9. Use of synthetic peptides in exploring and modifying complement reactivities

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Introduction

In recent years an impressive amount of information on synthetic peptides has accumulated in the literature. They are used extensively in different areas of immunochemistry and are valuable tools in elucidating the structure-activity relationships of peptides and proteins. Synthetic peptides are used: (1) as analogs to define the structural features of a peptide which are essential for a specific function and optimal biological activity, (2) as replicas to verify certain structures and bioactivities, (3) as models to study regions in proteins which are difficult to analyze using the native proteins, (4) as models to study the conformational aspects and folding rules of proteins, (5) as immunogens to produce antibodies of predetermined specificity with possible use as vaccines, and (6) as tools to study the details of antigen-antibody interactions (for details see ref. [1–11]).

Although the conformation of a binding region in a protein is influenced by long-range interactions, short peptides have been found to mimic some of the relevant features of native proteins. Many binding regions have been isolated from the native protein and synthesized in active form. The increasing availability of long synthetic peptides, sometimes approaching the length of small proteins, as well as the design of synthetic peptides with tailor made conformational features [3, 5, 7, 9, 11], make the use of synthetic peptides an attractive approach in the study of protein-protein interactions.

The complement system, characterized by its multiple protein-protein interactions, consists of a group of proteins which function in host defense as effector molecules and as mediators of several immunological processes (for review see ref. [12–24]). Despite the fact that the complement proteins have been purified to homogeneity and a great deal of information on their multiple reactivities has accumulated, the structural elements involved in these interactions, as related to function, are largely unknown. One of the complement proteins which has been the subject of intensive research is the third component of complement (C3), probably one of the most versatile and multifunctional molecules known to date. C3 plays a very important role not only in
complement activation but also in several cellular responses (for review see ref. [19, 74–77]). This review covers the recent work on the use of synthetic peptides in complement research and, in particular, on their use in studying the multifunctional role of C3 (see Tables 1 and 2). Some older work, mainly on the use of synthetic peptides to study the structure and function of anaphylatoxins, will also be reviewed briefly (for more see ref. [15, 28, 29]).

**Strategy for Identifying the C3-ligand Interaction Sites**

Although in this chapter we are primarily concerned with the use of synthetic peptides in understanding protein-protein interactions within the complement system, we would like to outline briefly a general strategy for identifying the sites involved in these interactions (Figure 1). The different approaches offer a substantial amount of information on the structural elements involved in the different interactions, and they may provide useful tools to study the functions of different complement receptors [14, 18, 19, 23, 24, 26, 27]. The latter is especially important for the C3 molecule since its different fragments have the ability to interact simultaneously with different receptors [24, 26, 27]. Synthetic peptides have been used successfully in studying several of the protein-protein interactions of the complement system. However, a great deal of information is needed before one can begin synthesizing a peptide that will be able to mimic the binding properties of the native molecule and, due to the cost and time involved, a systematic approach is needed. Currently, our laboratory uses a general strategy which approaches the problem at hand from three different directions (Figure 1). For example, C3 is degraded enzymatically or chemically into small fragments which are then analyzed for binding to the various C3 binding proteins. Following purification of the fragment of interest, sequence determination is performed and the location of the fragment within the C3 sequence is obtained. Overlapping as well as analog peptides within the identified area are synthesized and analyzed for binding to the ligand of interest. The second approach, which originates at the DNA level, involves construction of the expression vector Jag11[30–32]. First the C3 cDNA is digested with DNase and the fragments with nucleotide sequences of approximately 200–300 bases are cloned into the expression vector Jag11[30–37]. This library is then screened with antibodies against C3 or with various proteins that bind C3. The reactive clones are then sequenced and their location within the C3 sequence enables the mapping of a specific site. This approach can be a fast and efficient method for screening numerous monoclonal antibodies. We have screened a number of monoclonal antibodies which differ in their specificity for surface bound or fluid phase C3 and the resulting epitope map yielded a great deal of information in the structural and conformational changes that occur upon C3 activation and deposition [30–32]. At the DNA level we have also expressed the whole human C3 molecule and we are performing domain exchange (chimeric molecules) and site directed
Table 1. Summary of synthetic peptides used in complement studies

