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# Activation of the complement system generates antibacterial peptides

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The complement system represents an evolutionary old and significant part of the innate immune system involved in protection against invading microorganisms. Here we show that the anaphylatoxin C3a, and its inactivated derivative C3a-desArg are antibacterial, demonstrating a previously unknown direct antimicrobial effect of complement activation. The C3a peptide, as well as functional epitopes in the sequence, efficiently killed the Gram-negative bacteria *Escherichia coli*, *Pseudomonas aeruginosa*, and the Gram-positive *Enterococcus faecalis*. In mice, a C3a-derived peptide suppressed infection by Gram-positive *Streptococcus pyogenes* bacteria. Fluorescence and electron microscopy demonstrated that C3a binds to and induces breaks in bacterial membranes. C3a was also found to induce membrane leakage of liposomes. These findings provide an interesting link between the complement system and antimicrobial peptides, two important branches of innate immunity.

In contrast to adaptive immunity, innate immune effectors, such as phagocytes, complement, chemokines, and antimicrobial peptides (AMPs), provide a rapid and non-specific host-response (1). Antimicrobial peptides, originally described in silk worms (2), are important in the initial clearance of bacteria at biological boundaries susceptible to infection (3-5). However, many AMPs, such as defensins and LL-37, also possess chemotactic activity against leukocytes, T-cells (6) and mast cells (7). Conversely, chemokines, which attract and activate specific leukocyte populations during inflammation and infection, were recently shown to exert direct antimicrobial functions (8). Thus, it is becoming increasingly clear that various protein and peptide mediators bridge the innate and adaptive immune systems.

In vertebrates, the complement system is activated by the classical, alternative, and lectin pathways, each converging at the step of C3 with release of multiple proteolytic fragments, including the anaphylatoxin C3a (9). C3a exerts multiple proinflammatory functions, involving histamine release from mast cells, smooth muscle contraction, increased vascular permeability and chemoattraction against mast cells (9). The biological effects of C3a are regulated by the plasma protease carboxypeptidase B, which cleaves off the C-terminal arginine to generate the inactive C3a-desArg peptide (9). Many AMPs, such as the human cathelicidin LL-37 (5, 10, 11) are able to interact with bacterial surface components like lipopolysaccharides (LPS), teichoic acid, peptidoglycans and phospholipid groups (11). These interactions promote conformational changes, such as formation of an amphipatic helix, which in turn facilitates hydrophobic membrane interactions, oligomerization, and finally, membrane destabilization and bacterial inactivation (11). The human C3a molecule (77 aa, 9083 Da) is cationic (pI 11.3) and contains four  $\alpha$ -helical regions (9). The structural features of C3a, which resemble those of AMPs, prompted us to investigate whether C3a and its inactivated form C3a-desArg exerted antimicrobial effects. In this report, we show that C3a,

C3a-desArg, and functional epitopes in the C3a-sequence function as “classical” AMPs, demonstrating a previously unknown direct antimicrobial effect of complement activation.

## Materials and Methods

**Biological materials.** Sterile wound fluids (WF) were obtained from surgical drainages after mastectomy. Collection was for 24 h, 24 to 48 h after operation. WFs were centrifuged, aliquoted and stored at  $-20^{\circ}\text{C}$ . The use of human WF was approved by the Ethics Committee at Lund University (LU 708-01). Polymorphonuclear leukocytes (PMN) were obtained from blood samples drawn from healthy volunteers.

**Peptides/Proteins.** C3a and C3a-desArg were obtained from Calbiochem (San Diego, CA). The protein C3 was obtained from Reasearch Diagnostics, Inc (Flanders, USA). The peptides: LTE21, LRK26, LGE27, CNY21, CNY20, LGL5 (Fig. 3B), CNY21(S): CNYITELSSQHASASHLGLAR (Fig. 5A) and tetramethylrhodamine (TAMRA) conjugated LRK26, LGE27 and CNY21 were synthesized by Innovagen AB, Lund, Sweden. The purity (>95%) and molecular weight of these peptides was confirmed by mass spectral analysis (MALDI.TOF Voyager).

**Microorganisms.** *Escherichia coli* 37.4, *Enterococcus faecalis* 2374 and *Pseudomonas aeruginosa* 27.1 isolates, were obtained from patients with chronic venous ulcers. *Streptococcus pyogenes* AP1 (40/58) of the M1 serotype was provided by the WHO (World Health Organisation) Streptococcal Reference Laboratory in Prague, Czech Republic.

