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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; 2% 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 Cetavlon 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 3-3, 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-

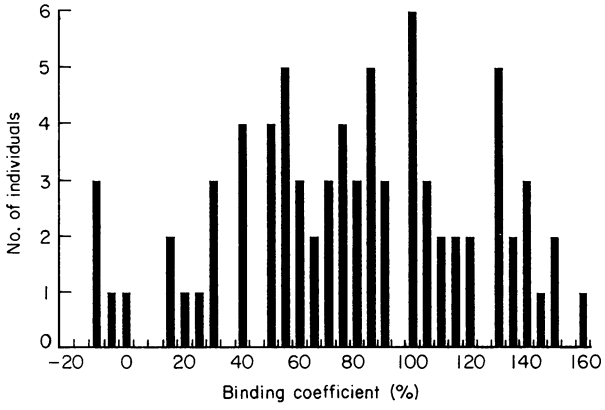

Fig. 1. C3 fragment-zymosan binding coefficients for 77 sera obtained from healthy blood donors. Data generated using the C3c elution technique. Results are expressed as a binding coefficient using sera previously determined to have high- and low-binding characteristics. These defined the 100% and 0% 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-C3bi antibody and are not included in the figure.

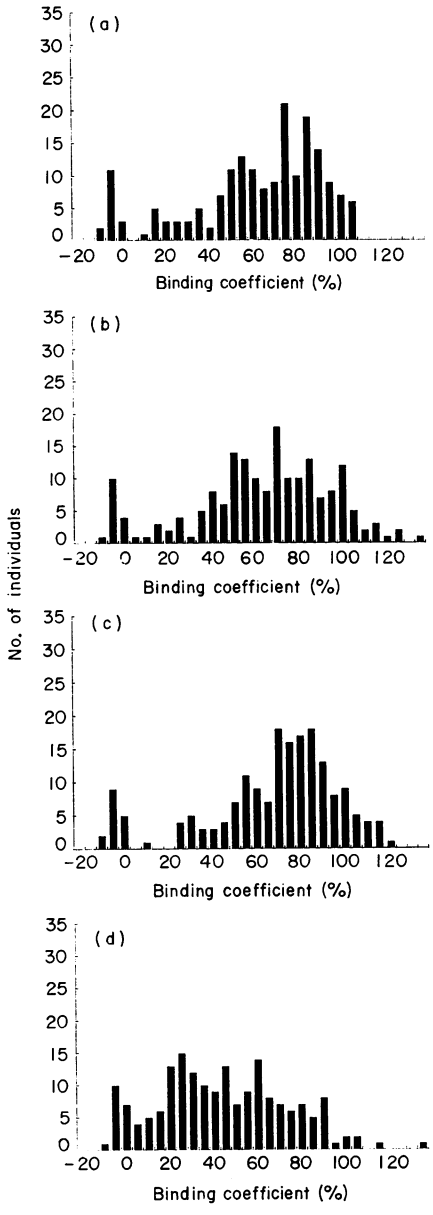

Fig. 2. Binding of various complement proteins to mannan-coated ELISA plates. Comparative study of 179 sera obtained from healthy blood donors. Plates developed with specific antibodies as described in Materials and Methods to reveal the following: (a) C3bi (C3g specificity); (b) factor B; (c) properdin;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

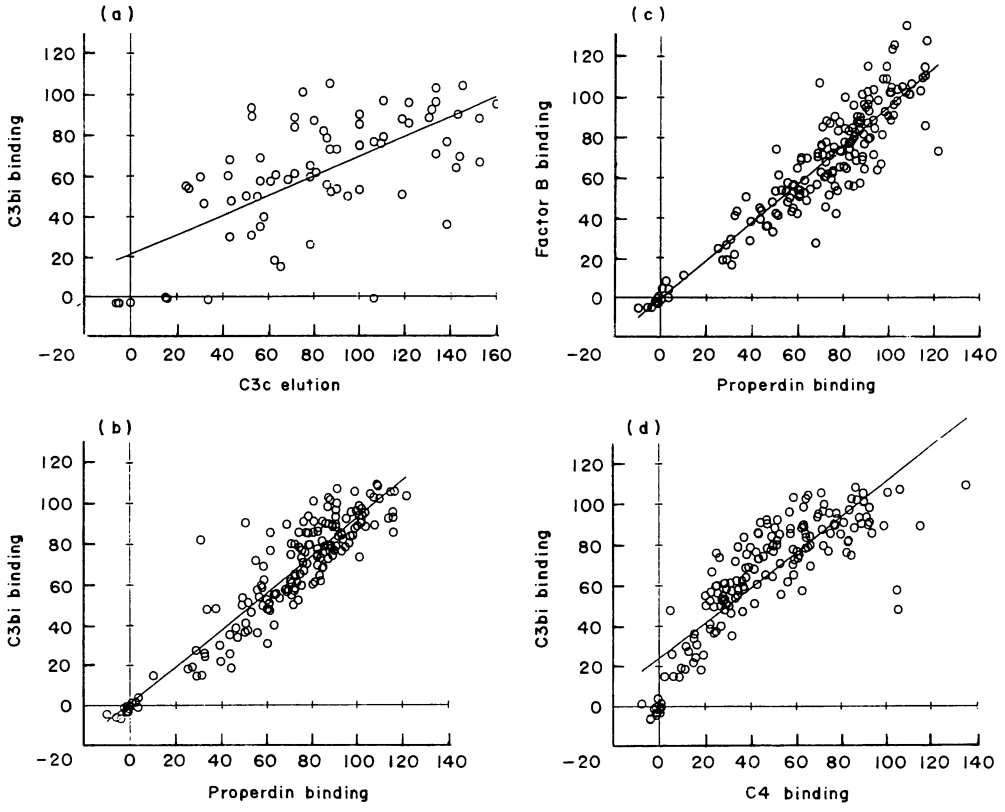

Fig. 3. (a) Correlation between C3 fragment-zymosan binding coefficients determined by the C3c elution technique and C3bi mannan-binding coefficients determined in 77 sera; (b) Correlation between C3bi mannan binding coefficients and properdin-binding coefficients determined in 179 sera; (c) Correlation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

