N/A	#N/A	172.2
42	56	56.25	73.79	104.86	69.42	57.74	107.39	85.27	31.6	138.4	130.2
43	57	118.75	103.48	57.49	82.81	88.17	74.7	59.74	22.6	20	11.2
44	58	50	84.82	20.24	60.61	50.37	50.89	35.33	36	16.4	45.6
45	59	56.25	94.27	39.04	58.24	69.31	56.98	59.92	89.8	441.6	31.6
46	60	106.25	95.86	74.45	61.41	77.05	70.29	72.03	22.6	38.4	91
47	61	118.75	81.23	29.24	60.97	51	52.27	19.53	31.4	64.6	49
48	62	62.5	55.37	18.17	25.02	18.31	24.8	9.68	164	51.4	163.2
49	63	56.25	72.47	31.57	43.29	35.35	45.23	8.35	13.6	8.2	18.4
50	*65	106.25	-8.8	-2.2	-2.47	-1.03	-2.14	-1.4	65.2	240	33
51	66	143.75	95.7	66.2	82.4	69.8	83.74	31.38	53	356	58
52	*67	-6.25	-15.01	0.48	-1.4	-3.11	-2.98	0.939	31.8	105.2	32.4
53	69	42.11	92.26	24.96	80.26	60.38	66	36.28	53.8	15.8	66.8
54	70	110.53	99.19	83.75	102.27	97.2	96.79	75.19	11.6	29.6	8.6
55	71	31.58	77.72	31.06	52.82	46.57	54.06	42.36	13.4	8.6	46
56	72	57.89	76.17	26.72	62.95	40.15	58.97	20.52	22.4	7.4	18
57	73	100	95.86	38.07	84.96	75.57	83.14	67.48	21	38.4	48.2
58	74	131.58	106.12	78.5	116.09	92.92	110.9	98.02	120.4	100.2	29.2
59	75	68.42	89.47	37.04	72.42	58.49	61.1	60.48	23.2	5.4	191.8
60	76	94.74	76.37	26.66	48.82	50.09	48.1	55.81	13	29.2	145.6

	A	B	C	D	E	F	G	H	I	J	K
61	77	142.11	87.28	37.79	72.56	64.28	88.58	94.3	53	22.4	51.4
62	78	152.63	94.01	22.84	76.3	67.01	56.02	51.82	30.6	14.6	18.6
63	*79	15.79	3.26	0.384	3.44	-0.85	0	0.998	255.2	17.4	230
64	*80	-5.26	-7.18	-1.44	-0.76	-3.04	-2.61	3.99	115.4	112.8	164.4
65	81	63.16	87.22	42.71	70.92	60.85	71.6	43.69	37	4.4	37.8
66	82	110.53	100.54	34.32	88.52	79.49	70.88	91.57	32.6	5.2	458.6
67	*83	-5.26	-11.97	-0.96	-1.75	-2.97	-2.54	-0.12	60.8	411.8	28.4
68	84	84.21	97.9	51.52	93.84	82.51	84.83	63.72	140.2	10.6	109.2
69	85	52.63	92.16	77.49	90.76	89.67	115.61	115.2	84	84.6	106.8
70	*86	15	-2.28	-1.35	-1.39	-0.22	-0.44	-5.69	28.4	82.8	95.4
71	87	90	88.4	27.1	49.03	53.68	33.19	32.99	40.2	10.8	140.4
72	88	80	101.3	71.56	99.19	87.6	109.37	83.16	33.4	35.6	9.2
73	89	145	106.6	65.25	106.02	104.43	105.44	76.65	204.6	628	99.6
74	90	160	110.68	78.09	115.92	95.5	115.08	53.8	30.8	53.2	66.8
75	92	55	82.47	30.22	71.61	50.04	60.6	13.54	13	119.4	28.6
76	93	30	88.15	36.62	75.28	59.78	62.66	56.57	21.6	8.6	155.2
77	*94	0	-5.37	-0.86	-0.76	-2.9	-2.63	6.28	126.6	357.8	59
78	95	90	101.23	43.17	83.85	73.43	85.19	71.31	218.8	74.8	116.6
79	96	100	94.2	61.23	82.39	75.13	74.58	67.84	62	13.8	16
80	101		109	49.94	96.65	80.46	67.27	75.74	#N/A	308.8	137.8
81	102		95.87	49.81	78.48	79.83	65.66	68.89	109.6	46.2	75.8
82	103		98.9	42.24	93.37	76.67	71.9	59.03	#N/A	#N/A	#N/A
83	104		94.8	65.15	90.92	79.04	79.66	73.91	47.6	13.6	72.8
84	105		102.11	64.09	116.14	85.66	86.19	53.42	193.6	137.2	103.4
85	106		73.46	36.28	61.02	47.28	54.02	55.84	#N/A	39.6	121.6
86	107		88.71	63.4	86.07	90	90.82	116.88	63.6	12.8	26
87	108		103.21	85.97	90.83	88.34	87.7	83.29	94.2	47.2	23.8
88	109		95.13	59.23	87.3	72.97	57.71	53.01	78.4	93.8	50.2
89	110		105.81	33.161	54.9	72.12	53.37	62.69	#N/A	#N/A	#N/A
90	111		88.06	37.72	85.01	68.87	72.19	54.96	46.4	64.2	22.8

	A	B	C	D	E	F	G	H	I	J	K
91	112		88.98	84.6	70.46	75.02	76.06	88.9	39.4	67.8	#N/A
92	114		105.31	66.2	72.32	80.01	78.1	70.66	22	115	31.2
93	115		96.97	90.02	114.99	105.44	110.03	112.4	278.6	24	73.8
94	*116		10.54	0.807	2.4	1.67	8.43	-12.49	11.6	#N/A	9.2
95	117		49.22	19.2	43.47	25.93	39.64	34.42	50.6	48.8	306.2
96	118		98.35	48.75	82.36	77.62	77.48	52.59	78.8	41	53
97	119		83.38	22.38	72.13	56.97	45.7	15.52	19.4	42.6	39.2
98	120		92.41	27.56	70.76	54.96	71.98	33.18	16.2	43.8	34.2
99	121		87.42	83.48	30.69	82.35	29.52	87.46	37.8	57	45.6
100	122		46.46	14.85	39.42	22.06	38.41	32.06	24.6	17.4	43.8
101	123		39.67	11.28	44	18.68	43.9	26.1	14.6	33.6	55
102	124		87.24	30.92	70.19	61.54	63.1	59.73	26.4	6.8	53.2
103	125		91.55	64.09	74.16	78.59	71.52	77.62	48	17.8	111.6
104	126		96.86	99.13	89.08	89.32	96.78	99.55	71.8	39	28.4
105	127		99.35	93.89	74.05	85.7	87.38	81.44	44.6	16.6	111.4
106	128		105.74	50.53	103.97	88.45	104.21	65.47	171.6	69	78.8
107	129		91.02	58.49	77.71	70.63	83.66	66.37	31.8	14	26.4
108	130		94.05	76.82	101.68	90.21	123.89	100.34	19.2	8.2	21
109	131		83.23	33.66	70.31	62.6	76.73	67.4	7.2	4.4	92.8
110	132		103.14	69.4	102.52	93.73	125.99	90.34	208	289.4	49
111	133		98.59	60.86	101.22	73.71	105.2	54.86	29.4	309	45.2
112	134		88.64	36.08	58.47	62.6	56.31	43.6	20.6	149.8	30
113	135		69.59	22.02	50.38	41.14	42.45	13.71	30	42.4	23.8
114	136		66.56	22.11	46.11	38.72	36.01	33.52	26.2	78	63.6
115	137		100.32	91.56	78.4	93.62	74	82.84	15.2	129.8	19.6
116	138		86.04	25.26	68.7	56.55	70.79	43.97	353.6	24.8	81.2
117	*139		-16.67	-3.73	-5.8	-5.9	-4.95	-19.25	107	11.2	7.2
118	140		91.45	83.17	84.5	81.52	96.53	82.29	45.4	29.6	54.4
119	*141		-18.83	-3.88	-3.97	-6.6	-4.95	-16.36	40.2	55	32.2
120	142		100.43	36.91	91.83	83.93	80.2	65.81	13.6	81.2	46

	A	B	C	D	E	F	G	H	I	J	K
121	143		104.76	71.24	116.72	105.61	127.97	115.38	387.4	505.2	33.6
122	144		36.15	9.6	27.02	19.36	19.06	-5.05	12.2	23.6	17.4
123	145		59.52	24.59	51.37	37.62	41.58	11.87	32.2	14.2	8.4
124	146		84.4	28.53	37.29	48.33	50.66	46.49	26.6	242	41.2
125	147		73.36	21.99	59.75	52.62	42.3	34.79	46.8	66.8	19.8
126	148		34.01	8.74	28.86	14.77	19.23	6.67	222	#N/A	82
127	*149		-17.01	-1.28	-9.91	-4.28	-5.34	-13.97	45	27.4	35.6
128	150		85.78	28.01	73.27	61.3	62.15	55.15	43	33.6	121.2
129	*151		6.32	0.94	0.24	-0.26	-0.98	-4.02	73.2	15.2	51.6
130	152		95.18	47.1	94.97	78.58	64.29	38.69	20.2	37.8	31.4
131	153		91.65	49.5	83.31	64.64	78.18	49.83	18.6	7.4	52.8
132	154		105.04	51.75	113.99	92.39	103.56	75.23	20.6	113.6	108.2
133	155		100.17	51.45	70.76	80.28	85.64	99.26	222.4	253	815.6
134	156		102.71	69.91	103.7	95.33	98.49	71.49	25.4	61.2	109.2
135	157		41.84	6.18	31.05	15.15	16.56	19.12	92	251.4	81.6
136	158		83.81	41.55	51.55	51.33	61.59	56.45	61.6	27.2	102
137	159		91.57	34.33	68.36	58.13	56.9	40.1	#N/A	462	17.8
138	*160		9.1	-0.55	3.62	4.03	4.27	-12.27	35.6	90.2	28
139	161		83.97	49.91	75.47	77.05	72.75	44.4	9.4	12	17
140	162		58.31	15.09	46.78	34.14	36.24	34.7	66.6	257.4	340.8
141	163		92.85	26.17	82.31	74.04	56.72	52.68	420.8	143.6	315.8
142	164		96.01	63.26	101.1	98.59	102.85	87.86	67.8	93.8	653.4
143	165		78.33	20.08	62.97	55.18	52.71	16.04	15.6	11	42
144	166		103.71	64.32	107.49	102.8	102.8	101.69	16.2	125	81
145	167		95.33	81.33	76.18	90.98	90.92	111.2	63.8	83.4	34.2
146	168		95.78	45.43	89.26	74.51	77.28	71	145.4	33.2	670.8
147	169		99.77	52.69	93.24	85.36	88.96	82.85	65.8	52.3	33.6
148	170		102.11	72.5	91.24	99.88	93.6	98.89	38.6	123.8	28.6
149	171		101.6	88.27	90.68	93.43	103.2	111.09	76.2	102.8	16.4
150	172		92.55	90.2	80.66	100.96	100.4	107.7	201.6	202.6	71.4