**Radial diffusion assay.** Essentially as described earlier (12), bacteria were grown to mid-logarithmic phase in 10 ml of full-strength (3% w/v) trypticase soy broth (TSB) (Becton-

Dickinson, Cockeysville, MD). The microorganisms were washed once with 10 mM Tris, pH 7.4.  $4 \times 10^6$  bacterial colony forming units (CFU) were added to 5 ml of the underlay agarose gel (0.03% (w/v) TSB, 1% (w/v) low electroendosmosis type (EEO) agarose (Sigma, St Louis MO) and 0.02% (v/v) Tween 20 (Sigma)). The underlay was poured into a Ø85 mm petri dish. After agarose solidification, 4 mm-diameter wells were punched and 6 µl of test sample was added to each well. Plates were incubated at 37°C for 3 hours to allow diffusion of the peptides. The underlay gel was then covered with 5 ml of molten overlay (6% TSB and 1% Low-EEO agarose in dH<sub>2</sub>O). Antimicrobial activity of a peptide is visualized as a zone of clearing around each well after 18-24 h of incubation at 37°C. The activity of the complement peptides C3a, C3a-desArg (50 and 10 µM), the C3 holoprotein (10 µM) (Fig. 1A), or the synthetic C3a-derived peptides LTE21, LRK26, LGE27, CNY21, and LGL5 (50 µM) were compared with the activity of the peptide LL-37. MIC-values of C3a against *P. aeruginosa* and *E. coli* and of LL-37 against *P. aeruginosa* were determined by triplicate experiments using 5-serial two-fold dilutions (starting at 32 µM) of peptides. The logarithmic concentrations of peptides were plotted versus the respective diameter of inhibition zone. Linear regression using least squares was used to estimate MIC-values. The antimicrobial activities of CNY21 and CNY20 were compared by measuring zones of clearance of 50 µM of respective peptide (n = 15). There was no statistical difference in activity between the two peptides. Inhibitory effects of heparin were examined by adding equimolar amounts (~50 µM) of heparin to the peptides.

**Viable count analysis.** *E. faecalis*, *P. aeruginosa* and *S. pyogenes* bacteria were grown to mid-logarithmic phase in Todd-Hewitt (TH) medium. Bacteria were washed and diluted in 10 mM Tris, pH 7.4 containing 5 mM glucose (Fig. 1B, 3C, 4A), or in 10 mM MES, pH 5.5 containing 5 mM glucose (Fig. 4A), with or without the addition of 0.15 M NaCl. Activity of

10  $\mu\text{M}$  C3a and LL-37 was also tested against *P. aeruginosa* diluted in buffer +/- 20% human wound fluid. Significance was determined by using the Holm-Sidak method; one way repeated measures analysis of variance (ANOVA) and the statistical software used was SigmaStat, (SPSS Inc., Chicago, IL) (Fig. 4B). The control peptide CNY21(S) was not active against *S. pyogenes* whereas CNY21 killed the bacteria at 10  $\mu\text{M}$  ( $P = 0.001$ ,  $n = 7$ ) (Fig. 5B). Bacteria (50  $\mu\text{l}$ ;  $2 \times 10^6$  CFU/ml) were incubated, at 37°C for 2 h, with the complement peptides, C3a, C3a-desArg, the holoprotein C3 or LL-37 at concentrations ranging from 0.003 to 6  $\mu\text{M}$  or with the synthetic peptides LTE21, LRK26, LGE27, CNY21, and LGL5 at concentrations ranging from 0.03 to 60  $\mu\text{M}$ . To quantify the bactericidal activity, serial dilutions of the incubation mixture were plated on TH agar, followed by incubation at 37°C overnight and the number of colony-forming units was determined.

**Heparin-binding assay.** The complement peptides C3a, C3a-desArg, C3 (Fig. 1C) and the synthetic peptides, LTE21, LRK26, LGE27, CNY21, LGL5 (Fig. 3D), were tested for heparin-binding activities. LL-37 was used as positive control. C3a, C3a-desArg, C3 (2  $\mu\text{g}$ ) and the synthetic peptides (1, 2, 5  $\mu\text{g}$ ) were applied onto nitrocellulose membranes (Hybond<sup>TM</sup>-C, Amersham Biosciences). Membranes were blocked (PBS, pH 7.4, 3% bovine serum albumin) for one hour and incubated with radiolabeled heparin (~10  $\mu\text{g}/\text{ml}$ ) (13). Unlabeled heparin (6 mg/ml) was added for competition of binding (Fig. 3D). The membranes were washed (3 x 10 min in 10 mM Tris, pH 7.4). A Bas 2000 radioimaging system (Fuji) was used for visualization of radioactivity.

**Electron microscopy.** *P. aeruginosa* 27.1 ( $16 \times 10^6$  /sample) were incubated for 2 h at 37°C with the complement factors C3a and C3a-desArg at ~50% of their required bactericidal concentration (0.3  $\mu\text{M}$ ). The protein C3 (0.3  $\mu\text{M}$ ) was included as a control. Each sample was

gently transferred onto poly-L-lysine-coated Nylaflo® (GelmanSciences, MI) nylon membranes. The membranes were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate, pH 7.2, for 2 h at 4°C, and subsequently washed with 0.15 M cacodylate, pH 7.2. They were then postfixed with 1% osmium tetroxide (w/v) and 0.15 M sodium cacodylate, pH 7.2, for 1 h at 4°C, washed, and subsequently dehydrated in ethanol and further processed for Epon embedding. Sections were cut with a microtome and mounted on Formvar coated copper grids. The sections were postfixed with uranyl acetate and lead citrate and examined in a Jeol 1200 EX transmission electron microscope operated at 60 kV accelerating voltage.

