The Journal of Immunology

Complement Inhibitor of C5 Activation from the Soft Tick *Ornithodoros moubata*

Miles A. Nunn, Ankit Sharma, Guido C. Paesen, Sharon Adamson, Olga Lissina, Anthony C. Willis and Patricia A. Nuttall

J Immunol 2005; 174:2084-2091; ;
doi: 10.4049/jimmunol.174.4.2084
http://www.jimmunol.org/content/174/4/2084

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Complement Inhibitor of C5 Activation from the Soft Tick *Ornithodoros moubata*

Miles A. Nunn,[1]* Ankit Sharma,[†] Guido C. Paesen,* Sharon Adamson,[‡] Olga Lissina,* Anthony C. Willis,[§] and Patricia A. Nuttall*

Blood-feeding ticks must control C activation or be damaged by the host inflammatory response. We report the characterization and expression of a novel, relatively small, broad-acting C inhibitory protein (termed OmCI) from the soft tick *Ornithodoros moubata*. The native 17-kDa nonglycosylated protein inhibits both human and guinea pig classical and alternative C activation pathways. The IC_{50} values for each pathway were 12 and 27 nM, respectively, in hemolytic assays using human serum diluted 40-fold. The cDNA encodes a protein of 168 aa, including an 18-aa secretion signal sequence that is absent in the mature form. The inhibitor has 46% amino acid identity with moubatin, a platelet aggregation inhibitor also from *O. moubata* that is an outlying member of the lipocalin family. Native OmCI had no inhibitory effect on the addition of C8 and C9 to preformed C5b-C7 and C5b-C8 to form the membrane attack complex and no effect on the rate of C3a production by the C3 convertase enzymes C4bC2a, C3(H_2O)Bb, or C3bBb. Both recombinant and native OmCI abolish production of C5a by human classical (C4bC3bC2a) and alternative (C3bC3bBb) C5 convertases. Addition of excess C5 but not C3 competes away the inhibitory activity of OmCI, indicating that OmCI targets C5 itself rather than inhibiting the C5 convertase C4bC3bC2a itself. Direct binding of OmCI to C5 was demonstrated by Western blotting and gel filtration chromatography using ^{125}I-labeled proteins. OmCI is the first lipocalin family member shown to inhibit C and also the first natural inhibitor that specifically targets the C5 activation step. ***The Journal of Immunology,*** **2005, 174: 2084–2091.**

The two activation pathways (classical and alternative) of C comprise parallel cascades that converge on the C3 protein and use similar catalytic C3 and C5 convertases (1). The classical pathway C3 and C5 convertases comprise C4bC2a and C4b,C3b,C2a, respectively. The alternative pathway C3 and C5 convertases comprise C3(H_2O),Bb or C3b,Bb and C3b,C3b,Bb, respectively. The homologous proteins C2a and Bb are the catalytic components of each structure. C activation results in the release of the acute inflammatory peptide mediators C3a and C5a (called anaphylatoxins) by the C3 and C5 convertases, respectively, and formation of the terminal membrane attack complex (MAC[2]; C5b-C9). The C3a and C5a peptides induce damage by recruiting and stimulating granulocytes to release proinflammatory mediators, tissue degradative enzymes, and oxygen free radicals (2). The anaphylatoxins also increase adhesion molecule and inflammatory cytokine expression (3). The MAC can disrupt foreign or disordered cells and has proinflammatory activity mediated indirectly by induction of self-cell activation by causing up-regulation of adhesion molecules, tissue factor, and chemokines. Additional effects of C activation include opsonization and phagocytosis of pathogens via deposition of C4b and C3b, clearance of immune cell complexes by recruitment of macrophages, and increased efficiency of Ag presentation to B cell receptors.

The potency of activated C demands tight control to prevent damage to the body's own tissues, and in humans, >14 serum and cell surface proteins are involved in its functional regulation. Many parasites also express specific inhibitory proteins or produce physical barriers and/or sequester host regulatory proteins to counteract C activity (4–8). C is one of the first immune defense systems activated by ticks when they take a blood meal. Failure to control C activation may result in damage to the tick mediated by the host's inflammatory response. Tick-resistant guinea pigs treated with cobra venom factor (CVF), which depletes C activity, exhibited significantly reduced resistance (9). Host resistance appears to depend on the alternative pathway of C activation in the case of *Dermacentor andersoni* feeding on a strain of guinea pigs deficient in the classical pathway factor C4 (10). Levels of C3 increase as hosts develop resistance, and C3 is deposited in the midgut of feeding ticks and also in epidermal vesicles that develop in the host beneath the point of tick attachment (11, 12).

Tick salivary proteins that inhibit components of the host immune system may have an impact on the transmission of vector-borne pathogens (13). *Ornithodoros moubata* and other *Ornithodoros* sp. soft ticks are vectors of both African swine fever virus, which causes a lethal, hemorrhagic disease in domestic pigs, and human tick-borne relapsing fever caused by various species of *Borrelia*. Both pathogens are susceptible to C-mediated lysis in vitro (14, 15), although C3 and C5 deficient mice can clear relapsing fever spirochetemia (16).

An 18.5-kDa protein from the hard tick *Ixodes scapularis* that inhibits the alternative pathway of C activation has been cloned

*Centre for Ecology and Hydrology, Oxford, Oxford, United Kingdom; [†]School of Biochemistry and Microbiology, University of Leeds, Leeds, United Kingdom; [‡]Ministry of Agriculture and Forestry, Wellington, New Zealand; and [§]Immunochemistry Unit, Department of Biochemistry, Medical Research Council, Oxford, United Kingdom

Received for publication August 19, 2004. Accepted for publication November 2, 2004.

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] Address correspondence and reprint requests to Dr. Miles A. Nunn, Centre for Ecology and Hydrology, Mansfield Road, Oxford, OX1 3SR U.K. E-mail address: amn@ceh.ac.uk

[2] Abbreviations used in this paper: MAC, membrane attack complex; OmCI, *Ornithodorus moubata* C inhibitor; rOmCI, recombinant OmCI; nOmCI, native OmCI; CVF, cobra venom factor; NHS, normal human serum; SGE, salivary gland extract; RaHBP, *Rhipicephalus appendiculatus* histamine-binding protein; pI, isoelectric point; IEF, isoelectric focusing; CH50, classical hemolytic assay; AH50, alternative hemolytic assay; EA, sensitized sheep erythrocyte; PNGaseF, peptide *N*-glycosidase F; RP-HPLC, reversed phase-HPLC; TSGP, tick salivary gland protein.

0022-1767/05/$02.00

and expressed (17, 18). A distinct salivary anaphylatoxin inactivating activity has been reported in the same species (19). Classical and alternative pathway C inhibitory activity has previously been recognized in *O. moubata* salivary gland extract (SGE) but the active components have not been identified (20).

In this paper we describe the cloning, expression, and characterization of a C inhibitor (termed OmCI) from *O. moubata.* OmCI is the first natural protein identified that specifically targets the C5 activation step without affecting C3 activation.

Materials and Methods

Materials

Sheep and rabbit RBC were from Tissue Culture Services. Hemolysin, pooled normal human sera (NHS), depleted human sera, and gelatin veronal buffered saline (GVB^{2+}) were all obtained from Sigma-Aldrich. Guinea pig serum and control proteins (*Rhipicephalus appendiculatus* histamine-binding proteins 2 and 3 (RaHBP2 and RaHBP3)) were derived in house. Pure C3, C4, C5, C8, and C9, and anti-human C3a rabbit polyclonal antisera were purchased from Calbiochem. *Naja naja* CVF was from Quidel. The C5a ELISA detection kit was purchased from Immunobiological Laboratories.

