Gene frequency and partial protein characterization of an allelic variant of mannan binding protein associated with low serum concentrations

P. GARRED, S. THIEL*, H. O. MADSEN, L. P. RYDER, J. C. JENSENIUS* & A. SVEJGAARD
*Tissue Typing Laboratory of the Department of Clinical Immunology, Rigshospitalet, State University Hospital, Copenhagen,
and *Institute of Medical Microbiology, Department of Immunology, Århus University, Århus, Denmark*

SUMMARY

Low plasma concentration of mannan binding protein (MBP) has been shown to be the basis for a common opsonic deficiency and suggested to be caused by a single nucleotide substitution at base 230 of exon 1 in the MBP gene. This substitution causes a replacement of glycine (codon GGC) with aspartic acid (codon GAC). Of 123 healthy Danish individuals investigated by polymerase chain reaction performed on exon 1, followed by restriction fragment length polymorphism or allospecific probing, 93 were homozygous (75·6%) for GGC, 28 heterozygous (22·8%), and two homozygous for GAC (1·6%). The gene frequency of the GAC allele was found to be 0·13. DNA sequencing of the cloned exon 1 from one GAC homozygous individual revealed no other substitution. The median MBP concentration in the group containing the GAC allele was 6·4 times lower than in the GGC homozygous group (195 and 1234 $\mu g/l$ respectively). However, the range in plasma concentrations of MBP was wide and overlapping between the groups. MBP protein was detected in both the GAC homozygotes (9 and 387 $\mu g/l$). Furthermore, no difference in relative mass and biological activity (mannan binding) was found when sera containing the two forms of MBP were investigated. Accordingly, it can be concluded that the GAC allele is able to produce a functional MBP protein which may be detected in serum at low concentrations.

Keywords mannan binding protein   deficiency   complement   lectins   polymorphism

INTRODUCTION

A low plasma concentration of human mannan binding protein (MBP) is closely associated with a decreased opsonizing capacity of serum [1]. The frequency of this deficiency in the general population has been estimated to be between 5% and 10% [2]. The deficiency has been reported to be significantly more common in infants with recurrent respiratory tract infection, otitis media and chronic diarrhoea [3].

MBP is a liver-synthesized lectin with binding specificity towards mannose and N-acetylglucosamine. It is able to activate the complement system by the classical pathway upon binding to a mannose-rich surface [4]. The relevant carbohydrates are found on a number of micro-organisms, which might activate the complement system without the involvement of specific antibodies. Deficiency of MBP might thus be regarded as a new complement defect. The MBP protein consists of a mixture of trimers, tetramers, pentamers and hexamers of a 96-kD structural subunit [5]. This subunit is based on the three MBP chains of 32 kD. Only the pentamers and hexamers seem to activate complement.

Correspondence: Dr Peter Garred, Rigshospitalet, Tissue Typing Laboratory-7631, Tagensvej 20, 2200 Copenhagen N, Denmark.

Recently, sequence analysis of the MBP gene in three unrelated children with recurrent infections, opsonic deficiency and low serum MBP concentrations has been reported [6]. No abnormalities in the MBP gene were observed except for a point mutation at base 230 of exon 1 which caused a shift of codon 54 from GGC to GAC. Investigation of 16 relatives of the probands provided evidence that low MBP serum concentration is a result of this mutation.

The aim of this study was to establish the frequency of the two MBP alleles in the general population. Furthermore, we wished to determine whether this point mutation alone could explain low serum concentrations of MBP.

MATERIALS AND METHODS

Blood samples were obtained with consent from 123 Danish blood donors (69 women, 54 men). The median age was 40 years (range 21–64 years). Samples were drawn in 0·5 mM ethylene diamine tetra-acetate (EDTA) tubes. Plasma separated from the cells was frozen at −70°C and tested within 2 weeks.

