Specific Inhibition of the Classical Complement Pathway by C1q-Binding Peptides

Anja Roos, Alma J. Nauta, Daniël Broers, Maria C. Faber-Krol, Leendert A. Trouw, Jan Wouter Drijfhout and Mohamed R. Daha

http://www.jimmunol.org/content/167/12/7052

References
This article cites 33 articles, 13 of which you can access for free at:
http://www.jimmunol.org/content/167/12/7052.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Specific Inhibition of the Classical Complement Pathway by C1q-Binding Peptides

Anja Roos,† Alma J. Nauta,* Daniël Broers,* Maria C. Faber-Krol,* Leendert A. Trouw,* Jan Wouter Drijfhout,† and Mohamed R. Daha*

Undesired activation of the complement system is a major pathogenic factor contributing to various immune complex diseases and conditions such as hyperacute xenograft rejection. We aim for prevention of complement-mediated damage by specific inhibition of the classical complement pathway, thus not affecting the antimicrobial functions of the complement system via the alternative pathway and the lectin pathway. Therefore, 42 peptides previously selected from phage-displayed peptide libraries on basis of C1q binding were synthesized and examined for their ability to inhibit the function of C1q. From seven peptides that showed inhibition of C1q hemolytic activity but no inhibition of the alternative complement pathway, one peptide (2J) was selected and further studied. Peptide 2J inhibited the hemolytic activity of C1q from human, chimpanzee, rhesus monkey, rat, and mouse origin, all with a similar dose-response relationship (IC50 2–6 μM). Binding of C1q to peptide 2J involved the globular head domain of C1q. In line with this interaction, peptide 2J dose-dependently inhibited the binding of C1q to IgG and blocked activation of C4 and C3 and formation of C5b-9 induced via classical pathway activation, as assessed by ELISA. Furthermore, the peptide strongly inhibited the deposition of C4 and C3 on pig cells following their exposure to human xenoreactive Abs and complement. We conclude that peptide 2J is a promising reagent for the development of a therapeutic inhibitor of the earliest step of the classical complement pathway, i.e., the binding of C1q to its target. The Journal of Immunology, 2001, 167: 7052–7059.

Activation of the complement system plays a dual role in disease. On the one hand, complement activation has many protective functions in immunity, both as a first line defense mechanism against invading pathogens and as a potentiator of acquired immunity. On the other hand, complement activation is a major cause of tissue injury in many pathological conditions (1). Thus far, three different pathways have been identified via which the complement system can be activated, i.e., the classical pathway, the alternative pathway, and the lectin pathway. Activation of the classical complement pathway is involved in tissue damage resulting from deposition of autoantibodies and immune complexes, which may occur in autoimmune diseases such as systemic lupus erythematosus, myasthenia gravis, and Goodpasture’s syndrome (1–3). Furthermore, classical pathway activation is responsible for tissue injury in hyperacute xenograft rejection, triggered by the direct binding of preformed host Abs to the graft endothelium (4). Inappropriate complement activation is also an important mediator of ischemia/reperfusion injury occurring, for example, in stroke and myocardial infarction and after major surgery (5–7). Activation of the classical complement pathway in this type of tissue damage can occur via Ab-dependent as well as Ab-independent mechanisms, which in the latter case may involve the direct binding of C1q to damaged cells and in situ deposited acute phase proteins (6, 7).

The therapeutic application of complement inhibitors, to prevent undesired effects of complement activation, is currently under development. For example, C1 inhibitor, a physiological inhibitor of the serine proteases C1r and C1s of the classical pathway, has been preliminarily used in man (8, 9). These studies have underscored the value of anticomplement strategies in the treatment of, e.g., acute myocardial infarction and sepsis. Next to inhibiting the C1 complex, C1 inhibitor also affects the lectin pathway of complement activation and the contact system (8, 10). Recent experiments indicate that high-dose i.v. Ig, which is frequently used as a broad anti-inflammatory treatment in patients, is also able to inhibit classical pathway activation in vivo (11). Other studies explored the use of soluble recombinant complement receptor 1 (CR1) as a therapeutic complement inhibitor. CR1 (CD35) is a membrane-bound receptor that binds the complement components C1q, C4, and C3. The soluble protein is able to prevent complement activation and complement-related damage in vivo (9). However, the disadvantage of this treatment is the lack of specificity, i.e., because soluble CR1 blocks C3, complement activation via the alternative pathway and the lectin pathway and the classical pathway are inhibited, all to the same extent. Recently, a peptide inhibitor of C3 has been developed, named compstatin (12–15). Compstatin has promising properties for therapeutic complement inhibition both in vitro and in vivo.