**Liposome preparation and leakage assay.** Dry lipid films were prepared by dissolving dioleoylphosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) (60 mol%) and cholesterol (Sigma, St Louis, MO) (40 mol%) in chloroform, and then removing the solvent by evaporation under vacuum overnight. Subsequently, buffer (10 mM Tris, pH 7.4) was added together with 0.1 M carboxyfluorescein (CF) (Sigma, St Louis, MO). After hydration, the lipid mixture was subjected to eight freeze-thaw cycles consisting of freezing in liquid nitrogen and heating to 60°C. Unilamellar liposomes, of about Ø100 nm were generated by multiple extrusions through polycarbonate filters (pore size 100 nm) mounted in a LipoFast miniextruder (Avestin, Ottawa, Canada) at 22°C. Untrapped carboxyfluorescein was then removed by two gelfiltrations (Sephadex G-50) at 22°C, with the Tris buffer as eluent.

In the liposome leakage assay, self-quenching of CF was used. Thus, at 100 mM CF is self-quenched, and the recorded fluorescence intensity from liposomes with entrapped CF is low. On leakage from the liposomes, released CF is dequenched, and hence fluoresces. The CF release was determined by monitoring the emitted fluorescence at 520 nm from a liposome dispersion (10mM lipid in 10 mM Tris pH 7.4). An absolute leakage scale is obtained by



disrupting the liposomes at the end of the experiment through addition of 0.8 mM Triton X100 (Sigma, St Louis, MO), thereby causing 100% release and dequenching of CF. A SPEX-fluorolog 1650 0.22-m double spectrometer (SPEX Industries, Edison, NJ) was used for the liposome leakage assay.

**Fluorescence microscopy.** *P. aeruginosa* 27.1 bacteria were grown to mid-logarithmic phase in TH medium. The bacteria were washed twice in 10 mM Tris, pH 7.4. The pellet was dissolved to yield a suspension of  $5 \times 10^6$  cfu/ml in the same buffer. Two hundred microliter of the bacterial suspension was incubated with either 1  $\mu$ l TAMRA-LGE27, TAMRA-LRK26 or TAMRA-CNY21 (2 mg/ml) on ice for 5 min and washed twice in 10 mM Tris, pH 7.4. The bacteria were fixed by incubation on ice for 15 min and in room temperature for 45 min in 4% paraformaldehyde. The suspension was applied onto Poly-L-lysine coated cover glass and bacteria were let to attach for 30 min. The liquid was poured away and the cover glass was mounted on a slide by Dako mounting media, Dako (Carpinteria, CA). This was performed using a Nikon Eclipse TE300 inverted fluorescence microscope equipped with a Hamamatsu C4742-95 cooled CCD camera, a Plan Apochromat 60X objective, and a high N.A. oil-condenser.

**Bacterial dissemination to the spleen.** *S. pyogenes* AP1 bacteria were grown to early logarithmic phase ( $OD_{620} \sim 0.35$ ), harvested, washed in 10 mM Tris, pH 7.4, diluted in the same buffer to  $2 \times 10^6$  cfu/ml, and kept on ice until injection. Five hundred microliter of the bacterial suspension were injected intraperitoneally (ip) into female BALB/c mice. Ten minutes after the bacterial injection, 0.5 mg CNY21 or CNY21(S) in 10 mM Tris, pH 7.4, was injected ip into the mice. This was repeated after 6 hours. After 24 hours, the mice were killed, the spleens were removed, homogenised in 10 mM Tris, pH 7.4, and the number of

colony-forming units was determined. The P-value was determined with the Mann-Whitney U-test.

**Generation of C3a-like peptides in wound fluid.** Neutrophils were prepared by routine procedures (14) (polymorphprep<sup>TM</sup>, AXIS-SHIELD PoC AS, Oslo, Norway) from blood of healthy human donors. The cells were disrupted by freeze-thawing and addition of 0.3% Tween 20. The amount of neutrophils corresponding to  $9.4 \times 10^4$  cells were incubated at 37° with 5 µl of human wound fluid (WF) for different time periods (10 min, 30 min, 60 min, 3 h and 17 h). Separate incubations (60 min) of neutrophils and WF were used as controls. The C3a (0.5 µg) peptide was added for size comparison. The materials were analyzed on 16.5% precast sodium dodecyl sulfate polyacrylamide (SDS-PAGE) Tris-Tricine gels (BioRad, Hercules, CA) under reducing conditions. Proteins/peptides were transferred to nitrocellulose membranes (Hybond<sup>TM</sup>-C, Amersham Biosciences). Membranes were blocked by 3% (w/v) skimmed milk, washed, and incubated for 1 h with rabbit polyclonal LGE27 antibodies (1:1500) (Innovagen AB, Lund, Sweden), washed again, and subsequently incubated (1 h) with HRP-conjugated secondary antibodies (1:1000). C3a proteins/fragments containing whole or parts of the LGE27 sequence were visualized using the ECL developing system (Amersham Biosciences).