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Reactivity/Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3</td>
<td>EDLVGKLYVSA</td>
<td>Binds CR2</td>
<td>[72]</td>
</tr>
<tr>
<td>C3197-200</td>
<td>CNYTELIRQHARASHLGLAR</td>
<td>Binds to C3a-receptor</td>
<td>[15, 28]</td>
</tr>
<tr>
<td>C3196-216</td>
<td>13/8/5/3'</td>
<td>Inhibits complement activation</td>
<td>[15]</td>
</tr>
<tr>
<td>C3192-199</td>
<td>GLARSNI</td>
<td>Binds CR1</td>
<td>[58]</td>
</tr>
<tr>
<td>C3172-176</td>
<td>6'</td>
<td>Inhibits H and B binding to surface bound C3b</td>
<td>[120, 121]</td>
</tr>
<tr>
<td>C3170-179</td>
<td>SNLDEDEIAEENIVSRSEFESWLWNVEDLKKEPPK3GISTKL</td>
<td>Binds B</td>
<td>[27]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3174-182</td>
<td>DEDIIAEINI</td>
<td>Binds CR2</td>
<td>[72]</td>
</tr>
<tr>
<td>C3174-182</td>
<td>EFPSWLWNVED</td>
<td></td>
<td>[71]</td>
</tr>
<tr>
<td>C3174-182</td>
<td>FPESWLWNVE</td>
<td>Binds C3a-receptor</td>
<td>[150]</td>
</tr>
<tr>
<td>C3174-182</td>
<td>VYHHFISGGVRKSLKVYPEGIRMNKTVAVR</td>
<td>Binds CR2</td>
<td>[71]</td>
</tr>
<tr>
<td>C3174-182</td>
<td>TLDPERLGR</td>
<td>Binds H</td>
<td>[27]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3174-182</td>
<td>TLDPERLREGVQKEDPPADLSQVDPDTESETRLGLGTPV</td>
<td>Induces leukocytosis</td>
<td>[58]</td>
</tr>
<tr>
<td>C3174-182</td>
<td>AKDKRNEWDPPQKLFRQETALLQKLKDFDVPPVWRILNEQXYYSGGQGSTQA</td>
<td>Enhances vascular permeability</td>
<td>[156]</td>
</tr>
<tr>
<td>C3174-182</td>
<td>KFLLTAKDKRNEWDPPQKLFRQETALLQKLKDFDVPPVWRILNEQXYYSGGQGSTQA</td>
<td>Binds H and CR2</td>
<td>[72]</td>
</tr>
<tr>
<td>C3174-182</td>
<td>DKRNEWDPPQKLFRQETALLQKLKDFDVPPVWRILNEQXYYSGGQGSTQA</td>
<td>Binds H and CR2</td>
<td>[72]</td>
</tr>
<tr>
<td>C3174-182</td>
<td>16'</td>
<td>Binds H</td>
<td>[72]</td>
</tr>
<tr>
<td>C3174-182</td>
<td>SSKITHRHWESASLLR</td>
<td></td>
<td>[151]</td>
</tr>
<tr>
<td>C3174-182</td>
<td>TMILEEITATYRGDQDATMSI</td>
<td>Binds Properdin</td>
<td>[128]</td>
</tr>
<tr>
<td>C3174-182</td>
<td>GVDRYSKVELDRKAFSDRNTLHLYLDSVSHEDD</td>
<td></td>
<td>[146]</td>
</tr>
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mutagenesis experiments. Studies with chimeric molecules have proven very useful in assessing the role of the different domain(s) of multifunctional proteins [33–38]. The use of site directed mutagenesis to study structure-function relationships requires structural information about the protein under investigation [39]. Structural data, when X-ray crystallographic or NMR spectroscopic data are not available, can be obtained from homologies in protein sequences [39, 40]. This represents the third approach and deals with the evolution and conservation of binding sites within C3 from different species and other homologous proteins such as C4, C5, and α2M. First, C3 from different species is purified and tested for its ability to bind the various human C3 ligands [41]. These proteins are then sequenced at the DNA level, thus allowing analysis of the conservation of binding sites between different species. This, together with the conserved sequences of other homologous proteins that do or do not bind the 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 [42], mouse [43, 44] rat [45], lamprey [46], hagfish [47] and trout C3 (Lao, Pang, Alszenz and Lambris, submitted), and partial sequences of rabbit [48], xenopus [49], axolotl [50] cobra [51] and chicken C3 (Mavroidis, Wang and Lambris, unpublished) have been resolved recently.

Identification of binding sites within C3 and modulation of C3 reactivities by synthetic peptides

The CR1 binding site in C3
Complement receptor type one (CR1) binds to the C3b fragment of C3 (and the C4b fragment of C4) and this binding is responsible for the immune adherence phenomenon between cells carrying the receptor and microorganisms that have fixed C3b to their surface [52]. CR1 is expressed on various cell types including erythrocytes, neutrophils, monocytes, macrophages, lymphocytes, gomeral podocytes, and Schwann cells [13, 18, 23, 24]. The fixation of numerous C3b molecules onto the activating surface suggests that C3b-multivalency may be important in the CR1 mediated functions. This is also suggested by the fact that dimeric C3b [53] has an affinity (Ka = 7 × 10^7 M^-1) for CR1 that is approximately 50–100 times greater than that of monomeric C3b (appKa = 0.5 × 10^5 – 2 × 10^6 M^-1) [53, 54]. In addition to C3b, CR1 has also been shown to bind to iC3b [55] (appKa = 5 × 10^6 M^-1) [56] and C3c [57, 58] but with lower affinity. Recently, we have further localized the CR1 binding site within the C3c fragment of C3 [58]. Using elastase, four different fragments of C3c were generated which varied in their reactivity toward CR1 and in the size of their N-terminal '27Kd' x' chain fragment. Based on carbohydrate analysis, which allowed us to estimate the C-terminus of this fragment, N-terminal sequence data, and the differential binding of these four C3c fragments to CR1, the binding site for CR1 was
C5a (porcine)  
C5a1-74  
MLQKKEEAAK/YAMLKCCYDGPVR/NDEFCEERAA/IKIGPKC/VKAFKDCYIANQ/RAEQSHK/NIQLGR  
Similar to C5a  
[162]

C5a (human)  
C5a8-27  
HKDMQLGR  
Binds C5a receptor  
[165, 166]

C5a  
C5a19-30  
KKCCYDGC/VNN  
Binds C5a receptor  
[167, 168]

C4a  
C4a70-73  
KQGAGLQR  
Binds to C3a-receptor  
[15, 28]

Factor B  
Factor B1-237  
E/QKRKIV  
Inhibits C3/C5 convertase  
[157, 188]

Factor B1-234  
GHPGEQQKR  
Inhibits B cell proliferation  
[160]

Factor B1-234  
KEELLPAQNIKALFVSEEKLLTRKEVYIKNGDK  
Binds Ca2+, Mg2+, Ni2+  
[182]

C2 (C2b)  
C2119-223  
ALGTSFSHMLGMATNPTQKTFESLGR  
Spasmogenic and enhances vasopermeability  
[161]

gp350 protein of EBV  
gp35019-31  
TGEDPGFFNVEI  
Binds to CR2  
[82, 88]

Interferon a  
Interferon a  
IFNa87-98  
FTELQQLNDLEA  
Binds to CR2  
[74]

CR  
CR262-99  
CEYFNKYSSCP/P/VPGGYKRGSTPYR-I/GDSVFTFC  
Inhibits EBV binding  
[37]

CR259-133  
YRHDVTFACKTNFSMNGKSVWCNGANMMGPTLRPTVSVFPLE  
Inhibits EBV binding  
[37]

IgG  
IgG238-292  
KFNWY/DGVQVHNAKTKPI  
Binds C1  
[169, 171]