	A	B	C	D	E	F	G	H	I	J	K
156	173		97.66	46.89	95.28	92.46	98.84	69.78	21.2	57	11.6
157	174		91.99	29.22	57.63	58.71	56.2	28.18	11.6	64.2	#N/A
158	175		73.36	23.74	50.14	36.79	53.63	6.48	8.2	10.6	35
159	176		95.87	74.81	97.6	95.6	109.37	91.68	26.2	55.8	72.8
160	177		104.98	82.62	86.02	76.36	88.21	59.82	45.8	81	137.4
161	178		112.14	49.09	98.31	84.31	93.21	60.21	231.6	153.8	20.2
162	179		102.02	44.13	84.75	91.06	80.72	60.01	#N/A	#N/A	#N/A
163	*180		7	-7.53	0.706	1.44	4.61	-25.03	7	7.8	34.8
164	181		61.38	15.82	32.49	24.51	41.45	38.88	324.4	64.2	146.2
165	183		102.51	92.13	87.46	89.92	90.61	105.98	97.8	#N/A	46.6
166	184		97.64	84.82	87.42	102.7	88.91	115.34	12	6.6	10.4
167	185		95.07	105.39	59.76	48.02	65.06	53.84	358	152	48.2
168	187		91.43	23.69	57.94	49.69	45.11	17.68	52.8	52.2	28.2
169	191		111.39	87.22	110.11	102.08	106.87	87.71	104.8	84	15
170	192		103.87	58.84	72.3	65.49	68.19	39.6	294.4	45.4	31.6
171	194		105.02	62.85	68.66	89.71	72.49	55.2	76	28.6	18.2
172	195		104.81	62.5	67.85	57.59	27.49	47.2	37	102	156
173	196		107.73	34.32	85.64	79	68.84	49.86	24.4	11.4	9
174	198		101.04	72.1	78.97	79.73	73.4	91.76	21	251.2	163.8
175	199		105.54	92.75	80.79	91.06	84.75	101.45	25.6	96.4	32.6
176	200		110.45	64.3	97.17	83.89	81.75	65.32	19.8	491.6	74.8
177	201		105.33	92.75	87.06	98.23	87.48	81.7	19.4	15.6	60.8
178	202		100.84	79.9	78.36	85.45	88.01	109.44	95.6	46	103.2
179	203		87.36	28.25	60.36	48.23	51.63	45.95	94.8	503.2	39.8
180	204		107.84	106.35	91.3	107.07	99.35	104.34	36	51.2	54.6
181	205		104.91	58.63	88.88	77.96	82.14	67.82	157.4	63.8	45.2
182	206		102.72	49.52	87.06	79.63	84.09	72.74	124.4	127.6	43.2
183	207		111.91	86.67	109.45	108.32	101.83	102.37	33.8	38	277.4
184	208		67.82	12.78	28.61	27.65	26.6	6.74	15.4	11.2	39.6
185	209		104.08	75.35	61.38	85.76	69.1	78.85	137.4	77.6	16.2
186	210		107.01	135.38	108.96	109.11	101.83	108.77	17.4	36	22

APPENDIX 4: Levels of complement complements, mannose binding proteins and immunoglobulin subclasses binding to mannan-coated plates from the sera of 30 healthy adult blood donors with the highest levels of total IgG or IgM anti-mannan antibodies. The levels shown in Columns B-J are the mean values of duplicate assays:

Column A	Code Number of serum
Column B	C3bi binding (expressed as Binding Coefficient %)
Column C	C4 binding (expressed as Binding Coefficient %)
Column D	Properdin binding (expressed as Binding Coefficient %)
Column E	Factor B binding (expressed as Binding Coefficient %)
Column F	MBP binding (antibody capture) (expressed as Binding Coefficient %)
Column G	MBP binding (mannan capture) (expressed as Binding Coefficient %)
Column H	IgG1 binding (expressed as % of serum standard)
Column I	IgG2 binding (expressed as % of serum standard)
Column J	IgG3 binding (expressed as % of serum standard)

Note: Sera from individuals with defective opsonic function (defined as $< 10\%$ binding coefficient in the C3bi mannan capture assay) are indicated by an asterisk.

	A	B	C	D	E	F	G	H	I	J
1	Sera	C3bi	C4	Properdin	Factor B	MBP(ab)	MBP man	IgG1	IgG2	IgG3
2	45	15.04	2.34	10.08	11.34	#N/A	19.48	47	31.6	6
3	47	96.24	57.01	99.84	91.19	#N/A	82.34	40.6	39.8	24.2
4	52	90.57	89.22	50.48	74.51	#N/A	80.86	11.6	7.4	1.8
5	53	52.37	27.84	73.76	51.73	#N/A	24.73	132.6	16.8	98
6	59	69.31	39.04	58.24	56.98	#N/A	59.92	13.6	46.6	7.4
7	*65	-1.03	-2.2	-2.47	-2.14	#N/A	-1.4	24.8	61.6	5.8
8	66	69.8	66.2	82.4	83.74	#N/A	31.38	115.8	18.4	48.6
9	79	-0.85	0.38	3.44	0	#N/A	1	21.4	14.6	87.6
10	82	79.49	34.32	88.52	70.88	#N/A	91.57	21.6	11.6	51.4
11	*83	-2.97	-0.96	-1.75	-2.54	#N/A	-0.12	53.8	32.2	16.4
12	89	104.43	65.25	106.02	105.44	#N/A	76.65	189.4	67.4	80.4
13	*94	-2.9	-0.86	-0.76	-2.63	#N/A	6.28	156	86.4	118.6
14	101	80.46	49.94	96.65	67.27	85.26	75.74	53.2	46.6	#N/A
15	112	75.02	84.6	70.46	76.06	92.07	88.9	28.4	22.4	141.8
16	117	25.93	19.2	43.47	39.64	26.46	34.42	81.6	63	24.2
17	132	93.73	69.4	102.52	125.99	97.74	90.34	125.2	49.2	#N/A
18	133	73.71	60.86	101.22	105.2	53.82	54.86	500	71.6	30
19	143	105.61	71.24	116.72	127.97	110.07	115.38	44.2	79.4	100
20	146	48.33	28.53	37.29	50.66	48.85	46.49	11	25.2	42
21	155	80.28	51.45	70.76	85.64	106.36	99.26	20	49	201.4
22	157	15.15	6.18	31.05	16.56	4.1	19.12	8.2	55.6	1
23	159	58.13	34.33	68.36	56.9	25.52	40.1	231	26.8	66.4
24	162	34.14	15.09	46.78	36.24	22.55	34.7	12.6	11	14.8
25	163	74.07	26.17	82.31	56.72	74.35	52.68	14.6	3.8	178
26	164	98.59	63.26	101.1	102.85	111.63	87.86	215.4	29	93.2
27	168	74.51	45.43	89.26	77.28	75.59	71	16.8	3.6	16.4
28	198	79.73	72.1	78.97	73.4	100.22	91.76	60.4	28.6	258.4
29	200	83.89	64.3	97.17	81.75	50.79	65.32	48	16.6	81.2
30	203	48.23	28.25	60.36	51.63	28.14	45.95	74.4	62.8	245.8
31	207	108.32	86.67	109.45	101.83	108.17	107.37	47.8	24.6	73.2

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in differences in exposure to asphyxia. There is no other obvious explanation for the difference in the rates of neonatal seizures—the use of oxytocin was similar in the two groups; labour was somewhat shorter in the EFM group but this finding is of questionable clinical significance; trauma was, if anything, increased in the EFM group, by the higher instrumental delivery rate. Furthermore, the contrast between the groups was greatest for early-onset (< 48 h) seizures,[4] the subgroup that seems most likely to reflect intrapartum asphyxia.[5,6]

A second possible explanation for the lack of any striking difference in cerebral palsy rates is that the type or degree of intrapartum asphyxia sufficient to cause seizures, and preventable by continuous fetal heart rate monitoring, is not the same as that which causes cerebral palsy. Compared with the neonate, the fetus is remarkably resistant to intrapartum asphyxia.[7-9] To cause disability, asphyxia must be both prolonged and severe, and nearly lethal.[8] Follow-up of cohorts of children resuscitated after birth with Apgar scores of zero, one, or two, has demonstrated that the great majority of these babies either die or survive apparently intact. The few who are impaired usually have severe motor disability.[9] The cases in our study associated with intrapartum eclampsia and intrapartum placental separation, neither of which seems likely to be affected by the method of fetal heart rate monitoring used, could be examples of such severe asphyxia; but in both instances there could also have been an underlying cause common to both the intrapartum abnormality and cerebral palsy (see below).

A third possible explanation is that cerebral palsy occurring after neonatal seizures is not due to intrapartum asphyxia. 1 of the 6 cases of cerebral palsy following neonatal seizures (late-onset seizures after preterm birth and intraventricular haemorrhage) seems likely to reflect neonatal problems. The other 5 had clinical signs suggestive of intrapartum asphyxia, but some also had other abnormal features (twin-to-twin transfusion, subarachnoid haemorrhage), which suggests that an underlying problem may have caused both the signs suggestive of asphyxia, and the cerebral palsy.[5,6,10] Furthermore, among cases of early-onset seizures, those almost certainly not caused by asphyxia seem to be far more likely to lead to serious disability in childhood.[11,12]

The extent to which severe asphyxia due to factors unaffected by the differential effects of monitoring contributed to these 6 cases is therefore uncertain. 2 cases may reflect trauma rather than asphyxia, and 1 other may involve a secondary effect of asphyxia by meconium aspiration. At least 1 case (the twin-to-twin transfusion) seems almost certain to have been due to an underlying problem and not intrapartum asphyxia; other underlying problems may remain unrecognised amongst the others. Furthermore, even if some of these 6 cases were caused by intrapartum asphyxia, it is questionable whether any could have been prevented by different clinical management.

