Structural and Functional Analysis of C3 Using Monoclonal Antibodies

J. Alsenz 1*, J. D. Becherer 1, B. Nilsson 2, and J. D. Lambris 1

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1 Introduction

Monoclonal antibodies (MoAbs) have greatly facilitated the structural and functional analysis of proteins in general and of the third protein of complement (C3) in particular. Various aspects of the structure and functions of C3 have been addressed using MoAbs; these include: (a) the study of conformational changes occurring in the C3 molecule and its fragments during complement activation, (b) the analysis of the interactions of C3 with other complement components and receptors as well as with proteins of foreign origin, and (c) the detection of C3 activation products in biological fluids. The purpose of this review is to summarize the contribution that MoAbs have made in understanding the structure and functions of C3.

2 Generation of Anti-C3 Monoclonal Antibodies

Although the generation of MoAbs has become a routine procedure, a few interesting points have been observed concerning the nature of the immunizing antigen and the subsequent specificity of the anti-C3 MoAb. In the most commonly used immunization

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<table>
<thead>
<tr>
<th>MoAb</th>
<th>Fragment recognized</th>
<th>Neoantigen expressed in</th>
<th>Inhibition of the C3 interaction with</th>
<th>References</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>H</td>
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<td>C3a</td>
<td>C3*/C3a</td>
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<td>C3a</td>
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<td>H453, H454</td>
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<td>C3-1</td>
<td>C3c</td>
<td>C3*/C3b/C3c</td>
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<tr>
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<td>C3*/C3b/C3c</td>
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<td>C3b/C3c</td>
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<tr>
<td>bh6</td>
<td>C3c</td>
<td>C3b/C3b/C3c</td>
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<tr>
<td></td>
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<td></td>
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<tr>
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<td>sb° C3b/C3b</td>
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<td>Ab 84</td>
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<td>311</td>
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<td>C3d</td>
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<td>C3d</td>
<td>-</td>
<td>+</td>
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</tr>
<tr>
<td>H11</td>
<td>β-chain</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>H2</td>
<td>β-chain</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

C3* = C3(H2O); sb° = surface bound.

Other anti-C3 MoAbs reacting with:
- C3: anti-FG11 (Ma et al. 1985); HAV-4 (Koch and Behrendt 1986)
- C3a: H13 (Burger et al. 1987); MoAb 868 (Klos et al. 1988)
- C3b: MoAb 755 (Klos et al. 1988)
- C3c: clone 4 (Lachmann et al. 1980); N-7A (Iida et al. 1987a); WM-1 (Whitehead et al. 1981); MoAb-BRL (Agudo et al. 1985); anti-C3c (22 different MoAb) (Dobbee et al. 1987)
- C3d: Fc 112, Fc 283; Ortho (Chaplin and Monroe 1986); anti-C3c (nine different MoAbs) (Dobbee et al. 1987); 3D4H3 (Ruddy et al. 1983); BRIC 8 (Holt et al. 1985)
have been identified in C3b, one in C3d and another in C3c (GANU and MÜLLER-
EBERHARD 1985; LAMBRIS et al. 1988; BECHERER et al. 1989a; LAMBRIS et al. 1989;
BECHERER et al. 1989c). The H binding site in C3d is associated with the CR2 site
while the site in C3c is within the domain of C3 containing the B and CR1 binding
sites. It is interesting that the latter site contains a sequence (between residues 744
and 755) that is similar to the sequence of the CR2 binding site in C3d (for more
details see BECHERER et al. 1989a). Furthermore, a third sequence similar to the CR2
binding site has been identified in the β-chain of C3 (between residues 295 and 306),
and a peptide spanning this sequence has been found to bind to CR2 (ESPAZZA et
al. 1989). Although a direct binding of CR1, H, or B to this peptide has not yet been
determined, this may explain the finding that MoAbs to the β-chain of C3 have also
been found to inhibit H, CR1, and B binding to C3b (WÖRNER et al. 1989).

The selective inhibition by MoAbs of either B (498, 105, 111) or H binding (311,
Ab 12, 14, 72, and 84) to C3b (TAMERIUS et al. 1982; BURGER et al. 1982) suggests
the presence of distinct binding sites for B and H on C3b. This is not necessarily in
contrast to the above findings and might be attributed to the interaction of these
molecules with C3b via multiple sites. In this context, it has been observed that
MoAb 130 inhibits CR2 but not H binding to C3d; both sites are located within
residues 1187–1249 of C3. The latter fact further supports the findings with synthetic
peptides which have shown that these two sites are related but are not the same
(LAMBRIS et al. 1985, 1988). Of interest also is the recent finding that MoAbs to C3
which binding H were also found to inhibit the binding of glycopolypeptide C(gC) of herpes
simplex virus type 1 (HSV-1) to C3b (HUEMER et al. 1989). However, the inhibition
of H but not gC binding to C3b by other antibodies (Table 1) may explain the
functional differences between these two molecules: H has both cofactor and
decay-accelerating activity while gC has only the latter.

