

Neoantigens in complement component C3 as detected by monoclonal antibodies

Mapping of the recognized epitopes by synthetic peptides

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The different fragments of the third complement component, C3, generated upon complement activation/inactivation have the ability to bind to several other complement components and receptors as well as to proteins of foreign origin. These multiple reactivities of C3 fragments are associated with a series of conformational changes occurring in the C3 molecule during its degradation. The conformations acquired by the different C3 fragments are also associated with the exposure of neoantigenic epitopes that are specific for (a) particular fragment(s). In order to study these epitopes and thus the conformational changes occurring in C3, monoclonal antibodies (mAbs) recognizing such epitopes were produced in Balb/c mice after immunization with denatured human C3. Two of the three antibodies (7D84.1 and 7D264.6) presented in this study recognized predominantly surface-bound iC3b, and one mAb (7D323.1) recognized both surface-bound and fluid-phase iC3b. Although none of the mAbs recognized any other fluid-phase C3 fragment, all three antibodies detected micro-titre-plate-fixed C3b and iC3b, but not C3c or C3d. In addition to the reaction with human C3, mAb 7D323.1 also bound to micro-titre-plate-fixed rabbit C3. The epitopes recognized by the three mAbs were further localized by using synthetic peptides that were designed on the basis of the differential binding of the mAbs to the C3 fragments. All three antibodies reacted with C3-(924–965)-peptide, which represents the region of C3 between the kallikrein-cleavage site (923–924) and the elastase-cleavage site (965–966). On the basis of the binding of the mAbs to five different overlapping peptides spanning the region between residues 924 and 965 of the human C3 sequence, and the sequence similarity between human C3 and rabbit C3 within this area, the epitopes recognized by these antibodies are mapped. The contribution of the individual amino acid residues in the formation of the epitopes is discussed.

INTRODUCTION

Complement component C3 is one of 30 complement proteins recognized to date. The C3 molecule is comprised of two polypeptide chains with molecular masses of 110 kDa (α -chain) and 75 kDa (β -chain), which are linked by one disulphide bond and non-covalent forces (Janatova, 1986; Matsuda *et al.*, 1985). It contains two *N*-linked carbohydrate moieties at residues 63 and 917 (de Bruijn & Fey, 1985; Hirani *et al.*, 1986). Cleavage of C3 between residues 726 and 727 (Arg-Ser) of the α -chain, by either the classical-pathway C3 convertase or the alternative-pathway C3 convertase, leads to the generation of the C3a and the C3b fragments. The nascent C3b molecule transiently acquires the ability to bind to target molecules through an ester or an amide bond (Gadd & Reid, 1981; Law *et al.*, 1980). In contrast with native C3, C3b expresses multiple binding sites for other complement components and receptors. Binding of Factor B and properdin to C3b leads to the generation of the alternative-pathway C3 convertase and C5 convertase, the latter of which initiates the formation of the membrane-attack complex. C3b is inactivated by Factor I in three different steps, each of which requires one of the several cofactors (MCP, CR1, CR2, H) (Pangburn *et al.*, 1977; Fujita & Nussenzweig, 1979; Medof *et al.*, 1982; Ross *et al.*, 1982; Seya *et al.*, 1986; Mitomo *et al.*, 1987). The cleavage of the α' -chain of C3b, first between residues 1281 and 1282 (Arg-Ser) and secondly between residues 1298 and 1299 (Arg-Ser), liberates the 2 kDa C3f fragment and yields iC3b. The third Factor-I-mediated cleavage, between residues

932 and 933 (Arg-Glu), generates C3d.g with the concomitant liberation of C3c. The generated C3 fragments bind specifically to several cell-surface receptors, known as CR1, CR2, CR3, CR4, CR5 and the C3a receptor, which mediate various biological responses [for reviews on C3 functional sites see Lambris (1988) and Lambris & Müller-Eberhard (1986)].