Tick rearing and preparation of SGE

O. moubata ticks were reared according to Jones et al. (21). Salivary glands were dissected under a microscope, rinsed briefly in cold PBS, and stored at −70°C. Protein extracts were prepared by disruption of 30 pairs of frozen salivary glands in 500 μl of PBS using a 1-ml Dounce homogenizer and clarified by centrifugation (12,000 × *g* for 2 min). The supernatant (referred to as SGE) was tested for C inhibitory activity or fractionated. Protein concentration was determined by the Bradford assay (Bio-Rad).

Purification and N-terminal sequencing of native C inhibitor

A total of 150 μl of SGE (nine pairs salivary glands) was diluted in 5 ml of 25 mM sodium phosphate buffer (pH 6.8) and 50 mM NaCl and fractionated on a 1-ml Q-Sepharose high performance anion exchange column (Pharmacia) at a flow rate of 1 ml/min using a 40-min 0.05–0.75 M NaCl gradient. One percent of each 1-ml fraction was assayed for C inhibitory activity in 200 μl of classical hemolytic assays (CH50; see below). Centricon 3 filtration units (Millipore) were used for buffer exchange to PBS and concentration of fractions to 50 μl before reducing SDS-PAGE. Active fractions were applied to a Superdex 75 column (Pharmacia) at a flow rate of 0.5 ml/min using 20 mM Tris (pH 7.6) and 200 mM NaCl as running buffer. Five microliters of each 0.5-ml fraction was assayed by CH50 and visualized by reducing SDS-PAGE.

Active and inactive fractions were resolved by isoelectric focusing (IEF) electrophoresis (pH 3–7 gel; Invitrogen Life Technologies) and electroblotted to a Immobilon-P polyvinylidene difluoride membrane (Millipore) using 0.7% acetic acid (1 mA/cm^2 for 1 h). After staining with Ponceau-S, major bands were excised and eluted in 200 μl, 50 mM Tris (pH 8), and 2% Triton X-100 by vortexing for 1 min and centrifuging for 10 min at 21,000 × *g*, three times. Triton-X100 was removed by chromatography over Q-Sepharose before exchanging the buffer to PBS (as above).

Twenty microliters of the active fraction eluted from the IEF-resolved protein were run on a Jupiter C4 column (150 × 1.0 mm), a gradient 10–40% acetonitrile, 0.1% trifluoroacetic acid, a flow rate of 1 ml/min with 0.5% acetonitrile/min increments, and monitored at 215 nm. Peaks were transferred to Immobilon-P membrane, and N-terminal sequences were obtained using an Applied Biosystems MiniBlott cartridge.

CH50 and alternative hemolytic assays (AH50)

Sheep RBC were sensitized using rabbit hemolysin (Sigma-Aldrich) titrated as described previously (22). Classical pathway assays were conducted in a total volume of 200 μl using 100 μl of diluted NHS or guinea pig serum in GVB^{2+} as a source of C and 100 μl of 2×10^8 sensitized sheep erythrocytes (EA) ml^{-1}. Rabbit RBC were prepared in 50 ml of GVB/mg (10 mM) EGTA buffer as described previously (22). Alternative pathway assays were conducted in a total volume of 200 μl using 100 μl of NHS diluted in GVB/Mg EGTA buffer as a source of C and 100 μl of 2×10^8 rabbit erythrocytes ml^{-1}.

All lytic assays were set up on ice. SGE, C inhibitor, or the negative control RaHBP2, which is similar to OmCI in size structure and isoelectric point (pI; Ref. 23), was added last, and reactions were incubated at 37°C. At the end of the time course (up to 60 min), whole cells were removed (12,000 × *g* for 5 s) and hemolysis measured spectrophotometrically at 412 nm.

Lytic assays using sera depleted in specific C components

Volumes and dilutions of pure C components that gave 50–90% lysis with depleted human sera (Sigma-Aldrich) were determined empirically. Standard assays (50–70% lysis) using C8- and C9-depleted sera comprised 10 μl of C8- or 4 μl of C9-depleted sera, 20 μl of 1/250-diluted NHS (1/2500 final), 70 or 76 μl of GVB^{2+} buffer, and 100 μl of EA. The final volume was adjusted to 200 μl with GVB^{2+} buffer. Reactions were incubated for 30 min at 37°C. In experiments examining inhibition, OmCI and the negative control RaHBP2 and all other components were added and incubated at room temperature for 3 min before adding diluted sera and incubating for 30 min at 37°C.

Standard assays (80–90% lysis) using C3- and C5-depleted sera comprised 2.5 μl of C3- or C5-depleted sera, 0.25 μg of pure C3 or 0.0062 μg of pure C5, and 25 μl of EA and GVB^{2+} buffer to a final volume of 50 μl. Reactions were incubated for 30 min at 37°C.

For competitive assays between OmCI and C5 or C3, the smallest amount of OmCI that inhibited the standard assays by 90% was used: 0.05 and 0.0125 μg for the C3 and C5 assays, respectively. OmCI and negative control (RaHBP2) were added last, and reactions were incubated at 37°C for specified times (0, 2, 6, 12, and 22 min).

Detection of C3a and C5a production

CH50/AH50 assays were set up in a total volume of 200 μl using a 1/50 final dilution of NHS or guinea pig serum with 0.5 μg of native OmCI (nOmCI) or recombinant (rOmCI) or 4 μg of RaHBP2-negative control protein. Reactions were removed from the 37°C waterbath at specified time points (2, 4, 8, 16, and 32 min for C3a and 32 min only for C5a), spun at 12,000 × *g* for 10 s, and supernatants were removed for subsequent analysis by immunoblotting (C3a) or ELISA (C5a).

C3a was detected using anti-human C3a rabbit polyclonal antisera (Calbiochem) on immunoblots of 10 μl of each reduced supernatant sample. Confirmation of equal loading and even transfer to all lanes was obtained from the intensity of the serum albumin band following Ponceau staining. A C5a ELISA kit (Immunobiological Laboratories) was used to detect cleavage of C5a from C5 in accordance with the manufacturer's instructions. To prevent cross-reaction with uncleaved C5, the C5 present in 100 μl of supernatant from each hemolytic assay was precipitated using the reagent provided in the kit. The measuring range of the kit extends from 0.1–10 μg/L, with a lower limit of detection of 0.02 μg/L.

Decomplementation of sera with CVF

A total of 0.25 μg of *N. naja* CVF (1 μg/μl stock) and either 2 μg of nOmCI or 4 μg of RaHBP2 was added to 10 μl of NHS and incubated for 1 h at 37°C. Half of the CVF-treated sera (5 μl, giving a 1/40 final dilution) was added to 95 μl of GVB^{2+} and 100 μl of EA. After incubation (20 min, 37°C), the amount of lysis and concentration of C5a in reaction supernatants were determined by ELISA as described above.

Detection of C5 binding to OmCI

For Western blotting, 0.5 μg of nOmCI and 5 μg of RaHBP2 were subjected to nonreducing SDS-PAGE, then transferred to nitrocellulose and blocked overnight in PBS, 0.05% Tween 20, and 5% nonfat dried milk (PBSTM). C3 and C5 were labeled with ^{125}I using Iodogen in accordance with the manufacturer's instructions (Pierce). Blots were incubated with 2 μg of ^{125}I-labeled C3 (1440 kcpm/min) and 2 μg of ^{125}I-labeled C5 (2160 kcpm/min) in 15 ml of PBSTM for 4 h at room temperature. After three 20-min washes in PBSTM at room temperature, the nitrocellulose membranes were dried and autoradiogrammed.

For gel filtration chromatography, 0.07 μg of ^{125}I-labeled OmCI (1687 kcpm/min) were incubated with 2 μg of pure C3 or C5 or 23.8 μl of NHS or C3- or C5-depleted serum. PBS was added to a total volume of 100 μl, and the mixture was incubated for 10 min before chromatography through a Superose 12 10/30 column at a flow rate of 1 ml/min PBS. One-milliliter fractions were collected, and cpm was measured at a set distance from a hand held Geiger counter.