**Degradation of C3a with human neutrophil elastase.** C3a (2 µg, 0.2 mg/ml) was incubated with neutrophil elastase (40 mU) for increasing time periods (5, 15, 30, 60 and 120 min) at 37°C and were then analyzed by SDS-PAGE, 16.5% Tris-Tricine gel (BioRad). For control 0.5 µg C3a was used.

**Definition of C3a cleavage products.** The final degradation products were reduced and alkylated by subsequent addition of 10-20  $\mu$ l of 10 mM dithiothreitol (55°C, 30 min) and 10-20  $\mu$ l of 110 mM iodoacetamide (20°C, 30 min). Peptides were purified using C18 Ziptips (Millipore). Precrystallized 2,5-dihydroxybenzoic acid was provided on an Anchorchip target (Bruker Daltonik GmbH). The purified peptides were eluted directly onto this matrix using 50% acetonitrile, 0.1% trifluoroacetic acid and allowed to co-crystallize. Spectrometry was carried out on a Bruker Reflex III matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer. The instrument was operated at an acceleration voltage of 26 kV in the positive ion/delayed extraction mode and detection in the reflector mode. Autolysis fragments of trypsin were used for calibration of the spectra that were summed of 75-100 single-shot measurements each. Peaks were assigned using the FindPept tool ([www.expasy.org/tools/findpept.html](http://www.expasy.org/tools/findpept.html)). The detected signals corresponded to masses of 1247.69, 2177.97, and 3068.47 Da and the peptides VQLTEKRMDK or QLTEKRMDKV, SLGEACKKVFLDCCNYIT, and SLGEACKKVFLDCCNYITELRRQHA, respectively. The cleavage products were also blotted from a 16.5% Tris-Tricine gel onto PVDF membranes (Hybond<sup>TM</sup>-P, Amersham Biosciences). One major fragment (Fig. 5E, indicated by an arrow) was cut out and sent for N-terminal sequencing at the Protein Analysis Center (KI, Stockholm, Sweden). One fragment was identified by both methods,

<sup>44</sup>SLGEACKKVFLDCCNYITELRRQHA<sup>68</sup>.

## Results and Discussion

To elucidate if the anaphylatoxin C3a and its inactivated variant C3a-desArg possess antibacterial activity, we initially investigated effects on *Escherichia coli* (Fig. 1A). C3a, as well as C3a-desArg, were antibacterial in radial diffusion assays (RDA). Noteworthy, C3a yielded a larger inhibition zone against *E. coli* than the classical AMP LL-37 in the low-salt conditions used. In viable count assays, both C3a and C3a-desArg showed 80-100% killing at  $\sim 0.6 \mu\text{M}$ , of the Gram-positive species *Enterococcus faecalis* and the Gram-negative *Pseudomonas aeruginosa* (Fig. 1B). In these experiments, the holoprotein C3 did not exert any antibacterial effect. For comparison, the activity of the human antimicrobial peptide LL-37 is shown (Fig. 1A, B). In RDA the minimum inhibitory concentrations (MIC) of C3a were determined to  $0.70 \mu\text{M}$  and  $0.75 \mu\text{M}$  against *P. aeruginosa* and *E. coli*, respectively. This was comparable to the MICs obtained for LL-37 ( $1.03 \mu\text{M}$  for *E. coli*). The heparin-binding ability of the peptides was tested and the results showed that both C3a and C3a-desArg bind heparin (Fig. 1C). As recently shown, amphipaticity, cationicity, and helix structure are features that characterize heparin-binding peptides but also confer antimicrobial properties to this group of molecules (13). Therefore, the finding that C3a interacts with heparin at physiological conditions provides an additional link between C3a and many cationic AMPs.

To examine whether C3a interacts with and generates breaks in bacterial plasma membranes, *P. aeruginosa* was incubated with C3a peptides at  $\sim 50\%$  of the required bactericidal concentrations, and analyzed by electron microscopy. Clear differences in the morphology of peptide-treated bacteria (Fig. 2C, D) in comparison with the control (Fig. 2A) and the holoprotein C3 (Fig. 2B) were demonstrated. C3a caused local perturbations and breaks along *P. aeruginosa* plasma membranes, and occasionally, intracellular material was found extracellularly. These findings were similar to those seen after treatment with the

antimicrobial peptide LL-37 (13). To further analyze the effects of C3a on membranes, we used a liposome model to study membrane binding and permeabilisation (Fig. 2E). The activity of C3a was recorded by measuring fluorescence release of carboxyfluorescein from dioleoylphosphatidylcholine liposomes. Addition of C3a caused increased fluorescence, and 100% liposome leakage was noted at  $\sim 0.1 \mu\text{M}$  peptide. LL-37 yielded similar effects at  $\sim 0.5 \mu\text{M}$  (Fig. 2E). Kinetic analysis showed that  $\sim 80\%$  of the maximum fluorescence was reached at  $\sim 17$  min for both peptides (at  $0.5 \mu\text{M}$ ) (not shown).