Sequential exposure of binding sites for different complement components and receptors on the C3 fragments suggests that the molecule undergoes a series of conformational changes. This has been confirmed by spectroscopic and solution scattering analyses of C3 and its fluid-phase fragments C3b, iC3b, C3c and C3d.g (Molenaar *et al.*, 1975; Isenman & Cooper, 1981; Isenman, 1983; Perkins & Sim, 1986). In addition, conformational changes occur upon binding of C3 fragments to different surfaces, as is evident from their ability to bind to complement components and receptors with higher affinity as compared with their corresponding soluble fragments (DiScipio, 1981; Pangburn & Müller-Eberhard, 1983; Ross & Medof, 1985; Becherer *et al.*, 1989). Since the analysis of the conformational changes of surface-bound C3 fragments by physicochemical techniques is hampered by technical difficulties, monoclonal and polyclonal antibodies have been used to show that the conformation of C3 differs depending on its fragmentation stage (Lachmann *et al.*, 1982; Tamerius *et al.*, 1982; Burger *et al.*, 1982, 1988; Aguado *et al.*, 1985; Kanayama *et al.*, 1986; Iida *et al.*, 1987; Garred *et al.*, 1988). In this context, the use of mAbs to study protein conformational changes has proved to be more sensitive than low-resolution spectroscopic methods such as c.d. (Collawn

Abbreviations used: C3, the third component of complement; C3b, iC3b, C3c, C3d.g, C3g and C3d, proteolytic degradation fragments of C3; EAC3b and EAC3bi, C3b and iC3b bound to sheep erythrocytes; mAb, monoclonal antibody.

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et al., 1988). Even though several mAbs have been reported to recognize a specific fragment of C3 (Lachmann *et al.*, 1982; Tamerius *et al.*, 1982; Burger *et al.*, 1982, 1988; Aguado *et al.*, 1985; Kanayama *et al.*, 1986; Iida *et al.*, 1987; Garred *et al.*, 1988), only three epitopes recognized by anti-C3 mAbs have been mapped. The first one to be described was mAb 130, which binds to an epitope that is exposed concomitantly with the binding site of CR2 in the iC3b, C3d,g and C3d fragments of C3 (Tamerius *et al.*, 1985; Lambris *et al.*, 1985, 1990). The epitope recognized by mAb 130 and the receptor-binding site have been localized by means of overlapping synthetic peptides to amino acid residues 1199–1249 and 1199–1210 respectively (Lambris *et al.*, 1985, 1990). The other two antibodies, mAb H453 and mAb H454, recognize neoantigenic epitopes in the C3a fragment that reside within the eight C-terminal amino acid residues of C3a (Burger *et al.*, 1988).

Several neoantigens, designated C3(D), have previously been identified and found to be expressed solely by surface-bound C3 fragments and not by the corresponding soluble proteins (Nilsson & Nilsson, 1982; Nilsson *et al.*, 1989). The location of these epitopes within C3 as well as their nature have not been elucidated. In the present study we characterize three epitopes exposed specifically on the iC3b fragment of C3 by using mAbs and synthetic peptides. Two of the epitopes are expressed by surface-bound iC3b and one by both surface-bound and fluid-phase iC3b.

MATERIALS AND METHODS

Materials

Trypsin was purchased from Worthington Corp. (Freehold, NJ, U.S.A.). Sephadex G-100 and Protein A–Sepharose were obtained from Pharmacia AB (Uppsala, Sweden). Biotin-aminohexanoate *N*-hydroxysuccinimide ester was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Horseradish-peroxidase-conjugated anti-(mouse Ig) antibody was purchased from Dako Immunoglobulins (Copenhagen, Denmark). Micro-titre plates (Immunoplates IIF) and micro-titre plate strips were obtained from Nunc (Roskilde, Denmark). Enzymobeads were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Carrier-free Na¹²⁵I was purchased from Amersham International (Amersham, Bucks., U.K.).

Complement components

Human C3 was purified as described by Hammer *et al.* (1981). Factor I (Fearon & Austen, 1977) and Factor H (Nilsson *et al.*,

1975) were isolated from serum as previously described. C3b was generated by incubating purified C3 with 1% (w/w) trypsin for 2 min at room temperature followed by gel-filtration on a Sephadex G-100 column (2.5 cm × 80 cm) equilibrated with phosphate-buffered saline (0.154 M-NaCl/20 mM-sodium phosphate buffer, pH 7.4) iC3b was produced by incubating C3b (1 mg/ml) with Factor I (33 µg/ml) and Factor H (170 µg/ml) in phosphate-buffered saline for 60 min at 37 °C. C3c and C3d were generated by incubating C3 with 2% (w/w) trypsin for 60 min at 37 °C as described by Eggertsen *et al.* (1985). Rabbit C3 was purified as previously described (Horstmann & Müller-Eberhard, 1985).

Preparation of erythrocytes coated with C3 fragments

EAC14^{oxy}23b (EAC3b) and EAC14^{oxy}23bi (EAC3bi) were prepared and the amount of C3 fragments bound to the cells was calculated by using ¹²⁵I-labelled native C3 as previously described (Nilsson & Nilsson, 1985).