Because the classical pathway in many cases is for a major part responsible for complement-related tissue damage, a specific and effective inhibitor of the classical pathway is desirable. Such an approach does not affect the alternative pathway and the lectin pathway, pathways known to play a key role in innate immunity against pathogens. Given that such an anticomplement treatment

1 Departments of Nephrology and Immunohematology and Bloodtransfusion, Leiden University Medical Center, Leiden, The Netherlands
2 Address correspondence and reprint requests to Dr. Anja Roos, Department of Nephrology, Leiden University Medical Center, D3P, Postbox 9600, 2300 RC Leiden, The Netherlands. E-mail address: A.Roos@LUMC.NL

Received for publication July 20, 2001. Accepted for publication October 19, 2001.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by grants from the Dutch Organization for Scientific Research (901-12-094), the European Community (Biotech; Bio4-CTD97-2242), and the Dutch Kidney Foundation (PC95).

2 Address correspondence and reprint requests to Dr. Anja Roos, Department of Nephrology, Leiden University Medical Center, D3P, Postbox 9600, 2300 RC Leiden, The Netherlands. E-mail address: A.Roos@LUMC.NL

3 Abbreviations used in this paper: CR1, complement receptor-1; dig, digoxigenin-3-O-methylcarbonyl-ε-aminocaproic acid-N-hydroxysuccinimide ester; EA, Ab-coated erythrocyte; VBS, Veronal-buffered saline; CH50, hemolytic activity of the classical component pathway; AP50, hemolytic activity of the alternative complement pathway; ghB, B chain of the globular head region of C1q.
will potentially be useful in immune-compromised patients, the first line antimicrobial defense is of great importance to prevent life-threatening infections. Therefore, we aimed at the development of novel inhibitors of C1q, the recognition unit of the classical complement pathway.

Recently, Lauvrak et al. (16) reported the sequences of 42 peptides that were selected from phage display libraries on the basis of binding to human C1q. In the present study, we explored the effects of these peptides on complement activation. We selected one peptide that inhibits the classical pathway but not the alternative pathway and we present its mechanism of action. Because this peptide inhibits C1q from human, primate, and rodent origin, we propose that this peptide is a promising candidate for further development as a therapeutic C1q inhibitor.

Materials and Methods

Peptide synthesis

Peptide sequences were obtained from Lauvrak et al. (16), who selected 42 peptide sequences from five different phage-displayed peptide libraries (unconstrained and cysteine-constrained) on the basis of phage binding to human C1q. In this study, these 42 peptides were synthesized. Synthetic peptides were made by solid phase technology on Tentagel resin (Rapp, Tübingen, Germany) using N-Fmoc-t-butyI-protected amino acids, piperidine deprotection, and PyBOP/NMethylmorpholine activation. For the synthesis of peptide 2I-3, Fmoc-2-aminobutyric acid was used to introduce Xε. Peptide 2cy was obtained by first introducing an N-terminal bromoacetyl moiety (Xε), trifluoroacetic acid-induced cleavage and deprotection, ether precipitation of the cleaved product, and subsequent immediate cyclisation of the peptide in Na-phosphate pH 8 solution for 1 h at room temperature. Synthetic peptides were analyzed by analytical reversed phase HPLC and matrix-assisted laser desorption ionization time of flight mass spectrometry.

Peptides were dissolved in DMSO at a concentration of 10 mM and stored at −80°C. From this stock solution, appropriate dilutions were made in the different assay buffers as described below.