We chose to concentrate formal follow-up on children with abnormal neurological signs in the neonatal period because, like Freeman and Nelson,[3] we believed that children without such signs have not suffered "substantial asphyxia". The normal obstetric and paediatric records for most of the 15 additional cases identified from specialist remedial clinics support the view that intrapartum asphyxia is not the cause of most cases of cerebral palsy.[3]

When the 16 children with cerebral palsy whose mothers had not been in the trial were taken into account, the estimated cerebral palsy rate for the hospital during the period of the trial was 2·2 per 1000—higher than the rate for an earlier period in the east of Ireland[13] and similar to those reported from elsewhere.[14-18] Underascertainment among children who were not formally followed-up is therefore likely to be small. Furthermore, there is no reason for thinking that the within-trial comparisons were biased by differential ascertainment rates for the two trial groups. The relatively high rate of cerebral palsy among non-participants in the trial (16 out of 4224 liveborn children or 3·6 per 1000, compared with 1·7 per 1000 participants) could have been related in some to the reason for ineligibility for the trial—2 were delivered before 29 weeks' gestation, 4 had congenital anomalies, and 1 was associated with prelabour caesarean delivery for eclampsia.

Our findings are consistent with reports that cerebral palsy rates have changed little over the past 30 years despite developments in perinatal care that have greatly reduced the risk of death;[14,18] that intrapartum fetal heart rate patterns do not seem to correlate with later cerebral palsy;[19,20] that suboptimum intrapartum care, in particular failure to respond appropriately during labour to an abnormal fetal heart rate pattern, has been shown to be associated with about a 6-fold increase in the risk of very early (< 48 h) neonatal seizures[6,21,22] but not with an increased risk of cerebral palsy;[21] and that less than 10 per cent of cases of cerebral palsy are likely to be related to intrapartum asphyxia.[23,24]

Obstetric practice is beset by worries about medical negligence. Our results indicate that preventable intrapartum asphyxia is a much less common cause of cerebral palsy than is often supposed.

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ASSOCIATION OF LOW LEVELS OF MANNAN-BINDING PROTEIN WITH A COMMON DEFECT OF OPSONISATION

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Summary Failure to opsonise bakers' yeast (*Saccharomyces cerevisiae*) is a defect found in 5–7% of the general population. In this study, the presence of the defect was linked with low levels of mannan-binding protein (MBP), a calcium-dependent serum lectin. Purified MBP corrected the defect in a dose-dependent way in an in-vitro assay measuring the deposition of complement moieties on a mannan-coated surface. There was a highly significant correlation between the serum MBP level and the generation of C3b opsonins in a population of healthy blood donors. The median MBP level of ten children previously shown to have the functional opsonic defect was 4·9 μg/l (range 2·5–35·0 μg/l) compared with 143 μg/l (range 2·5–880 μg/l) for a paediatric control group.

Introduction

THE failure of serum to opsonise bakers' yeast (*Saccharomyces cerevisiae*) for phagocytosis by normal polymorphonuclear leucocytes was first described in an infant with severe recurrent infections, diarrhoea, and failure to thrive.[1] This functional defect was subsequently reported in a series of children with frequent unexplained infections,[2] in association with chronic diarrhoea of infancy,[3] and in association with otitis media in infants.[4] A link with allergic illness has also been reported.[4,5] The defect is surprisingly common (5–7%) in the general population,[2,6,7] and it may be a factor reducing the immune potential of people with the defect throughout life.

In attempts to define the molecular mechanisms underlying this defect, we established an association with the deposition of suboptimum amounts of C3b/C3bi opsonic fragments on the yeast surface[8] and proposed that an unidentified opsonic cofactor was absent or inactive in people with the defect.[9]

With the yeast cell wall component, mannan, as a substrate for binding, we have obtained evidence that, under the assay conditions normally used to study opsonisation, an antibody-independent cleavage of C4 occurs.[10] We further established that such cleavage was probably regulated by mannan-binding protein (MBP), a calcium-dependent serum lectin, which in rats activates complement through the classic pathway.[11] We report here evidence linking this common defect of opsonisation with low levels of MBP.

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Subjects and Methods

Blood samples were obtained with consent from 186 blood donors. Serum was separated from the cells within 2 h, and aliquots were frozen rapidly to −70°C. Serum samples were also obtained with parental consent from 59 healthy babies (aged 4·5 months), taking part in a prospective study of the development of cows' milk allergy, and from 19 children undergoing non-emergency operations for various non-immunological disorders (aged 3 months–13 years). Each aliquot was studied only once after thawing. Serum samples from 10 patients attending the Hospital for Sick Children, Great Ormond Street, with respiratory tract infections, pyrexia of unknown origin, or diarrhoea, and previously shown to have the opsonic defect, were similarly obtained and stored.

Four procedures were used sequentially to isolate MBP from three sources (a pool of 50 ml serum from 100 healthy adult donors, 8 ml serum from an individual showing high levels of C3b binding to yeast, and 8 ml serum from an individual with low C3b binding activity). Since MBP is a calcium-dependent lectin, EDTA displacement from a mannan-'Sepharose' affinity column was chosen as the principal purification step. Prior gel fractionation of serum (8 ml with ethylene diamine tetra-acetate added to 10 mmol/l) on a column of 'Sephacryl S300' (85 × 2·6 cm) equilibrated in phosphate-buffered saline (Oxoid) pH 7·3 containing 10 mmol/l EDTA, allowed us to reduce the load on the affinity chromatography column. For that step, sephacryl fractions corresponding to the molecular mass of MBP (700 kD) were dialysed extensively against 40 mmol/l imidazole/hydrochloric acid, pH 7·8, containing 1·25 mol/l sodium chloride. 1 mol/l calcium chloride was then added to a final concentration of 50 mmol/l and the fractions were loaded (at room temperature) onto a 3 ml mannan-sepharose affinity column which had been equilibrated with the imidazole/50 mmol/l calcium buffer. The column was washed with this buffer until the optical density at 280 nm of the effluent was less than 0·02 absorbance units. The bound fraction was then eluted with imidazole buffer containing 5 mmol/l EDTA; the eluate was depleted of IgM by passage through a sepharose-coupled anti-IgM affinity column. The IgM-depleted material was concentrated and dialysed against 20 mmol/l bis "tris", pH 6·5, by means of an Amicon stirred cell. The dialysis sample was loaded on to a 'Mono-Q' anion exchange column linked to the fast protein liquid chromatography (FPLC) system (Pharmacia UK Ltd) with a start buffer of 20 mmol/l bis "tris", pH 6·5, and a limit buffer of 20 mmol/l bis "tris", pH 6·5, 1 mol/l sodium choride.

Samples of MBP-enriched fractions were vigorously reduced by boiling in the presence of 40 mmol/l dithiothreitol and duplicate samples were subjected to electrophoresis on a 10% reducing sodium dodecyl sulphate polyacrylamide slab gel.[12] The gel was divided; half was silver-stained[13] and half was electroblotted by means of the BioRad 'Transblot' and probed with rabbit anti-MBP followed by iodine-125-labelled antibody to rabbit immunoglobulin (Amersham UK Ltd).

Antiserum to human MBP was raised in a rabbit by means of MBP prepared from 6 litres of outdated human plasma by affinity chromatography on a mannan-sepharose column, displacement with EDTA, repeated chromatography on a similar, smaller column, elution with mannose, and successive fractionation by 'Superose 6'-gel filtration and mono-Q ion exchange chromatography; contaminating IgM was removed from the MBP preparation by means of an anti-human-IgM affinity column.

IgG was isolated from the antiserum by sodium sulphate precipitation and passage of the redissolved precipitate through a

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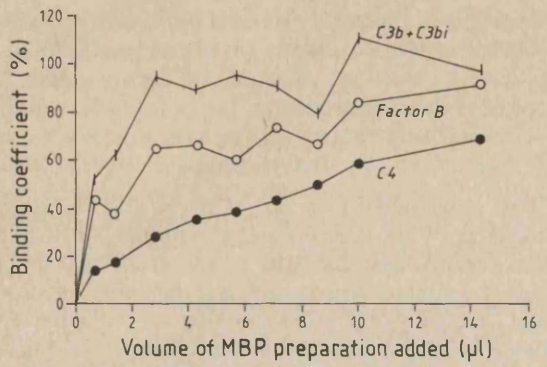

Fig 1—Titration of MBP (approximately 200 µg/l) into serum with low C3b binding activity.

Deposition of C3b/C3bi, factor B, and C4 on mannan-coated ELISA plates was measured as binding coefficients.

DE52 ion exchange column. The antibodies in this preparation were shown to be specific for MBP by probing on a western blot of whole serum. Further tests established that there was no crossreactivity with C1q.

The tryptic release of C3c fragments from zymosan previously incubated with human serum has been described in detail elsewhere[9] and is known to correlate well with other assays of yeast opsonisation. So that a binding coefficient could be calculated for each sample every assay included sera known to give high (HB) and low (LB) levels of C3b binding (C3b%) to zymosan.

$$\text{Binding coefficient} = \left[1 - \left(\frac{\text{HB C3b\% - Test C3b\%}}{\text{HB C3b\% - LB C3b\%}}\right)\right] \times 100\%$$

For measurement of complement components bound to mannan serum samples were diluted to 5% in veronal-buffered saline containing 5 mmol/l magnesium chloride and 5 mmol/l calcium chloride, added to the wells of mannan-coated enzyme-linked immunosorbent assay (ELISA) plates, and incubated for 30 min at 37°C. The complement fragments which bound to the solid phase were then detected with antibodies specific for C3bi (rat monoclonal anti-C3g; kindly provided by Prof Peter Lachmann, MRC, Cambridge), polyclonal anti-C3c (C3b and C3bi specific), anti-factor B, and anti-C4 (Serotec UK Ltd, Kidlington, Oxford).

Sera known to give high (HB) and low (LB) levels of C3b binding to yeast, zymosan, and mannan were included in every assay and used to calculate a binding coefficient for each test system (OD492 = optical density at 492 nm).

$$\text{Binding coefficient} = \left[1 - \left(\frac{\text{HB OD492 - Test OD492}}{\text{HB OD492 - LB OD492}}\right)\right] \times 100\%$$

In the correction assay, small volumes of an MBP preparation containing about 200 µg protein per litre were titrated into the serum with low C3b binding activity used at 5% in veronal-buffered saline with magnesium and calcium chloride. In each case adjustments were made with the buffer to give a constant volume and constant calcium and magnesium concentrations. The binding coefficients of complement proteins to mannan-coated ELISA plates were then measured as above.