* Number of residues starting from the C-terminus of the longer peptide with the same activity.
* unpublished observations
* Residues in ( ) do not exist in the protein sequence.
<table>
<thead>
<tr>
<th>Reference</th>
<th>Peptide Sequence</th>
<th>Specificity of the Antibodies</th>
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</thead>
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<tr>
<td>[181]</td>
<td>CEEC GCGR</td>
<td>CEEC GCGR</td>
</tr>
<tr>
<td>[181]</td>
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<td>CEEC GCGR</td>
</tr>
<tr>
<td>[181]</td>
<td>CEEC GCGR</td>
<td>CEEC GCGR</td>
</tr>
<tr>
<td>[165]</td>
<td>CEEC GCGR</td>
<td>CEEC GCGR</td>
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<tr>
<td>[155]</td>
<td>CEEC GCGR</td>
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<tr>
<td>[112]</td>
<td>CEEC GCGR</td>
<td>CEEC GCGR</td>
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**Table 2. Contd.**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Specificity of the antibodies</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Factor D</strong></td>
<td></td>
<td>Recognizes D fixed to ELISA plates but not fluid phase D&lt;sup&gt;a&lt;/sup&gt;</td>
<td>[180]</td>
</tr>
<tr>
<td>Factor D&lt;sup&gt;1-7&lt;/sup&gt;</td>
<td>(R)ILGGREA(Y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Factor B</strong></td>
<td></td>
<td>Recognizes B and Bb in ELISA and increases the half life of 3C3bBb&lt;sup&gt;a&lt;/sup&gt;</td>
<td>[182]</td>
</tr>
<tr>
<td>Factor B&lt;sup&gt;B94-627&lt;/sup&gt;</td>
<td>KEELLPAQINKLFVSEEEKLRKEVYIKNGDK</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>C5a</strong></td>
<td></td>
<td>Recognize C5a-des-arg&lt;sup&gt;a-d&lt;/sup&gt;</td>
<td><strong>e</strong></td>
</tr>
<tr>
<td>C5a&lt;sup&gt;1-21&lt;/sup&gt;</td>
<td>TLQKKIIEIAAKYHSVVKC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>C9</strong></td>
<td></td>
<td>Recognize C8 and C9 in immunoblots&lt;sup&gt;a&lt;/sup&gt;</td>
<td>[184]</td>
</tr>
<tr>
<td>C9&lt;sup&gt;101-111&lt;/sup&gt;</td>
<td>DNDGDFSDED</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Monoclonal antibodies.
<sup>b</sup> Polyclonal antibodies.
<sup>c</sup> For details see the corresponding section.
<sup>d</sup> Antibodies raised against the native molecule.
<sup>e</sup> Number of residues starting from the C-terminus of the longer peptide with the same activity. Residues in ( ) do not exist in the protein sequence.
Figure 1. Schematic representation of a general strategy for analyzing C3-ligand binding regions.

localized to a domain in the N-terminus of the α’ chain of C3b. It was found that the absence of eight amino acids from the N-terminus of the α’ chain was enough to abolish CR1 binding to C3c, suggesting that these residues are contained within the binding site or that they are essential for the conformational stability of the CR1 binding site. The involvement of this region in CR1 binding was confirmed by a synthetic peptide spanning the N-terminal 42 amino acids of the α’ chain of C3b (residues 727–768 of the C3 sequence). This peptide, C3727–768, and the corresponding antipeptide antibody, both inhibited
Figure 2. Inhibition of CR1 binding to C3c by C3\textsuperscript{1727-1766} peptide or anti-C3\textsuperscript{1727-1766} antibody.
A. 50 µl of tonsil lysates (2 x 10\textsuperscript{7} cells/ml) were preincubated for one hour at room temperature with 50 µl of synthetic peptides C3\textsuperscript{1727-1766} (C), C3\textsuperscript{1728-1833} (●), and C3\textsuperscript{21432-14449} (▲) at various concentrations (10 µM–700 µM). 50 µl of the incubation mixture was then transferred to microtiter wells precoated with C3c. Bound CR1 was detected using MoAb To5 followed by a peroxidase-conjugated rabbit anti-mouse lg. B. Serial dilutions of antipeptide antibodies C3\textsuperscript{1727-1766} (□) and C3\textsuperscript{21432-14449} (●) were incubated with C3c fixed to microtiter wells for 30 minutes at room temperature. Plates were washed and the CR1 binding was carried out as described above (Adapted from Becherer and Lambris [122]).
CR1 binding to C3b and C3c (Figure 2). Since the anti-C3\textsuperscript{727-768} was produced in rabbits and previous results have shown that rabbit C3 binds to human CR1 [41], it is therefore possible that the anti-C3\textsuperscript{727-768} does not bind to the CR1 binding site in C3b but inhibits only sterically. A greater degree of inhibition was seen when a second antibody specific for rabbit Ig was used to increase the steric hindrance surrounding the antibody binding site. Although the precise sequence comprising the CR1 binding site in C3 remain to be defined, the fact that the C3c fragment missing eight amino acids from its N-terminus could still bind the anti-C3\textsuperscript{727-768} antibody but not CR1 suggests that the CR1 binding site is localized to these N-terminal amino acids of the α chain of C3b. Experiments are now under way to further characterize the CR1 binding site by using overlapping synthetic peptides. Using the anti-C3\textsuperscript{727-768} antibody (affinity purified on C3b-sepharose) and several overlapping synthetic peptides it was found that residues 727–740 comprise an epitope that is exposed after cleavage of C3 to C3b (Becherer and Lambris, unpublished observations).

The CR2 binding site in C3
Complement receptor type 2 (CR2) is a glycoprotein of 140 000 dalton [59–62] expressed on B cells, pharyngeal epithelial cells, follicular dendritic cells, cervical epithelial cells, thymocytes and T cells [14, 18, 21, 23, 24, 63, 64]. CR2 has binding specificity for the iC3b, C3dg and C3d fragments of C3 [14, 23, 24, 65] and for the gp220/350 protein of Epstein-Barr virus [66, 67]. The C3d fragment of C3 produced from the cleavage of C3 by trypsin or elastase contains, in addition to the CR2-binding site [68], the C3 metastable (thiolester) site [69, 70] and binding sites for factor H [71] and CR3 [62, 72]. Binding studies using rosette assays or radiolabeled ligands have indicated that CR2 has a high affinity for iC3b, C3dg, and C3d and a low affinity for C3b [14, 62, 65].

