Inhibition of Human Complement by a C3-Binding Peptide Isolated from a Phage-Displayed Random Peptide Library[1]

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We have screened a phage-displayed random peptide library for binding to C3b, the proteolytically activated form of complement component C3, and have identified a novel peptide that suppresses complement activation. This phage-displayed peptide bound to C3, C3b, and C3c, but not to C3d, indicating that it binds to the C3c region of C3. A synthetic 27-amino acid peptide corresponding to the phage-displayed peptide also bound to C3 and C3 fragments and inhibited both the classical and alternative pathways of complement activation. The inhibition of complement activation was reversible. Studies with overlapping peptides indicated that the functional activity was located in the cyclic 13-amino acid N-terminal region (ICVVQDWGHHRCT) of the parent peptide. Reduction and alkylation of this 13-residue synthetic peptide destroyed its inhibitory activity. Analysis of the mechanism of inhibition revealed that the peptide inhibited C3 cleavage in normal human serum as well as when the alternative pathway was reconstituted with purified complement components, and the observed inhibition was not due to sterically hindered access to the C3a/C3b cleavage site. Further, the peptide did not inhibit the cleavage of factor B, indicating that it did not affect the interaction of C3b with factor B or the formation of C3b,Bb. The peptide also had no effect on the binding of properdin to C3, demonstrating that the observed inhibition of C3 cleavage in normal human serum was not due in part to its effect on the properdin-stabilized C3 convertase, C3b,Bb,P. These results indicate that the peptide we have identified interacts with C3 to inhibit its activation. *The Journal of Immunology,* **1996, 157: 884–891.**

The complement component C3[3] plays a pivotal role in the activation of classical and alternative pathways of complement activation (1). Proteolytic activation of C3 by classical (C4b,2a) or alternative (C3b,Bb) pathway C3 convertase leads to cleavage of C3 into an anaphylotoxic peptide C3a and an opsonic fragment C3b. Covalent attachment of metastable C3b to target cells undergoing complement attack results in generation of C5a and formation of C5b-9 membrane attack complex.

Although complement is an important line of defense against pathogenic organisms, its activation may lead to host cell damage. Complement-mediated tissue injury has been reported in a wide variety of diseases, including autoimmune diseases such as experimental allergic neuritis (2), type II collagen-induced arthritis (3), myasthenia gravis (4, 5), hemolytic anemia (6), glomerulonephritis (7), and immune complex-induced vasculitis (8). It has also been identified in the adult respiratory distress syndrome (9), stroke (10), heart attack (11), xenotransplantation (12, 13), multiple sclerosis (14), burn injuries (15), and extracorporeal dialysis and blood oxygenation (16).

The tissue injury that results from complement activation is directly mediated by the membrane attack complex, C5b-C9, and indirectly by the generation of anaphylotoxic peptides, C3a and C5a. These peptides induce damage through their effects on neutrophils (17) and mast cells (18). Previous studies have demonstrated that upon stimulation with C5a, neutrophils produce a serine elastase that causes tissue injury (19). C5a also triggers the generation of toxic oxygen-derived free radicals from neutrophils (17), and both C3a and C5a stimulate rapid and enhanced production of leukotrienes from IL-3-primed basophils (20).

Control of the activation process is mediated in vivo by a family of structurally and functionally related proteins, termed regulators of complement activation. The regulators of complement activation include both plasma proteins, factor H and C4-binding protein, and membrane proteins, primarily complement receptor 1, decay-accelerating factor, and membrane cofactor protein. These proteins inhibit the generation of C3a and C5a by inactivating the C3 and C5 convertases of the classical and alternative pathways. Inhibition of complement activation by these proteins is achieved by dissociation of the subunits of C3 and C5 convertases and/or by proteolytic inactivation of the subunits by factor I. A soluble form of complement receptor 1 has been tested and found to suppress complement in several complement-dependent disease models (20). However, it is desirable to identify smaller functionally active fragments of these proteins or peptides that functionally mimic these proteins.

Random peptide libraries are a rich source of structural diversity and have proved to be a useful tool in identifying the peptide epitopes recognized by mAbs (21) and the ligands for various proteins (22–25). In this study we have used C3b to screen a phage-displayed random peptide library in an attempt to identify a peptide that inhibits complement activation. Biopanning resulted in isolation of one such peptide. We have further demonstrated that a

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[3] Abbreviations used in this paper: C3, third component of complement; C3b, the proteolytically activated form of C3; C3c, 135,300 molecular weight fragment of C3 generated using elastase; C3d, 35,000 molecular weight fragment of C3 generated using elastase; NHS, normal human serum; GVB, gelatin veronal-buffered saline; GVB^{++}, gelatin veronal-buffered saline containing 0.5 mM $MgCl_2$ and 0.15 mM $CaCl_2$; GVBE, gelatin veronal-buffered saline containing 10 mM EDTA; MgEDTA, 0.1 M $MgCl_2$ and 0.1 M EGTA; TFA, trifluoroacetic acid; EA, sheep erythrocytes coated with Abs; Er, rabbit erythrocytes; ABTS, 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid).

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synthetic peptide corresponding to this C3-binding peptide can inhibit complement activation in normal human serum (NHS), but not in rat or mouse serum, by inhibiting the proteolytic activation of C3 at a concentration approximately twice that of human C3. This peptide may, therefore, be of therapeutic value in clinical situations involving complement-mediated tissue damage.

Materials and Methods

Chemicals and buffers

All chemicals and reagents used for peptide synthesis were purchased from Applied Biosystems (Foster City, CA), with the exception of F-moc amino acids, which were obtained from Nova Biochem (San Diego, CA). Veronal-buffered saline, pH 7.4, contained 5 mM barbital and 145 mM NaCl. Gelatin veronal-buffered saline (GVB) was veronal-buffered saline containing 0.1% gelatin, GVB^{++} was GVB containing 0.5 mM $MgCl_2$ and 0.15 mM $CaCl_2$, and GVBE was GVB with 10 mM EDTA. MgEGTA contained 0.1 M $MgCl_2$ and 0.1 M EGTA. PBS, pH 7.4, contained 10 mM phosphate and 145 mM NaCl. The blocking buffer was PBS containing 0.5% milk and 1% BSA.

Purified complement components

Human complement proteins C3 (26, 27), factor B (28), factor H (29), and factor I (30) were purified from normal human plasma as previously described. Factor D was kindly provided by Dr. J. Volanakis (Department of Medicine, University of Alabama-Birmingham). The C3 used in this study was a mixture of 72% native C3 and 28% C3(H_2O), as determined by running a sample of the protein on a Mono-S column (Pharmacia, Piscataway, NJ) (31). C3b was generated by limited trypsin cleavage of C3 and purified on a Mono-Q column (Pharmacia) (32). C3c and C3d were generated by elastase treatment of C3 and purified on a Mono-Q column (32). Aggregated C3 was made using glutaraldehyde as previously described (33). For iodination, native C3 was separated from C3(H_2O) on a Mono-S column and radiolabeled using ^{125}I and Iodogen (Pierce Chemical Co., Rockford, IL). The sp. act. of the labeled C3 varied from 1.0 to 2.5 μCi/μg.