After separation of plasma, DNA was isolated from blood cells according to a standard procedure including non-ionic detergent lysis of the cells and harvest of the nuclei [7]. The MBP

exon 1 sequences were amplified by polymerase chain reaction (PCR). As primers a pair of oligonucleotides 5'-GTA GGA CAG AGG GCA TGC TC-3' (upper strand) and 5'-CAG GCA GTT TCC TCT GGA AGG-3' (lower strand) were used as previously described [6]. PCR was performed in the presence of 1·5 mM $MgCl_2$ in the following 40 cycles: 30 s at 94°C, 1 min at 60°C and 2 min at 72°C. This was preceded by a 4-min denaturation step at 94°C and followed by 5 min extension at 72°C. The PCR product was digested with the BanI restriction enzyme (Stratagene, La Jolla, CA) recognizing 5'-G↓GP$_y$P$_u$CC-3'. Subsequently restriction fragment length polymorphism analysis (RFLP) was performed. pBR322 (Boehringer, Mannheim, Germany) digested with HaeIII (Amersham, Bucks, UK) was used as molecular weight marker. Alternatively, the PCR product was reacted with allele sequence-specific oligonucleotides recognizing GGC (5'-GG CGT GAT GGC ACC AAG G-3') and GAC (5'-GG CGT GAT GAC ACC AAG G-3') in the presence of tetramethylammonium chloride [8]. Sequencing of the whole exon 1 part of the MBP gene from one GAC homozygous individual was amplified with the means of the above mentioned primer set. For cloning purposes the primer set was modified by the addition of an EcoRI and a BamHI linker, respectively. The PCR product was digested with EcoRI and BamHI restriction enzymes (Amersham) and cloned into both of the M13 vectors mp18 and mp19 to obtain sequences in both orientations. The DNA sequencing was performed by the dideoxy-method [9] and the Sequenase DNA polymerase (USB, Cleveland, OH).

MBP plasma concentration was measured in a double enzyme immuno assay (EIA) essentially as described elsewhere [10]. Briefly, polystyrene wells were coated with polyclonal rabbit anti-MBP antibody. The detector antibody was biotinylated anti-MBP antibody from another rabbit. The assay standard curve was calibrated using a preparation of highly purified MBP. This standard has recently been exchanged with workers at the Institute of Child Health, London and MRC Immunochemistry Unit, Oxford, UK, and as a result it has been agreed that previously published MBP levels [1,5] were underestimated by a factor of approximately 10. The detection limit was found to be 2 μg MBP per litre. Reconstitution of MBP in MBP-deficient plasma confirmed the specificity. Both specific antibodies were shown to react exclusively with a band corresponding to MBP in a crude MBP preparation when tested in SDS-PAGE with Western blotting. Parallel plates were coated with equivalent amounts of normal rabbit IgG and processed as the anti-MBP plate. This was done to reveal the binding of rheumatoid factors, anti-animal immunoglobulins and unspecific binding of MBP interfering in the system. The background of each sample was subtracted from the readings in the corresponding anti-MBP plate.

Furthermore, in order to analyse the structure of the MBP captured in the EIA, MBP from five individuals was eluted from EIA wells and subjected to further analysis in SDS-PAGE with Western blotting. Briefly, EIA wells were incubated with 200 μl (10 μg/ml) rabbit anti-MBP or with 200 μl (10 μg/ml) normal rabbit IgG (Dako, Copenhagen, Denmark). Each sample to be analysed was diluted 1:10 and 200 μl were added to 12 wells coated with anti-MBP and to 12 wells coated with normal rabbit IgG and incubated. Between each step the plates were washed using Tris buffer with 0·05% Tween 20. To elute the bound MBP the first well was incubated with 200 μl SDS-PAGE sample buffer (4 M urea, 10% glycerol, 1·5% SDS, 0·5 M Tris, 1‰ bromophenol blue, pH 6·7). After 10 min incubation the sample buffer was transferred to the next well until the twelfth well was eluted. The eluates were reduced by addition of dithiothreitol before boiling. Electrophoresis on a 10% SDS-PAGE gel was followed by blotting onto immobilone (Millipore, Molsheim, France). The blot was developed using a biotin-labelled mouse anti-MBP MoAb (produced at the State Serum Institute, Copenhagen, Denmark) followed by alkaline phosphatase conjugated avidin phosphate (Sigma, St Louis, MO). The substrate was 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Sigma).