Monoclonal antibodies

Monoclonal antibodies (mAbs) was produced in BALB/c mice against SDS-denatured reduced C3 according to the procedure described previously (Nilsson *et al.*, 1987). The mAbs were purified by affinity chromatography on Protein A–Sepharose. Culture supernatants containing IgG mAbs were adjusted to 3 M-NaCl/1.5 M-glycine, pH 8.9, and applied to a 4 ml Protein A–Sepharose column. After a washing, the antibodies were eluted with 0.1 M-glycine, pH 2.8. The antibodies were designated 7D84.1, 7D264.6 and 7D323.1. The antibodies were shown by radial immunodiffusion to belong to the IgG1 subclass.

Protein iodination

Human C3 and mouse mAbs were labelled with ¹²⁵I by using Enzymobeads according to the manufacturer's recommendations (Nilsson & Nilsson, 1985). C3 retained its haemolytic activity after the labelling procedure, as determined by haemolytic titration with a C3-depleted serum (Nilsson & Nilsson, 1984).

Synthetic peptides

The synthesis of C3-(924–965)-peptide and C3-(1402–1435)-peptide has been previously described (Becherer & Lambris, 1988; Daoudaki *et al.*, 1988). These and other peptides (Fig. 1) were synthesized by using an Applied Biosystems 430A peptide synthesizer according to the Merrifield solid-phase method on a 4-methylbenzhydrylamine resin (Merrifield, 1963; Barany & Merrifield, 1980). Purification and analysis of peptides were

Peptide	Sequence
	Factor I
	↓
C3-(929–936)-peptide	RLGR EG VQ
C3-(938–946)-peptide	EDIPPADLS
C3-(933–946)-peptide	EGVQKEDIPPADLS
C3-(929–946)-peptide	RLGR EGVQKEDIPPADLS
C3-(924–946)-peptide	TLDPERLGR EGVQKEDIPPADLS
C3-(924–965)-peptide	TLDPERLGR EGVQKEDIPPADLSDQVPDTESETRILLQGTPV
C3-(1361–1381)-peptide	TMILEIATRYRGDQDATMSIL
C3-(1282–1298)-peptide	SSKITHRIHWESASLLR
C3-(1402–1435)-peptide	GVDRIYSKYELDKAFSDRNTLLIYLDKVSHSSEDD

Fig. 1. Amino acid sequences of the synthetic peptides used in this study

In the synthesis of C3-(1361–1381) the Cys-1367 residue was replaced by alanine. The numbering of the peptide residues is based on their position in the predicted amino acid sequence of C3 (de Bruijn & Fey, 1985) after subtraction of the signal peptide sequence (22 residues). The arrow between amino acid residues 932 and 933 corresponds to the third Factor-I-mediated cleavage of iC3b.

performed as previously described (Lambris *et al.*, 1988; Becherer & Lambris, 1988; Daoudaki *et al.*, 1988).

Assays for binding of mAbs to C3 fragments and synthetic peptides

Three different types of assays were employed in order to assess the binding of the mAbs to epitopes expressed by the solid-phase or the fluid-phase C3 fragments and synthetic peptides.

(1) The binding of mAbs to micro-titre-plate-fixed antigens was analysed by e.l.i.s.a. Micro-titre plates were coated with 50 µl of serial dilutions or constant amounts of C3, C3 fragments (highest concentration 20 µg/ml) or synthetic peptides (100 µg/ml) for 2 h at room temperature. After saturation with 1% (w/v) BSA, 50 µl of constant amounts of mAb (to serial dilutions of coated antigen) or serially diluted mAb (to constant amounts of coated antigen) in phosphate-buffered saline containing 1% (w/v) BSA was added to the plate and incubated for 30 min at room temperature. The binding of mAb was detected by horseradish-peroxidase-conjugated rabbit anti-(mouse Ig) antibody.

(2) The binding of mAbs to fluid-phase C3 and C3 fragments, synthetic peptides or C3 fragments fixed to erythrocytes was tested in an inhibition e.l.i.s.a. This was a modification of the e.l.i.s.a. described above. Briefly, 50 µl of serially diluted test antigen was preincubated with 50 µl of a constant amount of monoclonal anti-C3 antibody (0.5–1 µg/ml) for 60 min at room temperature. After centrifugation the supernatants were tested for binding to micro-titre-plate-fixed C3 in the above-described direct-binding e.l.i.s.a.