The CR2-binding site within C3 was localized by CNBr fragmentation of C3d. This generated a fragment of 8.6 Kd (amino acids 1177–1252 of the C3 sequence) which had the ability to bind CR2 [68]. The site responsible for this binding was further localized using several peptides covering residues 1187–1214 of the C3 sequence (Table 1). Based on the differential binding of these peptides to CR2 on Raji cells (Figure 3), the CR2 binding site in C3 was localized to residues 1205–1214 [68]. Two other sites sharing sequence homology with the CR2 binding site on C3d have been identified as residues 295–306 of the β chain and 744–755 of the α chain and peptides representing these segments have been found to bind to CR2 [73]. The existence of multiple CR2 binding sites in C3 may play a role in CR2 mediated responses (see below).

Recently it was shown that CR2, in addition to being the receptor for C3 and EBV, also serves as a receptor for interferon α (INFα), an interaction which is thought to be involved in the antiproliferative effects of this cytokine on B cells [74]. The binding site of interferon α for CR2 was localized to a segment
of \( \text{INF}_x \) spanning residues 92–99. The sequence similarity of this region to the CR2 binding sites on C3c and gp350 led to the suggestion that it binds to the same region on CR2 as C3d and EBV [74]. The amino acid sequence similarity, however, is low and further work is required to determine the relationship of the CR2 binding site on \( \text{INF}_x \) to those on C3d and EBV.

Proteolytic fragments of C3 have been reported to have both stimulatory and suppressive effects on different cellular functions [13, 14, 21, 23, 24, 75]. On B lymphocytes these effects were shown to be mediated via CR2 since cross-linking of the receptor by anti-CR2 antibodies [76–78] or particle-bound C3d/C3dg [79, 80] enhances B cell proliferation in the presence of T cell factors [76, 77]. In addition, C3 [73, 81, 82] or monoclonal antibodies to CR2 [83] support the growth of Raji cells at low density and in serum free culture conditions. The nature of the ligand that interacts with the receptor and leads to B cell activation is not easy to characterize because the C3 fragments also bind to receptors other than CR2. The use of synthetic peptides that mimic the native ligand-receptor interaction is useful in exploring the molecular events involved in such ligand-receptor interactions. Experiments using such synthetic peptides showed that the effect of soluble C3d on the B cell growth and on the maturation of B cell progenitors can be replaced by a CR2-binding peptide [84, 85]. In addition, we showed that CR2-binding peptides support the growth of the CR2 positive human B lymphoblastoid lines in a manner similar to that of C3 [73, 82]. CR2 binding peptides have been also found to stimulate phosphorylation of pp105 protein [86]. The fact that polyvalent C3\(^{1202-1214} \) but not monomeric C3\(^{1201-1214} \) stimulates the growth of Raji cells indicated that
cross-linking of CR2 is a necessary signal for B cell activation. Ligand valency is critical in this context [73, 82, 87]. This was confirmed by the observation that monomeric C31201-1214 inhibited the growth-supporting effect of polyvalent C31202-1214. Another peptide that could inhibit this growth-supporting effect was the gp35019-31 peptide (Table 1) from the gp220/350 of EBV. The region of gp220/350 from which this peptide was synthesized shows a high degree of amino acid similarity with, the C3 region spanning residues 1201-1214 [18, 66, 67]. This inhibitory effect suggests that the segment of gp350/220 represented by peptide gp35019-31 is a site of interaction between EBV and CR2 [82]. Similarly, Nemerow et al. have also used synthetic peptides to show that this segment of gp350/220 is involved in CR2 binding [88].

In addition to the effects of C3 fragments on B cells, C3d, C3dg, C3dK, or polyclonal antibody to the gp72 fragment of CR2 [89] inhibit mitogen-, antigen-, and alloantigen-stimulated T cell proliferation or lymphocyte proliferation induced by the mixed lymphocyte reactions [90-93]. The CR2 binding peptides from C3d have been shown to mimic several of these C3d effects (Lambris et al. unpublished observations).

The factor H binding site in C3

Factor H, a Mr 150,000 glycoprotein, plays a regulatory role in the activation of the alternative pathway by acting as (1) a decay accelerating factor by displacing the Bb fragment of factor B from the C3/C5-convertase, (2) an inhibitor of C3-convertase formation by preventing the interaction of C3b with factor B and C5 and, (3) a cofactor for factor I mediated inactivation of C3b to iC3b [94-97]. In addition, factor H binds to a cell surface receptor found on B cells [98, 99] and monocytes [100, 101] and induces several cell responses [98, 100-103]. Studies using monoclonal antibodies to factor H and purified tryptic fragments of factor H showed that both the C3b binding site and the factor I cofactor activity on H are located in its 38,000 dalton N-terminal tryptic fragment [104]. The C3b binding site in H was found to be rapidly destroyed by reducing agents and by further enzymatic degradation of the 38,000 dalton tryptic factor H fragment [105]. In addition, it was found that trypsin-cleaved factor H failed to bind to surface-bound C3b, but retained its ability to interact with fluid phase C3b [104, 106]. These results, as well as the direct binding of H to particle bound C3b (H to C3b ratio of 0.32-0.5 [107, 108]), suggest that two different binding sites for C3b may exist in factor H. For additional details on factor H, see the review by Vik et al. [109].

Factor H as well as CR1, CR2, and the membrane cofactor protein (MCP) belong to the family of C3b/C4b-binding proteins. All contain homologous repeating units of about 60 amino acids [13, 16, 109, 110] and serve as cofactors for the factor I mediated C3b cleavage of C3b [95, 111-116]. These similarities led to the speculation that similar structures in these molecules contribute to their interaction with C3b (directly or indirectly) and that these cofactor molecules may share similar binding sites within C3. The latter was
recently shown for three of these molecules (H, CR1, CR2) by using C3 synthetic peptides (see below).