Cloning cDNA encoding C inhibitor

Sixty pairs of *O. moubata* third and fourth feed nymphal salivary glands were excised and placed in 1 ml of RNA*later* (Ambion). mRNA was isolated using the FastTrack 2.0 mRNA isolation kit (Invitrogen Life Technologies), and cDNA was synthesized using a Stratagene random primed cDNA synthesis kit. cDNA were ligated into the Stratagene UniZAP XR

directional phage vector and packaged with Gigapack III Gold packaging extract.

Degenerate primers OF4 5′-GTACWSNGGNWSNGARCCNGT-3′ and OF5 5′-GTACGAYWSNGARWSNGAY-3′ were used with the vector-specific T7 primer to amplify the cDNA encoding the C inhibitor from the *O. moubata* library (PCR; 95°C for 1 min, 48.5°C for 45 s, 72°C for 1 min; 30 cycles). PCR products were gel purified (Qiaex II, Qiagen kit; Qiagen) and sequenced using ABI PRISM dye terminator cycle sequencing and a 373 ABI sequencer (PerkinElmer). A full-length cDNA was obtained using vector-specific T3 primer and a reverse primer (OR1 5′-GGGAGGCTT TCTGTATCC-3′). The 650-bp product was cloned into pGEM-T Easy (Promega), and sequencing was completed using cDNA-specific primers OR3 5′-CGTCCAATCGGTTGAAG-3′ and OF6 5′-GACTCGCAAAG TCATCAC-3′.

Sequence analysis

Analyses were conducted using the Expert Protein Analysis System proteomics server of the Swiss Institute of Bioinformatics (⟨http://expasy.hcuge.ch/⟩). Sequences were compared with the GenBank nonredundant protein database using the BlastX program (24) and searched against the Pfam (25) and SMART (26) protein domain database. Multiple sequence alignment were performed with Clustal X (27).

Yeast expression, purification, and deglycosylation of rOmCI

The OmCI coding region was PCR amplified (95°C for 30 s, 50°C for 30 s, and 72°C for 30 s; 18 cycles) using the forward primer OM1Y (5′-ATA GAGCTCAAAATGCTGGTTTTGGTGACC-3′) and the reverse primer OR7a (5′-ACTGAGCGGCCGCCTAGCAGTCCTTGTGGGG-3′). The primers add a *Sac*I site upstream of the start codon and a *Not*I site downstream of the stop codon. The product was ligated between the *Sac*I and *Not*I sites of the pMETα C transfer vector (Invitrogen Life Technologies) and transformed into the *Pichia methanolica* strain pMAD11 and grown according to the manufacturer's instructions (Invitrogen Life Technologies). C inhibitory activity in supernatant and cells of positive clones was assayed daily for 5 days.

After 96 h of growth, 500 ml of cell medium was centrifuged at 6500 × *g* for 15 min, and the protein was precipitated from the supernatant by addition of 30% (w/v) PEG-8000 and stirring on ice for 1 h. A pellet was recovered by centrifugation (23,700 × *g*, 1 h) and resuspended in 50 ml of 25 mM sodium phosphate buffer (pH 6.8) and 50 mM NaCl before centrifuging again (6,500 × *g*) to remove insoluble material. The clarified solution was applied to a 1-ml Q-Sepharose high performance anion exchange column and active fractions purified additionally by gel filtration.

Purified rOmCI was treated with peptide *N*-glycosidase F (PNGaseF) in accordance with the manufacturer's instructions (New England Biolabs). Deglycosylated rOmCI was repurified by gel filtration.

Results

Purification and identification of active fractions from O. moubata *SGE*

The C inhibitory activity present in the SGE of *O. moubata* eluted from a Q-Sepharose anion exchange column at ~0.15 M NaCl (Fig. 1*A*) ablating lysis of sheep RBC by the classical pathway (Fig. 1*B*). Reducing SDS-PAGE showed that the inhibitory activity was associated with a poorly resolved cluster of proteins with masses between 15 and 18 kDa (Fig. 2*A*). Estimates from Coomassie blue-stained gels indicated the average amount of these proteins was ~5 μg per adult (seventh feed) tick. By IEF gel electrophoresis, the 15- to 18-kDa proteins ran as a single band with a pI of ~4.2 (Fig. 2*B*). To show that the C inhibition was due to these proteins, an active (f15) and an inactive (f17) control fraction (comprising a reasonably similarly sized abundant protein) were eluted and purified from IEF-resolved protein blots (Fig. 2*B*) and assayed. Repurification was necessary to remove the Triton X-100 used to elute the proteins because the detergent causes spontaneous lysis of RBC. Fraction 15 abolished lysis in the CH50 assay, whereas fraction 17 had no effect on lysis.

Reversed phase-HPLC (RP-HPLC) of the polyvinylidene difluoride-eluted inhibitory fraction (f15) revealed four close running peaks at ~53 min (Fig. 2*C*). Related N-terminal sequences were obtained from the two largest peaks (labeled C and D in Fig. 2*C*),

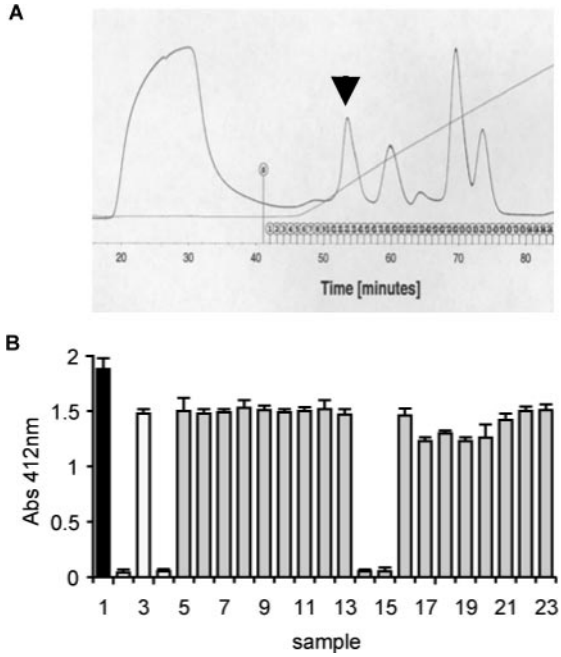

FIGURE 1. Purification of nOmCI. *A*, Anion exchange chromatography. The vertical arrow indicates the peak containing inhibitory activity. *B*, CH50 using a 1/80 final dilution of guinea pig serum. Sample 1 (■), 100% lysis; sample 2, 0% lysis; sample 3 (□), serum only; sample 4, serum plus 1 μl of SGE (0.06 of a salivary gland); samples 5–23 (▨), serum plus 10 μl of fractions 10–28 shown in *A*. Each value represents the average of three replicates.

suggesting the proteins in the four peaks are distinct but closely related. The peak C sequence was D S E S D Q L (6 aa), and peak D sequence was D S E S D X S G S E P V D/V A F Q A F R/S E (20 aa), where X denotes an unidentified residue. The N-terminal sequences enabled the design of two degenerate primers (OF4 and OF5) to amplify the cDNA encoding the C inhibitor(s) from an *O. moubata* salivary gland directional library constructed for this purpose. Five PCR products were gel purified and sequenced. The sequence of the most abundant product (~500 bp) derived using primer OF4 had a significant BlastX match with the C-terminal sequence of moubatin (28). The 5′-coding sequence of the cDNA was obtained with a reverse primer (OR1) matching a region beyond the cDNA stop codon- and vector-specific T3 primer. Translation of this region revealed an amino acid sequence that exactly matched the N-terminal amino acid sequence derived from the largest RP-HPLC, peak D (Fig. 2*C*). The protein encoded by the cDNA was called OmCI. The full-length sequence was submitted to GenBank (accession no. AY560803).