Construction of the phage library

The library consisted of 2×10^8 recombinants, each expressing the peptide sequence *SR* X_{12} (*S*, *P*, *T*, or *A*) *A* (*V*, *A*, *D*, *E*, or *G*) X_{12} *SR* at the N-terminus of pIII. Fixed and semifixed amino acids are italicized. The library was constructed by annealing and extending two long degenerate oligonucleotides with a short region of complementarity at their 3′ termini (34). The six nucleotides of complementarity, corresponding to the *Sac*II recognition sequence, encoded the tripeptides (S/P/T/A) A (V/A/D/E/G). This design, which fixes Ala as the central amino acid of every displayed peptide, permits subdividing of the long peptides for the analysis of binding residues by *Sac*II digestion of the DNA insert (35). The random amino acids were encoded by NNK, where N represents equimolar ratios of A, C, G, or T, and K represents G or T. The NNK coding scheme uses 32 codons to encode 20 amino acids; the frequency of each amino acids is once (C, D, E, F, H, I, K, M, N, Q, W, Y), twice (A, G, P, V, T), or three times (L, R, S) per codon.

Biopanning of the phage library

Long random peptides were considered to be a potential source of C3-binding peptides for a number of reasons; thus, we screened a random 27-mer library. We presumed that long peptides may adopt a secondary structure more conducive to binding than short peptides. In addition, a certain fraction of peptides would be expected to have pairs of cysteines that form intramolecular disulfide bonds during viral morphogenesis (34), thereby constraining the peptides in a potentially favorable conformation (36). The C3-binding phage was isolated by screening a library as described previously (34). Microtiter wells (Nunc, Inc., Naperville, IL) were coated overnight with 20 μg of C3b in PBS at 4°C and blocked with PBS containing 1% BSA for 30 min at 22°C. After washing, 6×10^{11} plaque-forming units of the library were added to each well and incubated overnight at 4°C. The wells were washed five times with PBS containing 0.1% Tween-20 and 0.1% BSA. Bound phage particles were eluted with 100 mM glycine-HCl, pH 2.3, and immediately neutralized with 100 mM Tris-HCl, pH 8.5. Recovered phage particles were amplified in DH5αF′ *Escherichia coli*, and the biopanning procedure was repeated twice as described above. The amplified phage mixture obtained after the third round of amplification was plated, and positive phages were identified by confirming their binding to C3b in an ELISA, in which bound phages were detected by peroxidase-labeled anti-M13 Ab (Pharmacia). DNA was prepared from positive phage stocks and subjected to dideoxy sequencing (37).

Synthesis and purification of peptides

A synthetic peptide corresponding to the phage-displayed C3-binding peptide and its analogues (peptides III, IV, and VI) were synthesized in an Applied Biosystem peptide synthesizer (model 431A, Foster City, CA) using F-moc amide resin. The side chain protecting groups were Cys(Trt), Asp(otBu), Arg (Pmc), Thr (tBu), Ser (tBu), Gln (Trt), Trp(Boc), His(Trt), and Asn(Trt). All peptides were cleaved from the resin by incubation for 3 h at 22°C with a solvent mixture containing 5% phenol, 5% thioanisole, 5% water, 2.5% ethanedithiol, and 82.5% trifluoroacetic acid (TFA). The reaction mixture was filtered through a fritted funnel, precipitated with cold ether, dissolved in 50% acetonitrile containing 0.1% TFA, and lyophilized. The crude peptides obtained after cleavage were dissolved in 10% acetonitrile containing 0.1% TFA and purified using a reverse phase C_{18} column (Waters Associates, Milford, MA). Disulfide oxidation of purified peptides I and IV was performed by stirring a 0.15-mM solution of peptide in 0.1 M ammonium bicarbonate, pH 8.0, and bubbling with oxygen at 22°C for 48 h. Purified peptides I and IV were reduced with 10 mM DTT and alkylated with 40 mM iodoacetamide. The identity and purity of all peptides were confirmed by laser desorption mass spectrometry (38, 39).

Peptide binding to C3 and C3 fragments

Binding of synthetic peptide to C3 and to various fragments of C3 was evaluated by means of an ELISA. Microtiter plates (Nunc) were coated for 1 h at 37°C with 100 μl of peptide I at 400 μg/ml or with 10 μg/ml of peptide I coupled to BSA (1/1, w/w), then saturated with blocking buffer for 30 min at 22°C. The plates were washed three times with PBS containing 0.05% Tween-20, and various amounts of C3 or C3 fragments were added. After incubation for 1 h at 22°C, the wells were washed and incubated at 22°C for 1 h with 100 μl of 2 μg/ml polyclonal rabbit anti-C3 Ab. Unbound anti-C3 Abs were removed by washing, and a 1/1000 dilution of peroxidase-conjugated goat anti-rabbit IgG was added and incubated for 1 h at 22°C. Color was developed by adding ABTS peroxidase substrate, and the optical density was read at 405 nm. Net binding was calculated by subtracting the readings obtained for nonspecific binding of C3 and C3 fragments to the plate in the absence of peptide.

Hemolytic assay

Inhibition of classical and alternative pathway activity by the peptides was measured as follows. To determine the effects of peptides on the classical pathway, various concentrations of peptide were mixed with 11 μl of NHS (diluted 1/10 in GVB^{++}), and 5 μl of EA (1×10^9/ml) and GVB^{++} was added to give a total volume of 250 μl. The reaction mixture was incubated at 37°C for 1 h and centrifuged. The percentage of lysis was determined by measuring the optical density of the supernatant at 414 nm. The effects of peptides on the alternative pathway were determined by measuring the lysis of Er in NHS. Various concentrations of peptide were mixed with 5 μl of NHS, 5 μl of MgEGTA, and 10 μl of Er (1×10^9/ml) and brought to a final volume of 100 μl in GVB. The reaction mixture was incubated at 37°C for 20 min and stopped by adding 200 μl of GVBE. After centrifugation, hemolysis was determined at 414 nm. The percentage of lysis was normalized by considering 100% lysis to be equal to lysis occurring in the absence of the peptide.