In 35 samples selected to encompass the whole range of MBP concentrations MBP was estimated by a functional EIA when biotinylated mannan was substituted for the detection antibody. In this assay the lower detection limit was found to be 10 μg MBP per litre and is thus approximately five times less sensitive than the double antibody EIA. Values are given as arbitrary units (AU/l) and refer to a pool of normal human plasma. Processing was performed with alkaline phosphatase conjugated avidin (Sigma). The subtrate was p-nitro-phenyl-phosphate (Boehringer).

The complement-activating capacity of a serum which derived from one GAC homozygous individual and 10 sera from GGC homozygous individuals was measured essentially as previously described [1]. Briefly, microtitre plates were coated with mannan (Sigma) overnight. Free binding sites were blocked with human serum albumin (1 h, 37°C). Serum samples were diluted 1:90 in a 4 mM barbital buffer containing 140 mM NaCl and 2 mM $CaCl_2$, pH 7·4 and incubated for 1 h at 37°C. Rabbit anti-human C4 (A065) from Dako diluted 1:5000 was then added and incubated overnight at 4°C. Finally, an alkaline phosphatase conjugated goat anti-rabbit antibody (A-8025) from Sigma diluted 1:5000 was added and incubated for 2 h at room temperature. Serum samples known to give high, medium and low levels of C4 binding to mannan were included on every plate. The negative control was the same as the positive one except that the sample was diluted in an EDTA containing buffer in order to block complement activation. All plates were read when the positive control reached an OD of 1·1 and the negative 0·09.

Statistical analysis

The Mann–Whitney test for unpaired group comparisons and the Spearman rank correlation test were used for statistical analysis. The Hardy–Weinberg equilibrium was examined by the χ^2-test with 1 degree of freedom without Yates correction.

RESULTS

Extracted leucocyte DNA was amplified by PCR as described in Materials and Methods, and digested with the restriction enzyme BanI. Upon gel electrophoresis, three distinct patterns could be observed (Fig. 1). The upper band represents the GAC allele which is resistant to BanI and the lower band represents the allele containing the GGC sequence which is split by BanI.