Primary structure of OmCI

The cDNA-inferred sequence of OmCI is 168 amino acids long and has a N-terminal secretion signal comprising the first 18 residues (Fig. 3). The N-terminal sequence that was obtained (above) indicates the signal peptide cleavage site is between Ala^{18} and Asp^{19}. The mature protein is thus 150 aa long, and its predicted molecular mass (16.77 kDa) and pI (4.3) are similar to the mass and pI determined by gel electrophoresis (Fig. 2, *A* and *B*). OmCI has two predicted *N*-glycosylation sites (Asn^{78} and Asn^{102}) and 12 potential phosphorylation sites. However, the native protein is not glycosylated (see below), and the phosphorylation sites have a high probability of chance occurrence, and it is possible that none are modified.

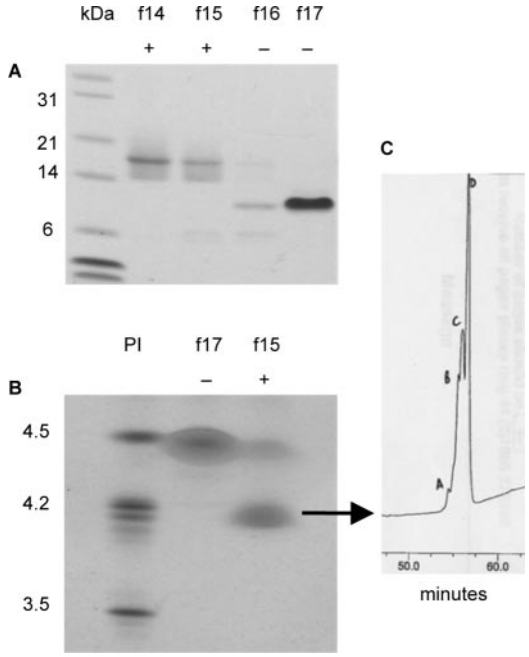

FIGURE 2. Analysis of purified nOmCI by reducing SDS-PAGE (*A*), IEF (*B*), and RP-HPLC (*C*). Gels are stained with Coomassie blue. Fractions f15 and f17 in *A* and *B* are the same. Activity of fractions is indicated above the gels (+, inhibitory; −, not inhibitory). Fraction f15 was recovered and analyzed by RP-HPLC (*C*). Size (kilodaltons) and pI markers are indicated.

The primary sequence of OmCI has no detectable sequence similarity to any known C inhibitors in public databases. However, it does have a 58% amino acid identity to tick salivary gland proteins 2 and 3 (TSGP2 and TSGP3) of the soft tick *Ornithodorus savignyi* (29) and a 49% identity to moubatin from *O. moubata* (28). Multiple sequence alignment indicates that all six cysteine residues, and therefore presumably the disulfide-bridging pattern, is conserved in these four proteins (Fig. 3). The alignment shows that OmCI has two short amino acid insertions, SESD at the N terminus and PD approximately two-thirds of the way through the sequence of the mature protein (Fig. 3), which may be of functional significance. Moubatin and TSGP2 and TSGP3 are members of the lipocalin family of β barrel-forming proteins that includes tick histamine-binding proteins (30, 31). Analyses of multiple sequence alignments of OmCI and its closest relatives with the tick histamine binding proteins show that the amino acid residues known to be involved in binding of histamine (30) are not conserved (data not shown).

Expression, purification, and activity of rOmCI

The positive yeast clones exhibited variable levels of rOmCI expression. In all cases, inhibitory activity per unit volume of supernatant gradually increased through to the final assay point on day 5 (data not shown). Control clones expressing other tick proteins had no inhibitory activity. Approximately 90% of the expressed protein was in the supernatant. The clone that gave the highest expression levels (~5 mg/L of media) was selected for protein production. After polyethylene glycol precipitation of the supernatant and two chromatography steps, partially purified active rOmCI appeared to be hyperglycosylated (Fig. 4*A*, fractions 9, 10, and 11). This was confirmed by removal of N-linked carbohydrate using PNGaseF, which converted a proportion of the hyperglycosylated form to a deglycosylated form that comigrated with nOmCI, which is not glycosylated (Fig. 4*B*). Deglycosylated

```
OmCI        .MLVLVTLIFSFSANIAYADSESDGSGSEPVDAFQAFSEGKEAYVLVRST
TSGP3       MMLVLATVILSFSASTALAD....GPTGKPTEAYVAFNEGKGAYILVRST
TSGP2       MMLVLATVILSFSASTALAD....GPTGKPTDAYVAFNEGQGAYILVKST
Moubatin    MMLVLTTLIFSFSASIAYAQSG..GSVSDPLDALKAFKDGAGTFLLQKST
             **** * * **** * *        *   *  *  **  *     *  **

OmCI        DPKARDGLKGEPAGEKQDNTLPVMMTFKNGTDWASTDWTFTLDGAKVTAT
TSGP3       NLNARDGLKGEATGKKEGNTLPVMMAFKDEGKWVSLPWTFTLDGPKVTAT
TSGP2       DLDARDGLKGSATGKKEGNKVPVMMAFKNEGQWVSLPWTFTLDGPKVTAT
Moubatin    DPQARDGLKGTPNGNRDGNTLPVTMTYKDDSKWVSLNWMFTLEGANIVAT
              *******   *    *  ** *  *    *  * *** *    **

OmCI        L.GNLTQNREVVYDSQSHHGHVDKVEKEVPDYEMWMLDAGGLEVEVEGGR
TSGP3       H.GQRTLKGEVVYDVPSHHGHIEKLESGA..YDMWMLEAGGLEVDIEGGN
TSGP2       D.GQRTLKREVVYDVASHHGHVEKLASGA..YEMWMLEAGGLEVDIEGGN
Moubatin    LEGKRKQRGELVYDVQSHDGHITKLSSGV..YQQWQSNGSADDKDIKGGD
              *       * ***  * **  *     *  *              **

OmCI        QKLEELASGRNQMYPHLKDG.....
TSGP3       KRYDELTSGQVVIRPQDKDG.....
TSGP2       KKYDELTSGQVVIRPQDKDG.....
Moubatin    EKFKELTSGIDYTKPQEKGGETSAK
             ** **     *  * *
```

FIGURE 3. Clustal X sequence alignment of OmCI (AY560803) with TSGP2 and TSGP3 and moubatin (AF452889, AF452889, and L04129, respectively). Identical residues have asterisks, and cysteines are highlighted in black.

rOmCI separated from hyperglycosylated rOmCI by gel filtration chromatography (Fig. 4*A*, fractions 12 and 13) had similar potency to nOmCI in lytic assays (Fig. 5*A*). The N-terminal sequence of deglycosylated rOmCI was M D S E S D *X* S G S E P V D A. The sequence is identical with that of nOmCI except for the N-terminal methionine.