C1q isolation

C1q was isolated from human donor plasma as described previously (17). In brief, a protein precipitate was made from recallfied human plasma by addition of polyethylene glycol 6000 (3% v/v; Sigma-Aldrich, St. Louis, MO) and incubation for 1 h on ice. After centrifugation, the precipitate was dissolved in a Tris-Veronal-buffered saline (VBS, 1.8 mM Na-5.5-diethylbarbital, 0.2 mM 5,5-diethylbarbituric acid, 145 mM NaCl) containing 10 mM EDTA and loaded on an affinity column consisting of Sepharose-coupled human IgG that was previously incubated with rabbit IgG directed against human IgG. After washing, the column was eluted using a NaCl gradient. The elution was assessed by analytical reversed phase HPLC and matrix-assisted laser desorption ionization time of flight mass spectrometry.

ELISA

In general, ELISA were performed using Maxisorb plates (Nunc, Roskilde, Denmark) that were coated with different proteins or peptides diluted in coating buffer (100 mM Na2CO3/NaHCO3, pH 9.6), either for 2 h at 37°C or overnight at room temperature. Nonspecific binding sites were blocked using incubation with 3% BSA in PBS for 1 h at 37°C. All subsequent steps were performed in PBS containing 0.05% Tween 20 and 1% BSA unless otherwise indicated, and each step was followed by washing for three times using PBS/0.05% Tween 20. Enzyme activity of HRP was assessed by addition of ABTS (Sigma-Aldrich) and H2O2. The OD at 415 nm was measured using a microplate biokinetics reader (EL312e, Bio-Tek Instruments, Winooski, VT).

To assess binding of C1q to peptides, peptides were coated on the plate at various concentrations, followed by a blocking step and addition of purified human C1q (0.1 μg/ml). Binding of C1q was detected using F(ab‘)2 from rabbit IgG anti-human C1q, conjugated to digoxigenin-3-O-methylcarbonyle-α-mannosacric acid-N-hydroxysuccinimide ester (dig; Boehringer Mannheim, Mannheim, Germany) according to instructions provided by the manufacturer, followed by HRP-conjugated F(ab‘)2, from goat IgG anti-dig (Boehringer Mannheim). To assess the effect of peptides on C1q binding to IgG, plates were coated with rabbit IgG (5 μg/ml) and purified human C1q (0.4 μg/ml) was added in the presence or absence of peptides in various concentrations. C1q binding was assessed using a biotinylated mouse mAb directed against human C1q (mAb 2214; kindly provided by Dr. C. E. Hack, Sanguin Blood Supply Foundation, Amsterdam, The Netherlands) (18), followed by HRP-conjugated streptavidin (Boehringer Mannheim). In some experiments, C1q was allowed to bind to coated rabbit IgG or peptide 2J in the presence or absence of purified mAb 2214 directed against the globular head portion of C1q or mAb 2214 directed against the collagenous portion of C1q (both mouse IgG; kindly provided by Dr. C. E. Hack) (18), followed by detection of C1q binding using dig-conjugated rabbit IgG F(ab‘)2, anti-human C1q, as indicated below.

ELISA-based assays to detect classical pathway complement activation were performed in plates coated with purified human IgM that were incubated with normal human serum (generally diluted 1/200) in the presence or absence of peptides diluted in gelatin Veronal buffer (VBS, 0.1% gelatin, 0.5 mM MgCl2, 2 mM CaCl2, 0.05% Tween 20). Complement activation was assessed using mouse mAb directed against deposition of activated C4 (C4a-anti-human C4d (19), from Dr. C. E. Hack, conjugated to dig), activated C3 (RFK22 anti-human C3 (20), conjugated to dig) and the membrane attack complex C5b-9 (unconjugated AE11 anti-C5b-9; kindly provided by Dr. T. E. Molinaes, Norland Central Hospital, Bodo, Norway). Ab binding was detected using either HRP-conjugated F(ab‘)2 anti-dig (Boehringer Mannheim) or HRP-conjugated goat anti-mouse IgGs (Dako, Glostrup, Denmark).