Studies have demonstrated that C3a contains four helical regions (8-15, 17-28, 36-43 and 47-66)(Fig. 3A) (9). To explore whether these helical epitopes of C3a were responsible for the antibacterial effects, we synthesized peptides spanning the four helices, including a peptide known to exert full anaphylatoxic activity, CNY21 (15), its inactive variant, CNY20, and the smallest peptide with any activity at all, LGL5 (16) (Fig. 3B). The experiments showed that these peptides indeed were antibacterial against *E. faecalis* and *P. aeruginosa*. Concentrations killing 80-100% of *E. faecalis* were 3-6  $\mu\text{M}$  for LRK26, LGE27 (previously presented (13)) and CNY21. LTE21 was less effective against this bacterium, and LGL5 showed no antimicrobial activity at all. Similar results were obtained for *P. aeruginosa* (Fig. 3C). Thus, in comparison with C3a, the C3a-derived peptides required a  $\sim 10$  times higher concentration for efficient killing of *E. faecalis* (Fig. 1B and Fig. 3C). Notably CNY21, which has random conformation in aqueous solution (9, 15), adopts an  $\alpha$ -helical and amphipatic conformation in trifluoroethanol/water (15), adding another structural and functional link between helical AMPs and this C3a-derived peptide. The antimicrobial activities of CNY21 and its desArg variant, CNY20 (devoid of anaphylatoxin activity), were compared in RDA by measuring zones of clearance using  $50 \mu\text{M}$  of respective peptide (not shown). There was no

difference in activity between the two peptides, suggesting that the antimicrobial and anaphylactic functions are separate.

Like C3a and C3a-desArg, the C3a-derived antibacterial peptides bound radiolabeled heparin, and the binding was inhibited by an excess of unlabeled heparin (Fig. 3D). The binding of heparin to peptides LRK26 and LGE27 has been presented previously (13), but is shown for completeness. Three peptides, LRK26, LGE27, and CNY21, were labeled with the fluorescent dye TAMRA, and incubated with *P. aeruginosa*. As demonstrated by fluorescence microscopy analysis, the peptides were bound to the bacterial surface, and the binding was completely blocked by heparin (Fig. 3E). In antibacterial assays (RDA) heparin at equimolar amounts was able to abolish the antibacterial activities of the peptides (not shown).

Activities of AMP depend on salt concentration, pH, and the presence of plasma proteins. For example, the antimicrobial activities of defensins are inhibited by the presence of physiological salt (17), while the cathelicidin LL-37 is inhibited by plasma (18). Other AMPs, such as azurocidin and magainins, are potentiated by acidic conditions likely to occur in biological fluids following oxidative burst response of leukocytes (19). Hence, we examined the activities of C3a and related peptides in physiological salt. A minor decrease in the killing of *P. aeruginosa* was noted for C3a in 0.15 M NaCl at both pH 7.4 and pH 5.5 (Fig 4A). No inhibition of bacterial killing in physiological salt was noted for CNY21 (Fig 3C and 4A). LGE27 and LRK26 exerted only weak antibacterial activities in the same buffers (not shown). *P. aeruginosa* bacteria were also subjected to 10  $\mu$ M C3a at physiological salt conditions in the presence of human wound fluid (20%). For comparison, LL-37 was used at the same concentration (Fig. 4B). The peptides were active in both the absence and the presence of wound fluid, but C3a was significantly more potent than LL-37 in the presence of wound fluid. These experiments demonstrated that C3a has potent antibacterial effects under

physiological conditions. However, the molecule exerts multiple proinflammatory functions, thus making it hard to dissect out a direct antibacterial role *in vivo*. Thus, the CNY21 peptide (Fig. 5A) and a variant, CNY21(S), in which three arginine residues were replaced with serines, rendering it totally devoid of antibacterial activity (Fig. 5B), were injected into mice infected by the Gram-positive bacterium *Streptococcus pyogenes*. Compared with the control peptide, treatment with CNY21 yielded significantly lower bacterial numbers in the spleen of the animals ( $P = 0.006$ ) (Fig. 5C). This, and the fact that both peptides contained the terminal anaphylatoxin determinant LGLAR (9), demonstrated a direct antibacterial role of a well-defined and helix-forming segment (15) of the C3a-molecule.

Many AMPs, such as defensins and LL-37, are found at epithelial surfaces.

Furthermore, Pasch *et al* (20) have shown that keratinocytes constitutively produce C3 and that the production is up-regulated by inflammatory cytokines. In addition, neutrophil elastase and mast cell tryptase both release C3a-like peptides (21, 22). Acute human wound fluid was therefore incubated with lysed neutrophils for various periods of time. Western blot analysis using an anti-LGE27 antibody showed that C3a-like peptides were generated over time (Fig. 5D). We also explored whether neutrophil elastase could further degrade C3a (Fig. 5E). C3a was treated with the enzyme for increasing periods of time, and the resulting peptide fragments were analyzed by MALDI-TOF mass spectrometry and Edman degradation. The results showed, that neutrophil elastase degraded C3a into several fragments. One fragment,  $^{44}\text{SLGEACKKVFLDCCNYITELRRQHA}^{68}$ , containing a helical region of the C3a peptide, was identified by both methods. This peptide was synthesized and found to exert similar antibacterial effects as CNY21 and the other C3a-derived peptides (not shown). This indicates that, apart from C3a, additional antibacterial fragments of C3 may be generated during inflammation.