Mechanism of action of OmCI

OmCI can inhibit the classical pathway entirely, but the alternative pathway is inhibited by at most 80% (Fig. 5*A*). The IC_{50} values

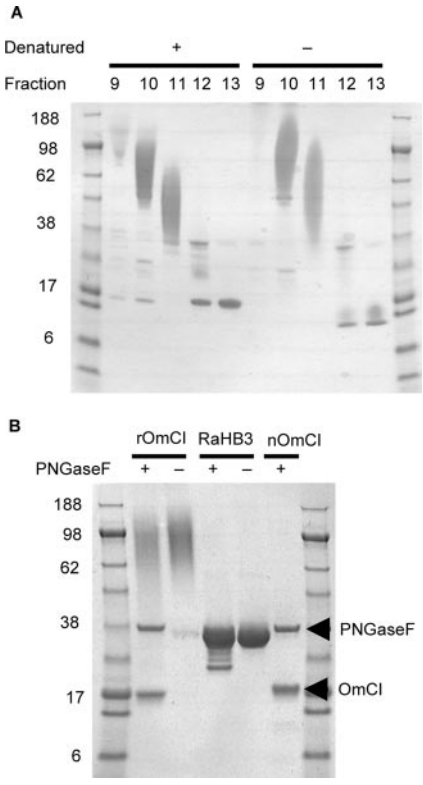

FIGURE 4. Purification (*A*) and deglycosylation (*B*) of yeast cell-expressed rOmCI. *A*, SDS-PAGE of fractions 9–13 of glycosylated (9–11) and PNGaseF deglycosylated (12 and 13) rOmCI separated on a Superdex-75 gel filtration column. *B*, Effect of PNGaseF on mobility of hyperglycosylated rOmCI. Arrows indicate PNGaseF (*upper arrow*) and native OmCI (*lower arrow*). The positive control RaHBP3 is distantly related to OmCI and is known to be glycosylated. Size markers (kilodaltons) are indicated at *left* of panel.

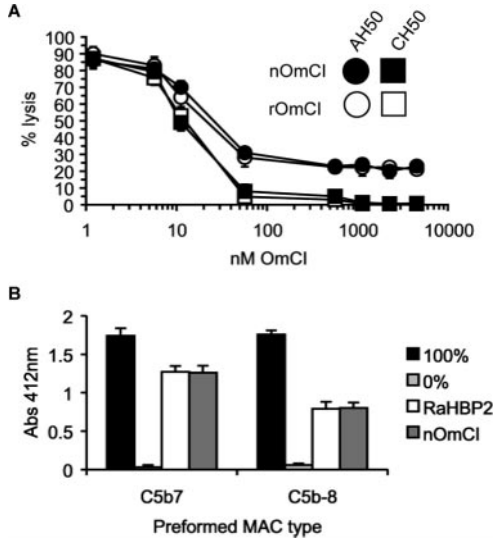

FIGURE 5. Potency C inhibition by OmCI (*A*) and lack of effect on partially formed MAC (*B*). *A*, Classical (CH50) and alternative (AH50) pathway inhibition by nOmCI and deglycosylated rOmCI. NHS used at a final dilution of 1/40. Average of four replicates. *B*, Effect of nOmCI on addition of C8 and C9 to partially formed MAC. Absorbance due to 100 and 0% lysis and in absence (4 μg of RaHBP2) and presence (2 μg of nOmCI) is shown. Average of six replicates.

estimated from this plot are 12 and 27 nM for the classical and alternative pathways, respectively, using diluted (1/40 final) human serum. Inhibition of both C activation pathways suggests OmCI functions after convergence of the two pathways, i.e., at or after formation of the C3 convertase. Thus, we did not look for any effects of the inhibitor on the C cascade before formation of the C3 convertase.

Noting that OmCI and C component C8γ are both members of the lipocalin superfamily and that lysis of RBC is reduced by 85% in the absence of C8γ (32), we first tested whether OmCI somehow competes with C8γ, thereby inhibiting MAC formation. However, lytic experiments using depleted sera show addition of excess OmCI before addition of C8 or C9 has no effect on incorporation of C8 and C9 into preformed C5b-7 or C5b-8, respectively (Fig. 5*B*).

The rate of C3a and C5a production was measured to examine whether OmCI inhibits the formation or action of the classical and alternative C3 (C4bC2a or C3bBb) and C5 (C4bC3bC2a or C3bC3bBb) convertases. The inhibitor had no effect on the rate of C3a production by either guinea pig (Fig. 6*A*) or human (data not shown) C3 convertases. Whereas both nOmCI and deglycosylated rOmCI (data not shown) prevent the C5 convertase-mediated production of C5a from C5 (Fig. 6*B*) and thus subsequent formation of the MAC, *N. naja* CVF-treated sera were inactive in lytic assays (data not shown). Excess OmCI did not prevent such decomplementation of sera or prevent production of C5a by CVF (Fig. 6*B*).

To examine whether OmCI inhibits the formation or action of the C5 convertase, we set up a competition experiment. The assay comprised sensitized sheep RBC-, C3-, and C5-depleted sera and pure C3 and C5. The smallest amount of OmCI needed to inhibit the lowest concentration of pure C3 and C5 that was used in the assay was determined empirically. This quantity of OmCI was used throughout the experiment, and increasing amounts of pure

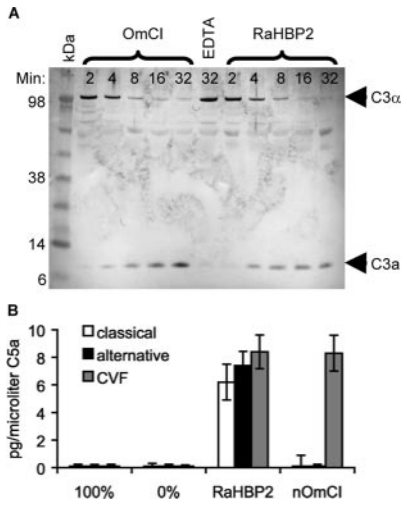

FIGURE 6. Effect of OmCI on cleavage of C3a (*A*) and C5a (*B*). *A*, Representative time course showing absence of effect of OmCI on classical pathway cleavage of C3a from C3α analyzed by immunoblot with C3a-specific antisera. Minutes since the start of the reaction has been indicated. Reactions performed using 1/50 final dilution of guinea pig sera with (0.25 μg of nOmCI) or without (4 μg of RaHBP2) inhibitor or in presence of 10 mM EDTA. Size markers (kilodaltons) indicated at *left* of panel. *B*, Effect of nOmCI on classical, alternative, and CVF C5 convertase cleavage of C5a from C5α analyzed by ELISA. Picograms per microliter C5a released was measured after 100% lysis of sheep RBC with water, 0% lysis in GVB^{2+} only, and reactions with (0.25 or 2 μg of nOmCI for C5 or CVF convertases, respectively) or without (4 μg of RaHBP2, negative control) inhibitor. Average of four replicates.

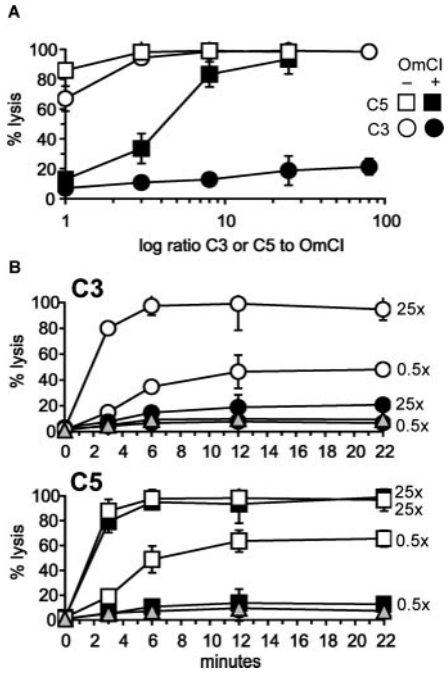

FIGURE 7. Effect of increasing amounts of C3 and C5 on C inhibition (*A*) and rate of RBC lysis mediated by the classical pathway (*B*). *A*, Effect of addition of pure C3 (●, ○) and C5 (■, □) to C3- and C5-depleted sera in the presence (+, filled symbols) or absence (−, open symbols; 2 μg of RaHBP2) of a minimal amount of nOmCI (termed log ratio 1) that completely inhibited lysis in standard assays. The concentrations of purified proteins in the standard assays at log ratio 1 were 27 nM C3, 58.8 nM nOmCI and 0.66 nM C5, 14.7 nM nOmCI for the C3 and C5 assays, respectively. *B*, Effect of excess C3 and C5 on rate of lysis. The minimal inhibitory dose of nOmCI was used together with 0.5 or 25× the amount of pure C3 and C5 that were added to the standard assay. Grey triangles indicate lysis caused by incubation of EA with depleted sera only. Each value represents the average of four replicates.