Measurement of C3 convertase-mediated cleavage of C3

Inhibition of C3 cleavage by peptide IV in NHS was studied as follows. Seven microliters of 35% NHS containing 0.5 μCi of [^{125}I]C3 and 14.3 mM MgEGTA was mixed with graded concentrations of peptide IV and 4 μl of 50% zymosan. The total volume of the reaction mixture was adjusted to 20 μl by adding GVB. Samples were incubated at 37°C for 30 min, mixed with 10 μl of 30 mM EDTA, and centrifuged. The supernatant obtained was mixed with SDS sample buffer containing 10 mM DTT, analyzed on a 7.5% SDS-PAGE gel (40), and subjected to autoradiography. Radioactive bands were excised and counted to calculate the percentage of C3 cleaved. The percentage of [^{125}I]C3 cleaved was normalized by considering 100% [^{125}I]C3 cleaved to be equal to the [^{125}I]C3 cleaved in the absence of the peptide. Controls were incubated in the presence of 10 mM EDTA.

To study the effect of peptide IV on C3 cleavage by purified complement components, 2 μg of C3 were incubated with various concentrations of the peptide at 37°C for 15 min. Thereafter, 2 μg of factor B and 0.04 μg of factor D were added in the presence of 5 mM MgEGTA in a total volume of 20 μl to activate the pathway. After 2 h at 37°C, samples were run on a 7.5% SDS-PAGE gel, stained, and scanned for densitometric

analysis, and the percentage of C3 cleaved was calculated. The data obtained were normalized by considering 100% C3 cleavage to be equal to the amount of C3 cleaved in the absence of the peptide. Controls were incubated in the presence of 10 mM EDTA.

Measurement of factor B cleavage

The effect of peptide IV on factor B cleavage was studied by quantitating the limited cleavage of factor B by factor D. C3b (2 μg) was preincubated for 15 min at 37°C with different concentrations of the peptide. The reaction mixture was then incubated at 37°C for 30 min with 2 μg of factor B and 0.06 ng of factor D in a total volume of 20 μl veronal-buffered saline containing 5 mM MgEGTA. The percentage of factor B cleaved was determined by electrophoresis of samples on a 7.5% SDS-PAGE gel under reducing conditions and densitometric analysis of the stained gel. Controls contained 10 mM EDTA instead of 5 mM MgEGTA.

ELISA for measurement of C3 binding to properdin

Binding of properdin to C3 was determined by ELISA as previously described (41). Briefly, microtiter wells were coated with 50 μl of C3 (20 μg/ml) by incubation at 37°C for 1 h. After coating, wells were saturated with 200 μl of blocking buffer at 22°C for 30 min and incubated for 1 h at 22°C with 50 μl of NHS diluted 1/50 in PBS, pH 7.4, containing 10 mM EDTA. To determine the effect of peptide IV, various concentrations of the peptide were added to the reaction mixture. The amount of properdin bound to C3 was quantitated by adding 50 μl of polyclonal goat anti-properdin Ab (10 μg/ml), followed by 50 μl of peroxidase-conjugated anti-goat Ab diluted 1/1000 in PBS (Bio-Rad Laboratories, Hercules, CA). Each Ab was incubated at 37°C for 1 h and washed with PBS, pH 7.4, containing 0.05% Tween-20. Color was developed by adding ABTS peroxidase substrate, and optical density was measured at 405 nm.

Results

Isolation and characterization of C3b-binding phages

To isolate C3b-binding phages, we screened a phage-displayed random peptide library that contains 2×10^8 unique clones expressing random peptides 27 amino acids in length. Phage particles expressing C3b-binding peptides were affinity purified by plating on a microtiter plate coated with C3b. After a third round of biopanning, individual phages isolated were tested for binding; 14 of 16 clones bound to C3b. DNA was isolated from all positive clones, and the nucleotide sequence of each was determined. All 14 positive clones had an identical sequence, indicating that this clone was specific and had been amplified during the second and third rounds of biopanning.

Binding results obtained with representative positive (clone 9) and negative (clone 1) clones showed that clone 9 bound to immobilized C3, C3b, and C3c, but not to C3d (Fig. 1). The binding strength of the positive clone followed the order C3 > C3b > C3c, possibly reflecting differences in affinity. To demonstrate that the binding of clone 9 was specific, a competition ELISA was performed (Fig. 1). Aggregated C3 (250 μg/ml) significantly inhibited the binding to C3, C3b, and C3c.

To confirm these results, a synthetic peptide corresponding to the deduced amino acid sequence of the phage-displayed peptide was tested in a binding assay. The synthetic peptide (peptide I) was coated on a microtiter plate, and its binding to C3 and C3 fragments was analyzed by ELISA. Immobilized peptide I bound to C3 and C3b; however, no binding to C3d was detected (Fig. 2). Peptide I also failed to bind to C3c (Fig. 2), suggesting that the peptide's binding site is buried when C3c is present in its native conformation.

Inhibition of complement activation by the C3-binding peptide

We hypothesized that the binding of peptide I to C3 or C3b might affect the interaction of these proteins with other complement proteins and inhibit complement activation. To test this possibility we examined the effect of peptide I on complement activation. Inhibition of the alternative pathway was measured by using rabbit erythrocytes in the presence of MgEGTA (Fig. 3*A*); inhibition of lysis of Ab-coated sheep erythrocytes was used as an indicator of inhibition of the classical pathway (Fig. 3*B*). Peptide I inhibited both classical and alternative pathways, with IC_{50} values of 65 and 19 μM, respectively.

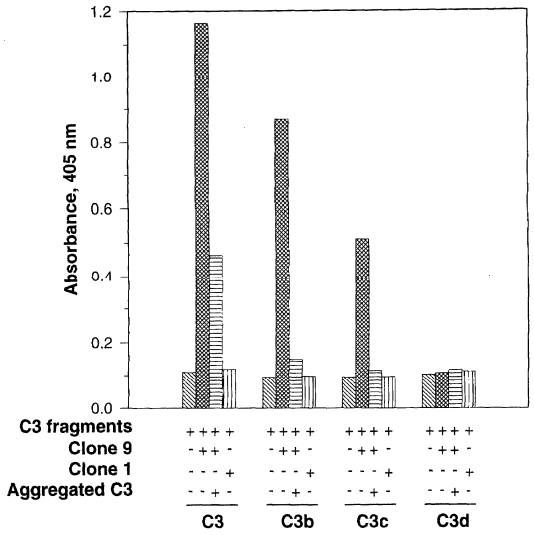

FIGURE 1. Specific binding of a C3-binding clone isolated from a phage-displayed random peptide library to C3 and C3 fragments. Microtiter wells were coated with 2 μg of C3, C3b, C3c, or C3d; saturated with blocking buffer; washed with PBS, pH 7.4, containing 0.05% Tween-20; and incubated for 1 h at 22°C with a positive (clone 9) or a negative (clone 1) clone. Binding was inhibited by adding 250 μg/ml of aggregated C3 in PBS, pH 7.4. Bound M13 phage particles were detected by peroxidase-coupled anti-M13 Ab and ABTS peroxidase substrate.