Significant amounts of C3a and C3a-desArg have been detected during sepsis (up to 0.5  $\mu\text{M}$  in blood) (23), acute media otitis ( $\sim 0.7 \mu\text{M}$  in ear secretions) (24), and in epithelial lining fluids of patients at risk for adult respiratory distress syndrome ( $\sim 1.3 \mu\text{M}$ ) (25). Most likely even higher concentrations of C3a peptides occur at the site of complement activation. In blood the concentration of C3 is 5-11  $\mu\text{M}$ , and local complement activation should therefore induce generation of C3a and C3a-desArg at antibacterial concentrations. During wound healing and inflammation, local synthesis of C3 by monocytes and keratinocytes may constitute a significant additional source of C3a at epithelial surfaces (20, 26). Indeed, analogously to other antimicrobial peptides such as defensins and LL-37 (27), C3a is present in psoriatic skin (28), which in part may explain the lower occurrence of bacterial infections in patients with psoriasis than in patients with atopic dermatitis (29). The fact that C3 deficiency is connected with increased susceptibility to bacterial infections in humans (30) as well as in animal models (31), is also compatible with the antibacterial effect of C3a revealed here. C3a homologues are found in lower organisms lacking an adaptive immune system (32), and it has been reported that a C3a-like peptide is proteolytically generated in serum from the tunicate *Pyura stolonifera*, after subjection to bacterial lipopolysaccharide (33). These findings together with the data presented here, suggest that direct bacterial killing could be an original function of C3-related peptides. If so, the present study shows that this function has been conserved during evolution, whereas the complement system of vertebrates has evolved to act in concert with adaptive immunity.



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## FIGURE LEGENDS

**Fig. 1.** Antibacterial and heparin-binding effects of C3a and C3a-desArg. (A) Peptides were tested in radial diffusion assay in low-salt conditions. *E. coli* isolate 37.4 ( $4 \times 10^6$  cfu) was inoculated in 0.1% TSB agarose gel. Each 4 mm-diameter well was loaded with 6  $\mu$ l of peptide at the indicated concentration. The zones of clearance correspond to the inhibitory effect of each peptide after incubation at 37 °C for 18-24 h. A negative control, containing buffer (10 mM Tris, pH 7.4) was included in the well at the top left of the plate. This clear zone corresponds to the 4 mm well. (B) In viable count assays antibacterial activities were seen against both *E. faecalis* isolate 2374 (—●—) and *P. aeruginosa* isolate 27.1 (—□—).  $2 \times 10^6$  cfu/ml of bacteria were incubated in 50  $\mu$ l with peptides at concentrations ranging from 0.003 to 6  $\mu$ M. For C3, the two graphs overlap. (C) C3a and C3a-desArg were both able to bind heparin, whereas only weak binding was seen with C3. C3a and C3a-desArg (2  $\mu$ g), C3, and LL-37 (2 and 5  $\mu$ g) were applied to nitrocellulose membranes. These were then incubated in PBS (containing 3% bovine serum albumin) with iodinated ( $^{125}$ I) heparin. LL-37 was used as positive control.

**Fig. 2.** C3a interacts with and generates breaks in bacterial plasma membranes. *P. aeruginosa* 27.1 was incubated with the holoprotein C3 and C3a peptides at 0.3  $\mu$ M and analyzed with electron microscopy. (A) Control, (B) C3, (C) C3a, and (D) C3a-desArg. Scale bar represents 0.5  $\mu$ m. (E) Effects of C3a and LL-37 on liposomes. The membrane permeabilizing effect was recorded by measuring fluorescence release of carboxyfluorescein from liposomes. Values represents mean of double samples. A representative experiment out of three is shown.

**Fig. 3.** Activities of synthetic C3a-derived peptides. (A) Structure of the anaphylatoxin C3a peptide, modified from Hugli 1990 (9). (B) The synthetic peptides used in this work are indicated in the sequence. (C) Viable count assays were performed using both *E. faecalis* 2374 (—●—), and *P. aeruginosa* 27.1 (—□—).  $2 \times 10^6$  cfu/ml of bacteria were incubated in 50  $\mu$ l with peptides at concentrations ranging from 0.03 to 60  $\mu$ M. For LGL5, the two graphs overlap. (D) Heparin-binding activity of the C3a-derived peptides. Peptides at indicated concentrations were applied to nitrocellulose membranes followed by incubation in PBS (containing 3% bovine serum albumin) with iodinated ( $^{125}$ I) heparin. LL-37 was used as positive control. Excess of unlabeled heparin (+) inhibited the binding of peptides to  $^{125}$ I-heparin. (E) Binding of TAMRA-labeled peptides to *P. aeruginosa* 27.1 and inhibition of binding by excess of heparin. Panel 2 shows red fluorescence of bacteria ( $1 \times 10^7$  ml $^{-1}$ ) stained with TAMRA-conjugated peptides (10  $\mu$ g ml $^{-1}$ ) and panel 4 shows bacteria incubated with heparin and TAMRA-conjugated peptides. Images in 2 and 4 were recorded using identical instrument settings. The corresponding Nomarski images are shown in panel 1 and 3. Scale bar represents 10 $\mu$ m.