(3) The determination of duplicate antibodies (recognizing the same epitope) was done by a radioimmunoassay. A 200 µl portion of C3 (12.5 µg/ml) in phosphate-buffered saline was adsorbed on micro-titre plate strips overnight at 4 °C. Then 50 µl of serially diluted competing monoclonal anti-C3 antibody was

incubated with 50 µl of a constant amount of ¹²⁵I-labelled mAb 7D84.1 or mAb 7D323.1 in the wells for 60 min at room temperature. The wells were rinsed with phosphate-buffered saline containing 0.1% Tween 20, then cut out, and the radioactivity was monitored with a γ-radiation counter.

RESULTS

Binding of monoclonal anti-C3 antibodies to C3 fragments

The monoclonal anti-C3 antibodies 7D84.1, 7D264.6 and 7D323.1 were tested for their ability to bind to C3 fragments fixed to micro-titre plates. A constant amount of the three monoclonal anti-C3 antibodies (3–5 µg/ml) was allowed to bind to serially diluted C3, C3b, iC3b, C3c or C3d (20–0.28 µg/ml). All antibodies bound to C3, C3b and iC3b with similar affinity, but no binding to C3c or C3d was observed. Furthermore, no significant differences in binding strength between the antibodies could be seen (Table 1).

The reactivity of the three mAbs for fluid-phase C3, C3b, iC3b and C3c and for EAC3b and EAC3bi was analysed in the inhibition e.l.i.s.a. (Fig. 2 and Table 1). The binding of mAbs 7D84.1 and 7D323.1 was inhibited by both EAC3bi cells and fluid-phase iC3b, whereas that of mAb 7D264.6 was inhibited only by EAC3bi. The doses of fluid-phase iC3b required for a 50% inhibition of the binding of mAbs 7D323.1 and 7D84.1 to micro-titre-plate-fixed C3 were respectively approximately 2-fold and 64-fold higher than that required for a 50% inhibition by erythrocyte-bound iC3b. Fluid-phase iC3b even at a dose of 4 pmol only partially inhibited binding of mAbs 7D84.1 and 7D323.1 to C3 (by 60% and 20% respectively). None of the other fluid-phase C3 or C3 fragments affected the binding of any of the antibodies. EAC3b cells (having fixed 4 pmol of C3b) only inhibited the binding of mAb 7D84.1 (by 40%).

In order to determine whether the three mAbs bound to the

Table 1. Binding of the monoclonal anti-C3 antibodies to C3 fragments and synthetic peptides

Antigen	Binding		
	mAb 7D84.1	mAb 7D264.6	mAb 7D323.1
Direct-binding e.l.i.s.a.			
Component C3	+	+	+
Fragment C3b	+	+	+
Fragment iC3b	+	+	+
Fragment C3c	–	–	–
Fragment C3d	–	–	–
C3-(929–936)-peptide	–	+	–
C3-(938–946)-peptide	–	–	–
C3-(933–946)-peptide	–	–	–
C3-(929–946)-peptide	+	+	+
C3-(924–946)-peptide	+	+	+
C3-(924–965)-peptide	+	+	+
Inhibition e.l.i.s.a.			
Component C3	–	–	–
Fragment C3b	–	–	–
Fragment iC3b	+(64*)	–(> 16*)	+(2*)
Fragment C3c	–	–	–
Fragment EAC3b	(+)	–	–
Fragment EAC3bi	+	+	+
C3-(929–936)-peptide	–	–	–
C3-(938–946)-peptide	–	–	–
C3-(933–946)-peptide	–	–	–
C3-(929–946)-peptide	+	+	–
C3-(924–946)-peptide	+	+	–
C3-(924–965)-peptide	+	+	–

* The difference in dose between the soluble and the corresponding bound test antigen in the inhibition e.l.i.s.a.