For factor H, three different interaction sites have been identified in the α-chain of C3 using: (1) anti-idiotypic anti-H antibodies (aaH) [71, 117], (2) monoclonal antibodies to C3 [118, 119], and (3) synthetic peptides covering various regions of the α-chain of C3 [71, 120, 121]. Two of these binding sites were found to reside in the C3c fragment [117, 120, 121] and one in the C3d fragment of C3 [71]. The C3d binding site appears to be discontinuous and spans residues 1187–1249. Both factor H and an antiidiotypic anti-H antibody were shown to bind to C3b and C3d but not to C3c. An 8600 dalton CNBr fragment of C3d covering the amino acid sequence 1198–1275 also reacted with H and aaH. Among the various peptides synthesized from this region of the C3 molecule (Table 1), peptides C3\textsuperscript{1187–1214} and C3\textsuperscript{1234–1249}, covering residues 1187–1214 and 1234–1249 of the C3 sequence respectively, were found to interact with H. Furthermore, both peptides were shown to block completely the factor H cofactor activity in the factor I mediated cleavage of fluid phase C3b. Peptide C3\textsuperscript{1187–1214} completely inhibited the binding of H to C3d and partially to C3b; thus it suggests that at least two binding sites are involved in factor H binding to C3b. The two site model for factor H on C3b is also confirmed by using either anti-C3c or anti-C3d monoclonal antibodies which inhibited both factor H binding to C3b [118, 119, 121] and factor H cofactor activity (Lambris et al. unpublished observations). The fact that the C3\textsuperscript{1187–1214} synthetic peptide is also involved in CR2 binding (see CR2 binding site) and that both H and CR2 are cofactors of factor I suggests that the CR2 and H molecules induce similar conformational changes in C3b which is necessary for the factor I mediated cleavage of C3b.

A second factor H binding site in C3 has been reported to be located in the N-terminal 40 amino acids of the α′ chain of C3b (residues 727–768 of C3) [27, 120, 121]. Both peptides covering this region and rabbit antibodies to this peptide inhibited H binding to C3b-coated sheep erythrocytes (Tables 1 and 2). The C3\textsuperscript{727–768} peptide is also reported to inhibit B binding to surface bound C3b [120]. The localization of factor H [120, 121], factor B [120, 121], CR1 [122] and CR2 binding sites [73] within the same segment of C3 (residues 727–768) suggested that these molecules share binding sites. However, the findings that human CR1 and CR2, but not H, bind to Xenopus iC3, and that H, but not CR1 and DR2, interacts with Trout iC3 suggest that, although these three molecules recognize the same domains in human C3, their exact binding sites are different [41]. Similarly, the inability of human factor B to bind to either Xenopus or Trout C3 [41] suggests that its binding site on human C3b, located in the CR1 binding domain [27, 121], is different from that for H and CR1. Thus, the ability of H and CR1 to compete with B for binding to C3b may due to an allosteric or steric effect and not to competition for the same binding site(s). Recent data by Fishelson et al. [27] have sublocalized the H and B binding sites to residues 745–754 and 730–739 of C3 respectively. Our laboratory has localized a CR2 binding site within residues 744–755 of C3 [73].
Finally, by using an anti-idiotypic anti-H antibody derived from antisera against the α-chain of human C3, it was suggested that a third H binding site in C3c exists in the 39 500 dalton C-terminal fragment of the α-chain of C3 [117]. Although this αaH antibody paralleled some of the functional activities of factor H, the existence of the third interaction site in C3b requires further investigation and confirmation with other techniques since antibodies may change the conformation of C3 and indirectly influence its functions without directly interfering with the interaction site of H in C3b.

The properdin binding site in C3
Properdin is a plasma glycoprotein which participates in the regulation of the alternative pathway of complement activation by binding and stabilizing the C3bBb convertase [17]. Recent studies have suggested that, in addition to the stabilizing effect of properdin, it inhibits the action of factor I on surface bound, but not fluid phase, C3b [123]. Furthermore, it has been suggested that properdin exerts a negative regulation in the classical pathway by inhibiting the formation of the C5 convertase [124, 125]. The mechanism of the convertase stabilization by properdin is not yet known. It is not clear if properdin must bind to both C3b and Bb in order to exert its stabilizing effect, or if only properdin binding to C3b is enough to change the conformation of C3b and thus increase its affinity for Bb. Sedimentation equilibrium ultracentrifugation studies using C3 fragments have shown that, in addition to C3b, C3c also is able to bind properdin [126]. Further analysis of the interaction of properdin with C3 fragments have confirmed the above studies and further showed that the properdin binding site in C3c is located within its 40Kd C-terminal α-chain fragment [127]. Cleavage of the 40 Kd fragment by CNBr resulted in several fragments one of which (17 Kd, residues 1385–1541 of C3) retained the ability to interact with properdin [128]. The availability of the amino acid sequence of properdin binding proteins (human, mouse, and rabbit C3) and of related but non-properdin-binding proteins (human and mouse C4, C5, and α2 M) provided the necessary information for further analysis of the properdin binding site in C3b. The comparison of the sequences of these proteins indicated that the segment in C3 spanning residues 1402–1435 has low sequence similarity in the non-properdin-binding proteins. A synthetic peptide (C31402-1435) spanning residues 1402–1435 of the human C3 sequence has been shown to bind to properdin and to inhibit its binding to C3b (Figure 4). The amount of the synthetic peptide required to inhibit binding of properdin to ELISA plate fixed C3b was fifty fold that of fluid phase C3b. In addition to the direct inhibition of properdin binding to C3b, this peptide was shown to inhibit lysis of rabbit erythrocytes via the alternative pathway, presumably by inhibiting the stabilization of the alternative pathway convertase. The inhibition of the alternative pathway by the C31402-1435 peptide was confirmed by measuring the generation of the properdin-C3b complex in serum after activation of the alternative pathway with zymosan in presence or absence of the C31402-1435 or control peptide (Figure 5). The
inhibitory effect of C3^1402-1435 on the activation of alternative pathway clearly demonstrates the importance of properdin in the formation of an efficient alternative pathway convertase. This is also supported by the reduced alternative pathway hemolytic activity of properdin deficient serum [129-131]. Further characterization of the properdin binding site in C3b using overlapping synthetic peptides and analogues has shown that the His^{1431} and the Ser^{1432} are important for the binding of properdin to C3b [132].