To measure MBP in serum and column eluates the mannan-capture assay was used. Dynatech 'Immulon' microELISA plates were coated overnight at 4°C with mannan diluted to 0·5 mg/ml in carbonate-bicarbonate buffer (15 mmol/l Na_2CO_3, 35 mmol/l $NaHCO_3$, pH 9·6). The coated plates were washed three times with phosphate-buffered saline, pH 7·3, containing 0·05% 'Tween 20', once with phosphate-buffered saline without tween, and once with imidazole buffer. Sera were diluted to 5% in imidazole buffer (40 mmol/l imidazole/HCl pH 7·8 with 1·25 mol/l sodium chloride) containing 50 mmol/l calcium chloride, and 100 µl volumes were added in duplicate to the wells of the mannan-coated plate. The plates were incubated at 37°C for 2 h. After that incubation, and each subsequent incubation stage, the plates were washed four times with phosphate-buffered saline/tween. Rabbit antibody to human MBP, diluted to 1/500 in the buffer, was added to all wells, and the plates were incubated at 37°C for 2 h. After washing, the plates were incubated at 37°C for 2 h with horseradish peroxidase labelled sheep antibody to rabbit IgG (Serotech UK Ltd) at 1/500

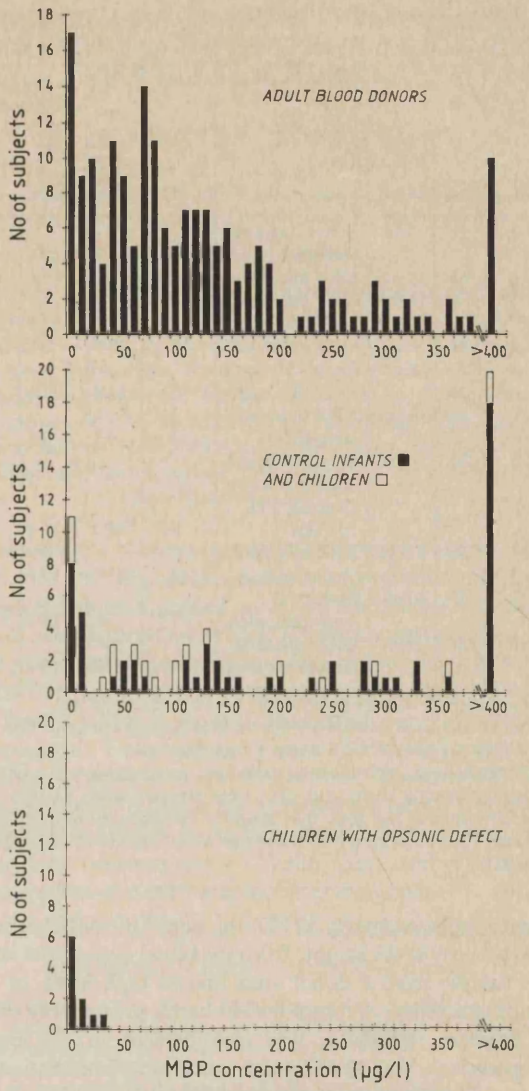

Fig 2—MBP levels in 186 healthy adult blood donors, 59 control infants aged 4·5 months and 19 control children aged 3 mo to 13 yr, and 10 children with the functional opsonic defect.

in the buffer, washed further, and then incubated at room temperature in the dark for 15–30 min with substrate solution (10 µg *o*-phenylenediamine in 20 ml citrate-phosphate buffer pH 5·2 [50 mmol/l citric acid plus 100 mmol/l Na_2HPO_4] plus 10 µl 30% hydrogen peroxide). The colour reaction was stopped by addition of 100 µl 2 mol/l sulphuric acid to each well. The plates were read at 492 nm with a Titertek 'Multiskan' plate reader. An MBP binding coefficient was then calculated as described above. In addition, a pool of serum from healthy adults, previously calibrated against a purified preparation of MBP, was serially diluted to provide a gravimetric standard curve, and unknowns were determined by reference to this curve. The detection limit of the assay was 2·5 µg/l.

MBP was also assayed by means of an antibody-capture sandwich ELISA in which the rabbit anti-MBP serum was used as both the capture and detector antibody. For use as the detector the antibody was biotinylated[14] and the assay developed with streptavidin peroxidase. Unknowns were assayed by reference to the secondary standard serum pool described above.

Correlation coefficients (non-parametric Spearman rank) were determined by means of the SAS statistical package (SAS Institute Inc, Cary, North Carolina, USA).

Results

The MBP-enriched preparations were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis under reducing conditions. On the silver-stained gel a major

LEVELS OF MBP IN CHILDREN PREVIOUSLY SHOWN TO HAVE OPSONIC DEFECT

Patient	Sex	Age*	Initial presentation†	BC (%)‡	MBP (μg/l)	BC (%) to mannan§ C3 fragments	BC (%) to mannan§ C4 fragments
1	F	3 yr	Severe diarrhoea, failure to thrive	0	9·4	−2·9	−0·6
2	M	15 mo	URT infections	5·9	4·9	−3·6	−0·9
3	M	16 mo	URT infections, IgG2 deficiency	−5·6	<2·5	−3·5	−0·8
4	M	9 mo	URT infections	0	13·9	0·5	−0·2
5	F	7 yr	Pyrexia of unknown origin	0	17·4	1·6	9·8
6	M	8 yr	URT infections, migraine	0	4·5	−2·0	−0·6
7	M	6 mo	Severe diarrhoea, failure to thrive, parotitis with pyrexia	5·3	35·2	−11·4	−0·9
8	M	2·5 yr	URT infections, bronchiectasis	0	23·9	13·9	1·7
9	F	10 mo	Pyrexia of unknown origin	5·3	2·8	−16·4	−1·3
10	M	9 yr	URT infections, failure to thrive	−5·0	<2·5	10·5	0

*At last hospital visit, when laboratory assays were carried out.
†No immunological abnormality was found in these patients other than IgG2 deficiency in patient 3. URT = upper respiratory tract.
‡C3c elution assay; BC = binding coefficient: normal range = 68 − 110%.
§Negative binding coefficients (BC) were obtained when ELISA colour development was less than that given by standard serum defining low binding. Normal range for C3 fragments 30–115%, and for C4 fragments 15–120%.

band of approximately 32 kD (the size of the MBP subunit) was present in the sample from the blood donor pool and in the sample from a donor with known high levels of C3b binding activity. A minor 64 kD band, presumably due to incomplete reduction, was also present in the pooled preparation. The 32 kD band was absent from the serum known to have low C3b binding activity.

Electroblotting and probing with the rabbit anti-MBP serum showed both the major 32 kD and the minor 64 kD bands in the preparations from the serum pool and the serum with high C3b binding, but there was no evidence of these bands in the sample from the serum with low C3b binding.

When the MBP-enriched material derived from the serum pool was used in the correction assay, it produced complement binding activity in the low C3b binding serum in a dose-dependent way (fig 1).

The median MBP level of the control group of children was 143 μg/l (range 2·5–880 μg/l), whereas the median for the larger adult control group was 92 μg/l (range 2·5–610 μg/l; fig 2). In contrast, the median level of MBP for the 10 children known to have the opsonic defect was 4·9 μg/l (range 2·5–35·0 μg/l). The C3c elution data defining poor opsonic function, the MBP levels, and the deposition of C3 and C4 fragments by serum from each patient are shown in the table. All of the values were either below, or low within, the normal range.

Further evidence linking levels of MBP with opsonic capacity in the population of blood donors was the extent of correlation between assay results. The results of the mannan-capture assay for MBP were significantly correlated with those of the functional opsonic assay by C3c elution (Spearman rank $[r_s] = 0{\cdot}4688$, $n = 76$, $p < 0{\cdot}0001$), the antibody-capture assay for MBP ($r_s = 0{\cdot}930$, $n = 102$, $p < 0{\cdot}0001$), the mannan-capture C3bi assay ($r_s = 0{\cdot}8744$, $n = 178$, $p < 0{\cdot}0001$), and the mannan-capture C4 assay ($r_s = 0{\cdot}8584$, $n = 178$, $p < 0{\cdot}0001$). These studies are reported in detail elsewhere.[10]

Discussion

The observation that rat MBP is able to activate complement[11] has been confirmed with human MBP.[10,15] Moreover, it is clear that MBP acts as an analogue to C1q, which it resembles structurally, and that, after interacting with C1r and C1s, it is able to promote the formation of C1 esterase[15] with the potential to cleave C2 and C4. There is also circumstantial evidence that MBP has a role in host defence. It has been reported to behave as an acute phase reactant in man[16] and it inhibits in-vitro infection of lymphoblasts by the human immunodeficiency virus.[17] The protein recognises mannose and N-acetylglucosamine, and the widespread occurrence of these sugars in the cell walls of pathogenic gram-negative bacteria, mycobacteria, and yeasts makes such organisms putative targets for serum lectins. An oganism with a cell wall rich in mannose groups could selectively concentrate MBP on its surface, which could then act as a focus for complement activation through the classic pathway. Kuhlman et al[18] reported that MBP may itself be opsonic, presumably through interactions with specific C1q-like receptors on the phagocyte surface. Whether this process occurs on a substantial scale in vivo remains to be established, since the levels of MBP used in these in-vitro assays exceeded the normal level of the protein in serum one hundred times. Our previous observations that people with the opsonic defect deposit lower levels of C3b not only on *Candida albicans*, but also on *Staphylococcus aureus* and *Escherichia coli*[19] suggest that low levels of MBP may result in suboptimal responses to a wide range of common bacterial infections.

By immunoblotting we showed that MBP-enriched preparations could be obtained from both the pool of serum and from the donor with high C3b binding. The material from pooled serum corrected the opsonic defect in the serum with low C3b binding in a dose-dependent way. In contrast, MBP was not detected in the immunoblot from the donor with low C3b binding. However, sensitive ELISA procedures detected 5–7 μg/l MBP in that serum. It is likely, therefore, that losses during purification account for the absence of signal from the low binding sample in the immunoblot, whereas a strong signal was still obtained with MBP derived from the donor with high C3b binding (MBP level 400 μg/l).

At a serum concentration of 5% the levels of C4 and C3 complement fragments binding to mannan were strongly correlated with the serum concentration of MBP. Furthermore, there was no correlation between these levels and the mannan-binding activity of various complement-fixing immunoglobulins (neither total IgG and IgM nor IgG1, IgG2, IgG3 subclasses).[10]

The ten patients with the opsonic defect had abnormal results in various mannan binding assays. The median MBP level for this group was 4·9 μg/l and 8 of the 10 had MBP levels below 20 μg/l.