The interaction of properdin with C3b, as investigated by MoAbs to either C3c
or C3d (Table 1; TAMERIUS et al. 1982), suggests a two-side interaction between these
two molecules. Since the site in C3c for P has recently been localized to residues
1402–1435 of C3 (DAOUDAKI et al. 1988), it will be of interest to see whether these
anti-C3c antibodies react with this region of C3, or whether the observed inhibition
is indirect (steric or allosteric effects, etc.). The presence of a second P interaction site
in C3b remains to be determined.

The epitopes recognized by several other MoAbs (Table 1) have not yet been
localized, and this makes it difficult to correlate the structural elements involved in
antibody binding to those involved in ligand binding. In addition, MoAbs which
bind close to a functional site in C3 (see also Fig. 1) were found not to inhibit its
reactivity with the ligand. However, this is not necessarily unexpected since it has
been shown that a peptide, 24 amino acids in length, is able to bind two different
antibodies simultaneously (JACKSON et al. 1988).

3.3 Mapping of the Epitopes Recognized by the Monoclonal Antibodies

A major breakthrough in understanding antibody-antigen interactions was provided
by the crystallographic studies of Fab fragments complexed to the protein antigens
lysozyme (AMIT et al. 1986) and influenza virus neuraminidase (COLMAN et al. 1987).
A. PROTEIN

C3
→ Enzymatic or chemical fragmentation
→ Fragment purification and analysis for their binding to MoAbs or C3-binding proteins
→ Sequencing of the fragment of interest
→ Synthesis of peptides corresponding to segments of the active fragment
→ Analysis of peptides for binding to MoAbs or C3-binding proteins

B. DNA

C3 cDNA
→ DNAase digestion to generate 200-300 nucleotide fragments
→ Construction of an expression cDNA library
→ Screening of the library with anti-C3 MoAbs or C3-binding proteins
→ DNA sequencing of the inserted fragments from the reactive clones

C. CONSERVATION OF THE LIGAND BINDING SITES AND MoAb-RECOGNIZED EPITOPES IN C3

Analysis of the MoAb binding to C3 from different species or of the C3-ligand interactions between different species.
→ Comparison of the amino acid sequence of C3 and other homologous proteins from different species.
→ Analysis of the amino acid similarities, in conjunction with the results from approach A and B, for determining the structural elements in C3 important for binding of MoAb and C3-binding proteins.

Fig. 2. Schematic representation of a general strategy to localize MoAb-recognized epitopes and ligand binding sites in C3

The third approach deals with the conservation of antigenic and functional sites within C3 from different species and other homologous proteins such as C4, C5, and α2-macroglobulin. First, C3 from different species is purified and tested for its ability to bind to MoAbs or to the various human C3 ligands (ALSENZ et al. 1989; BECHERER et al. 1987). These proteins can then be sequenced at the DNA level, thus allowing analysis of the conservation of epitopes or binding sites between different species. This, together with the conserved sequences of other homologous proteins that do or do not bind the MoAb or ligand of interest, offers a wealth of information on the structural features of the C3 molecule. Concerning this third approach, the complete amino acid sequences of human and mouse C3 (DE BRUIN and FEY 1985; LUNDWALL et al. 1984; WETSEL et al. 1984) and the partial sequences of rabbit (KUSANO et al. 1986) and Xenopus C3 (GROSSBERGER et al. 1989) have been resolved recently.
Using the above approaches, various epitopes recognized by anti-C3 MoAbs have been localized (Table 2). Several of the antibodies recognize epitopes located within or close to functional regions of C3 and therefore have been used as tools to analyze its functions. For example, the MoAb 130 was localized to a discontinuous epitope within residues 1192-1249 of C3 (LAMBRIS et al. 1989). This MoAb inhibits the binding of C3d to CR2 and assists in the localization of the CR2 binding site within this fragment to residues 1199–1210 (LAMBRIS et al. 1985).