The CR3 binding site in C3

Complement receptor type 3 (CR3) is a glycoprotein found on the cell surface of monocytes, macrophages, granulocytes, and large granular lymphocytes that binds the iC3b fragment of C3 (see ref. [14, 18, 23, 24, 133] for review). Also, CR3 has been reported to interact with the C3d fragment of C3 [72] and possess a binding site for polysaccharides [134, 135]. CR3 functions in the phagocytosis of particles opsonized with iC3b [14, 136] in addition to its role as an adhesive molecule in cellular interactions [137-139]. It is comprised of noncovalently associated α (Mr = 170,000) and β (Mr = 95,000) chains; the β chain of CR3 being identical to the β chain of two other leukocyte receptors, namely LFA-1 and p150-95 [140-142]. These three receptors form a family of receptors based on their identical β chains and on their homologous, but immunogenically
distinct, z chains. In turn, these receptors belong to a superfamily of cell adhesive molecules, termed integrins, with each subfamily characterized by the sharing of a common b chain in association with distinct z chains. Other receptors in this superfamily are the fibronectin and vitronectin receptors, platelet glycoprotein IIb/IIIa, and the VLA family of leukocyte markers (for review see [143, 144]). The characterized ligands that bind these integrins possess, as their receptor recognition site, a domain containing the sequence RGD. It has been shown, however, that the residues surrounding this RGD sequence are responsible for the specificity each ligand has for its receptor [145]. Since iC3b is the ligand for CR3 and an RGD sequence is located in the z chain of C3, a synthetic peptide from this region of C3 was tested for binding to CR3 [146]. A 21 amino acid peptide (C3<sub>1361-1380</sub>) (Table I) containing the RGD sequence, corresponding to residues 1361-1380 of the C3 sequence, was synthesized and coupled through its
N-terminus to myristic acid, thus allowing incorporation of the peptide into erythrocyte membranes. Subsequently, these erythrocytes were shown to bind to human monocytes and this binding could be inhibited by monoclonal antibodies directed against the iC3b binding site in CR3. In contrast to these findings are recent studies showing that the RGD sequence in C3 is not required for binding to CR3 [147]. This explains earlier results where an RGDS containing peptide that bound the fibronectin receptor showed no effect on iC3b binding to CR3 [148].

The C3a receptor binding site in C3
C3a is one of the three (C3a, C4a and C5a) anaphylatoxins generated during complement activation (for review see ref. [15, 28, 29]). Extensive studies on the responses elicited by the different anaphylatoxins have shown that, in contrast to C5a, C3a and C4a interact with the same receptor. The two major effects among the variety of cellular responses mediated by the anaphylatoxins and involving numerous cell types are the enhancement of vascular permeability and the induction of smooth muscle contraction (for details see ref. [15, 28, 29]). The elucidation of the primary structure of anaphylatoxins from different species, the crystallographic analysis of C3a [149], and the use of synthetic peptides (Table 1) have been instrumental in defining the structure-activity relationship of the anaphylatoxins. The use of C3a synthetic peptides in functional and conformational studies made possible the localization of the ‘active site’ and the characterization of the structural requirements necessary for the expression of the function of these molecules. Even though the full activity of C3a was seen with a 21 amino acid peptide (residues 57–77 of C3a) various degrees of activities were observed with smaller peptides. The smallest peptide possessing C3a activity is a tripeptide representing the three C-terminal residues of C3a (LAR), with Arg77 essential for binding activity [150]. The differences in activity between peptides of various lengths is believed to result from conformational differences between these peptides. Similar to C3a, the smallest C4a peptide possessing 0.1–0.2% of the activity of native molecule was found to be a peptide representing the eight C-terminal residues (KQGAGLQQR) of C4a. A similar activity to that of C3a, although to lesser extent (< 1%), has been also reported for the C3f fragment of C3 [151].

Use of synthetic peptides in other areas of complement

C3 peptides

Peptides inhibiting complement activation. A series of synthetic peptides spanning the convertase cleavage site in C3 were found to inhibit complement activation by both the classical and alternative pathways [152]. A six amino acid synthetic peptide (LARSNL, residues 746–751 of C3) inhibited both pathways equally well while a three amino acid peptide (LAR) was more
effective in inhibiting the classical pathway. Increasing the peptide length to 14 amino acids did not change its inhibitory activity. A seven amino acid peptide (GLARSNL) produced in our laboratory inhibited, by 50%, the lysis of rabbit erythrocytes by human serum at a concentration 2500 fold higher than the C3 concentration in serum. This is approximately three fold higher than the concentration reported in the above study, and it may be due to differences in experimental conditions. The inhibition of the complement activation by these peptides is due to substrate competitive inhibition. Although the above synthetic peptides represent relatively poor complement inhibitors, hybrid synthetic peptides, composed from the sequences at the cleavage sites of C4 by C1s and the C-terminus of anti-thrombin III, were forty times more effective in inhibiting classical pathway activation [152].

Leukocytosis inducing peptide. Studies in different laboratories showed that C3-derived fragments cause leukocytosis when injected intravenously in rabbits [153–155]. The nature and the location of these fragments within C3 is debatable. One of these fragments, known as C3dK, was purified to homogeneity, and, after sequencing, it was found that its N-terminus extends 9 amino acids from the N-terminus of C3dg [153]. This fragment had leukocytosis activity and this activity was reproduced by a synthetic peptide representing the 9 N-terminal amino acids of C3dK [156]. In the same study, it was also shown that an antibody to the C3e fragment of C3 [155] reacted weakly with this synthetic peptide, suggesting that this peptide may represent the active site of C3e.