Analysis of the PCR products from all the individuals with allele specific oligonucleotides confirmed the RFLP results (data not shown). The GAC specific oligonucleotide reacted with all the GAC homozygous and heterozygous, but not with the GGC homozygous. Conversely, the GGC specific oligonucleotide
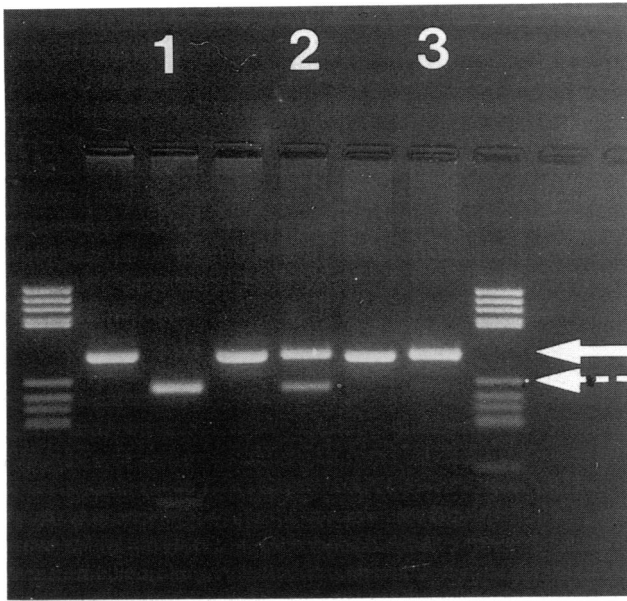
Fig. 1. Restriction fragment length polymorphism (RFLP) of polymerase chain reaction (PCR) products of exon 1 sequences of the mannan binding protein (MBP) gene digested with BanI. To the left and right are shown the molecular weight marker pBR322 digested with HaeIII. Unmarked lanes represent the undigested PCR products. The upper bands indicated by a solid arrow represent the GAC allele which is resistant to BanI and the lower bands indicated by a dotted arrow represent the allele containing the GGC sequence which is split by BanI. 1, GGC/GGC homozygote; 2, GGC/GAC heterozygote; 3, GAC/GAC homozygote.
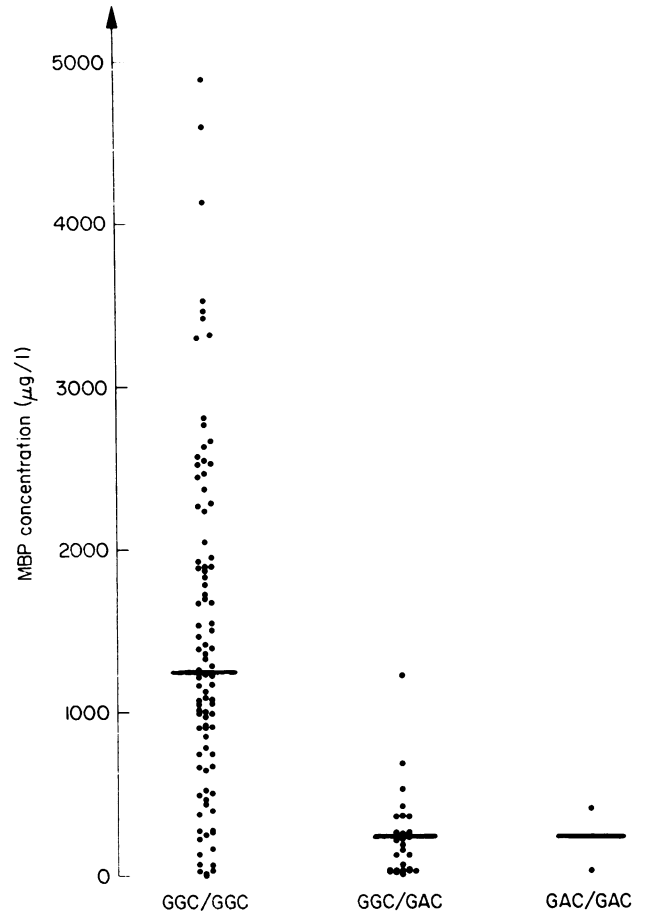

Fig. 2. Distribution of mannan binding protein (MBP) concentration in 93 GGC/GGC homozygous, 28 GGC/GAC heterozygous and two GAC/GAC homozygous healthy Danes. Median concentrations are indicated.

Table 1. Frequencies of mannan binding protein (MBP) genotypes in the Danish population

	No. of individuals		
	Observed (%)	Expected	χ^2
GGC/GGC	93 (75·6)	93·1	0·0001
GGC/GAC	28 (22·8)	27·8	0·0014
GAC/GAC	2 (1·6)	2·1	0·0050
Total	123	123·0	0·0065

Expected numbers were calculated assuming Hardy–Weinberg equilibrium and using frequencies of 0·87 and 0·13 for the GGC and GAC alleles, respectively. The very low χ^2 value of 0·0065 with 1 degree of freedom shows an almost perfect fit with expectations ($P = 0·90–0·95$).

reacted with the GGC homozygous and heterozygous, but not with the GAC homozygous.

The frequencies of the three genotypes in the population studied are shown in Table 1. Of the 123 tested individuals 93 were homozygous (75·6%) for GGC, 28 heterozygous (22·8%) and two homozygous for GAC (1·6%). The GAC homozygous individuals were retested on independent samples with same result. This gives a gene frequency of the GAC allele of 0·13. Table 1 also shows that there is an almost perfect fit to the Hardy–Weinberg expectations.