To determine whether the peptide inhibits rat or the mouse complement, we measured the effect on the complement-mediated lysis of Er by rat and mouse sera. Peptide I had no effect on mouse or rat complement activation. To confirm that the binding of the peptides was specific to human C3, we reconstituted the hemolytic activity of C3-deficient mouse serum by adding human C3 and looked for inhibition of hemolytic activity by the peptide. Peptide I inhibited the alternative pathway hemolytic activity of the reconstituted sera at concentrations similar to those found for human sera (data not shown).

Identification of the functional region of the C3 binding peptide involved in inhibition of complement activation

To localize the functional region of peptide I, we synthesized two overlapping peptides of 17 and 13 residues (peptides III and IV, respectively) and tested their abilities to inhibit 50% complement activity (Table I). Inhibitory activity was retained by the cyclic N-terminal region of the parent peptide (peptide IV). In contrast, peptide III showed no inhibitory activity, indicating that this region is not important for binding. To further characterize this peptide, we made a linear peptide (peptide V) by reducing and alkylating peptide IV. This reduction and alkylation destroyed the inhibitory activity of peptide IV and that of peptide I (to give peptide II) (Table I), suggesting that cysteines are important in maintaining the stable structures of peptides I and IV. The concentration of peptide I or IV that was required to inhibit the alternative pathway

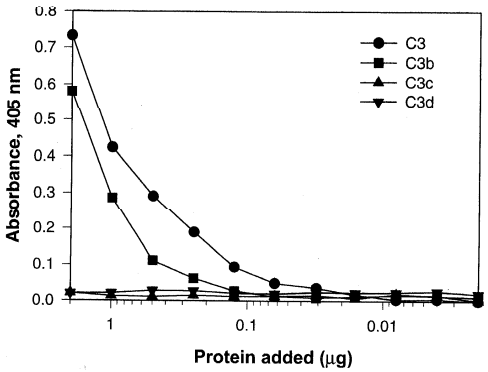

FIGURE 2. Binding of peptide I to C3 and C3 fragments. A microtiter plate was coated with peptide I, saturated with blocking buffer, washed with PBS, pH 7.4, containing 0.05% Tween-20, and incubated with a twofold dilution of C3, C3b, C3c, or C3d for 1 h at 22°C. The plate was then washed and incubated with a polyclonal rabbit anti-C3 Ab (2 μg/ml) and a 1/1000 dilution of peroxidase-coupled anti-rabbit Ab. Color was developed with the ABTS peroxidase substrate.

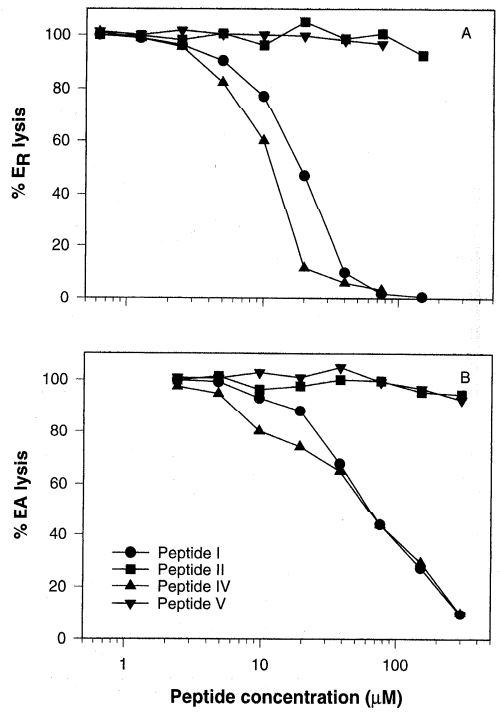

FIGURE 3. Inhibition of classical and alternative pathway-mediated lysis of erythrocytes by peptide I and its analogs. Cyclic (peptides I and IV) and reduced and alkylated (peptides II and V) peptides were tested for their effects on the alternative (*A*) and classical (*B*) pathways of complement activation as described in *Materials and Methods*.

was lower than that required to inhibit the classical pathway, perhaps because the alternative pathway is more sensitive to activation and deposition of C3 on the target cells. Peptide VI was 5.8-fold less inhibitory than peptide IV, indicating that flanking amino acid residues and/or Agr are important in enhancing the inhibitory activity of the peptide (Table I).

Reversible binding of C3-binding peptide to C3

To determine whether the inhibition of complement by C3-binding peptide is reversible, C3 (20 μg) was incubated with 100 μM peptide IV in 40 μl GVB at 37°C for 1 h. The C3-peptide mixture was spin-dialyzed over a G-25 column, diluted fivefold in GVB, and incubated at 37°C for 15 min. The hemolytic activities of C3 before and after dialysis were assayed by incubating C3 (6 μg) with 10 μl of rabbit erythrocytes (1×10^9/ml) and 25 μl of C3-deficient serum (Sigma Chemical Co., St. Louis, MO) in 100 μl of GVB at 37°C for 20 min. The hemolytic activity of treated C3, which was 2% that of untreated C3, was restored to 85% after dialysis followed by incubation at 37°C for 15 min.

Inhibition of C3 convertase-mediated cleavage of C3 by the C3-binding peptide

To verify the effect of the 13-residue peptide IV on complement activation, C3 cleavage was measured in NHS. This assay showed a concentration-dependent inhibition of C3 convertase C3b,Bb-mediated cleavage of C3 by peptide IV (Fig. 4). The peptide inhibited C3 cleavage with an IC_{50} of 10 μM, which correlated well with the concentration required to inhibit 50% of the hemolytic activity (Table I). During complement activation in serum, C3 convertase cleaves C3 into C3b, which is immediately inactivated by factors H and I to iC3b. The assay we used measures the amount of iC3b generated during activation, and the inhibition observed has been interpreted to be due to inhibition of C3 cleavage by C3 convertase. However, similar results could be obtained by inhibition of factor H- and I-mediated cleavage of C3b to iC3b. To rule out the second possibility, 5 μg of purified C3b was incubated with 1 μg of factor H and 0.04 μg of factor I in the presence of peptide IV. No inhibition of iC3b generation was observed, even at 300 μM, a concentration 30 times higher than the IC_{50} for C3 cleavage (data not shown). To examine the effect of peptide IV on purified C3 free of possible labeling artifacts, the alternative pathway was reconstituted with purified C3, factor B, and factor D (Fig. 5). As expected, peptide IV inhibited the proteolytic activation of C3 to C3b, with an IC_{50} of 28 μM. Peptide V, the reduced and alkylated form of peptide IV, had no effect on C3 cleavage.

Effect of the C3-binding peptide on the cleavage of C3 by trypsin

The data presented in Figure 5 suggest that peptide IV inhibits C3 cleavage by the convertase enzyme C3b,Bb. To explore the possibility that this inhibition is due to sterically hindered access to the C3a/C3b cleavage site, C3 (2 μg) was incubated in 20 μl of PBS with 800 μM of peptide VI (an analogue of peptide IV in which Arg was replaced with Ala; Table I) at 37°C for 15 min. The reaction mixture was then incubated either with 20 ng of trypsin at 22°C for 10 min or with factors B and D in the presence of MgEGTA (as described in *Materials and Methods*). Peptide VI failed to inhibit C3 cleavage by trypsin while completely inhibited the C3 conversion by C3b,Bb (Fig. 6).