Although 5% of the general population have impaired opsonic function in laboratory tests,[2,6,7] it is clear that disease is not inevitably associated with low levels of MBP and the importance of the association must be considered.

Hammerström and colleagues[20] have suggested that in early infancy much of the antipolysaccharide response may

be low-affinity IgG1 antibody. In most people isotype switching for polysaccharide antigens then occurs, resulting in a predominantly IgG2 subclass response. However, the antibody level observed in the minor IgG2 subclass (generally regarded as poor in complement activation) varies widely within the population. For example, after immunisation with a 23-valent polysaccharide vaccine, subjects homozygous for the G2m (n) allotype had about four times more IgG2 antibody than did those negative for G2m(n).[21]

We suggest that the contribution of MBP-activated classic pathway to opsonic processes is normally that of an accessory system. However, a deficiency of the lectin may become pathologically important in individuals whose IgG1 response to polysaccharides is of low affinity, in those with an isotype switch defect, or in the "low responders" having two G2m(n−) alleles. Infants aged 6–24 months would be most at risk, because there is no residual maternal antibody protection and, in most cases, the susceptibility would be transient because of maturation in the antibody repertoire. Maturation events, including the ability to make adequate responses in the complement-fixing IgG1 and IgG3 subclasses, would also explain the apparent compatibility of the defect with health in most older people. Although there are no published studies of subclass responses to mannan in relation to age, we have evidence of at least one adult with a low MBP level, high levels of IgG1 binding to mannan, and normal opsonic function as measured by the C3c elution assay.

Even in the subgroup of vulnerable children, intravascular responses, particularly alternative pathway activation mechanisms, would often be adequate. It is at extravascular sites, where such mechanisms may be less efficient, that difficulties might arise in the absence of an effective MBP compensatory mechanism. Secretory IgM is well known to have a similar compensatory role in the absence of secretory IgA.

The genomic organisation of the human MBP gene has been published[22] and consensus sequences probably involved in controlling the expression of human serum MBP have been identified in the promoter regions of the gene. It is possible that in individuals with persistently low levels of this acute phase protein there is an abnormality in one of these promoter regions which affects the expression of the protein. Further studies on both the immunopathology and genetics of this deficiency are now required.

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PRECURSOR LESIONS OF OESOPHAGEAL CANCER IN YOUNG PEOPLE IN A HIGH-RISK POPULATION IN CHINA

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Summary Young people (15–26 years) were selected from households in a population in China at high risk of oesophageal cancer on the basis of whether a case of oesophageal cancer had (166 participants) or had not (372 participants) occurred in a first-degree relative. In an endoscopic survey 43·5% of the male subjects and 35·9% of the female subjects showed histological signs of chronic oesophagitis. The presence of these precursor lesions was significantly associated in a multivariate logistic model with consumption of burning hot beverages, a family history of oesophageal cancer (including second-degree relatives), infrequent consumption of fresh fruit, and infrequent consumption of dietary staples other than maize.

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Introduction

SYMPTOMLESS chronic oesophagitis is very common among populations in Iran[1] and China[2] at high risk of oesophageal cancer. Findings from limited follow-up studies of progression of chronic oesophagitis to more severe lesions, the presence of the disorder in the part of the oesophagus where cancer subsequently occurs, and histological similarities to chemically induced lesions preceding oesophageal cancers have led to the recognition of symptomless chronic oesophagitis as a precursor lesion for oesophageal cancer. Observed associations with low levels of riboflavin, retinol, and zinc[3-5] led to a double-blind placebo-controlled intervention trial in a population in China.[6] The trial showed no effect of 13·5 months' supplementation with retinol, riboflavin, and zinc on the prevalence of chronic oesophagitis with or without atrophy or dysplasia (48·9% in the treated group, 45·3% in the placebo group). However, almost half the placebo-treated subjects also showed improvements in blood levels of retinol, probably owing to dietary changes; when these changes were taken into account, the prevalence of precancerous lesions was lower in subjects whose blood levels of vitamins and zinc did not change or improved during the study period than in subjects whose vitamin status deteriorated.[7] In addition, there was a significantly lower prevalence of micronucleated cells (an early endpoint) in the treated group than in the placebo group.[8] In view of the apparently early onset of precursor lesions, it has been suggested that investigations among children or adolescents are required to clarify further the role of vitamin deficiencies in the development of the precursor lesions.

We report here results from an endoscopic survey among young people, aged 15–26 years, in a high-risk area for oesophageal cancer in China.

Subjects and Methods

887 young people (aged 15–26 years) living in Meng Zhuang commune, Huixian county, Henan Province, China, were identified from households of cases of oesophageal cancer diagnosed after 1981 and from twice the number of randomly selected control households with no case of oesophageal cancer. 538 subjects (166 from case households, 372 from control households) agreed to take part in the study and gave informed consent in accordance with the Declaration of Helsinki.

The participants' weight, height, and blood pressure were measured: a 10 ml blood sample was taken for biochemical analysis of vitamins and trace elements; and overnight urine was collected from a random sample of 85 subjects for the analysis of N-nitroso compounds. The participants were interviewed with a questionnaire designed to obtain information from early childhood and from the past 5 years on dietary habits (frequency and duration of consumption of staples, fresh vegetables and fruits, different sources of animal protein, specialties such as pickled vegetables, and other mouldy foods), methods of food preparation, types of oil used, duration and amount of alcohol consumption and tobacco smoking, use of coal and other fuels, ventilation of cooking fumes, general family history of oesophageal cancer, occupation, and dental hygiene.

Endoscopic examination of the oesophagus and stomach was carried out with a slim fibreoptic forward-viewing oesophagogastroscope and two or more biopsy specimens were taken, one each from the middle and the lower third of the oesophagus or from macroscopic lesions. Slides of the biopsy specimens were studied by three pathologists (Q. S. L., N. M., P. C.) without knowledge of the clinical data or the case-control household status. The histological classification of oesophagitis as very mild, mild, moderate, or severe[1] was based on infiltration of the mucosa and submucosa or the presence of acute and/or chronic inflammatory cells as well as superficial elongation of the vascular papillae. Riboflavin, retinols, alpha-tocopherol, vitamin C, and selenium were measured by standard methods.[4]

Prevalence odds ratios were calculated initially for variables of interest at the beginning of the study by the Mantel-Haenszel method.[9] Variables identified in that evaluation were then included in a logistic regression analysis for the prevalence odds ratio.[10]

TABLE I—HISTOLOGICAL FINDINGS

—	n	% normal	% with oesophagitis		
			Very mild	Mild	Moderate
Male					
Total	354	56·5	31·6	10·7	1·1
15–20 yr	167*	57·5	32·3	8·4	1·8
21–26 yr	187	55·6	31·0	12·8	0·5
Female					
Total	184	64·1	30·4	4·3	1·1
15–20 yr	140*	66·4	28·6	3·6	1·4
21–26 yr	44	56·8	36·4	6·8	0

*Includes 1 subject aged 14 yr.

Results

Among the 354 male and 184 female subjects who underwent endoscopy, 43·5% and 35·9%, respectively, showed histological signs of chronic oesophagitis (table I). No cases of severe oesophagitis or dysplasia were seen. The rates observed among control households estimate the prevalence of oesophagitis among 15–26 year olds in Huixian (table II).

There was no difference in the prevalence of very mild oesophagitis among sex and age groups. Previous surveys have classified very mild oesophagitis as normal. Therefore, in subsequent case-control comparisons we took only subjects with mild or moderate oesophagitis as cases and those with very mild oesophagitis were included with those with normal findings as controls.

Adjusted for sex the prevalence odds radio for mild and moderate oesophagitis was 1·9 (95% confidence interval [CI] 1·0–3·4) for members of case households. Questionnaire-derived information on lifestyle factors was subjected to univariate and multivariate analysis, controlling for age and sex. Four variables showed significance in univariate analysis and gave odds ratio estimates with a 95% CI not containing the null value (1·0) in a final multivariate logistic model controlling for sex and age. This multivariate analysis showed that there was a greater than fourfold excess of mild and moderate oesophagitis among consumers of burning hot beverages (odds ratio 4·39 [95% CI 1·72–11·3]). Individuals who reported consumption of fresh fruits at least once a week had a lower prevalence of oesophagitis than those who ate less fresh fruit (odds ratio 0·29 [0·15–0·56]) and the prevalence was also lower in individuals consuming wheat flour products at least twice a day than in those who ate less (odds ratio 0·41 [0·22–0·75]). A family history of oesophageal cancer, including second-

TABLE II—HISTOLOGICAL FINDINGS BY HOUSEHOLD MEMBERSHIP

—	n	% normal	% with oesophagitis		
			Very mild	Mild	Moderate
Male					
Case household	99	41·4	40·4	16·2	2·0
Control household	255	62·4	28·2	8·6	0·8
Female					
Case household	67	64·2	29·9	4·5	1·5
Control household	117	64·1	30·8	4·3	0·9

The level of mannan-binding protein regulates the binding of complement-derived opsonins to mannan and zymosan at low serum concentrations

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SUMMARY

When sera diluted to 5% in a buffer containing calcium and magnesium were incubated with mannan-coated ELISA plates, C4 fragments, properdin and factor B were bound to the plates as well as the expected opsonic C3 fragments, C3b and C3bi. The calcium-dependent lectin mannan-binding protein, which is structurally similar to C1q, was also shown to bind in this assay and analysis of sera from 179 healthy blood donors revealed that the binding levels of all these proteins were highly significantly correlated. Results obtained with a previously described C3b opsonic assay using zymosan also correlated with the mannan-binding levels. When the sera were diluted to 5% in the presence of Mg-EGTA there was no detectable binding of complement proteins to the mannan surface, confirming that no alternative pathway activation occurred at this serum concentration. When sera were diluted to 5% in a buffer containing EDTA in order to study immunoglobulin binding in the absence of complement activation, the levels of bound IgG1, IgG2, IgG3, IgA and IgM antibodies were found to be completely unrelated to the C3bi binding levels previously observed. The results suggest that in this experimental system using low concentrations of serum, mannan-binding protein initiates an antibody-independent mechanism of cleavage of the classical pathway component C4, which subsequently regulates the degree of cleavage of C3 and recruitment of alternative pathway proteins.