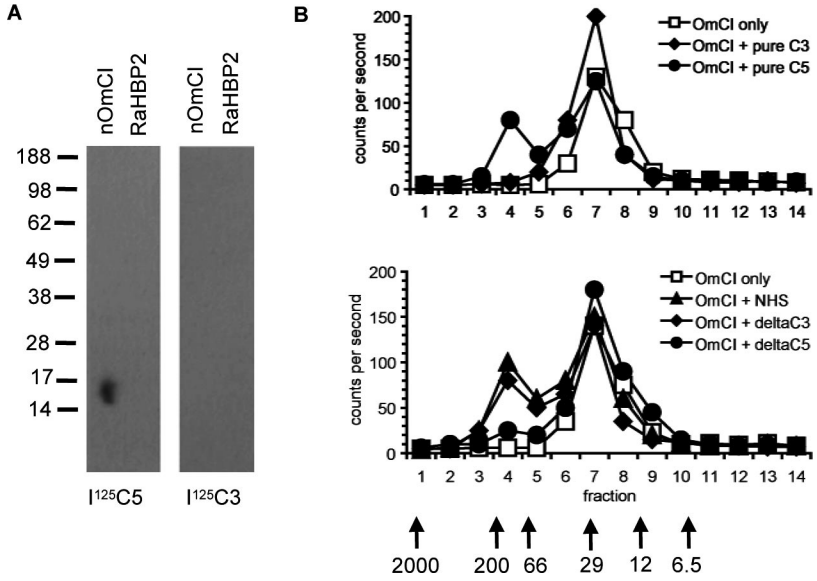

FIGURE 8. Demonstration of direct binding between OmCI and C5 by Western blotting (*A*) and gel shift (*B*). *A*, A total of 0.5 μg of nOmCI and 5 μg of RaHBP2 (control) transferred to nitrocellulose was probed with 2 μg of ^{125}I-labeled C3 or C5 and then autoradiogrammed. Protein size markers (kilodaltons) indicated at *left* of panel. *B*, Radiolabeled nOmCI (*A*) with or without purified C3 and C5 (pure C3/C5; *upper panel*) and with or without NHS, and C3- or C5-depleted sera (δ C3/C5; *lower panel*). Protein size markers (kilodaltons) indicated by arrows.

C3 and C5 were added to the depleted sera. The inhibitor does not appear to interfere with the formation of the C5 convertases because C5 is cleaved when added to the assay in large excess to OmCI (Fig. 7*A*). Excess C3 and C5 both caused substantial and similar increases in the rate of RBC lysis (Fig. 7*B*), which implies an increase in the rate of formation and/or absolute amount of C5 convertase present on the surface of sheep RBC. Despite this, excess C3 does not compete with the nOmCI inhibitor (Fig. 7*A*), which suggests that OmCI does not target the C5 convertase directly, but is more likely to target C5 itself.

This hypothesis led us to look for a direct interaction between OmCI and C5 by probing OmCI transferred to nitrocellulose with ^{125}I-labeled human C3 and C5. The results indicated direct binding of OmCI to C5 but not to C3 (Fig. 8*A*). Additional evidence for a direct interaction between OmCI and C5 was obtained by gel filtration chromatography. An apparent mass shift in a proportion of the ^{125}I-labeled nOmCI was observed in the presence of purified C5 but not C3 (Fig. 8*B*). A similar mass shift was evident in the presence of NHS and C3-depleted sera but not C5-depleted sera (Fig. 8*B*). The mass shift was maintained in the presence of 1 M NaCl but not 2 M NaCl, suggesting a strong electrostatic interaction exists between the inhibitor and C5 (data not shown).

Discussion

Activated C is a key effector mechanism in innate defense and has additional roles in the recruitment of both humoral and cellular immune responses. Although evidence supports C-mediated tick resistance (9–12) and the existence of C inhibitors in ticks (17–20), only one other C inhibitory molecule from the hard tick *I. scapularis* has previously been identified and expressed (18). The primary sequence of OmCI is unrelated to the *I. scapularis* protein and has no sequence similarity to the N-terminal sequence of *O. moubata* Ag 20A1 (33), which was proposed to be the factor responsible for C inhibition in SGE of *O. moubata* and *O. erraticus* (20). Indeed, OmCI has no detectable sequence similarity to any known C inhibitors in public databases. It's closest known relatives (Fig. 3) are TSGP2 and TSGP3 of the soft tick *O. savignyi* (29) and collagen-dependent platelet aggregation inhibitor, moubatin (28). However, the four closely migrating HPLC peaks and N-terminal sequence of peak C (Fig. 2*C*) suggest that *O. moubata* expresses three other close relatives of OmCI that may or may not be C inhibitors.

Neither moubatin or the TSGP proteins have been proposed to inhibit C. TSGP2 and TSGP3 are abundant in SGE, have 95% amino acid identity (29), and unlike moubatin do not inhibit platelet aggregation (31). TSGP2 is toxic to mice but TSGP3 is not (34). OmCI is highly unlikely to be a toxin because *O. moubata* does not cause toxicosis (20), whereas *O. savignyi* causes sand tampan toxicosis in a wide range of mammals (34). Furthermore, intradermal injection of 100 μg of purified nOmCI into guinea pigs, in the process of raising antisera, caused no obvious pathophysiological effects (M. Nunn, unpublished observation).

OmCI, TSGP2 and TSGP3, and moubatin are outlying members of the lipocalin family of proteins and are structurally related to the histamine-binding proteins isolated from hard ticks (30, 31). OmCI is the first lipocalin that has been shown to inhibit C, extending the diverse functions of this protein family in both vertebrates and invertebrates (35). The majority of lipocalins bind single, small, hydrophobic ligands within their characteristic β-barrel structure. The archetypal histamine-binding protein (RaHBP2) of the hard tick *Rhipicephalus appendiculatus* has significant structural differences from normal lipocalins that enable it to bind hydrophilic molecules in two separate internal binding sites (23). The critical histamine-binding residues (30) are not conserved in OmCI; however, the protein may bind another small ligand, and work is underway to address this possibility. Notably, structural data indicates MAC component C8γ (which is also a lipocalin) has a cavity for ligand binding (36), although the identity of the specific ligand or ligands is not yet known (32).

OmCI inhibits both the classical and alternative pathways and acts specifically at the C5 step of C activation because it prevents production of C5a from C5 but has no effect on C3a production. Thus, OmCI appears to work by an entirely different mechanism than the C inhibitor from *I. scapularis*, which is specific to the alternative pathway of C activation and prevents C3b deposition and C3a release (17)—probably by accelerating dissociation of factor Bb from the C3 convertase (18). Other tick species interfere with the C by mechanisms distinct from either of the aforementioned species (M. Nunn, unpublished data). It is possible that these C inhibitors differentially effect tick-borne pathogen transmission (13), and experimental studies examining the impact of C inhibitor on African swine fever virus and tick-borne relapsing fever transmission by *O. moubata* are feasible using rOmCI. Our estimates suggest adult (seventh feed) ticks have a 4-fold molar

excess of OmCI in SGE to C5 in their ~0.2-ml blood meal (assuming a C5 concentration of 70 μg/ml). If all the OmCI was secreted and taken up during feeding, it would probably be sufficient to fully inhibit the C5 present in the blood meal.