The concentration of MBP in all the individuals tested showed a wide range of MBP levels varying from 0 to 4889 $\mu g/l$ (Fig. 2). The median value was 992 $\mu g/l$ and the 2·5% and 97·5% limits were 10 and 4074 $\mu g/l$ respectively. In the GGC homozygous group the MBP levels ranged from 0 to 4889 $\mu g/l$. The median value was 1234 $\mu g/l$ and the 10% and 90% limits were 249 and 2768 $\mu g/l$ respectively. In the group containing the GAC allele including heterozygotes and homozygotes the MBP levels ranged from 0 to 1218 $\mu g/l$. The median value was 194 $\mu g/l$ and the 10% and 90% limits were 15 and 469 $\mu g/l$ respectively. The difference in MBP concentration between the two groups was highly significant ($P < 0·000\,01$). The MBP concentrations in the two GAC homozygous individuals were 9 and 387 $\mu g/l$ respectively. Further samples from the latter individual were obtained 1 month and 3 months later and the presence of MBP was confirmed (264 and 407 $\mu g/l$). SDS–PAGE with Western blotting of MBP detected by a anti-MBP MoAb showed that the GAC type contained a 32-kD polypeptide chain as found in the GGC type (Fig. 3). Furthermore, DNA sequencing of the whole exon 1 part of the MBP gene from the latter GAC homozygous
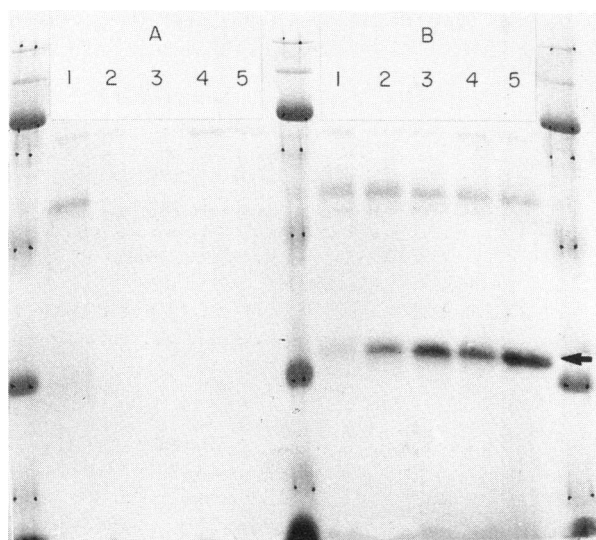
Fig. 3. Plasma from five individuals was eluted from (A) normal rabbit IgG and (B) polyclonal rabbit anti-mannan binding protein (MBP) IgG coated polystyrene wells and examined by SDS–PAGE and subsequent Western blotting. Detection antibody was a biotinylated MoAb against MBP. 1, GGC/GAC heterozygote (28 μg/l); 2, GAC/GAC homozygote (407 μg/l); 3, GGC/GGC homozygote (466 μg/l); 4, GGC/GAC heterozygote (679 μg/l); 5, GGC/GGC homozygote (3492 μg/l). Figures in brackets are the corresponding plasma concentrations of MBP in each sample. The arrow indicates the MBP 32-kD polypeptide chain. The high molecular weight bands represent non-specifically stained protein.

Fig. 4. Correlation between mannan binding protein (MBP) concentration detected in a double antibody enzyme immuno assay (EIA) and the MBP mannan binding capacity in which the detection antibody has been substituted with biotinylated mannan. O, GGC homozygotes; ●, GAC heterozygotes; □, GAC homozygotes.

individual revealed no substitution other than the replacement of GGC with GAC (data not shown).

In two individuals (1·6%), one from the GGC homozygous group and one from the GGC/GAC heterozygous group, MBP was undetectable. Very low concentrations of MBP (< 60 μg/l) were found in 12·2% of the subjects. These included four from the GGC homozygous group, 10 from GGC/GAC heterozygous group and one GAC homozygous individual.