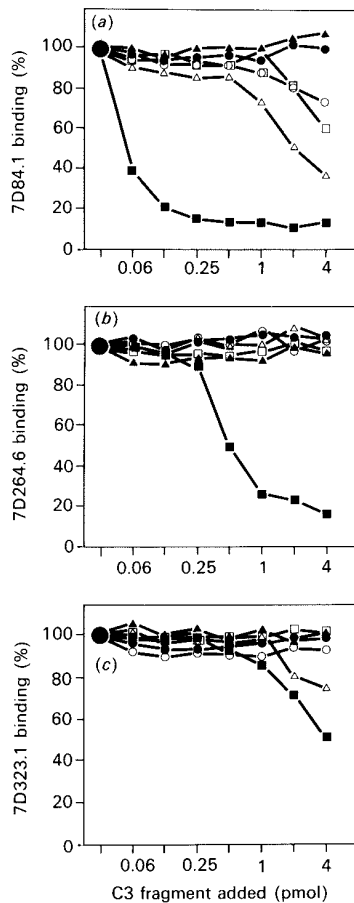


Fig. 2. Inhibition by C3 or C3 fragments of mAb binding to C3

Constant amounts of mAb 7D84.1 (a), mAb 7D264.6 (b) and mAb 7D323.1 (c) (3–6 pmol) preincubated with serially diluted C3 (○), C3b (●), iC3b (△), C3c (▲), EAC3b (□) or EAC3bi (■) (0.03–4 pmol) for 60 min at room temperature were incubated with C3 fixed to micro-titre plates (20 µg/ml). After incubation for 30 min at room temperature the bound antibody was detected by horseradish-peroxidase-conjugated rabbit anti-(mouse Ig) antibody.

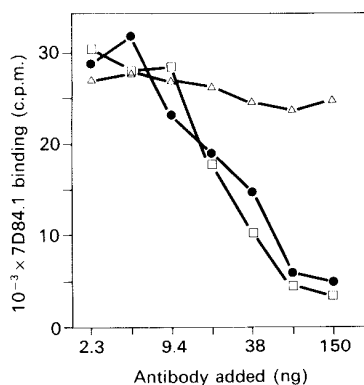


Fig. 3. Competitive inhibition by anti-C3 mAbs of the binding of ¹²⁵I-labelled mAb 7D84.1 to C3

A constant amount of ¹²⁵I-labelled mAb 7D84.1 was allowed to bind to C3 fixed to wells of micro-titre strips (12.5 µg/ml) in the presence of serially diluted non-labelled mAb 7D84.1 (□), mAb 7D264.6 (●) and mAb 7D323.1 (△). After the wells had been washed the radioactivity was monitored with a γ -radiation counter.

same or closely located epitopes, were performed cross-blocking experiments. To this end they were tested for their ability to inhibit the binding of ¹²⁵I-labelled mAb 7D84.1 (Fig. 3) or 7D323.1 (results not shown) to micro-titre-plate-fixed C3. Both mAb 7D84.1 and mAb 7D264.6 completely inhibited the binding of the ¹²⁵I-labelled 7D323.1 and 7D84.1 antibodies to C3, whereas mAb 7D323.1 completely inhibited the binding of ¹²⁵I-labelled mAb 7D84.1. These results showed that all three antibodies detect identical or closely located epitopes expressed by the bound and/or fluid-phase iC3b. The epitopes recognized by mAbs 7D264.6 and 7D84.1 are preferentially expressed by the bound form of the fragment.

Mapping of epitopes recognized by the anti-C3 mAbs by using synthetic peptides and rabbit C3

Since all three antibodies bound to micro-titre-plate-fixed C3b and iC3b but not to trypsin-generated C3c or C3d, we synthesized the peptide [C3-(924–965)-peptide] covering the segments of C3 between the kallikrein-cleavage site (923–924) and the elastase-cleavage site (965–966) (residues 924–965). This is the segment of C3 that is present in C3b and iC3b, but not in C3c or C3d.

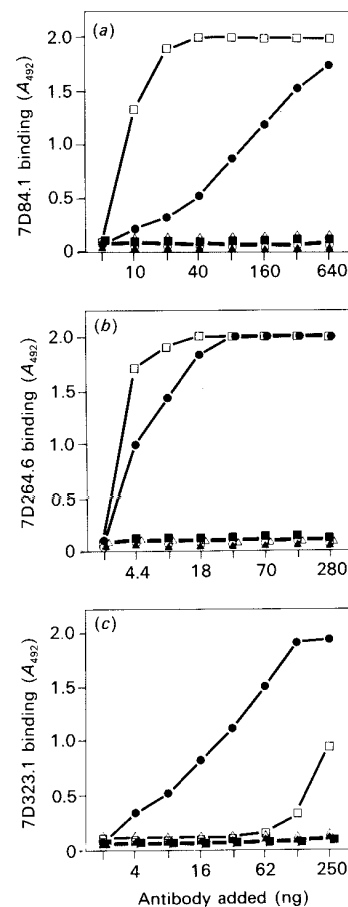


Fig. 4. Binding of mAbs to C3 and synthetic peptides

Serially diluted mAb 7D84.1 (a), mAb 7D264.6 (b) and mAb 7D323.1 (c) were incubated with micro-titre-plate-fixed C3 (●) (20 µg/ml) or synthetic C3-(924–965)-peptide (□), C3-(1402–1435)-peptide (△), C3-(1361–1381)-peptide (▲) and C3-(1282–1298)-peptide (■) (100 µg/ml) for 30 min at room temperature. The bound mAb was detected by horseradish-peroxidase-conjugated rabbit anti-(mouse Ig) antibody.