### Table 2. Summary of the monoclonal anti-C3 antibodies whose epitopes in C3 have been mapped

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>C3-fragment(s) recognized</th>
<th>Residues</th>
<th>References</th>
</tr>
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<tr>
<td>H11a,b</td>
<td>C3b/C3c/β-chain</td>
<td>1-645</td>
<td>WÖRNER et al. 1989</td>
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<tr>
<td>GV18b</td>
<td>β-chain/CHO</td>
<td>63*</td>
<td>GRIER et al. 1987</td>
</tr>
<tr>
<td>GV1.10b</td>
<td>γβ-chain, CHO</td>
<td>63*, 917*</td>
<td>GRIER et al. 1987</td>
</tr>
<tr>
<td>H7b</td>
<td>C3b/C3c/β-chain</td>
<td>89-645</td>
<td>WÖRNER et al. 1989</td>
</tr>
<tr>
<td>H11a,b</td>
<td>C3b/C3c/β-chain</td>
<td>98-293</td>
<td>WÖRNER et al. 1989</td>
</tr>
<tr>
<td>H2C</td>
<td>C3b/C3c/β-chain</td>
<td>98-293</td>
<td>WÖRNER et al. 1989</td>
</tr>
<tr>
<td>H2D</td>
<td>C3b/C3c/β-chain</td>
<td>98-293</td>
<td>WÖRNER et al. 1989</td>
</tr>
<tr>
<td>H453, H454a,i</td>
<td>C3a</td>
<td>718-725</td>
<td>BURGER et al. 1988</td>
</tr>
<tr>
<td>398.1d,i</td>
<td>sb C3b</td>
<td>741-758</td>
<td>BECHERER, NILSSON, LAMBRIS unpublished</td>
</tr>
<tr>
<td>406.4d,i</td>
<td>sb C3b</td>
<td>741-758</td>
<td>BECHERER, NILSSON, LAMBRIS unpublished</td>
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<td>7D 84.1d,i</td>
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<td>926-946</td>
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<td>7D 323.1d,i</td>
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<td>926-946</td>
<td>NILSSON et al. 1989a</td>
</tr>
<tr>
<td>Clone 9d,i</td>
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<td>933-946</td>
<td>MYINNS et al. 1989</td>
</tr>
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<td>7D 9.2d,i</td>
<td>sb C3b</td>
<td>1082-1118</td>
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<td>MoAb 130f,i</td>
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<td>sb C3b</td>
<td>1476-1510</td>
<td>NILSSON et al. 1989a</td>
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<tr>
<td>H215,i</td>
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<td>1476-1531</td>
<td>WÖRNER et al. 1989</td>
</tr>
</tbody>
</table>

* sb = surface bound;
* The MoAb binds to the carbohydrate moiety linked to this residue.

Immunization with:
- native C3;
- cobra venom factor;
- synthetic peptide C3169–76 coupled to KLH;
- SDS-denatured C3;
- inulin-fixed C3b/iC3b;
- trypsin-generated C3b;
- expressed C3 fragment.

Mapped by:
- enzymatic fragmentation;
- synthetic peptides;
- expressed C3 fragments
The segment of C3 spanning residues 929–946 contains at least four different overlapping epitopes (Table 2). These epitopes are expressed by surface-bound and/or fluid-phase iC3b and are within the segment of C3 which also contains: (a) the carbohydrate moiety mediating conglutinin binding (HIRANI et al. 1985), (b) the factor I cleavage site(s) (DAVIS and HARRISON 1982 and BECHERER et al. 1989a), and (c) the leukocytosis-inducing activity of the C3 molecule (MEUTH et al. 1983). Using these antibodies to localize the fragments generated upon cleavage of iC3b by factor I, it was found that factor I cleaves C3 within this region at three different positions (NILSSON-ENKDAHL et al. 1989; BECHERER et al. 1989a). The existence of several epitopes within such a limited amino acid sequence is not surprising since a segment as small as 19 amino acids has been shown to accommodate three different epitopes (FIESER et al. 1987).

Other epitopes in C3 have been mapped using either synthetic peptides or expressed C3 fragments (Table 2). These epitopes - several being expressed solely by surface-bound C3 fragments - have been located to residues 741–758 (four MoAbs), 1234–1294 (two MoAbs), and 1476–1531 (seven MoAbs) of the C3 sequence (for references see Table 2). The localization of these epitopes has been greatly facilitated by their expression in denatured C3 also. The possibility that some of these antibodies recognize the same epitope has not been excluded.

Related to the third approach (conservation of epitopes and binding sites), anti-C3 antibodies have been shown to cross-react with C3 from other species. Cross-reactivity studies using MoAbs have facilitated the identification of which residues are involved in the binding, and which MoAbs recognize the same or different epitopes. For example, from three MoAbs which recognize epitopes within residues 929–946 of human C3, only MoAb 7D323.1 and not 7D84.1 or 7D264.6 react with rabbit C3. Based on their reactivity with rabbit C3 and overlapping synthetic peptides, it was predicted that these MoAbs recognize different epitopes, and that residues Arg929, Arg932, and Glu933 are essential for the binding of MoAbs 7D84.1 and 7D264.6 but not that of 7D323.1 (NILSSON et al. 1989b).