We do not yet have an exact understanding the mode of action of OmCI; however, a number of possibilities can be ruled out and others ruled in. First, the inhibitor is unlikely to be a direct serine protease inhibitor of the convertase catalytic components C2a and Bb, or it would prevent C3a as well as C5a production (Fig. 6, *A* and *B*). Second, it is probably not factor H or I dependent, or it would be likely to inhibit the C3 as well as the C5 convertase. Third, OmCI is unlikely to act by binding to C3, permitting the C3 convertase to operate but preventing formation of a functional C5 convertase. Because the absolute amount of C5 convertase and inhibitor were held constant and the amount of C5 was increased, C5a was generated indicating the presence of functional C5 convertases (Fig. 7*A*). The latter experiments suggested OmCI binds C5 but not C3, and two independent lines of evidence (Fig. 8, *A* and *B*) strongly support direct and specific binding to C5. Thus, the simplest explanation for the activity of OmCI is direct binding to C5 that either prevents interaction with the C5 convertase or permits interaction but blocks the C5a cleavage site, which is akin to site blocking Ab TS-A12/22 (37). The latter seems less likely than the former because OmCI appears not to prevent C5a production by the CVF C3/C5 convertase (CVFBb; Fig. 6*B*). Therefore, OmCI may bind a different conserved site on C5 such as the C345C/Netrin module that is required for binding to the normal serum C5 convertases but which is not required for binding to CVF convertase (38). A difference in the binding mechanism between C5 and the classical (C4bC2aC3b) and alternative (C3bC3bBb) C5 convertases may also explain why excess OmCI inhibits RBC lysis by the alternative pathway by at most 80% (Fig. 5*A*). For example, $C3b^2Bb$ binding to C5 may not be entirely prevented by OmCI.

Whatever the precise binding mechanism, because OmCI is broad acting (inhibiting C activation in both human and guinea pig sera), it must bind to an element or elements of C5 that are conserved between species. This view is supported by a characteristic size mass shift (Fig. 8*B*) seen when OmCI is incubated with horse, goat, human, or mouse sera (data not shown). Such interspecies cross-reactivity appears to be a general feature of tick immunomodulatory proteins that may enable them to feed on a wide variety of hosts (13).

By impeding C5 cleavage, OmCI inhibits both pathways of C activation and prevents generation of C5a and MAC while preserving the immune clearance and opsonization functions of C that depend on C3b. Such a profile may be useful for therapeutic intervention in certain pathological conditions. Because no natural inhibitors of C5 activation have previously been reported, researchers have constructed inhibitory anti-C5 mAbs, inhibitory RNA aptamers, and synthetic peptides that target the C5a receptor (reviewed in Refs. 39 and 40). Early studies, using the anti-C5 mAb BB5.1 (41), established a pathological role for C5a and the MAC. Subsequent studies using this and other mAbs targeting C5 have demonstrated therapeutic effect in cardiopulmonary bypass patients (42) and in various disease models, including sepsis (43), immune complex nephritis (44), and animal models of arthritis (45). Classical and alternative C inhibitors may also be effective in treatment of hyperacute allo- and xenograft organ (heart and liver) rejection (46–48). Xenogeneic organ grafts are especially susceptible to C-mediated injury because C regulatory proteins, which normally protect cells from injury, function poorly in the heterologous environment. The broad activity of OmCI may make it particularly useful in this setting and should prove invaluable in testing and the development of animal models of C-mediated diseases where the species specificity of present C5 inhibitors has hampered in vivo studies that require different model species (49).

In summary, OmCI is novel small inhibitor of both C pathways that may have therapeutic potential and acts by binding directly to C5 preventing its cleavage by the C5 convertase. The precise binding mechanism, structure, and accessory roles (if any) played by serum factors are under investigation.

Acknowledgments

We thank Dr. Robert Sim for providing ^{125}I-labeled C3 and C5 and for reviewing the manuscript.

References

1. Law, S. K. A., and K. B. M. Reid. 1995. *Complement*. 2nd Ed. D. Male, ed. Oxford University Press, Oxford.
2. Ember, J. A., M. A. Jagels, and T. E. Hugli. 1998. Characterisation of complement anaphylatoxins and their biological responses. In *The Human Complement System in Health and Disease*. J. E. Volanakis and M. M. Frank, eds. Marcel Dekker, New York, p. 241.
3. Kohl, J. 2001. Anaphylatoxins and infectious and non-infectious inflammatory diseases. *Mol. Immunol. 38:175*.
4. Diaz, A., F. Irigoin, F. Ferreira, and R. B. Sim. 1999. Control of host complement activation by the *Echinococcus granulosus* hydatid cyst. *Immunopharmacology 42:91*.
5. Alitalo, A., T. Meri, L. Ramo, T. S. Jokiranta, T. Heikkila, I. J. Seppala, J. Oksi, M. Viljanen, and S. Meri. 2001. Complement evasion by *Borrelia burgdorferi*: serum-resistant strains promote C3b inactivation. *Infect. Immun. 69:3685*.
6. Inal, J. M., and J. A. Schifferli. 2002. Complement C2 receptor inhibitor trispanning and the β-chain of C4 share a binding site for complement C2. *J. Immunol. 168:5213*.
7. Pandiripally, V., E. Gregory, and D. Cue. 2002. Acquisition of regulators of complement activation by *Streptococcus pyrogenes* serotype M1. *Infect. Immun. 70:6206*.
8. Favoreel, H. W., G. R. Van-de-Walle, H. J. Nauwynck, and M. B. Pensaert. 2003. Virus complement evasion strategies. *J. Gen. Virol. 84:1*.
9. Wikel, S. K., and J. R. Allen. 1977. Acquired resistance to ticks. III. Cobra venom factor and the resistance response. *Immunology 32:457*.
10. Wikel, S. K. 1979. Acquired resistance to ticks: expression of resistance in C4-deficient guinea pigs. *Am. J. Trop. Med. Hyg. 28:586*.
11. Allen, J. R., H. M. Khalil, and J. E. Graham. 1979. The location of salivary antigens, complement and immunoglobulin in the skin of guinea pigs infested with *Dermacentor andersoni* larvae. *Immunology 38:467*.
12. Papatheodorou, V., and M. Brossard. 1987. C3 levels in the sera of rabbits infested and reinfested with *Ixodes ricinus* and in the midguts of fed ticks. *Exp. Appl. Acarol. 3:53*.
13. Wikel, S. K. 1999. Tick modulation of host immunity: an important factor in pathogen transmission. *Int. J. Parasitol. 29:851*.
14. Norley, S.G., and R. C. Wardley. 1982. Complement mediated lysis of African swine fever virus infected cells. *Immunology 46:75*.
15. McDowell, J. V., E. Tran, D. Hamilton, J. Wolfgang, K. Miller, and R. T. Marconi. 2003. Analysis of the ability of spirochaete species associated with relapsing fever, avian borreliosis, and epizootic bovine abortion to bind factor H and cleave C3b. *J. Clin. Microbiol. 41:3905*.
16. Connolly, S. E., and J. L. Benach. 2001. Cutting edge: the spirochetemia of murine relapsing fever is cleared by complement-independent bactericidal antibodies. *J. Immunol. 167:3029*.
17. Ribeiro, J. M. 1987. *Ixodes dammini*: salivary anti-complement activity. *Exp. Parasitol. 64:347*.
18. Valenzuela, J. G., R. Charlab, T. N. Mather, and J. M. Ribeiro. 2000. Purification, cloning, and expression of a novel salivary anticomplement protein from the tick, *Ixodes scapularis. J. Biol. Chem. 275:18717*.
19. Ribeiro, J. M., and A. Spielman. 1986. Ixodes dammini: salivary anaphylatoxin inactivating activity. *Exp. Parasitol. 62:292*.
20. Astigarraga, A., A. Oleaga-Perez, R. Perez-Sanchez, J. A. Baranda, and A. Encinas-Grandes. 1997. Host immune response evasion strategies in *Ornithodoros erraticus* and *O. moubata* and their relationship to the development of an antiargasid vaccine. *Parasite Immunol. 19:401*.
21. Jones, L. D., C. R. Davies, G. M. Steele, and P. A. Nuttall. 1988. The rearing and maintenance of ixodid and argasid ticks in the laboratory. *Anim. Technol. 39:99*.
22. Giclas, P. C. 1994. Classical and alternative pathway evaluation (sections 13.1 and 13.2). In *Current Protocols in Immunology*, Vol. 3. J. E. Coligan, A. M. Kruisbeek, D. H. Marguiles, E. M. Shevach, and W. Strober, eds. John Wiley and Sons, Inc., New York.
23. Paesen, G. C., P. L. Adams, K. Harlos, P. A. Nuttall, and D. I. Stuart. 1999. Tick histamine binding proteins: isolation, cloning, and three-dimensional structure. *Mol. Cell 3:661*.
24. Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. Lipman. 1997. Gapped BLAST and PSI-BLAST a new generation of protein database search programs. *Nucleic Acids Res. 25:3389*.
25. Bateman, A., E. Birney, R. Durbin, S. R. Eddt, K. L. Howe, and E. L. Sonnhammer. 2000. The Pfam protein families database. *Nucleic Acids Res. 28:263*.