In 35 plasma samples the capacity of MBP to bind mannan was tested. Irrespective of genotype a close correlation to the plasma concentrations of MBP was observed (r_s = +0·95; $P < 0·0001$) (Fig. 4). Moreover, there was no significant difference in the complement activating capacity of serum from the GAC homozygous individual with an MBP concentration of 387 μg/l compared with sera from 10 GGC homozygous individuals with comparable MBP protein concentrations (data not shown).

No differences in gene frequencies or protein concentrations were seen when women and men were compared ($P > 0·05$) (data not shown).

DISCUSSION

Our results show that the point mutation at base 230 in exon 1 which causes a change in codon 54 from GGC to GAC occurs with a remarkably high frequency of 24·4% in the normal Danish population (Table 1). The almost perfect fit to Hardy–Weinberg expectations show that this is a balanced polymorphism. Furthermore, we demonstrate that the point mutation is associated with a dramatic decrease in plasma concentration of MBP. The replacement of GGC (glycine) with GAC (aspartic
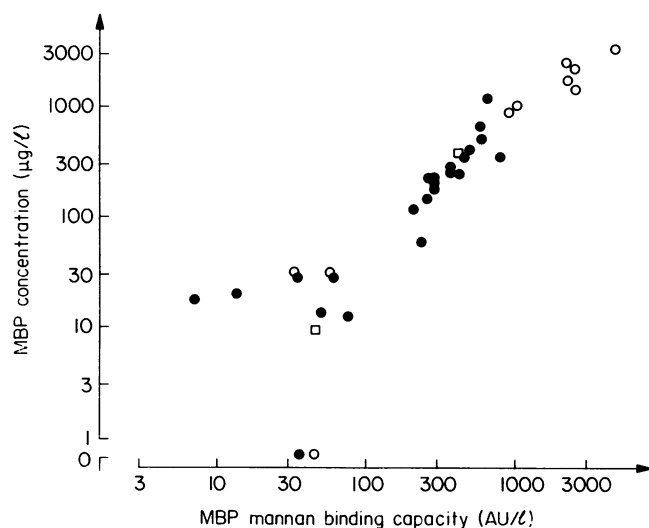
acid) has been suggested to prevent the formation of the normal triple helix found in each MBP subunit [2]. In the heterozygous state it was hypothesized that only one in eight of the triplets (1/2^3) would be normal and secreted, while the remaining 7/8 would be degraded intracellularly. The median concentration in the group containing the GAC allele was 6·4 times lower than in the GGC group, which seems to fit with the above assumption. However, the range in plasma concentrations of MBP was wide, with considerable overlapping between the two groups.

It has been suggested that the point mutation alone should be responsible for low MBP plasma concentrations [6]. Our results can only partly support this view. Largely in agreement with previously published results we found that 12·2% of the individuals had low MBP concentrations (< 60 μg/l) [2]. Nevertheless, 26·6% of these individuals were GGC homozygous. Furthermore, it was possible to detect MBP in both of the two GAC homozygous individuals. In one of them even relatively high amounts (387 μg/l) were found and SDS-PAGE with Western blot analysis revealed that the protein contained MBP polypeptide chains of normal relative mass. Sequencing exon 1 of the MBP gene in this individual revealed no other substitution which might compensate for the GAC influence. However, we cannot exclude the possibility of additional mutations in other parts of the MBP gene. Furthermore, irrespective of genotype there was a close correlation between the concentration of MBP measured as antigen in plasma and the amount of MBP estimated by binding to mannan. Moreover, serum from the GAC homozygous individual mentioned above activated the complement system to a similar degree to sera from GGC homozygotes with comparable MBP protein concentrations. It appears, therefore, that a functional MBP protein encoded by the GAC allele may be found at low concentrations in the circulation.

Several individuals had very low concentrations of MBP although they were homozygous for the GGC allele. It is therefore possible that there are other polymorphic variants of

the MBP gene than the one described so far and investigated in the present study.