Effect of the C3-binding peptide on the interaction of C3b with factor B

Binding of peptide IV to C3b could result in inhibition of its interaction with factor B (Fig. 7), H, or I. As described above, we observed that binding of peptide IV to C3b had no effect on the interaction of C3b with factor H or I. Because the interaction of C3b with factor B is important for the formation of C3 convertase (C3b,Bb), inhibition of this interaction by peptide IV could result in inhibition of C3 cleavage and, thus, of complement activation. We, therefore, examined the interaction of C3b with factor B in a fluid phase assay in which purified C3b, factor B, and factor D were incubated together with MgEGTA so as to generate C3b,Bb in the presence or the absence of peptide IV. The concentration of

Table I. *Amino acid sequences and functional activity of C3 binding peptide and its analogs*

Peptide/Clone	Amino Acid Sequence	Classical Pathway[a] IC_{50} (μM)	Alternative Pathway[b] IC_{50} (μM)
	1 15 31		
Clone 9	**S-S**-I-C-V-V-Q-D-W-G-H-H-R-C-T-A-G-H-M-A-N-L-T-S-H-A-S-A-I-**S-R**	ND	ND
Peptide I	I-C-V-V-Q-D-W-G-H-H-R-C-T-A-G-H-M-A-N-L-T-S-H-A-S-A-I	65	19
Peptide II[c]	I-C-V-V-Q-D-W-G-H-H-R-C-T-A-G-H-M-A-N-L-T-S-H-A-S-A-I	>300	>300
Peptide III	R-A-T-A-G-H-M-A-N-L-T-S-H-A-S-A-I	>300	>300
Peptide IV	I-C-V-V-Q-D-W-G-H-H-R-C-T	63	12
Peptide V[c]	I-C-V-V-Q-D-W-G-H-H-R-C-T	>600	>300
Peptide VI	C-V-V-Q-D-W-G-H-H-A-C	ND	70

[a] Classical pathway activity was determined using EA lysis assay.
[b] Alternative pathway activity was measured using Er lysis assay.
[c] In peptide II and V, cysteines were reduced and alkylated (see *Materials and Methods*). Bold-face residues were fixed in all library clones.

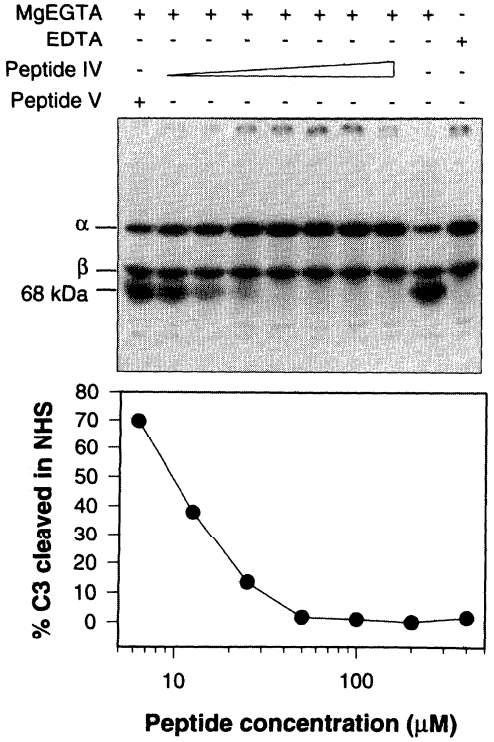

FIGURE 4. Inhibition of C3 cleavage by peptide IV during alternative pathway activation in NHS. Cleavage of C3 was measured by incubating NHS containing [^{125}I]C3 (0.5 μCi) with 5 mM MgEGTA, zymosan, and increasing concentrations of peptide IV for 30 min at 37°C. Samples were analyzed by 7.5% SDS-PAGE under reducing conditions, and the gel was subjected to autoradiography. Radioactive bands were cut out and counted. The data were normalized by considering 100% cleavage of C3 to be equal to the amount of C3 cleaved in the absence of peptide IV. Controls contained 10 mM EDTA. Peptide V (200 μM) was included as a control peptide.

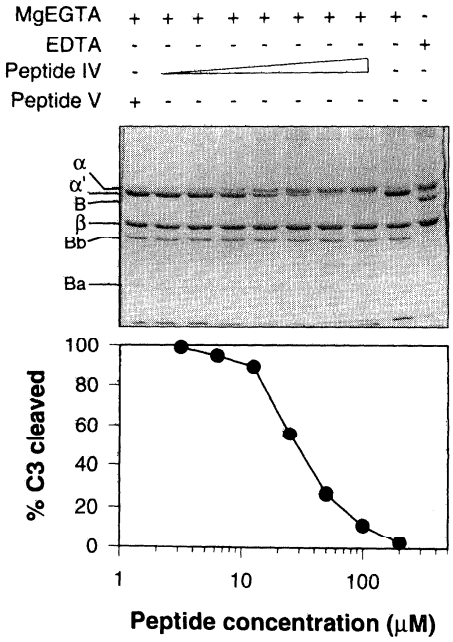

FIGURE 5. Inhibition of C3 cleavage by peptide IV during activation of the alternative pathway reconstituted with purified components. The alternative pathway was reconstituted by adding C3 and factors B and D, and various concentrations of the peptide IV were added. Samples were analyzed by 7.5% SDS-PAGE under reducing conditions. The gel was stained with Coomassie blue, and the intensity of each band was determined by densitometric analysis. Data were normalized by considering 100% cleavage of C3 to be equal to the cleavage observed in the absence of peptide IV. Controls contained EDTA. Peptide V (200 μM) was included as a control peptide.

factor D was adjusted to generate limited cleavage of factor B. Peptide IV showed no inhibition of factor B cleavage (Fig. 7), suggesting that this peptide has no effect on the interaction of C3b with factor B or on C3b,Bb formation.