26. Schultz, J., R. R. Copley, T. Doerks, C. P. Ponting, and P. Bork. 2000. SMART: a web based tool for the study of genetically mobile domains. *Nucleic Acids Res. 28:231*.
27. Jeanmougin, F., J. D. Thompson, M. Gouy, D. G. Higgins, and T. J. Gibson. 1998. Multiple sequence alignment with Clustal X. *Trends Biochem. Sci. 23:403*.
28. Waxman, L., and T. M. Connolly. 1993. Isolation of an inhibitor selective for collagen-stimulated platelet aggregation from the soft tick: *Ornithodoros moubata. J. Biol. Chem. 268:5445*.
29. Mans, B. J., J. D. Venter, P. J. Very, A. I. Louw, and A. W. Neitz. 2001. Identification of putative proteins involved in granule biogenesis of tick salivary glands. *Electrophoresis 22:1739*.
30. Paesen, G. C., P. L. Adams, P. A. Nuttall, and D. I. Stuart. 2000. Tick histamine-binding proteins: lipocalins with a second binding cavity. *Biochim. Biophys. Acta 1482:92*.
31. Mans, A. J., A. I. Louw, and A. W. H. Neitz. 2003. The major tick salivary gland proteins and toxins from the soft tick *Ornithodorus savignyi* are part of the tick lipocalin family: implications for the origins of tick toxicosis. *Mol. Biol. Evol. 20:1158*.
32. Schreck, S. F., C. Parker, M. E. Plumb, and J. M. Sodetz. 2000. Human complement protein C8γ. *Biochim. Biophys. Acta 1482:199*.
33. Baranda, J. A., R. Perez-Sanchez, A. Oleaga, R. Manzano, and A. Encinas-Grandes. 2000. Purification N-terminal sequencing and diagnostic value of the major antigens of *Ornithodoros erraticus* and *O. moubata. Vet. Parasit. 87:193*.
34. Mans, B. J., C. M. L. Steinmann, J. D. Venter, A. I. Louw, and A. W. H. Neitz. 2002. Pathogenic mechanisms of sand tampan toxicosis induced by the tick, *Ornithodoros savignyi*. *Toxicon 40:1007*.
35. Flower, D. R. 1996. The lipocalin protein family: structure and function. *Biochem. J. 318:1*.
36. Ortlund, E., C. L. Parker, S. F. Schreck, S. Ginell, W. Minor, J. M. Sodetz, and L. Lebioda. 2002. Crystal structure of human complement protein C8γ at 1.2A resolution reveals a lipocalin fold and distinct ligand binding site. *Biochemistry 41:7030*.
37. Marzari, R., D. Sblattero, P. Macor, F. Fischetti, R. Gennaro, J. D. Marks, A. Bradbury, and F. Tedesco. 2002. The cleavage site of C5 from man and animals as a common target for neutralizing human monoclonal antibodies: in vitro and in vivo studies. *Eur. J. Immunol. 32:2773*.
38. Sandoval, A., A. Rong, J. M. Ostresh, and R. T. Ogata. 2000. Distal recognition site for classical pathway convertase located in the C345C/Netrin module of complement component C5. *J. Immunol. 165:1066*.
39. Sahu, A., and J. D. Lambris. 2000. Complement inhibitors: a resurgent concept in anti-inflammatory therapeutics. *Immunopharmacology 49:133*.
40. Smith G. P., and R. A. G. Smith. 2001. Membrane-targeted complement inhibitors. *Mol. Immunol. 38:249*.
41. Frei, Y., J. D. Lambris, and B. Stockinger. 1987. Generation of a monoclonal antibody to mouse C5 application in an ELISA assay for detection of anti-C5 antibodies. *Mol. Cell. Probes 1:141*.
42. Fitch, J. C., S. Rollins, L. Matis, B. Alford, S. Aranki, C. D. Collard, M. Dewar, J. Elefteriades, R. Hines, G. Kopf, et al. 1999. Pharmacology and biological efficacy of a recombinant, humanized, single-chain antibody C5 complement inhibitor in patients undergoing coronary artery bypass graft surgery with cardiopulmonary bypass. *Circulation 100:2499*.
43. Huber-Lang, M. S., N. C. Riedeman, J. V. Sarma, E. M. Younkin, S. R. McGuire, I. J. Laudes, K. T. Lu, R. F. Guo, T. A. Neff, V. A. Padgaonkar, et al. 2002. Protection of innate immunity by C5aR antagonist in septic mice. *FASEB J. 16:1567*.
44. Wang, Y., Q. L. Hu, J. A. Madri, S. A. Rollins, A. Chodera, and L. A. Matis. 1996. Amelioration of lupus-like autoimmune disease in NZB/WF_1 mice after treatment with a blocking monoclonal antibody specific for complement component C5. *Proc. Natl. Acad. Sci. USA 93:8563*.
45. Wang, Y., S. A. Rollins, J. A. Madri, and L. A. Matis. 1995. Anti-C5 monoclonal antibody therapy prevents collagen-induced arthritis and ameliorates established disease. *Proc. Natl. Acad. Sci. USA 92:8955*.
46. Pratt, J. R., M. J. Hibbs, A. J. Laver, R. A. Smith, and S. H. Sacks. 1996. Effects of complement inhibition with soluble complement receptor-1 on vascular injury and inflammation during renal allograft rejection in the rat. *Am. J. Pathol. 149:2055*.
47. Tanaka, M., N. Murase, Q. Ye, W. Miyazaki, M. Nomoto, H. Miyazawa, R. Manez, Y. Toyama, A. J. Demetris, S. Todo, et al. 1996. Effect of anticomplement agent K76 COOH on hamster-to-rat and guinea pig-to-rat xenotransplantation. *Transplantation 62:681*.
48. Kroshus, T. J., C. T. Salerno, C. G. Yeh, P. J. Higgins, R. M. Bolman, and A. P. Dalmasso. 2000. A recombinant soluble chimeric complement inhibitor composed of human CD46 and CD55 reduces acute cardiac tissue injury in models of pig-to-human heart transplantation. *Transplantation 69:2282*.
49. Link, C., H. Hawlisch, A. Meyer zu Vilsendorf, S. Gyleruz, F. Nagel, and J. Kohl. 1999. Selection of phage-displayed anti-guinea pig C5 or C5a antibodies and their application in xenotransplantation. *Mol. Immunol. 36:1235*.