Assessment of complement deposition on PK15 cells

Complement activation induced by xenoreactivity of human serum to pig cells was assessed essentially as described previously (20). PK15 cells (pig kidney epithelial cell line, obtained from the American Type Culture Collection, Manassas, VA) were cultured in DMEM (Life Technologies, Breda, The Netherlands) supplemented with 10% FCS, penicillin (100 IU/ml) and streptomycin (100 μg/ml). Cells were detached by trypsinization, followed by incubation in normal human serum (5%) diluted in culture medium, for 30 min at 37°C, in the presence or absence at 100 μM of peptide inhibitors. Cells were washed with cold washing buffer (PBS, 1% BSA, 0.01% NaN3) followed by incubation with mAb directed against C4 (C4-4a and anti-human C4d (19), from Dr. C. E. Hack, conjugated to dig), activated C3 (RFK22 anti-human C3 (20), conjugated to dig) and the membrane attack complex C5b-9 (unconjugated AE11 anti-C5b-9; kindly provided by Dr. T. E. Molinaes, Norland Central Hospital, Bodo, Norway). Ab binding was detected using either HRP-conjugated F(ab‘)2 anti-dig (Boehringer Mannheim) or HRP-conjugated goat anti-mouse IgGs (Dako, Glostrup, Denmark).

Downloaded from http://www.jimmunol.org/ by guest on August 16, 2017

The Journal of Immunology
the surface expression of the major xenoantigen Galα1-3Gal or their response to exposure to human serum (20).

Results

Selection of peptides on the basis of C1q inhibition

To select useful C1q-inhibiting peptides from the 42 peptide sequences previously reported by Lauvrak et al. (16), we synthesized these peptides and tested them in four different assay systems (Table I). First, all peptides were tested in a sensitive C1q-dependent hemolytic assay. Of 42 peptides, 30 peptides showed inhibition of the hemolytic activity of C1q. Next, all peptides were tested for their ability to inhibit the classical complement pathway in a CH50 test. Of the 30 selected peptides, 20 peptides showed inhibition of the CH50 test. Furthermore, all peptides were examined for hemolytic assay. Of 42 peptides, 30 peptides showed inhibition of these peptides and tested them in four different assay systems (Table I). First, all peptides were tested in a sensitive C1q-dependent hemolytic assay (HA), in a CH50 test for the classical complement pathway, and in an AP50 test for the alternative pathway, as indicated. Furthermore, all peptides were examined for binding to C1q in ELISA plates (Fig. 1). As an additional test for the specificity of peptide 2J, we established that the peptide does not bind to mannan-binding lectin, which initiates the lectin pathway of complement activation (data not shown).

Peptide Sequence

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequencea</th>
<th>C1q-HA</th>
<th>CH50</th>
<th>AP50</th>
<th>C1q Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>WLGLGGGYGW</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>1B</td>
<td>NFPWLMDGWE</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>1C</td>
<td>YARLARVTGA</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>1D</td>
<td>YWMDIVSRNM</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>2A</td>
<td>FXYGGFNLNSLRLGW</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>2B</td>
<td>LRFLNPSLDGSGFW</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>2C</td>
<td>HSPFCLGLVECPGLV</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2D</td>
<td>TGCAFYLYHPFDICG</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2E</td>
<td>MQICLASHELYLPWC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2F</td>
<td>FFFQGSDQAFAPSMD</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>2G</td>
<td>NAVVRPRWSVPYFP</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2H</td>
<td>TCGYGFSLNSRCP</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>2I</td>
<td>GGWMGHYIYAGDFEG</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2J</td>
<td>CEGPFGDRHDLTCW</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2K</td>
<td>SYSVNSFPCDCGTCD</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2L</td>
<td>FPLRATFVVRITIG</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3A</td>
<td>PCVIDTSRSGWRCYL</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3B</td>
<td>HAAFEPRGDVRHTLL</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>4A</td>
<td>CRWDSGWGVECRC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4B</td>
<td>CYNWGTWGEAVC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4C</td>
<td>CMVVMNVGDVRC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4D</td>
<td>CFWHDNTGGSDC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4E</td>
<td>CYYDMWIFLAGSC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4F</td>
<td>CFRAGKFLGC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4G</td>
<td>CKDRWKEVECRC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4H</td>
<td>CWNRFFKMDRC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4I</td>
<td>CYNRWAWGTVFC</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>4J</td>
<td>CVLWDAVGWNC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4K</td>
<td>CQRWGFRAINGC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4L</td>
<td>CRVEGTALRGC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5A</td>
<td>RWFCPCNPKECCCSISV</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5B</td>
<td>RSTYCNKNDSCHEPE</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>5C</td>
<td>QPPQCICDKGFGVCIRV</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>5D</td>
<td>KGKCKCPEEHCPÑEPM</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>5E</td>
<td>NRRCSDDDGKLWHELH</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>5F</td>
<td>MGICGTMPPPLCLPAW</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>5G</td>
<td>TLTCNWPMPGDTTPPP</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>6A</td>
<td>PLGRFCPTCPALPS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6B</td>
<td>QRMRPCPSCLAPW</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>6C</td>
<td>WFSRCPSCPEVPFP</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6D</td>
<td>SCTXDCTCPCLPVPP</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>6E</td>
<td>GNTPCPLPCQPSQ</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