4 Analysis of the Complement Activation C3 Products

The selective binding of MoAbs to either native C3 or its degradation products, whether fluid-phase or surface-bound, has rendered possible the development of sensitive assays detecting these products in biological fluids. Most of these are simple (e.g., enzyme-linked immunosorbent assay, or ELISA) and available to laboratories that are not specialized in dealing with some of the tedious and complicated assays used in analyzing the complement system. To date three MoAbs have been found to react specifically with the C3a fragment of C3 (Table 1). The detection of this fragment is relevant for the diagnosis and/or prognosis of adult respiratory distress syndrome and various other diseases (e.g., rheumatoid arthritis, systemic lupus erythematosus; SLE) (for review see BITTER-SUERMANN 1988 and HUGLI 1989). Two versions of ELISA have been described for detecting C3a. In a competition ELISA using MoAb 4SD 17.1, which recognizes C3a but not native C3 (NILSSON et al. 1988), the amount of C3a in the test sample is quantitated based on its ability to inhibit the binding of
this MoAb to microtiter plate-fixed C3a. In the ELISA reported by BURGER et al. (1988), the C3a-containing sample is first incubated with an immobilized polyclonal anti-C3a antibody, and the bound C3a is detected by the MoAb H453. The sensitivity of both assays briefly described above is approximately 1 ng/ml.

In addition to the above antibodies which detect complement activation based on the generation of the C3a fragment, the MoAbs 130 (KANAYAMA et al. 1986), clone 9 (MOLLNES and LACHMANN 1987), and bH6 (GARRED et al. 1988a), all of which recognize neoantigens (Table 1), have also been used to develop assays that detect complement activation. These assays use either MoAbs fixed to microtiter plates (MOLLNES and LACHMANN 1987; GARRED et al. 1988a) or competition assays similar to that described for C3a.

The reactivity of several MoAbs with surface-bound, but not fluid-phase, C3 fragments led to the development of assays detecting and quantitating these fragments fixed to immune complexes (AGUADO et al. 1985; IIDA et al. 1987b) or particles and micro-organisms (NEWMAN and MIKUS 1985). A highly sensitive ELISA measuring C3 fragments in immune complexes has been developed using MoAbs 130 and 105. This assay detects as little as 6.26 µg aggregated human Ig/ml serum (AGUADO et al. 1985) and is comparable or superior to the most commonly used Raji (THEOFILOPOULOS et al. 1976) and C1q assays (ZUBER et al. 1976). Using this assay, increased levels of complement-fixing immune complexes were detected in plasma of patients with autoimmune diseases (rheumatoid arthritis, Sjögrens syndrome, systemic lupus erythematosus) and paracoccidioidomycosis (AGUADO et al. 1985). Interestingly, patients with paracoccidioidomycosis were found to have a markedly decreased degradation of immune complex bound C3b/iC3b to C3dg compared to patients with autoimmune diseases. This observation resulted from the differential specificities of MoAb 105, an antibody which binds preferentially to surface-bound C3b, and MoAb 130, an antibody specific for iC3b. In addition to the different fragments of C3 found on immune complexes, differences were also observed, using MoAb clone 9, in the fragments bound to the surface of complement activators (NEWMAN and MIKUS 1985). These findings emphasize the importance of monoclonal antibodies in distinguishing the C3 activation products in biological fluids.

5 Conclusions

MoAbs have been useful tools for studying the C3-ligand interactions as well as the conformational changes associated with its degradation. A great deal of information on the functional aspects of C3 has been obtained based on the ability of MoAbs differentially to inhibit certain C3 interactions. Since the results obtained with MoAbs are often due to steric or allosteric effects, and since most antibodies recognize conformation-dependent epitopes which are difficult to localize by present techniques, other approaches are necessary to confirm functional sites identified only by MoAbs. For diagnostic purposes, MoAbs directed against particular fragments of C3 have facilitated the development of assays detecting these fragments in biological fluids, and their use will augment our understanding of complement involvement, particularly the role of C3, in various diseases.
Acknowledgements. We thank Drs. D. Goundis and H. Huemer for helpful discussions and critical reading of the manuscript. Also, we thank Drs. J. Tamerius and B. Kolb (Cytotech) for generously providing several of the MoAbs used in our studies.

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