Factor B peptides
Peptides inhibiting complement activation. Similar to the C3 peptides, inhibition of the alternative pathway activation was observed by synthetic peptides which span the sequence of factor B surrounding the factor D cleavage site [157]. A six amino acid peptide from this region (Table 1) inhibited both fluid phase and cell bound C3/C5 convertase formation. This peptide, in contrast to the C3 peptides surrounding the convertase cleavage site, was not able to inhibit the generation of convertase in serum. This inefficiency was explained to be due to the rapid cleavage of this peptide by other serum proteases. In contrast to trypsin or C1s, purified factor D was not able to cleave this peptide.

A peptide inhibiting B cell proliferation. Using 125I-Ba [158, 159] and B cell proliferation assays [160], it was shown that Ba binds to a specific receptor on LPS-activated B cells with an appKa of 10^7 M^-1 [159]. Binding of Ba to B cells stimulated by Staphylococcus aureus and HMW-BCGF leads to inhibition of their proliferation [160] and a similar inhibition was obtained with a ten amino acid synthetic peptide (GHGPPGEQQKR) from the C-terminus of Ba [159].

C2 peptides
Several synthetic peptides representing the C-terminal 25 amino acids of the C2b fragment of the second complement component (C2) have been shown to have spasmogenic activity on rat uterus and to enhance the vascular
permeability of human and guinea pig skin [161]. These data with the synthetic peptides provided further evidence that kinin-like peptides may be derived from C2.

**Synthetic C5a**

Although the synthesis of long peptides is currently possible, the synthesis of the C5a molecule (74 amino acids in length) was difficult due to the presence of three disulfide bonds within C5a. Despite this problem, the complete C5a molecule was recently synthesized by fragment condensation [162]. Nine different fragments spanning residues 1–10, 11–16, 17–25, 26–32, 33–38, 39–50, 51–58, 59–63, and 64–74 of C5a were synthesized in solution using a strategy of maximum protection [163] and, after the removal of Boc or Pac groups by trifluoroacetic acid, the fragments were coupled together in a mixture of DMF, DMSO and N-methyl-pyrrolidone using 1-ethyl-3-(3′-dimethylaminopropyl)-carbodiimide and 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine. After completion of the synthesis and deprotection, the disulfide bonds were formed by air oxidation of the peptide (10⁻⁸ M) in acetate buffer (pH 7.5) at 5°C for five days. This synthetic C5a had similar activities to those reported for the natural C5a. Contraction of guinea pig ileum was observed at a concentration of 1 × 10⁻⁶ M of synthetic C5a which is two times higher than that reported for natural C5a (5 × 10⁻⁹) [164]. In the β-hexosaminidase releasing assay, the synthetic C5a was more active than the natural human C5a.

Attempts to localize the C5a-receptor binding site in C5a using synthetic peptides were originally unsuccessful. A peptide representing the eight C-terminal residues of C5a, as well as the fragment containing the N-terminal 69 residues of C5a were inactive in any functional C5a assay, thus suggesting that the intact conformation of C5a plays a role in the biological activity of this molecule [15]. However recently a synthetic peptide containing the eight C-terminal residues successfully mimicked the properties of C5a [165, 166]. This peptide inhibited the binding of¹²⁵Ι-C5a to its receptor and induced PMNL chemokinesis and lysosomal enzyme release [166]. A 1000-fold boost of binding affinity of C5a C-terminal octapeptide has been achieved by a single Phe for His substitution [165]. An additional C5a receptor binding site in C5a has been reported to be located within residues 19–30 of C5a [167, 168]

**CR2 peptides**

Several synthetic peptides representing the first two SCRs of complement receptor type two (CR2) as well as chimeric CR2 molecules constructed by exchanging mouse SCR1-4 have been used by Molina et al. [37] to show that CR2 has two different EBV binding sites. These same authors, as well as Martin et al. [38], using chimeric human/mouse CR2 molecules, were able to dissect the EBV, C3d, and OKB7 binding sites and to demonstrate that these sites are different (because these ligands inhibited each other it was thought that they bind to the same site).
IgG peptides

Activation of the classical pathway by IgG involves the binding of the C1q component of C1 to the antigen-IgG complex via the C,2 domain of IgG. The site on IgG involved in this interaction was localized by the use of synthetic peptides. Peptides spanning residues 274–292, as well as different analogues, were synthesized and tested for their ability to interfere with C1-IgG interaction [169–171]. These studies have suggested that residues His-285, Lys-288, Lys 290, and Arg-292, located on the surface of C,2 domain of IgG, are involved in C1q binding [171]. Mutagenesis studies, as discussed in chapter 2, indicate however that Glu318, Lys120 and Lys322 form the major part of the C1q binding site.

Anti peptide antibodies and mapping of the antibody recognizing epitopes using synthetic peptides

Extensive use of synthetic peptides in other areas of immunology has demonstrated that it is possible to raise antibodies to almost any region of a protein [172]. Such antibodies with pre-determined specificity are useful in studying (1) antigen-antibody interactions, (2) the structural elements involved in protein-protein interactions, and (3) the structure and function of proteins of newly discovered genes. In this section the use of antipeptide antibodies in complement research will be briefly reviewed (see Table 2).

Anti-C3 antibodies

MoAb 130. The monoclonal antibody 130 was generated against human C3 [118] and found to recognize an epitope on C3 which is expressed by iC3b and C3dg/C3d but not by C3b or native C3 [118, 173]. This antibody was found to inhibit the binding of C3 fragments to CR2, a property which was used to localize the CR2 binding site on C3d [68]. The neoantigenic site recognized by this antibody 130 was originally localized to residues 1195–1212 of the C3 sequence due to its ability to recognize peptides C31187-1214 and C31222-1249 but not C31205-1214 [68]. However, the binding of MoAb 130 to these synthetic peptides was lower than that to C3d, and it suggested that the C31187-1214 may not contain the complete antigenic site. This was proven to be the case since MoAb 130 recognizes peptide C31192-1249 and C3d equally well (Figure 6). The reactivity of MoAb 130 with peptide C31222-1249 suggests that the antigenic site recognized by this antibody is a discontinuous one. This site, however, is different from the H binding site (see H binding site) since H binds to C31187-1214, C31222-1249 and C31224-1249 peptides while the MoAb 130 binds only to C31187-1214, C31222-1249.