*a All peptides were synthesized according to sequences previously published by Lauvrak et al. (16). The numbers used in the nomenclature refer to different screening rounds of various peptide libraries.

*b Peptides were tested for their inhibitory capacity in a C1q-dependent hemolytic assay (HA), in a CH50 test for the classical complement pathway, and in an AP50 test for the alternative pathway, as indicated. Furthermore, they were tested for binding of C1q in ELISA. Inhibition of complement activation and binding of C1q are indicated by +.
Structural variants of peptide 2J

Peptide 2J is a 15-meric peptide containing cysteine residues at positions 1 and 14 (Table I). Depending on the redox status of the environment, such a sequence may form a cyclic structure upon oxidation. We examined whether a cyclic structure is required for the C1q-inhibiting properties of peptide 2J. Therefore, five variants of peptide 2J were synthesized. Four variants were obligatory linear due to a deletion of either one cysteine (2J1,2) or both cysteines (2J4), or due to the replacement of both cysteine residues by 2-aminobutyric acid, an isosteric homolog of cysteine (Xa ) that cannot form S-S bridges (2J3) (Table II). These variants were tested for C1q inhibition in a C1q-dependent hemolytic assay. Peptide 2J inhibited C1q hemolytic activity with an IC50 of \( \mu \text{M} \), but the linear variants did not show inhibition of C1q when applied at a concentration of up to \( \mu \text{M} \) (Fig. 2A). Furthermore, these linear variants did not bind C1q in ELISA, suggesting that the circular structure is required for the interaction of peptide 2J with C1q. Alternatively, a stable cyclic variant of peptide 2J (2Jcy) was constructed by replacing the N-terminal cysteine with bromoacetic acid, which was used to cyclise the peptide (N terminus attached to the cysteine side chain). Peptide 2Jcy inhibited the C1q hemolytic activity in a dose-dependent way, but the IC50 was approximately eight times higher than that of the original peptide 2J (Fig. 2B). Apparently, the two cysteines in the original peptide 2J are required for optimal activity.

Peptide 2J inhibits the classical complement pathway by blocking the binding of C1q to its ligand

Inhibition of the different steps of the classical complement pathway by peptide 2J was further studied by ELISA. Incubation of IgM-coated ELISA plates with human serum resulted in deposition of activated C4 and C3 as well as the terminal complement complex C5b-9 on the plate. Incubation of human serum in the presence of peptide 2J inhibited activation of C4 and C3 in a dose-dependent way, whereas the linear peptide 2J-3 had no effect (Fig. 3). Furthermore, peptide 2J showed a dose-dependent inhibition of the binding of purified human C1q to coated IgG (Fig. 4A), suggesting an interaction of peptide 2J with the globular head domain of C1q. To further characterize the interaction between C1q and peptide 2J, an experiment was performed using mAb directed against various domains of C1q. A mAb directed against the head portion of C1q blocked the binding of C1q to peptide 2J and to IgG, whereas a mAb against the collagenous part of C1q did not affect the binding of C1q to either IgG or peptide 2J (Fig. 4B).