Effect of the C3-binding peptide on binding of properdin to C3

It is possible that during complement activation, peptide IV inhibits the interaction of C3 with multiple complement proteins. Inhibition by peptide IV of properdin binding to C3 could inhibit the stabilization of C3 convertase by properdin in serum, an event that could, in turn, lead to inhibition of C3 cleavage. We, therefore, used a competition ELISA to examine the effect of peptide on properdin binding to C3 (Fig. 8). Peptide IV had no significant effect on properdin binding to C3, suggesting that the observed

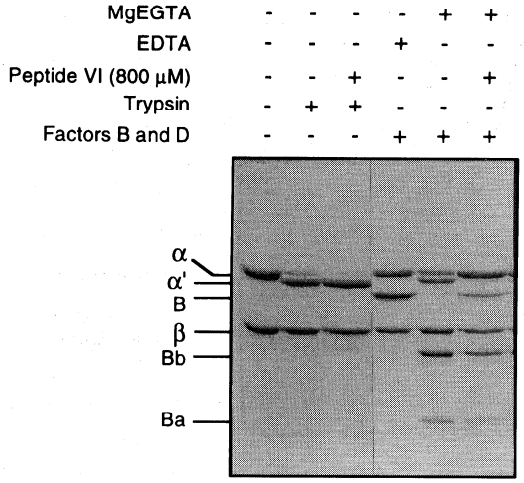

FIGURE 6. Effect of peptide VI on trypsin-mediated conversion of C3 to C3b. C3 was incubated in the presence or the absence of peptide VI with trypsin at 22°C for 10 min. Samples were analyzed by 7.5% SDS-PAGE under reducing conditions. Controls were incubated with factor B, factor D, and MgEGTA instead of trypsin.

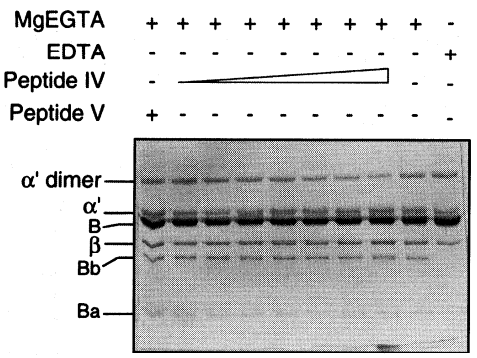

FIGURE 7. Effect of peptide IV on factor B cleavage. Factor B cleavage was quantitated by incubating C3b with factor B, factor D, and various concentrations of peptide IV (6–400 μM) in the presence of MgEGTA for 30 min at 37°C. Samples were analyzed by 7.5% SDS-PAGE in the presence of DTT. The gel was stained and scanned for densitometric analysis. Peptide V (200 μM) was included as a control peptide. Controls were set by adding 10 mM EDTA.

inhibition of C3 cleavage in NHS is not due to disruption of the properdin-stabilized C3 convertase, C3b,Bb,P.

Discussion

The importance of complement-mediated tissue injury in a variety of disease states underscores the growing need for a specific complement inhibitor. In the past, various conventional approaches have been used to identify such an inhibitor. These include targeting the serine proteases with peptides (42, 43) or chemical compounds (44–46) and, more recently, targeting the thioester of C3 (47). In this study we used a phage-displayed random peptide library to identify a peptide that inhibits complement. This approach has a unique advantage over other methods because it can rapidly open unexpected leads for designing therapeutically useful compounds.

Because C3b has a relatively low affinity for other complement proteins (48), half-physiologic ionic strength buffers have traditionally been used to study these interactions. In an attempt to increase the probability of selecting phage with high affinity, we used buffers of physiologic ionic strength for biopanning. With this strategy we achieved limited success. Three rounds of biopanning yielded positive clones, with all positive clones having an identical sequence. We found that a synthetic peptide analogous to the identified phage-displayed C3-binding peptide was able to inhibit the alternative pathway of complement activation at a concentration that was only twofold greater than the concentration of C3 in NHS (Fig. 3 and Table I), a finding that strongly suggests its potential as a therapeutic agent. To our knowledge, salicyl hydroxamate was previously found to be the most potent inhibitor of C3 (47, 49). This compound inhibited complement by reacting with the thioester of C3. The 50% inhibitory concentrations required for the inhibition of classical and alternative pathway-mediated hemolytic activities were 280 and 33 μM, respectively (47) (compared with 63 and 12 μM for peptide IV). Unfortunately, salicyl hydroxamate may produce systemic lupus erythematosus-like syndrome as its toxic side-effect (50).

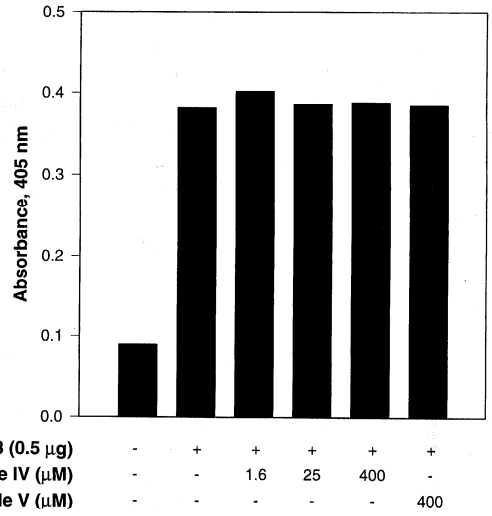

FIGURE 8. Effect of peptide IV on properdin binding to C3 coated to a microtiter plate. C3 (20 μg/ml) was coated onto microtiter wells and incubated with NHS containing 10 mM EDTA and graded concentrations of peptide IV for 1 h at 22°C. Binding of properdin was detected by a polyclonal goat anti-properdin Ab (10 μg/ml) and a 1/1000 dilution of peroxidase-conjugated anti-goat Ab. Peptide V was included as a control peptide.

The binding data (Figs. 1 and 2) suggest that the binding site for synthetic inhibitory peptide I is located in the C3c region of C3 and that a functional domain exists in this region that is important in C3 activation. It is important to note that while peptide I bound to C3 and C3b both in solution and in an immobilized state, we found that binding of peptide I to C3c occurred when the C3c was immobilized on a microtiter plate (Fig. 1), but not when the peptide was adsorbed to the plate and C3c was presented in native form in solution (Fig. 2). It, therefore, appears that the binding epitope is not exposed in C3c unless the conformation of C3c is altered in some way (e.g., by adsorbtion to a solid support).

We were able to identify a cyclic 13-amino acid N-terminal region (peptide IV) of the 27-amino acid peptide I that retained the inhibitory activity of the parent peptide. Reduction and alkylation of peptides I and IV destroyed their inhibitory activity, strongly suggesting that oxidation of cysteine residues is required for activity and maintenance of the preferred structure of the inhibitory

peptides. Previous studies have demonstrated that cysteine residues in phage-displayed peptides are disulfide bridged due to the oxidizing environment of the bacterial periplasmic space (51, 52). When we submitted the sequence of peptide I to a protein database for sequence similarity with known proteins, no significant similarity was found to any known proteins. Preliminary efforts to identify the protein in NHS using a polyclonal anti-peptide Ab have also failed.