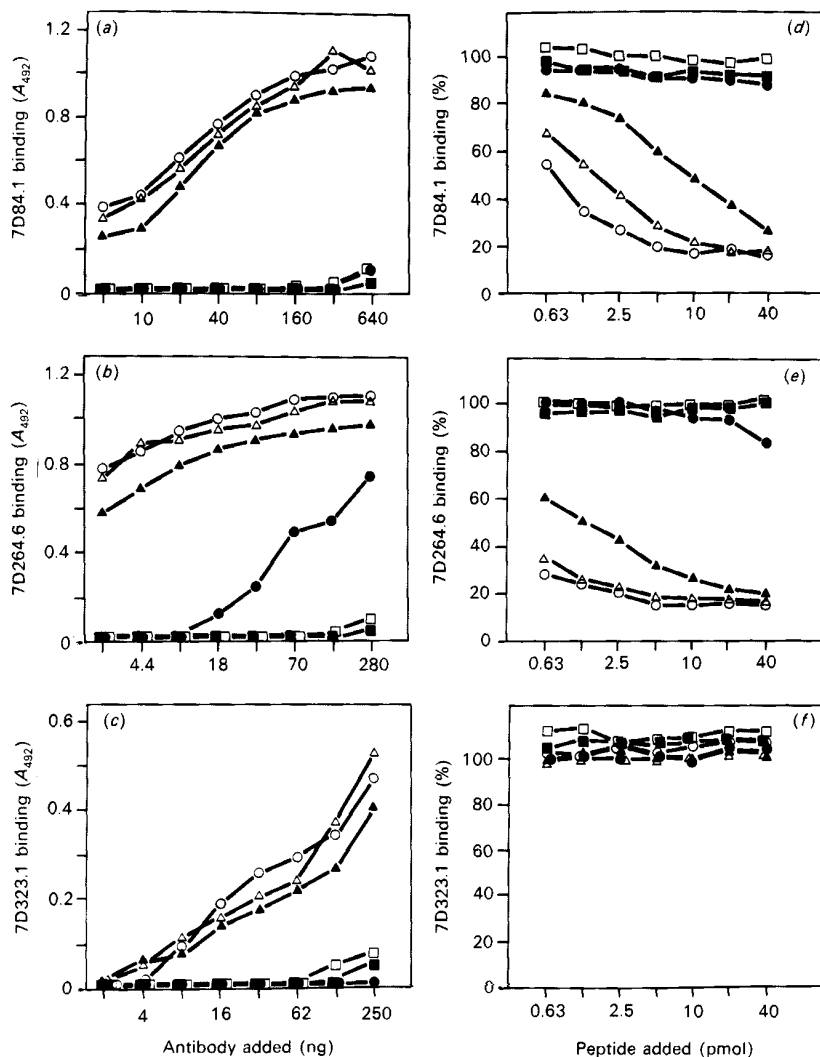


Fig. 5. Binding of mAbs to overlapping synthetic peptides

(a)–(c) Direct binding of mAbs to synthetic peptides. The binding of mAbs 7D84.1 (a), mAb 7D264.6 (b) and mAb 7D323.1 (c) to synthetic C3-(929–936)-peptide (●), C3-(938–946)-peptide (□), C3-(933–946)-peptide (■), C3-(929–946)-peptide (△), C3-(924–946)-peptide (▲) and C3-(924–965)-peptide (○) was analysed as in Fig. 4. (d)–(f) Inhibition by C3 synthetic peptides of mAb binding to C3. C3 was fixed to micro-titre plates. Constant amounts of mAb 7D84.1 (d), mAb 7D264.6 (e) and mAb 7D323.1 (f) (0.5–1 μ g/ml) preincubated with serially diluted (0.63–40 pmol) C3-(929–936)-peptide (●), C3-(938–946)-peptide (□), C3-(933–946)-peptide (■), C3-(929–946)-peptide (△), C3-(924–946)-peptide (▲) and C3-(924–965)-peptide (○) for 60 min at room temperature were tested for binding to C3 as in Fig. 2.