---

---

**FIGURE 1.** Selection of specific peptide inhibitors of the classical complement pathway. Various concentrations of the peptides 2J, 4B, and 2L, as indicated, were tested in different complement activation tests (A–C) and in ELISA (D). A. Peptides were preincubated with purified human C1q and tested in a C1q-dependent hemolytic assay. B. Peptides were preincubated with normal human serum and tested in a CH50 test for classical pathway activity. C. Peptides were preincubated with normal human serum and tested in an AP50 test for alternative pathway activity. D. Peptides were coated on ELISA plates followed by incubation with purified human C1q (0.1 \( \mu \text{g/ml} \)) and detection of C1q binding. A–C. Data represent the mean ± SD of duplicate analysis. Results are representative of three independent experiments.

**FIGURE 2.** The C1q-inhibiting activity of structural variants of peptide 2J. Various concentrations of peptide 2J, its linear variants (2J1–4, A) and a stable cyclic variant (2Jcy, B) were preincubated with human C1q and tested in a C1q-dependent hemolytic assay. Peptide 2L was included as a negative control. Data are presented as the mean ± SD from duplicate analysis. Similar results were obtained in at least two independent experiments.
Peptide 2J inhibits human, primate, and rodent C1q

Peptide 2J was selected from a phage display library on the basis of binding to human C1q. To examine the potency of peptide 2J for inhibition of C1q from other species, hemolytic assays were performed in which human C1q-deficient serum was reconstituted with limiting amounts of C1q from human, chimpanzee, rhesus monkey, rat, and mouse origin. Peptide 2J dose-dependently inhibited the hemolytic activity of C1q from all the species tested with a comparable strength (Fig. 5), suggesting that this peptide binds to a region of C1q that has a high degree of similarity among the various species.

Peptide 2J inhibits xenoreactivity of human serum to pig cells

Hyperacute rejection of xenografts is initiated by activation of the classical complement pathway following the binding of preformed xenoreactive Abs from the host to the graft endothelium. Accordingly, when PK15 cells (pig kidney epithelial cells) are exposed to human serum, binding of human anti-pig Abs results in complement activation and surface deposition of C3, as detected by flow cytometry (Fig. 6). C3 deposition was clearly detectable on the total cell population, as indicated by a shift of the fluorescence. Furthermore, a subpopulation of the cells showed particular strong fluorescence (C3 bright, fluorescence intensity > 200, 15.5%; Fig. 6, upper panel). Incubation of PK15 cells with human serum in the presence of peptide 2J, but not the linear variant peptide 2J-3, strongly inhibited the deposition of C3 on PK15 cells, as compared with the control (Fig. 6). This inhibitory effect of peptide 2J was apparent from the mean and the median fluorescence, from a shift of the major fluorescent peak as well as from a decrease of the
likely a matter of sensitivity. Two peptides showed inhibition of the CH50 assay but not of the C1q-dependent hemolytic assay. These peptides did not show C1q binding in ELISA, and they may interfere in the complement activation test at a different level, or in a nonspecific way. Furthermore, a group of peptides appeared to inhibit not only the classical pathway but also the alternative pathway, as demonstrated for peptide 4B. Peptide 4B, as well as other peptides from this group (4D, 4E, 2C, 2D, 2E, 6B), showed a clear binding to C1q in ELISA. It is possible that the complement-inhibiting potential of these peptides is due to (additional) interactions at another step in the complement activation cascade that is currently unidentified. Because the alternative pathway assay was performed in a calcium-free buffer, the inhibitory potential of 4B and related peptides in this assay is probably independent of its interaction with C1.

Some of the peptides examined did not show any interaction with C1q in our assays, neither functionally nor in ELISA, such as peptide 2L. Lauvrak et al. (16) reported a similar binding strength to C1q for peptides 2J and 2L, whereas we could demonstrate strong C1q binding for the former peptide only. This apparent contradiction may be related to the fact that Lauvrak et al. (16) assessed binding of phage-displayed peptides to coated C1q, whereas our binding assays used coated peptides and fluid phase C1q. The conformation of peptides in soluble form may differ from that of peptides displayed on a phage.