The fact that higher concentrations of peptides I and IV were required to inhibit the classical pathway than to inhibit the alternative pathway prompted us to study the effects of the peptides on the initial phases of alternative pathway activity, since these steps are more sensitive to activation and deposition of C3 onto the activators. Because peptide IV inhibited the cleavage of C3 to iC3b in NHS containing MgEGTA (Fig. 4) but did not affect the conversion of C3b to iC3b by factors H and I, we infer that the primary site of action is either upon the activation of C3 or upon the formation of C3b,Bb. Reconstitution of the alternative pathway with C3 and factors B and D in the presence of the peptide inhibited the conversion of C3 to C3b (Fig. 5), further confirming this hypothesis. To dissect its effect on C3 activation, as opposed to C3b,Bb formation, we incubated peptide IV with purified C3b, factor B, and factor D and allowed convertase to form. The peptide had no effect on B cleavage, indicating that it predominantly affects the proteolytic activation of C3. Parallel experiments performed with peptide I showed similar results. It is possible that binding of either peptide I or IV to native C3 causes a local steric effect or a conformational change in the C3 structure that makes the Arg^{726}-Ser^{727} bond unavailable to C3b,Bb. Alternatively, peptide binding could inhibit the interaction of native C3 with C3b,Bb. The fact that peptide VI failed to inhibit C3 conversion by trypsin (Fig. 6) rules out the first possibility.

The proteolytically activated form of C3, C3b, binds to more than 20 serum and membrane proteins, most of which belong to a superfamily of structurally and functionally related molecules (53). However, in native C3, the binding sites are buried for these proteins and become available only after the conformational change that occurs upon the cleavage of C3 to C3b. The data presented in Figures 1, 2, and 5 make it clear that peptides I and IV bind to native C3 and inhibit its activation. Thus, the inhibition of complement with these inhibitory peptides would inhibit not only the generation of C5a but also that of C3a. To our knowledge these are the only peptides known to bind and inhibit activation of native C3.

In summary, we have identified a peptide of 13 amino acids that binds specifically to human C3 and inhibits the activation of the classical as well as the alternative pathway of complement activation by inhibiting C3 convertase-mediated cleavage of C3. The molar concentration of the peptide required to inhibit 50% of alternative pathway-mediated complement activity was only twofold higher than the concentration of C3 in NHS, suggesting that this peptide has potential therapeutic use in diseases involving complement-mediated damage.

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References

1. Lambris, J. D. 1993. Chemistry, biology and phylogeny of C3. *Complement Profiles 1:16 (Rev.).*
2. Vriesendorp, F. J., R. E. Flynn, M. A. Pappolla, and C. L. Koski. 1995. Complement depletion affects demyelination and inflammation in experimental allergic neuritis. *J. Neuroimmunol. 58:157.*
3. Watson, W. C., and A. S. Townes. 1985. Genetic susceptibility to murine collagen II autoimmune arthritis. *J. Exp. Med. 162:1878.*
4. Nakano, S., and A. G. Engel. 1993. Myasthenia gravis: quantitative immunocytochemical analysis of inflammatory cells and detection of complement membrane attack complex at the end-plate in 30 patients. *Neurology 43:1167.*
5. Barohn, R. J., and R. L. Brey. 1993. Soluble terminal complement components in human myasthenia gravis. *Clin. Neurol. Neurosurg. 95:285.*
6. Schreiber, A. D., and M. M. Frank. 1972. Role of antibody and complement in the immune clearance and destruction of erythrocytes. I. In vivo effects of IgG and IgM complement-fixing sites. *J. Clin. Invest. 51:575.*
7. McLean, R. H. 1993. Complement and glomerulonephritis: an update. *Pediatr. Nephrol. 7:226.*
8. Cochrane, C. G. 1984. The role of complement in experimental disease models. *Springer Semin. Immunopathol. 7:263.*
9. Robbins, R. A., W. D. Russ, J. K. Rasmussen, and M. M. Clayton. 1987. Activation of the complement system in the adult respiratory distress syndrome. *Am. Rev. Respir. Dis. 135:651.*
10. Vasthare, U. S., R. H. Rosenwasser, F. C. Barone, and R. F. Tuma. 1993. Involvement of the complement system in cerebral ischemic and reperfusion injury. *FASEB J. 7:A424.*
11. Kilgore, K. S., G. S. Friedrichs, J. W. Homeister, and B. R. Lucchesi. 1994. The complement system in myocardial ischaemia/reperfusion injury. *Cardiovasc. Res. 28:437.*
12. Wang, M. W., P. S. Johnston, L. J. Wright, S. M. L. Lim, and D. J. G. White. 1992. Immunofluorescent localization of pig complement component-3, regardless of the presence or absence of detectable immunoglobulins, in hyperacutely rejected heart xenografts. *Histochem. J. 24:102.*
13. Leventhal, J. R., A. P. Dalmasso, J. W. Cromwell, J. L. Platt, C. J. Manivel, R. M. Bolman, and A. J. Matas. 1993. Prolongation of cardiac xenograft survival by depletion of complement. *Transplantation 55:857.*
14. Williams, K. C., E. Ulvestad, and W. I. Hickey. 1994. Immunology of multiple sclerosis. *Clin. Neurosci. 2:229.*
15. Gallinaro, R., W. G. Cheadle, K. Applegate, and H. C. J. Polk. 1992. The role of the complement system in trauma and infection. *Surg. Gynecol. Obstet. 174:435.*
16. Pekna, M., L. Nilsson, K. Nilssonekdahl, U. R. Nilsson, and B. Nilsson. 1993. Evidence for iC3 generation during cardiopulmonary bypass as the result of blood-gas interaction. *Clin. Exp. Immunol. 91:404.*
17. Ehrengruber, M. U., T. Geiser, and D. A. Deranleau. 1994. Activation of human neutrophils by C3a and C5a: comparison of the effects on shape changes, chemotaxis, secretion, and respiratory burst. *FEBS Lett. 346:181.*
18. Ellati, S. G., C. A. Dahinden, and M. K. Church. 1994. Complement peptides C3a- and C5a-induced mediator release from dissociated human skin mast cells. *J. Invest. Dermatol. 102:803.*
19. Smedly, L. A., M. G. Tonnesen, R. A. Sandhaus, C. Haslett, L. A. Guthrie, R. B. J. Johnston, P. M. Henson, and G. S. Worthen. 1986. Neutrophil-mediated injury to endothelial cells: enhancement by endotoxin and essential role of neutrophil elastase. *J. Clin. Invest. 77:1233.*
20. Kalli, K. R., P. Hsu, and D. T. Fearon. 1994. Therapeutic uses of recombinant complement protein inhibitors. *Springer Semin. Immunopathol. 15:417.*
21. Scott, J. K., and G. P. Smith. 1990. Searching for peptide ligands with an epitope library. *Science 249:386.*
22. Sparks, A. B., L. A. Quilliam, J. M. Thorn, C. J. Der, and B. K. Kay. 1994. Identification and characterization of Src SH3 ligands from phage-displayed random peptide libraries. *J. Biol. Chem. 269:23853.*
23. Blond-Elguindi, S., S. E. Cwirla, W. J. Dower, R. J. Lipshutz, S. R. Sprang, J. F. Sambrook, and M. H. Gething. 1993. Affinity panning of a library of peptides displayed on bacteriophages reveals the binding specificity of BiP. *Cell 75:717.*
24. Dedman, J. R., M. A. Kaetzel, H. C. Chan, D. J. Nelson, and G. A. J. Jamieson. 1993. Selection of targeted biological modifiers from a bacteriophage library of random peptides: the identification of novel calmodulin regulatory peptides. *J. Biol. Chem. 268:23025.*
25. Devlin, J. J., L. C. Panganiban, and P. E. Devlin. 1990. Random peptide libraries: a source of specific protein binding molecules. *Science 245:404.*
26. Hammer, C. H., G. H. Wirtz, L. Renfer, H. D. Gresham, and B. F. Tack. 1981. Large scale isolation of functionally active components of the human complement system. *J. Biol. Chem. 256:3995.*
27. Alsenz, J., D. Avila, H. P. Huemer, I. Esparza, J. D. Becherer, T. W. Kinoshita, Y. S. Oppermann, and J. D. Lambris. 1992. Phylogeny of the third component of complement, C3: analysis of the conservation of human CR1, CR2, H, and B binding sites, Concanavalin A binding sites, and thiolester bond in the C3 from different species. *Dev. Comp. Immunol. 16:63.*
28. Lambris, J. D., and H. J. Muller-Eberhard. 1984. Isolation and characterization of a 33,000-dalton fragment of complement factor B with catalytic and C3b binding activity. *J. Biol. Chem. 259:12685.*
29. Alsenz, J., T. F. Schulz, J. D. Lambris, R. B. Sim, and M. P. Dierich. 1985. Structural and functional analysis of the complement component factor H with the use of different enzymes and monoclonal antibodies to factor H. *Biochem. J. 232:841.*
30. Hsiung, L.-M., A. N. Barclay, M. R. Brandon, E. Sim, and R. R. Porter. 1982. Purification of human C3b inactivator by monoclonal-antibody affinity chromatography. *Biochem. J. 203:293.*
31. Pangburn, M. K. 1987. A fluorometric assay for native C3: the hemolytically active form of the third component of human complement. *J. Immunol. Methods 102:7.*
32. Becherer, J. D., and J. D. Lambris. 1988. Identification of the C3b receptor-binding domain in third component of complement. *J. Biol. Chem. 263.14586.*