Keywords complement C3 fragments mannan-binding protein opsonisation C4 fragments

INTRODUCTION

Many commonly used assays of opsonization employ heat-killed baker's yeast (*Saccharomyces cerevisiae*) or the yeast cell wall extract zymosan (Miller *et al.*, 1968; Soothill & Harvey, 1976; Roberton *et al.*, 1981; Richardson, Larcher & Price, 1982, 1983; Kerr *et al.*, 1983). Using such assays we and others have reported a relatively high frequency (5–7%) of poor opsonic function in the normal population (Soothill & Harvey, 1976; Levinsky, Harvey & Paleja, 1978; Kerr *et al.*, 1983), and an increased frequency in association with frequent infections (Soothill & Harvey, 1976; Richardson *et al.*, 1983). We have shown that this deficiency is correlated with the deposition of sub-optimal amounts of C3b/C3bi fragments on the zymosan surface (Turner, Mowbray & Roberton, 1981) and subsequently presented evidence for the absence or inactivity of an unidentified co-factor in the sera with poor opsonic function (Turner *et al.*, 1985).

Zymosan is known to consist almost entirely of two types of carbohydrate polymer, namely β-D-glucans and α-D-mannans (Phaff, 1963; Bacon *et al.*, 1969). The wells of microtitre plates can be readily coated with mannan and such coated plates were used by Yeaman & Kerr (1987) in a study of anti-yeast mannan IgA which had opsonic activity. We have modified this procedure in the present study to permit measurements of the various proteins that become bound after activation of the complement system and relate the results to those obtained with sera from individuals of known opsonic potential. At the same time we have also measured the binding of IgM and IgG subclasses and of mannan-binding protein (MBP) a putative activator of the complement system (Ikeda *et al.*, 1987).

These investigations have shown that in assays using 5% serum the binding of MBP is strongly correlated with the subsequent binding of both opsonic C3 fragments and C4 fragments, whereas the levels of specific anti-mannan antibodies with potential complement-fixing activity show no such correlation.

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MATERIALS AND METHODS

Human sera

Blood samples were obtained, with consent, from 179 blood donors attending the West End Donor Centre (with the kind

permission of the Director of the North London Blood Transfusion Service, Dr M. Contreras). Serum was separated from the cells within 2 h of bleeding and sub-aliquots frozen rapidly to −70°C. Aliquots were used once only after thawing.

Functional opsonization (C3c elution) assay

The tryptic release of C3c fragments from zymosan previously incubated with human serum has been described in detail elsewhere (Turner *et al.*, 1985) and is known to correlate well with other assays of yeast opsonization. Briefly, zymosan particles (prepared according to Lachmann & Hobart, 1978) were incubated in glass bacteriological tubes for 30 min at 37°C with human serum diluted to 17% in veronal-buffered saline (VBS) (145 mM NaCl, 4·4 mM diethylbarbituric acid, 1·8 mM sodium barbitone, pH 7·2) containing 5 mM $MgCl_2$ and 5 mM $CaCl_2$. The deposition of C3 fragments on the particles was terminated by the addition of ice-cold EDTA–VBS, pH 7·4. After washing, the particles were partially dried and incubated at 37°C for 15 min with trypsin (Difco; E. Molesey, Surrey, UK; 1% solution in 0·01 M EDTA–VBS). The total contents of the tube were then added to the well of a single radial diffusion plate containing sheep anti-human C3c antiserum (Scipac; Sittingbourne, UK) at a final dilution of 1/2000. After diffusion overnight at room temperature in a humid atmosphere, C3c fragments were quantitatively measured with reference to a C3 standard. It was possible to calculate a binding coefficient (BC) for each sample by including in every assay a serum known to give high levels of C3b binding (HB) to zymosan and a serum known to give low binding (LB) in the same system, using the formula:

$$BC = \left(1 - \frac{HB_{C3\%} - Test_{C3\%}}{HB_{C3\%} - LB_{C3\%}}\right) \times 100\%$$

Measurement of complement components binding to mannan

The binding of various complement proteins to mannan-coated ELISA plates was studied using the following protocol. The wells of Immulon (Dynatech, Plochingen, FRG) micro-ELISA plates were filled with 100 μl volumes of mannan (Sigma, Poole, UK; Code No. M-3640 prepared from *S. cerevisiae* by the Retavlon method) at 0·5 mg/ml in carbonate/bicarbonate buffer, pH 9·6 (1·59 g Na_2CO_3, 2·93 g $NaHCO_3$, 0·2 g NaN_3 made up to 1 l). After incubation overnight at 4°C, the mannan-coated plates were washed three times with phosphate-buffered saline (PBS) pH 7·3 (Oxoid, London, UK) containing 0·5% (v/v) Tween 20 (PBS-T), once with PBS (without Tween 20) and once with VBS.

The serum samples were diluted in Micronic tubes (Flow Laboratories) to 5% in VBS containing 5 mM $CaCl_2$ and 5 mM $MgCl_2$. Duplicate aliquots (100 μl) were then loaded into the wells of the mannan-coated ELISA plates and the plates incubated at 37°C for 30 min. The plates were then washed four times with PBS-T and bound ligands detected by incubation at room temperature for 1 h with the following indicator antibodies diluted in PBS-T: (i) horseradish peroxidase labelled polyclonal sheep anti-human C3c, C4, factor B and transferrin obtained from Serotec (Oxford, UK). The anti-C3c and anti-C4 reagents were used at dilutions of 1/10 000, and the anti-factor B and anti-transferrin antibodies were used at 1/2000; (ii) anti-properdin antibody. This was a mouse monoclonal (clone HYB 22-3, kindly provided by Dr Claus Koch, Statens Seruminstitut, Copenhagen). It was biotinylated by the method of Guesdon, Ternynck & Avrameas (1979) and used at a dilution of 1/4000; and (iii) anti-C3bi reagent. This rat monoclonal anti-C3g (clone 9, kindly provided by Professor Peter Lachmann, MRC, Cambridge) was used at 1/1000.

Following incubation with the indicator antibodies the plates were washed four times with PBS-T. The plates that had been incubated with the anti-properdin and anti-C3g monoclonal antibodies were then further incubated for 1 h with streptavidin–peroxidase (Serotec, Oxford, UK) at 1/4000 and peroxidase-labelled sheep anti-mouse IgG (Sigma) diluted to 1/500 in PBS-T, respectively. Following this incubation step, the plates were washed four times with PBS-T. Colour was developed following incubation for 15–30 min in the dark at room temperature with 100 μl/well of a solution of 10 μg of *o*-phenylene diamine in 20 ml phosphate-citrate buffer, pH 5·2 (prepared from 10·5 g citric acid, 14·2 g Na_2HPO_4 dissolved in 1 l) containing 10 μl 30% H_2O_2. The colour reaction was stopped by the addition of 4 N H_2SO_4 (100 μl/well) and the optical densities (OD) evaluated at 492 nm using a Titertek Multiskan ELISA plate reader and the Titersoft Program (Flow).

A serum known to give HB levels of C3b to zymosan and serum known to give LB in the same system were included in every assay and used to calculate a BC for each test system:

$$BC = \left(1 - \frac{HB_{OD492} - Test_{OD492}}{HB_{OD492} - LB_{OD492}}\right) \times 100\%$$

A selected panel of sera was also analysed in duplicate at both 5% and 30% concentration using a dilutent of VBS containing 7 mM $MgCl_2$ and 10 mM EGTA.

Measurement of MBP

Sera were diluted to 5% in imidazole buffer (40 mM imidazole/HCl, pH 7·8, with 1·25 M NaCl and 50 mM $CaCl_2$) and 100-μl aliquots were added in duplicate to the wells of Immulon micro-ELISA plates pre-coated with mannan as above. The plates were then incubated at 37°C for 2 h, washed four times with PBS-T, and rabbit anti-human MBP (kindly provided by Dr S. Thiel) diluted to 1/500 in PBS-T was added before a further incubation at 37°C for 2 h. The plates were washed four times with PBS-T and then incubated at 37°C with horseradish peroxidase–sheep anti-rabbit IgG conjugate (Serotec) at 1/500 in PBS-T. The plates were further washed four times with PBS-T and colour developed as above. An MBP binding coefficient was then calculated as described for the binding of complement components.

MBP was also assayed using an antibody-capture sandwich-ELISA in which the rabbit anti-BMP was used as both the capture and detector antibody. When used as the detector the antibody was biotinylated by the method of Guesdon *et al* (1979) and the assay developed with streptavidin peroxidase. An MBP binding coefficient was then calculated as above.

Measurement of immunoglobulins bound to mannan

A modification of the method described by Yeaman & Kerr (1987) was used. Dynatech micro-ELISA plates were coated with mannan as previously described. Sera were diluted to 5% using PBS-T containing 10 mM EDTA. Duplicate aliquots (100 μl) of the diluted sera were incubated in the plates for 2 h at 37°C. The plates were then washed four times with PBS-T before the addition of the appropriate indicator antibody. Peroxidase-

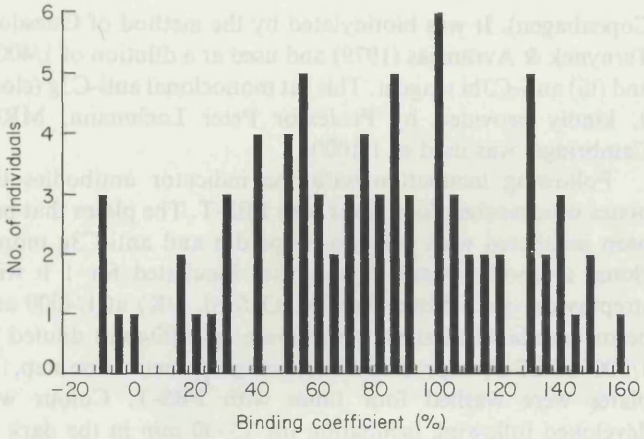

Fig. 1. C3 fragment-zymosan binding coefficients for 77 sera obtained from healthy blood donors. Data generated using the C3c elution technique. Results are expressed as a binding coefficient using sera previously determined to have high- and low-binding characteristics. These defined the 100% and 0% values.

labelled goat anti-human IgA, IgG and IgM (Sigma) were diluted 1/1000 in PBS-T and 100-μl volumes were added to each well. After a 2-h incubation at 37°C the plates were washed four times with PBS-T and colour developed as above. Unknowns were interpolated using a logistic curve fitting programme from Titersoft (Flow). The amount of bound immunoglobulin was related to a standard curve constructed with doubling dilutions (0·07–20%) of a normal human serum pool prepared from 100 healthy adult donors. The binding patterns of IgG1, IgG2 and IgG3 subclass antibodies to mannan were also investigate using biotinylated mouse monoclonal reagents purchased from Cambridge Bioscience (Cambridge, UK) at working dilutions of 1/500, 1/1000 and 1/250, respectively. After a further incubation with streptavidin peroxidase used at either 1/1000 (IgG2) or 1/250 (IgG1 and IgG3) the plates were developed as above and binding expressed as percentage of a normal human serum pool.