Based on the strength of the inhibitory capacity of the peptide for C1q and based on the specificity, peptide 2J was selected as the most promising candidate for a specific inhibitor of the classical complement pathway. This peptide inhibits the recognition phase of the classical pathway, namely the binding of C1q, resulting in inhibition of subsequent activation of C4 and C3, formation of the membrane attack complex, and erythrocyte lysis. Peptide 2J is a 15-meric peptide containing two cysteines at positions 1 and 14. Our results show that linear variants of the peptide are not active. In contrast, a stable cyclic variant showed C1q inhibitory activity and C1q binding. These results strongly suggest that peptide 2J is acting as a circular structure. Alternatively, it could be possible that peptide 2J forms active dimers or multimers via intermolecular S-S bridges. However, because the nonactive linear peptides 2J-1 and 2J-2 are able to form similar structures, whereas the active cyclic peptide 2Jcy is unable to form dimers, we consider this possibility as unlikely. Peptide 2Jcy is less active than the original peptide 2J, indicating that the two cysteines are important for the activity of peptide 2J, either by affecting the basic structure or size of the molecule or by a direct interaction with C1q. Interestingly, 31 of the 42 previously identified C1q-binding peptides contain at least two cysteines, and most C1q-binding peptides were identified in cysteine-constrained libraries (16).

Several other peptides have been described that bind C1q. An 11-meric peptide derived from the constant region of IgG1 inhibits the lysis of pig erythrocytes by human serum in the millimolar range (21). Furthermore, a dimeric peptide WY, derived from the C\textsubscript{H}2 domain of human IgG, inhibits lysis of EA, also in the millimolar range (22). Multimeric forms of these peptides had a strongly increased potency. The reported activity of the monomeric peptides is much less than that of peptide 2J, which is active in the micromolar range (22). Two different motifs were identified in C1q-binding peptides, i.e., the NPF motif ([N/S]PxFxL) and the SHY motif, of which the former motif was also present in some peptides identified by Lauvrak et al. (Ref. 16;
peptides 1B, 2B, and 2C, Table I). Peptides containing the NPF motif were shown to induce complement activation and consumption in the fluid phase (24).

Inhibition of C1q activity can also be accomplished using purified or recombinantly expressed natural C1q-binding proteins, such as the endothelial C1q receptor (17), calreticulin (25), the globular C1q receptor (26), and a C1q-binding protein derived from Escherichia coli (27). Furthermore, C1q-binding peptides derived from the globular C1q receptor (26) and from calreticulin (25) have been shown to inhibit C1q hemolytic activity. A related strategy for the development of C1q inhibitors is the synthesis of protein parts of the C1q molecule that can act as a competitive inhibitor of ligand binding. For this purpose, a 12-meric peptide was developed from the B chain of the globular head region of C1q (ghB) that inhibits complement-mediated lysis of EA with an IC50 of 130 μM (22). Furthermore, the ghB expressed as a fusion protein with maltose binding protein inhibits the lysis of EA (28, 29), and slightly better results were obtained with a trimerized ghB protein (29). These recombinant proteins have the potency to be developed into a therapeutic C1q inhibitor. In comparison with peptides, the production and administration of such recombinant proteins is more complicated and expensive. Therefore, the possible applicability of peptides for therapeutic complement inhibition is something worthwhile to be considered.

Recently a peptide was identified that can inhibit the complement system at the level of C3 (12). Compstatin is a 13-meric cyclic C3-binding peptide that inhibits complement activation in vitro and in vivo. Treatment with compstatin significantly prolonged the survival of pig kidneys perfused with human blood (15). Furthermore, compstatin blocked complement activation in baboons administered with recombinant human heparin-protein complexes (14). Recent data indicate that the cyclic structure of compstatin is important to prevent its breakdown by plasma proteases (30). The cyclic structure of 2J is required for its interaction with C1q but may in a similar way also be an advantage for its stability in plasma.