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

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 (Fig. 3a). Moreover, there were strong correlations between all of the mannan binding coefficients for complement proteins determined using sera from the larger population of 179 individuals (Fig. 3b, c, d; Table 1).

Levels of MBP

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

When the MBP binding coefficients obtained in the capture assay are compared with the levels of C3bi and C4 bound to the plates using the same sera, highly significant correlations are observed (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 ($P > 0·05$).

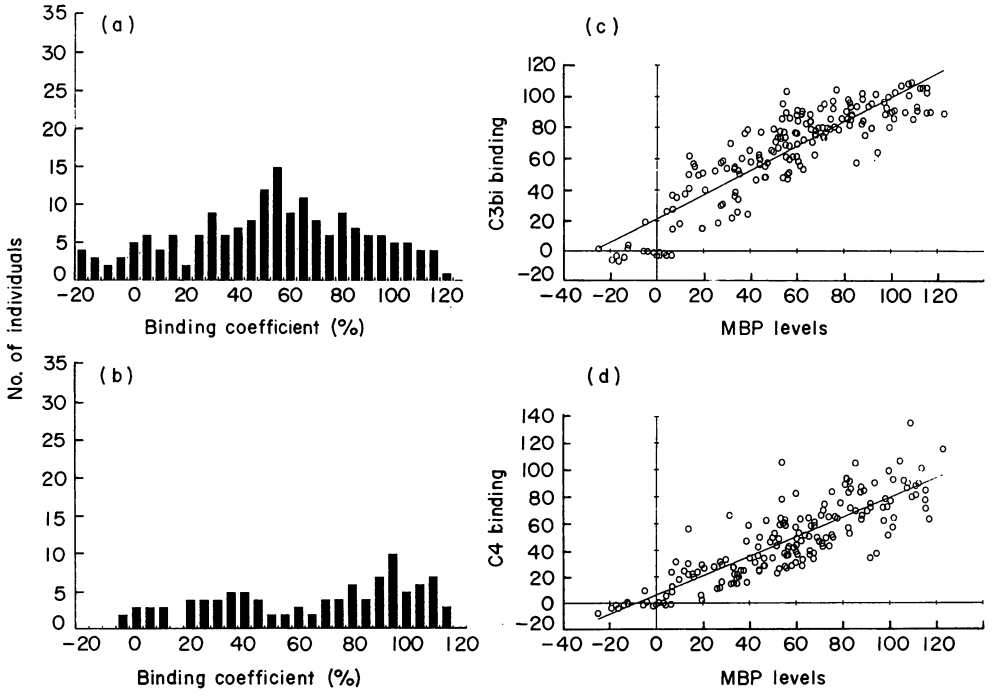

Fig. 4. (a) Levels of mannan-binding protein (MBP) determined in 179 sera from blood donors using the mannan capture assay; (b) Levels of MBP determined in 102 sera from blood donors using the antibody capture assay; (c) Correlation between MBP level determined with the mannan capture assay and C3bi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

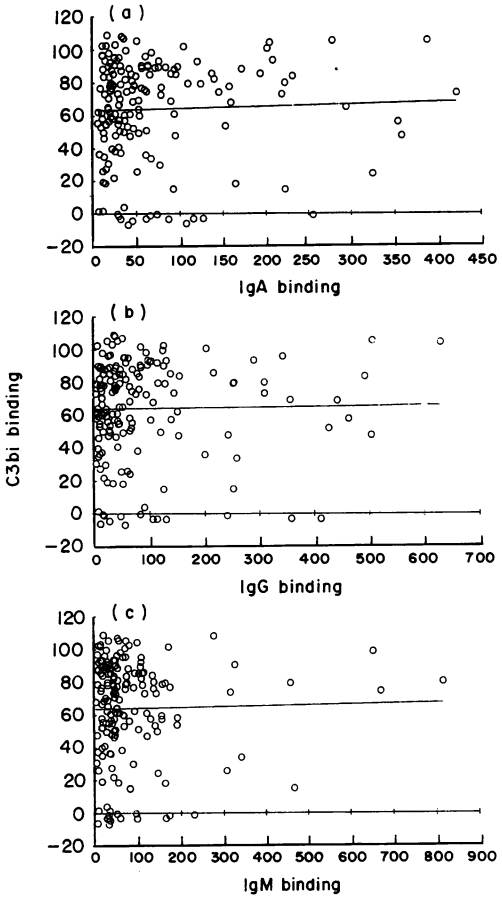

Fig. 5. Correlations between immunoglobulin binding to mannan-coated plates in the presence of EDTA and C3bi binding with the same (unchelated) sera. ELISA plates were developed to reveal: (a) IgA anti-mannan antibodies; (b) IgG anti-mannan antibodies; and (c) IgM anti-mannan antibodies. C3bi binding 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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