The reactivity and the specificity of the monoclonal anti-C3 antibodies for C3-(924–965) and for other C3 peptides were tested by e.l.i.s.a. Fig. 4 and Table 1 show that all three antibodies bound to peptide C3-(924–965)-peptide but not to C3-(1282–1298)-peptide, C3-(1361–1381)-peptide or C3-(1402–1435)-peptide. When the binding of the mAbs to C3-(924–965)-peptide was compared with that to C3, it was found that mAb 7D264.6 bound equally well to both of them whereas mAb 7D84.1 was 32-fold more reactive with the peptide and mAb 7D323.1 was 8-fold more reactive with C3.

In order to identify the amino acid residues within C3-(924–965)-peptide involved in the binding of each of the monoclonal anti-C3 antibodies, five different overlapping peptides were synthesized [C3-(929–936)-peptide, C3-(938–946)-peptide, C3-(933–946)-peptide, C3-(929–946)-peptide and C3-(924–946)-peptide] (Fig. 1), and their ability to react with the mAbs was

analysed by e.l.i.s.a. All three antibodies bound to C3-(929–946)-peptide and C3-(924–946)-peptide but not to C3-(938–946)-peptide or C3-(933–946)-peptide (Fig. 5a and Table 1). The mAb 7D264.6 also reacted with C3-(929–936)-peptide. In addition to the direct binding of mAbs to the peptides, the ability of the peptides to inhibit the binding of mAbs to C3 was analysed. Fig. 5(b) and Table 1 show that the binding of mAbs 7D84.1 and 7D264.6 was inhibited by C3-(929–946)-peptide, C3-(924–946)-peptide and C3-(924–965)-peptide, but not by C3-(938–946)-peptide, C3-(933–946)-peptide or C3-(929–936)-peptide. The binding of mAb 7D323.1 was not affected by any of the synthetic peptides.

In order to analyse further the fine specificity of the three antibodies, we tested their ability to bind to micro-titre-plate-fixed rabbit C3 (its primary sequence is known). Fig. 6 demonstrates that only mAb 7D323.1 bound to rabbit C3.

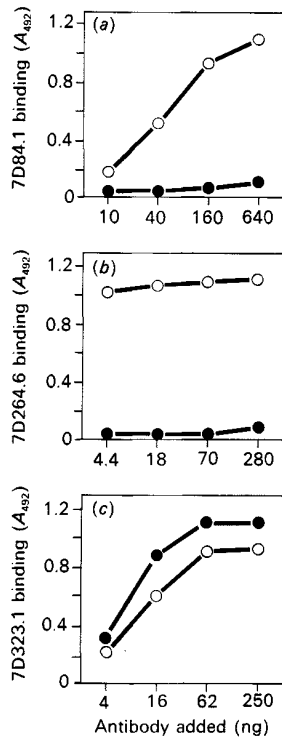


Fig. 6. Binding of mAbs to human C3 and rabbit C3

Serially diluted mAb 7D84.1 (a), mAb 7D264.6 (b) and mAb 7D323.1 (c) were incubated with micro-titre-plate-fixed human C3 (○) (20 μ g/ml) or rabbit C3 (●) (20 μ g/ml) for 30 min at room temperature. The bound mAb was detected by horseradish-peroxidase-conjugated rabbit anti-(mouse Ig) antibody.

DISCUSSION

During degradation of C3, its fragments sequentially acquire the ability to bind to other complement components and receptors. This ability of C3 fragments is associated with a series of conformational changes occurring in the C3 molecule and its fragments. The analysis of these conformational changes by means of physicochemical techniques, especially in surface-bound C3 fragments, is hampered by technical difficulties. Since mAbs have been shown to be excellent probes for analysing conformational changes in a protein (Collawn *et al.*, 1988), we produced mAbs specific for the iC3b fragment of C3 and mapped the epitopes recognized by these antibodies by using synthetic peptides.

The epitopes recognized by mAbs 7D84.1, 7D264.6 and 7D323.1 are expressed specifically by the iC3b fragment of C3. The mAbs 7D84.1 and 7D264.6 recognize predominantly bound iC3b, whereas mAb 7D323.1 recognizes equally well fluid-phase

and surface-bound iC3b. It is possible that the difference in specificity for soluble and bound iC3b is in fact even more prominent, since the preparation of soluble iC3b is likely to contain dimers of iC3b (Arnaout *et al.*, 1981), which may react as EAC3bi.