Antibodies recognizing C3b or iC3b fragments. Among the several monoclonal antibodies generated against denatured C3, three of them were found to react specifically with surface bound iC3b [174, 175]. C3 synthetic peptides were used to map the antigenic epitopes recognized by these antibodies. One of
these antibodies was found to recognize an octapeptide spanning residues 929–936 of the C3 sequence, while the other two antibodies recognize a peptide spanning residues 929–946 [175]. Since peptide 929–936 covers the junction of the C3dk–C3dg fragments (the third factor I cleavage site), these antibodies may be very useful tools in studying the third factor I mediated cleavage of C3, an issue which is still debatable [18, 19, 24]. Using synthetic peptides we have mapped another iC3b/C3dg specific epitope, recognized by clone 9, to residues 933–946 of C3 [176].

C3b specific epitopes localized by synthetic peptides have been also identified. Four MoAb anti-C3 antibodies were localized to a region of C3 spanning residues 741–758 (Becherer, Nilsson and Lambris, unpublished observations). A neoantigenic epitope expressed upon cleavage of C3 to C3b has been characterized by antibody to C3727–768 peptide [121].

Anti-C3a antibodies. Two monoclonal anti-C3a specific antibodies were recently produced after immunization with a synthetic octapeptide (RASHLGLA) spanning amino acids C3718–725 of the C3 sequence (69–76 of
C3a) [177]. Both antibodies were found to react with C3a but not C3, and this suggested that the C-terminus of C3a represents a neoantigenic determinant. Since these antibodies recognize only C3a they have been used in the development of a sandwich type ELISA to determine the levels of C3a in plasma. This assay may facilitate the determination of C3a (sensitivity 1–5 ng/ml) in samples from patients where C3a determination may have diagnostic and prognostic value.

**Anti-C3a antibodies**

Recently, eight different mouse MoAbs against human C5a were produced after immunization with purified C5a [178]. All the antibodies reacted with C5a-des-Arg and some of them also reacted with C5a and/or C5 [178]. One of these antibodies recognized only C5a-des-Arg and was used to develop an ELISA for measuring the levels of C5a-des-Arg in plasma. In order to localize the epitopes recognized by these antibodies, two synthetic peptides spanning residues 1-21 and 55-74 of human C5α were tested for binding to the MoAbs. The C5α-des-Arg specific MoAbs (six) were found to react with the 55-74 peptide, and this suggested that the C-terminus of C5, in a similar way to C3a [178], represents a neoantigenic determinant (Takeda, Kinoshita and Lambris, unpublished observations). The antibodies recognizing C5α and C5, in addition to C5α-des-Arg, did not react with any of the peptides. This suggests that they either recognize a conformational epitope or an epitope within residues 20-37 of C5α. Another group has reported that this latter region is involved in the binding of nine other MoAb to C5α [179].

**Anti-factor D antibodies**

One MoAb antibody to the seven N-terminal amono acids of Factor D was produced using a peptide (RILGGREAY)-BSA conjugate as immunogen. Using this antibody it was shown that the N-terminus of Factor D is buried inside the native molecule and it is only expressed upon its binding to microtiter plates [180].

**Anti-DAF antibodies**

Antipeptide antibodies have been extremely useful in detecting the protein products of cloned genes. Three different polyclonal antipeptide antibodies were generated against the deduced peptide sequence of the cDNA of the decay accelerating factor (DAF) (Table 2). All three antibodies recognized DAF in western blots confirming that the isolated cDNA encodes DAF [181].

**Anti-factor B antibodies**

Several monoclonal antibodies recognizing both B and Bb in an ELISA were produced after immunization with a highly charged peptide from factor B (amino acid residues 594-627) [182]. Because one of the generated monoclonal antibodies was found to increase the half-life of the EC3bBb convertase and the peptide itself was found to bind Ca⁺⁺, Mg⁺⁺, and Ni⁺⁺, it
was suggested that this peptide is involved in the interaction of Bb to C3b. Earlier studies have shown that the fragment of factor B spanning residues 445–739 binds to C3b and inhibits the activation of the alternative pathway [183].

Anti-C9 antibodies
One anti-peptide antibody specific for C9 was raised in rabbits after immunization with a synthetic peptide (ovalbumin conjugated) spanning residues 101–111 (DNDCGDFSDED) of the C9 sequence [184]. This antibody, in addition to recognizing C9 in western blots, also reacts with the α chain of C8; thus an antigenic crossreactivity exists between C8 and C9. In the same study, a similar crossreactivity between C8 and C9 was shown using MoAb raised against C9 [184].

Concluding remarks
Complement plays a very important role in the natural host defense mechanisms. However, in certain situations such as in transplantation rejection, in autoimmune and other diseases, complement may be disadvantageous to the host. Attempts to control the activation of complement using inhibitors have been limited due to the toxicity and non-specific action of the inhibiting agents [185, 186]. Therefore, an ideal inhibitor should be specific for a particular complement component and have low toxicity and antigenicity. Due to the central role of C3 in the complement system, its interactions with the other proteins represent potential sites for pharmacological manipulation. For example, inhibition of C3a binding to its receptor by an antagonist may abrogate the inflammatory effects of C3a whereas inhibition of factor B binding to C3 may block the formation the alternative pathway convertase and therefore the generation of C3a and C5a anaphylatoxins. The elucidation of molecular features involved in the C3-ligand interactions as well as in the interactions of the other complement components will greatly facilitate the design of specific inhibitors for the different steps of complement activation. Such inhibitors will not only broaden our knowledge on the molecular details of these interactions, but they may also become medically important.

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