33. Erdei, A., F. Melchers, T. Schulz, and M. Dierich. 1985. The action of human C3 in soluble or cross-linked form with resting and activated murine B lymphocytes. *Eur. J. Immunol. 15:184.*
34. Kay, B. K., N. B. Adey, Y.-S. He, J. P. Manfredi, A. H. Mataragnon, and D. M. Fowlkes. 1993. An M13 phage library displaying random 38-amino acid peptides as a source of novel sequences with affinity to select targets. *Gene 128:59.*
35. Adey, N., and B. Kay. 1996. Identification of calmodulin-binding peptide consensus sequences from a phage-displayed random peptide library. *Gene 169:133.*
36. Kay, B. K. 1995. Mapping protein-protein interactions with biologically expressed random peptide libraries. *Persp. Drug Discovery Design 2:251.*
37. Sanger, F. S., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA 74:5463.*
38. Moore, W. T. 1993. Integration of mass spectrometry into strategies for peptide synthesis. *Biol. Mass Spectrom. 22:149.*
39. Angeletti, R. H., L. Bibbs, L. F. Bonewald, G. B. Fields, J. S. McMurray, W. T. Moore, and J. T. Stults. 1996. Formation of disulfide bond in an octreotide-like peptide: a multicenter study. In *Techniques in Protein Chemistry*, Vol. 7. D. R. Marshak, ed. Academic Press, San Diego, p. 261.
40. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 277:680.*
41. Daoudaki, M. E., J. D. Becherer, and J. D. Lambris. 1988. A 34-amino acid peptide of the third component of complement mediates properdin binding [published erratum appears in *J. Immunol. 141:1788*].
42. Glover, G., C. S. Schasteen, W. S. Liu, and R. P. Levine. 1988. Synthetic peptide inhibitors of complement serine proteases. I. Identification of functionally equivalent protease inhibitor sequences in serpins and inhibition of C1s and D. *Mol. Immunol. 25:1261.*
43. Schasteen, C. S., R. P. Levine, S. A. McLafferty, R. F. Finn, L. D. Bullock, J. C. Mayden. and G. I. Glover. 1991. Synthetic peptide inhibitors of complement serine proteases. III. Significant increase in inhibitor potency provides further support for the functional equivalence hypothesis. *Mol. Immunol. 28:17.*
44. Kam, C. M., T. J. Oglesby, M. K. Pangburn, J. E. Volanakis, and J. C. Powers. 1992. Substituted isocoumarins as inhibitors of complement serine proteases. *J. Immunol. 149:163.*
45. Aoyama, T., Y. Ino, M. Ozeki, M. Oda, T. Sato, Y. Koshiyama, S. Suzuki, and M. Fujita. 1984. Pharmacological studies of FUT-175, nafamstat mesilate. I. Inhibition of protease activity in in vitro and in vivo experiments. *Jpn. J. Pharmacol. 35:203.*
46. Laura, R., D. J. Robison, and D. H. Bing. 1980. (*p*-Amidinophenyl) methanesulfonyl fluoride, an irreversible inhibitor of serine proteases. *Biochemistry 19: 4859.*
47. Sahu, A., and M. K. Pangburn. 1996. Investigation of mechanism-based inhibitors of complement targeting the activated thioester of human C3. *Biochem. Pharmacol. 51:797.*
48. Becherer, J. D., J. Alsenz, and J. D. Lambris. 1989. Molecular aspects of C3 interactions and structural/functional analysis of C3 from different species. *Curr. Top. Microbiol. Immunol. 153:45 (Rev.).*
49. Sim, R. B., T. M. Twose, D. S. Paterson, and E. Sim. 1981. The covalent-binding reaction of complement component C3. *Biochem. J. 193:115.*
50. Sim, E., E. W. Gill, and R. B. Sim. 1984. Drugs which induce systemic lupus erythematosus inhibit complement component C4. *Lancet 2:422.*
51. Luzzago, A., F. Felici, A. Tramontano, A. Pessi, and R. Cortese. 1993. Mimicking of discontinuous epitopes by phage-displayed peptides. I. Epitope mapping of human H ferritin using a phage library of constrained peptides. *Gene 128:51.*
52. Kremser, A., and I. Rasched. 1994. The adsorption of filamentous phage fd: assignment of its disulfide bridges and identification of the domain incorporated in the coat. *Biochemistry 33:13954.*
53. Holers, V. M., T. Kinoshita, and H. Molina. 1992. The evolution of mouse and human complement C3-binding proteins: divergence of form but conservation of function. *Immunol. Today 13:231.*