All three antibodies were shown to bind to the synthetic peptides C3-(924-965)-peptide, C3-(929-946)-peptide and C3-(924-946)-peptide, but not to C3-(938-946)-peptide or C3-(933-946)-peptide. Furthermore, mAb 7D264.6 reacted with C3-(929-936)-peptide. This suggests that all antibodies require residues 929-932 in order to react with iC3b. This reactivity and the finding that only mAb 7D323.1 reacted with rabbit C3 suggest (a) that one or more of the residues Arg-929, Arg-932 and Glu-933 are essential for binding of mAbs 7D84.1 and 7D264.6, and (b) that the above-mentioned residues as well as Asp-939, Pro-942 and Leu-945 are not essential for binding of mAb 7D323.1 (Fig. 7). Furthermore, this reactivity, in conjunction with the differential reactivity of the mAb with synthetic peptides and fluid-phase versus surface-bound iC3b, showed that each antibody had a distinctive specificity. In addition to the three epitopes described here, one further epitope between residues 929 and 946 has been localized by using the monoclonal anti-iC3b antibody clone 9, which recognizes both fluid-phase and bound iC3b (Lachmann *et al.*, 1982). The epitope recognized by clone 9 has been shown to reside between residues 933 and 946 of C3 (Myones *et al.*, 1989). Thus the region of C3 between residues 929 and 946 contains at least four different overlapping epitopes. The existence of different epitopes within such a limited amino acid sequence is not very surprising, since similar observations have been made earlier showing that three different epitopes exist within an α -helix region of myohaemerythrin (residues 69-87) (Fieser *et al.*, 1987).

Cleavage of C3b to iC3b by Factor I leads to the appearance of iC3b specific sites. One of these is for complement receptor 3 (CR3) and another is for conglutinin (Lambris *et al.*, 1990). The binding site for conglutinin has been localized to the carbohydrate moiety linked to residue 917 of C3 (de Bruijn & Fey, 1985; Hirani *et al.*, 1986). This, taken together with the position of the epitopes specifically expressed in the iC3b fragment, suggests that the segment covered by residues 917-946 of C3 is masked in C3b but exposed when it is cleaved to iC3b. This is in agreement with the appearance of (a) new Factor-I-cleavage site(s) upon generation of the iC3b fragment. The cleavage of iC3b by Factor I occurs between residues 932 and 933 and generates C3c and C3d,g (Davis *et al.*, 1984; de Bruijn & Fey, 1985). A fragment similar to C3d,g is generated upon cleavage of iC3b with kallikrein (Meuth *et al.*, 1983; Davis *et al.*, 1984; de Bruijn & Fey, 1985). This fragment, designated C3dk, extends nine amino acid residues from the N-terminus of C3d,g and has been shown to induce leucocytosis and increased vascular permeability. Similar leucocytosis activity has been reported for the peptide covering these nine residues of C3 [C3-(924-932)-peptide] (Hoeprich *et al.*, 1985). Although C3d,g has been reported not to have leucocytosis activity (Hoeprich *et al.*, 1985), a C3d,g-like



Fig. 7. Amino acid sequence similarity between human C3 and rabbit C3 in the region recognized by the mAbs

The deduced amino acid sequence between residues 924 and 946 of human C3 (de Bruijn & Fey, 1985) and the corresponding amino acid sequence of rabbit C3 (Kusano *et al.*, 1986) are aligned. Identical amino acid residues are indicated by asterisks (*) and the Factor-I-cleavage site by an arrow.

fragment produced by Factor I has been shown to express similar activities to those of C3dk (Seya & Nagasawa, 1985). This latter finding suggests that Factor I, in addition to cleaving iC3b between residues 932 and 933, may also cleave it at another site, generating fragments with C3dk-like activity. By using the above-described mAbs to detect the Factor-I-generated fragments, it was found that Factor I indeed cleaves iC3b at three different positions (Nilsson Ekdahl *et al.*, 1990).

In addition to using the described antibodies for the study of conformational changes and other structural features in C3, they are also likely to be used in diagnostic medicine. For example, they could be used for the detection of the C3dk fragment in plasma of patients with hereditary angioneurotic oedema. It has been reported that plasma from these patients contains an increased amount of C3dk fragment as a consequence of decreased inhibition of kallikrein (Seya *et al.*, 1985). Furthermore, since the antibodies have a specificity similar to that of conglutinin, i.e. to bind iC3b, they could be used in the detection of C3-bearing immune complexes in plasma specimens from patients with immune complex disease.

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