Statistical analysis

Pearson product moment correlations between variables were calculated using simple linear curve fit regression analysis and plotted with the Microsoft Chart software (Redmond, WA).

Non-parametric Spearman Rank correlation coefficients were determined using the SAS statistical package (SAS Institute, Cary, NC).

RESULTS

C3b opsonization determined with C3c elution assay

A random selection of 77 serum samples from the 179 blood donors was used in the C3c elution technique and five individuals (7%) were found to show evidence of poor C3b opsonization (Fig. 1).

Mannan binding complement components

One-hundred and seventy-nine serum samples were available for the determination of mannan binding coefficients using antibodies specific for C3bi, factor B, properdin and C4, and the results obtained are plotted in Fig. 2. The results obtained with the anti-C3c antibody (recognizing both C3b and C3bi determinants) were very similar to those obtained with the anti-C3b antibody and are not included in the figure.

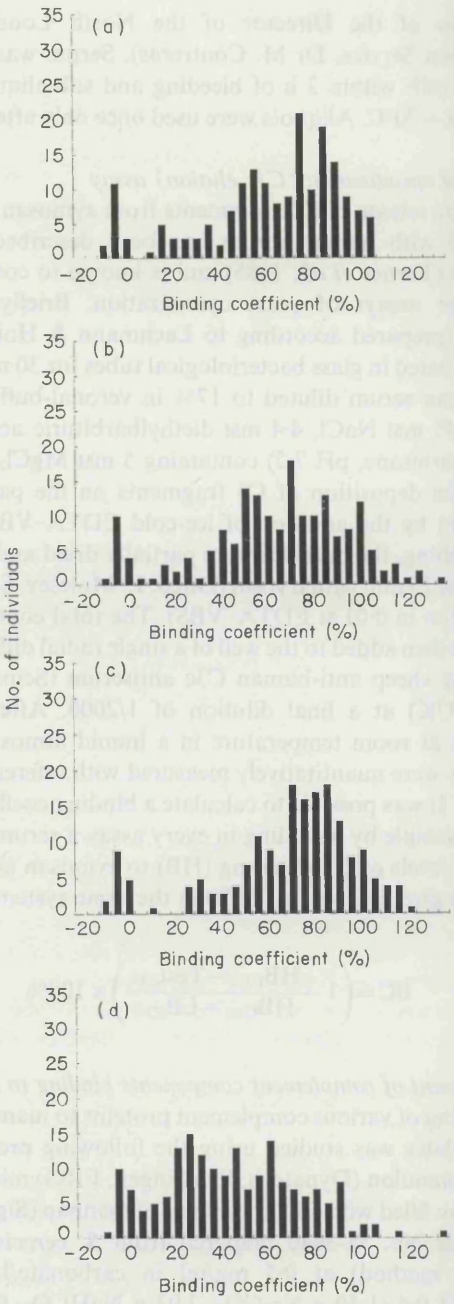

Fig. 2. Binding of various complement proteins to mannan-coated ELISA plates. Comparative study of 179 sera obtained from healthy blood donors. Plates developed with specific antibodies as described in Materials and Methods to reveal the following: (a) C3bi (C3g specificity); (b) factor B; (c) properdin; and (d) C4. All results expressed as binding coefficients.

In each case there was a broadly similar profile of binding activity with a sub-population of individuals in whom the binding coefficients were close to or below zero. Using the anti-C4 antibody, which presumbly recognized covalently bound C4b fragments, individuals with low-binding coefficients were again observed but these were less clearly defined within the population profile.

The assay for transferrin revealed no evidence of binding by this transport protein to the mannan-coated plates (data not shown). When the assays were repeated using 40 sera diluted to a

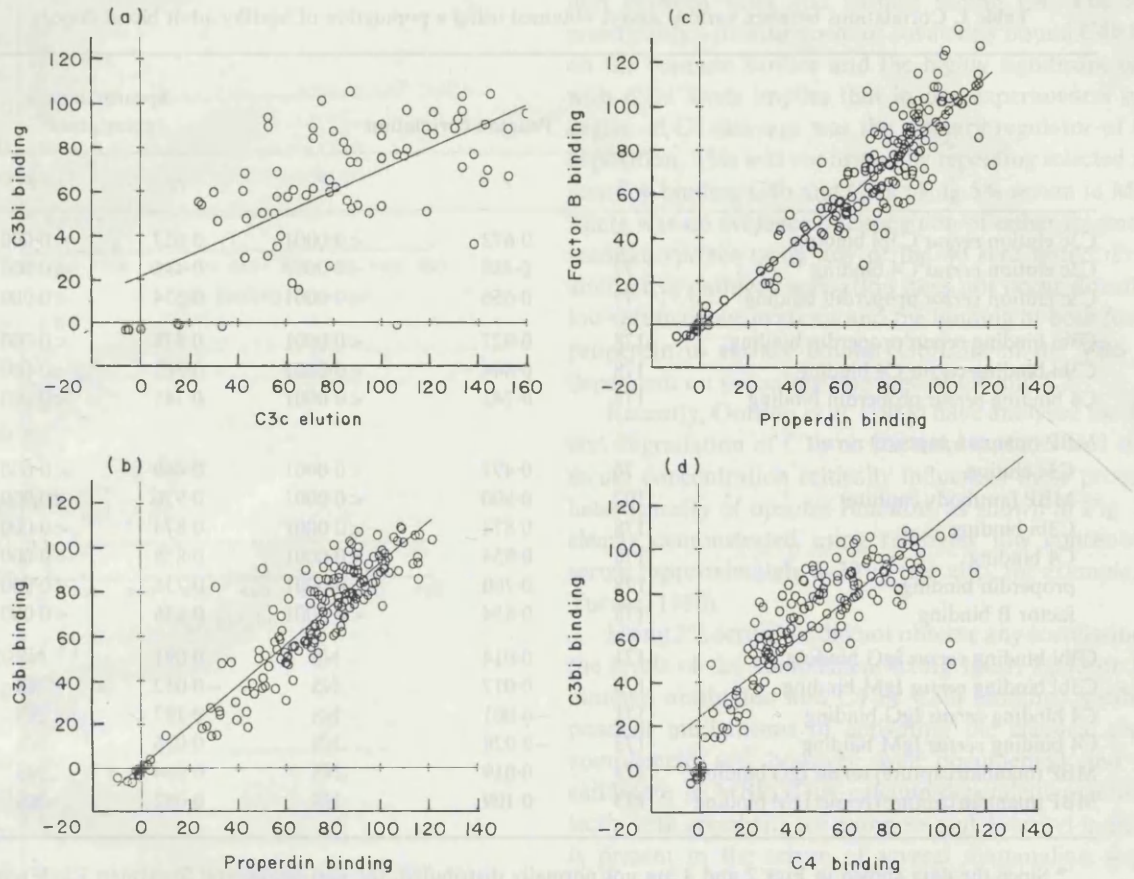

Fig. 3. (a) Correlation between C3 fragment-zymosan binding coefficients determined by the C3c elution technique and C3bi mannan-binding coefficients determined in 77 sera; (b) Correlation between C3bi mannan binding coefficients and properdin-binding coefficients determined in 179 sera; (c) Correlation between factor B mannan-binding coefficients and properdin mannan binding coefficients determined in 179 sera; and (d) Correlation between C3bi mannan binding coefficients and C4 mannan-binding coefficients determined in 179 sera.

oncentration of 5% in VBS containing Mg-EGTA, there was o significant binding of any of the complement components, cluding C4 (data not shown). Using serum diluted to 30% in e same buffer there was again no detectable binding of C4, though C3bi, factor B and properdin were bound in each case ata not shown). However, the levels bound using 30% serum Mg-EGTA were unrelated to those observed using 5% serum VBS containing divalent cations.

The levels of C3 fragments binding to zymosan in the C3c ution assay were found to correlate significantly with the C3bi i-annan binding coefficients when these values were compared as the subpopulation of 77 individuals (Fig. 3a). Moreover, ere were strong correlations between all of the mannan nding coefficients for complement proteins determined using ra from the larger population of 179 individuals (Fig. 3b, c, d; able 1).

evels of MBP

sing the mannan capture ELISA system MBP binding efficients were measured in 179 serum samples from blood nors and sufficient sample was available from 102 of the nors to make similar measurements with the antibody pture assay. These results are illustrated in Fig. 4a, b.

When the MBP binding coefficients obtained in the capture say are compared with the levels of C3bi and C4 bound to the ates using the same sera, highly significant correlations are served (Fig. 4c, d; Table 1).

Mannan-binding immunoglobulins

The serum samples used for the study of mannan-binding complement components were also investigated for specific total IgA, IgG and IgM binding in assays which were performed in the presence of EDTA in order to eliminate complement binding and possible steric hindrance. Significant binding (> 100% of pooled serum standard) was observed in all three classes for several sera including some with poor C3bi mannan-binding activity but for none of the isotypes did the levels correlate with the C3bi deposition (Fig. 5). The thirty individuals showing the highest levels of IgG binding were selected for further analysis of their IgG1, IgG2 and IgG3 binding characteristics and correlations were again sought with the levels of C3bi, properdin, factor B, C4 and MBP bound to the plates. In this subpopulation there was again a highly significant correlation between all of the non-immunoglobulin proteins studied but no correlation between the binding of any of the subclasses and that of any other protein studied (data not shown).

DISCUSSION

The binding of various complement components to mannan-coated ELISA plates following exposure to dilute serum was readily measured using specific ELISA procedures. Since both yeast and zymosan are known to be efficient activators of the alternative pathway of complement, it was anticipated that

Table 1. Correlations between various assays obtained using a population of healthy adult blood donors

	Pearson correlation			Spearman rank correlation*	
	n	*r*	*P*	r_s	*P*
C3c elution *versus* C3bi binding	77	0·672	<0·0001	0·627	<0·0001
C3c elution *versus* C4 binding	77	0·488	<0·0001	0·495	<0·0001
C3c elution *versus* properdin binding	77	0·656	<0·0001	0·574	<0·0001
C3bi binding *versus* properdin binding	178	0·927	<0·0001	0·878	<0·0001
C3bi binding *versus* C4 binding	178	0·844	<0·0001	0·867	<0·0001
C4 binding *versus* properdin binding	178	0·743	<0·0001	0·741	<0·0001
MBP (mannan capture) *versus*					
C3c elution	76	0·497	<0·0001	0·469	<0·0001
MBP (antibody capture)	102	0·890	<0·0001	0·930	<0·0001
C3bi binding	178	0·874	<0·0001	0·874	<0·0001
C4 binding	178	0·854	<0·0001	0·858	<0·0001
properdin binding	178	0·780	<0·0001	0·736	<0·0001
factor B binding	178	0·854	<0·0001	0·836	<0·0001
C3bi binding *versus* IgG binding	171	0·014	NS	0·091	NS
C3bi binding *versus* IgM binding	173	0·017	NS	−0·012	NS
C4 binding *versus* IgG binding	171	−0·001	NS	0·107	NS
C4 binding *versus* IgM binding	173	−0·028	NS	0·016	NS
MBP (mannan capture) *versus* IgG binding	171	0·019	NS	0·064	NS
MBP (mannan capture) *versus* IgM binding	173	0·109	NS	0·092	NS

* Since the data shown in Figs 2 and 4 are not normally distributed, the non-parametric Spearman Rank test of correlation has also been used.