Another explanation of our results might be found in the well known regulation of liver protein synthesis by cytokines [11]. Consensus sequences in the promoter region of acute phase proteins are also found upstream of the MBP gene, suggesting a similar regulation of MBP [12]. In support of this notion it has been shown that MBP levels are increased following surgery [10], a procedure known to induce acute phase reactions. Genetic factors outside the MBP gene could thus contribute to the control of the basal plasma level of MBP.

The high frequency (0·13) of the abnormal GAC allele indicates that it cannot just be subject to negative selective pressure, but might also confer some advantage to those carrying it. The nature of such advantage is entirely unknown at present. Nevertheless, it has been argued that some complement deficiencies or polymorphic variants might protect against some of the potential detrimental consequences of complement activation. In particular, it has been suggested that late complement deficiencies might offer a selective advantage in protection against atherosclerosis and septicaemic shock [13–15].

In conclusion, we have shown that a substitution at base 230 of exon 1 in the MBP gene causing a replacement of GGC with GAC has a frequency of 0·13. The substitution dramatically influences the MBP protein concentration. However, a wide and overlapping range in protein concentration was seen between the individuals with and without the substitution. Accordingly, this allelic polymorphism does not explain all cases of MBP deficiency.

ACKNOWLEDGMENTS

REFERENCES

1 Super M, Thiel S, Lu J, Levinsky RJ, Turner MW. Association of low levels of mannan-binding protein with a common defect in opsonisation. Lancet 1989; **2**:1236–9.

2 Turner MW. Deficiency of mannan binding protein—a new complement deficiency syndrome. Clin Exp Immunol 1991; **86**(Suppl.1): 53–56.

3 Turner MW, Super M, Levinsky RJ, Summerfield JA. The molecular basis of a common defect in opsonization. In: Chapel HM, Levinsky RJ, Webster ADB, eds. Progress in immune deficiency III. London—New York: Royal Society of Medicine Services, 1991:177–83.

4 Ikeda K, Sannoh H, Kawasaki N, Kawasaki T, Yamashina I. Serum lectin with known structure activates complement through the classical pathway. J Biol Chem 1987; **262**:7451–4.

5 Lu J, Thiel S, Wiedemann H, Timpl R, Reid KBM. Binding of the pentamer/hexamer forms of mannan-binding protein to zymosan activates the proenzyme C1r2Cls2 complex, of the classical pathway of complement, without involvement of C1q. J Immunol 1990; **144**:2287–94.

6 Sumiya M, Super M, Tabona P *et al.* Molecular basis of opsonic defect in immunodeficient children. Lancet 1991; **337**:1569–70.

7 Böhme J, Owerbach D, Denaro M, Lernmark Å, Peterson PA, Rask L. Human class II major histocompatibility antigen β-chains are derived from at least three loci. Nature 1983; **301**:82–84.

8 Wood WI, Gitschier J, Lasky LA, Lawn RM. Base composition-independent hybridization in tetramethylammonium chloride: a method for oligonucleotide screening of highly complex gene libraries. Proc Natl Acad Sci USA 1985; **82**:1585–8.

9 Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 1977; **74**:5463–7.

10 Thiel S, Holmskov U, Hviid L, Laursen SB, Jensenius JC. The concentration of C-type lectin, mannan-binding protein, in human plasma increases during an acute phase response. Clin Exp Immunol 1992; **90**:31–35.

11 Dinarello CA. Interleukin-1 and the pathogenesis of the acute-phase response. N Engl J Med 1984; **311**:1413–8.

12 Ezekowitz RAB, Day LE, Herman GA. A human mannose-binding protein is an acute-phase reactant that shares sequence homology with other vertebrate lectins. J Exp Med 1988; **167**:1034–46.

13 Geertinger P, Sørensen H. On the reduced atherogenic effect of cholesterol feeding in rabbits with congenital complement (C6) deficiency. Artery 1975; **1**:177–84.

14 Bhakdi S, Hugo F, Tranum-Jensen J. Functions and relevance of the terminal complement sequence. Blut 1990; **60**:309–18.

15 Morgan BP, Walport MJ. Complement deficiency and disease. Immunol Today 1991; **12**:301–6.