**Fig. 4.** Antibacterial activities of peptides under physiological conditions. (A) Viable count analysis of C3a and CNY21 in different buffers; 10 mM Tris pH 7.4 (—●—) and 10mM MES pH 5.5 (—□—), both containing 0.15 M NaCl. *P. aeruginosa* 27.1 ( $2 \times 10^6$  cfu/ml) were incubated in 50  $\mu$ l with peptides at concentrations ranging from 0.03 to 6  $\mu$ M for C3a and 0.03 to 60  $\mu$ M for CNY21. (B) *P. aeruginosa* bacteria were subjected to 10  $\mu$ M C3a in 10 mM Tris pH 7.4 containing 0.15 M NaCl in the presence or the absence of 20% wound fluid (WF). For comparison, LL-37 was used at the same concentration.  $P < 0.001$  (\*\*\*) ,  $P < 0.01$  (\*\*).

**Fig. 5.** Antibacterial activities of CNY21 *in vivo* and generation of C3a-fragments *ex vivo*. (A) Space-filling model of CNY21 arranged as an  $\alpha$ -helix. The arrows indicate positions of arginine residues that are replaced by serine residues in CNY21(S). (B) *S. pyogenes* bacteria were subjected to 10  $\mu$ M CNY21(S) or CNY21 in 10 mM Tris pH 7.4. At this dose CNY21 was lethal to *S. pyogenes* whereas CNY21(S) had no effect, (\*\*\*)  $P < 0.001$ ,  $n = 7$ . (C) C3a-derived peptides suppress bacterial dissemination to the spleen. Mice were injected ip with *S. pyogenes* bacteria, followed by ip injection with CNY21(S) (closed circles) or CNY21 (open circles). The mice were sacrificed 24 hours after injection and the total number of cfu in the spleen was determined for each mouse. Treatment with CNY21 yielded significantly lower bacterial numbers than treatment with CNY21(S), (\*\*)  $P = 0.006$ ,  $n = 14$ . The lines represent the median value in each group. (D) Human wound fluid (5  $\mu$ l) was incubated at 37 °C with lysed (0.3% Tween 20) neutrophils (corresponding to  $9.4 \times 10^4$  cells) for the indicated time periods. Western blot analysis identified cleavage products recognized by polyclonal antibodies against the C3a-derived peptide LGE27. No C3a fragments were detected in wound fluid (WF) or neutrophils (PMN). Molecular mass markers are indicated to the left. (E) Neutrophil elastase degrades C3a into several fragments. C3a (2  $\mu$ g) was treated with human neutrophil elastase (40 mU) for indicated time periods at 37°C and analyzed by SDS-PAGE (16.5% Tris-Tricine gel). For control 0.5  $\mu$ g C3a was used. Molecular mass markers are indicated to the left. The fragment indicated with an arrow was analyzed by N-terminal sequencing.