C3c elution, functional opsonic assay (see Materials and Methods); MBP, mannan-binding protein; NS, not significant ($P > 0·05$).

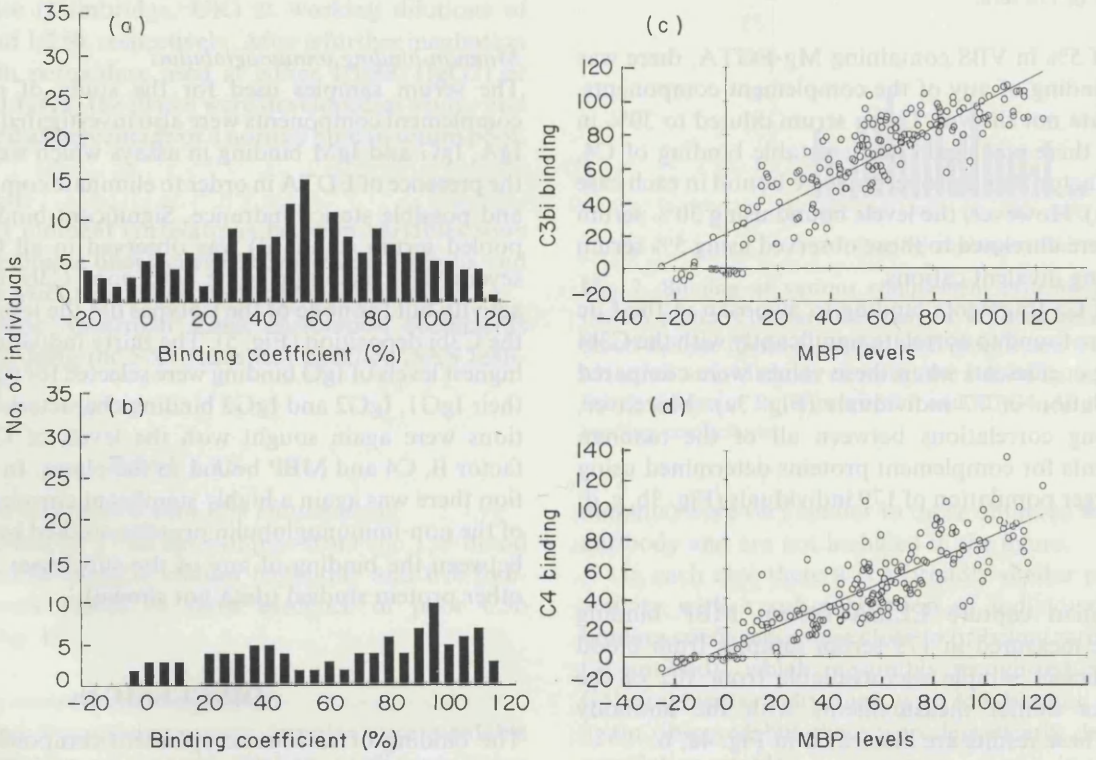

Fig. 4. (a) Levels of mannan-binding protein (MBP) determined in 179 sera from blood donors using the mannan capture assay; (b) Levels of MBP determined in 102 sera from blood donors using the antibody capture assay; (c) Correlation between MBP level determined with the mannan capture assay and C3bi binding coefficients; and (d) Correlation between MBP level determined with the mannan capture assay and C4 binding coefficients.

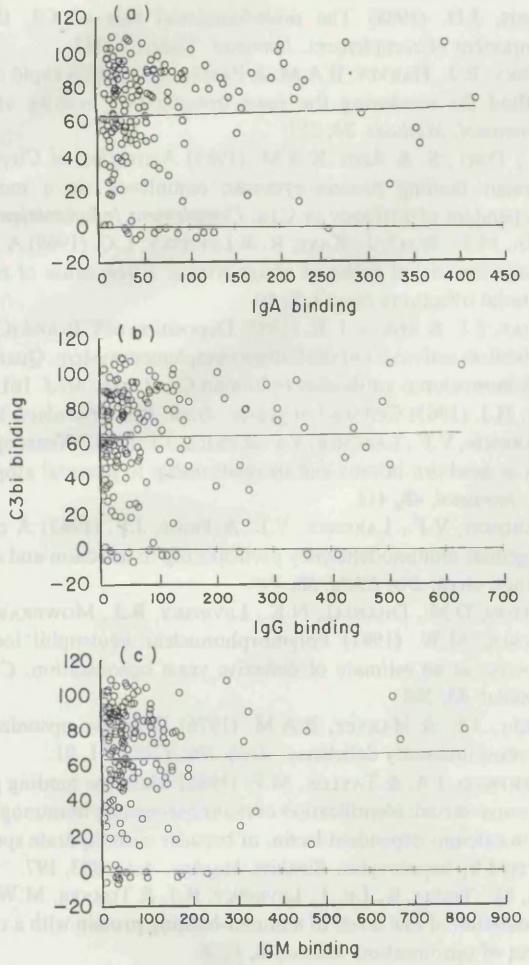

Fig. 5. Correlations between immunoglobulin binding to mannan-coated plates in the presence of EDTA and C3bi binding with the same (unchelated) sera. ELISA plates were developed to reveal: (a) IgA anti-mannan antibodies; (b) IgG anti-mannan antibodies; and (c) IgM anti-mannan antibodies. C3bi binding is expressed as a binding coefficient (%) and IgA, IgG and IgM binding is expressed as % of the binding of a pooled human serum standard.

coupling the major zymosan constituent mannan to a solid phase would provide a better-defined substrate for further studies of opsonic processes involving C3 moieties. The surface attachment of C3b fragments through covalent amide and ester bonds follows an internal activation of a thio-ester group in the fluid phase C3 at the time of C3a–C3b cleavage. Subsequently there is frequently proteolytic cleavage of the C3b to the slightly smaller C3bi fragment which is also opsonic. Newman & Mikus (1985) have claimed that most C3b on yeast surfaces is rapidly converted to C3bi.

The C3 molecule is known to interact with many different proteins (reviewed by Lambris, 1988) and when the positive feedback amplification loop is recruited both factor B and properdin bind to independant sites of the α-chain of the C3b molecule. Our observed correlation between the levels of C3bi and those of factor B and properdin binding to the mannan surface are in agreement with those of DiScipio (1981) who found a stoichiometric ratio of approximately 1:1:1 for C3:Bf:properdin interactions with zymosan using radio-labelled isolated components. More surprising was the correlation between C3bi and surface bound C4. The latter was presumably a measurement of covalently bound C4b fragments on the mannan surface and the highly significant correlation with C3bi levels implies that in our experimental system the degree of C4 cleavage was the primary regulator of C3b/C3bi deposition. This was confirmed by repeating selected assays for mannan binding C4b and C3bi using 5% serum in Mg-EGTA. There was no evidence of deposition of either fragment on the mannan surface using any of the 40 sera tested. Presumably alternative pathway activation does not occur directly at such low serum concentrations and the binding of both factor B and properdin to surface bound C3b/C3bi in the VBS system is dependent on preceding cleavage of C4 and C3.

Recently, Gordon *et al.* (1988) have analysed the deposition and degradation of C3b on bacterial surfaces and shown that serum concentration critically influences these processes. The heterogeneity of opsonic function, as shown in Fig. 1, is most clearly demonstrated using relatively low concentrations of serum (approximately 5–20%) (see also, for example, Kemp & Turner, 1986).

Using 5% serum we did not observe any correlation between the levels of the complement-fixing IgG1, IgG3 or IgM anti-mannan antibodies and C4 or C3bi binding. Antibody-independent mechanisms of activating the classical pathway of complement are, however, well documented and one such candidate is MBP. This calcium-dependent macromolecular lectin with specificity for mannose and *N*-acetyl-D-glucosamine is present in the serum of several mammalian species. The molecule comprises multiple subunits of approximately 32 kD, each with three domains: a cysteine-rich NH_2 domain, a collagenous region and a carboxyterminal globular domain containing the residues responsible for carbohydrate recognition (Summerfield & Taylor, 1986; Drickamer & McCreary, 1987; Thiel & Reid, 1989). The molecule shares many features with C1q and closely resembles it under the electron microscope (Thiel & Reid 1989). Following the report by Ikeda *et al.* (1987) that rat MBP was able to activate complement, Lu, Thiel & Reid (1989) were able to show that the protein interacts with C1r and C1s and thereby promotes the formation of C1 esterase, the C4-cleaving enzyme of the classical pathway. The highly significant correlations observed in our study between the bound levels of MBP and the deposition of various complement proteins strongly suggest that this antibody-independent mechanism of activating the classical pathway of complement may be largely responsible for the C3 fragmentation occurring at these low serum concentrations. Moreover, we have obtained evidence that individuals with the frequently observed functional opsonic deficiency have low serum levels of MBP (Super *et al.*, 1989) and are apparently unable to upregulate its synthesis. Addition of purified MBP to their serum corrects the defect *in vitro*.

We suggest that MBP is an important factor in host defence against Gram-negative bacteria, mycobacteria and yeasts, all of which abundantly express mannose and/or *N*-acetyl-D-glucosamine on their surface. At extravascular sites alternative pathway activation of complement by such organisms may be inefficient because of lower concentrations of serum proteins and the most common antibody may be of IgG2 subclass (preferentially elicited by polysaccharide antigens and poor in the classical pathway activation of complement). The lectin would be concentrated on the surface of the organism and could then act as an opsonic ligand in its own right as recently

demonstrated by Kuhlman, Joiner & Ezekowitz (1989) or, more efficiently, activate the classical pathway for subsequent generation of C3b opsonins.

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