A major advantage of the use of C1q-specific peptides for complement inhibition in vivo is the specificity for classical pathway inhibition only. Activation of the complement system via the alternative pathway and via the lectin pathway is a major mechanism in innate immunity and takes place upon direct contact with microorganisms, without the need of Ab production. Therefore, these pathways are more than the classical pathway directly involved in prevention of infections. In view of the potential treatment of severely ill and immune-compromised patients with complement inhibitors, it is of great importance to allow activation of these pathways.

Treatment with peptide 2J will inhibit the earliest step of undesired classical pathway activation, i.e., the binding of the head domain of C1q to its target. This is of relevance in view of the proinflammatory effects of early products of the complement activation cascade, such as C4a (1). Furthermore, C1q binding may trigger effects mediated via C1q receptors, which are present on, e.g., phagocytes and endothelial cells (31). For the potential treatment of hyperacute rejection of xenografts, early inhibition of complement activation is advantageous, because endothelial cell activation induced by complement is most likely involved in various phases of graft rejection. Therefore, complement inhibition at the level of C1q, using fluid phase inhibitors, could be used in combination with transgenic expression of molecules, such as CD46, CD55, and CD59, which affect the complement system at a later stage.

The potential efficacy of peptide 2J in xenotransplantation is illustrated in this study by showing that the peptide strongly inhibits complement deposition on pig cells exposed to human serum. Following their exposure to human serum, C3 deposition on the pig cells shows marked heterogeneity, which is possibly related to heterogeneous expression of xenografts on these cells as well as variability in sensitivity to complement activation. Addition of peptide 2J significantly decreased complement activation, resulting in C3 binding of low intensity in a more homogenous pattern. This may indicate that activation of human complement on the pig cell surface is not completely dependent on the classical pathway. Alternatively, it could be a matter of peptide efficacy and/or stability. Assessment of the resistance of peptide 2J against degradation by plasma proteases, which is important for potential in vivo applications, requires experiments of longer duration (30).

Information from C1q-deficient patients and mice indicates that a lack of C1q is directly involved in the induction of systemic lupus-like autoimmunity (32). Recent discoveries point to a role of C1q in the clearance of apoptotic and injured cells, thus leading to persistence of damaged self material in the case of C1q deficiency. C1q binds to apoptotic cells via its head domain (Ref. 33)4. Therefore, it is important to consider the effects of potential C1q inhibitors on the complement-mediated clearance of self material. However, treatment of patients with complement inhibitors will in most cases take place on a short term to overcome a severe injury, the initial consequences of an acute disease, or an exacerbation of a chronic disease. We consider that the induction of autoimmunity could be a long-term consequence of C1q-targeted complement inhibition, requiring proper testing in animal models before patient studies should be undertaken. In this respect, it is a major advantage that peptide 2J inhibits C1q from rodent, primate, and human origin, which will facilitate experimental and preclinical testing of its efficacy and safety.

Although activation of the classical complement pathway is causally involved in a number of situations of harmful complement activation, other pathways are likely to be involved as well. The alternative pathway amplifies the complement activation cascade induced via any pathway at the level of C3. In addition, recent experiments in C1q knockout mice suggest that at least part of the glomerular complement deposition that accompanies the renal disease in these animals is induced via the alternative pathway (32). Furthermore, the lectin pathway can be involved in complement activation after endothelial oxidative stress (34). A peptide inhibitor of mannan-binding lectin that has been developed recently was able to attenuate endothelial complement binding induced by hypoxic treatment in vitro (35). Therefore, further definition of the contribution of the various complement pathways in specific pathological situations is of major importance for the development of an effective, specific, and safe treatment.

In conclusion, peptide 2J has promising properties for therapeutic complement inhibition because it specifically inhibits the classical complement pathway at the earliest possible level. Further studies will be undertaken to develop this peptide into an effective drug for in vivo use.

Acknowledgments

We thank Isabelle van der Borch tot Verwolde and Willemien Benchijouen for excellent technical assistance. Dr. W. Bogers, Dr. C. E. Hack, and Dr. T. E. Mollnes are acknowledged for providing valuable reagents and advice.

4 A. Nauta, M. Daha, O. Tijsma, R. Nieuwland, C. Hack, and A. Roos. C1q binding to apoptotic cells and cell blebs induces complement activation. Submitted for publication.
References