Figure 1

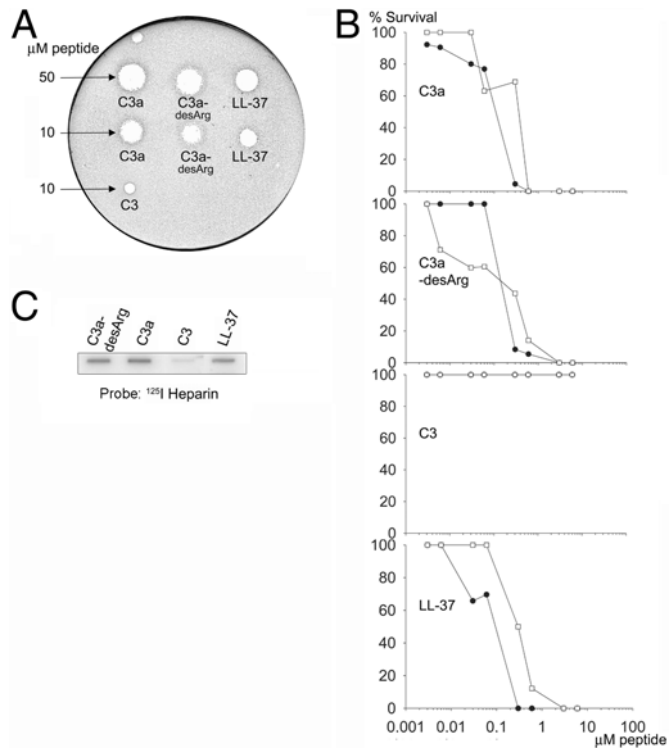


Figure 2

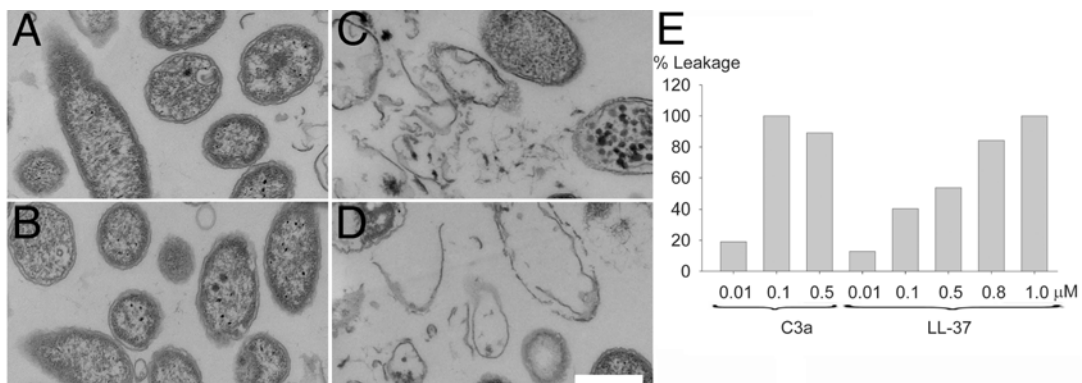




Figure 3

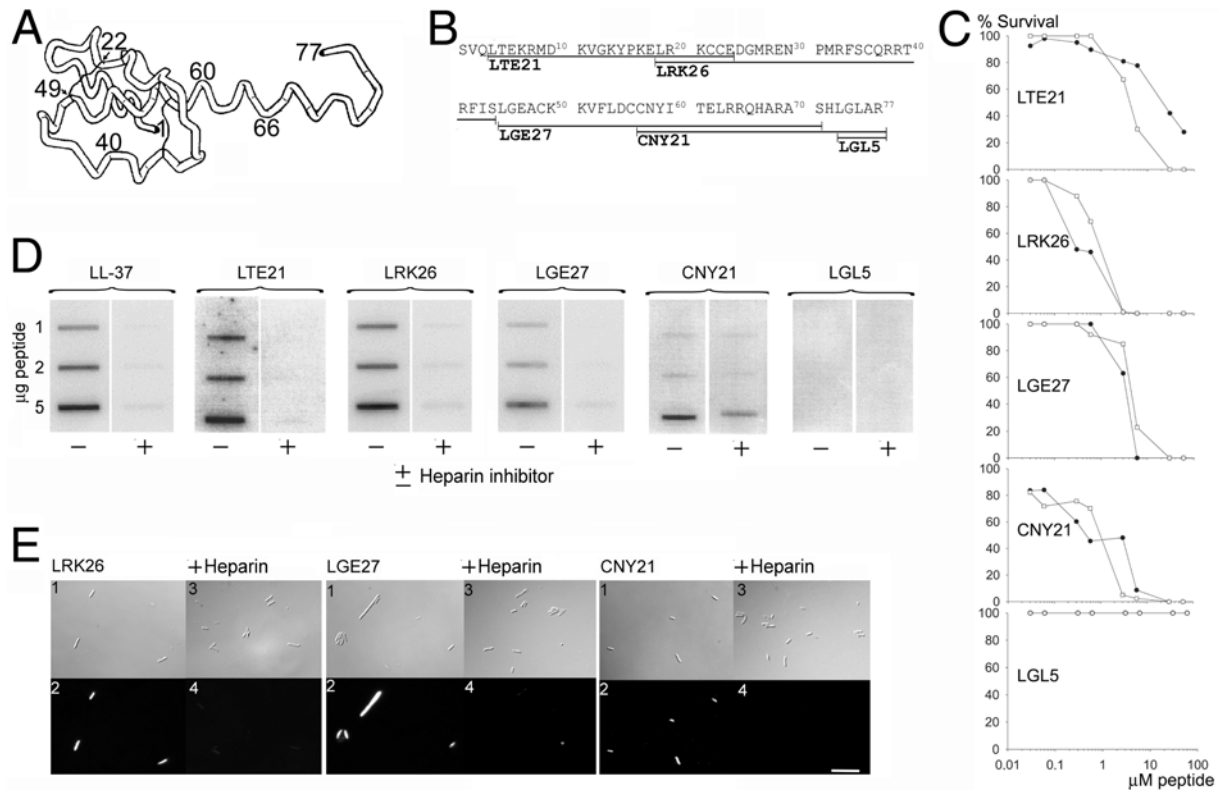


Figure 4

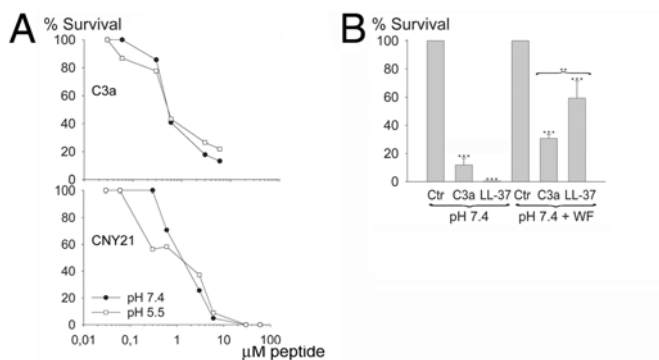


Figure 5

