The contact system—a novel branch of innate immunity generating antibacterial peptides

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Activation of the contact system has two classical consequences: initiation of the intrinsic pathway of coagulation, and cleavage of high molecular weight kininogen (HK) leading to the release of bradykinin, a potent proinflammatory peptide. In human plasma, activation of the contact system at the surface of significant bacterial pathogens was found to result in further HK processing and bacterial killing. A fragment comprising the D3 domain of HK is generated, and within this fragment a sequence of 26 amino acids is mainly responsible for the antibacterial activity. A synthetic peptide covering this sequence kills several bacterial species, also at physiological salt concentration, as effectively as the classical human antibacterial peptide LL-37. Moreover, in an animal model of infection, inhibition of the contact system promotes bacterial dissemination and growth. These data identify a novel and important role for the contact system in the defence against invasive bacterial infection.

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Introduction

Antimicrobial peptides (AMPs) were originally isolated from human leukocytes (Zeya and Spitznagel, 1963), but later identified in invertebrates (Steiner *et al*, 1981) and cold-blooded vertebrates (Zasloff, 1987). AMPs represent an important branch of innate immunity. They are ubiquitously present at biological boundaries prone to infection, where they provide a rapid and nonspecific defence against potentially invasive microorganisms (for references, see Lehrer and Ganz, 1999; Zasloff, 2002; Bevins, 2003; Boman, 2003; Peschel and Sahl, 2006), and the therapeutic potential of

AMPs in clinical medicine has recently been further emphasized (Mygind *et al*, 2005). A large number of AMPs have been identified, and molecules previously not regarded as AMPs have been found to have antimicrobial activity: chemokines (Cole *et al*, 2001), neuropeptides (Vouldoukis *et al*, 1996; Goumon *et al*, 1998; Shimizu *et al*, 1998; Kowalska *et al*, 2002; Brogden *et al*, 2005), peptide hormones (Mor *et al*, 1994; Kowalska *et al*, 2002) and C3a of complement (Nordahl *et al*, 2004).

The contact system (for references, see Bhoola *et al*, 1992; Colman and Schmaier, 1997; Joseph and Kaplan, 2005) comprises three serine proteinases: coagulation factors XI and XII, and plasma kallikrein, and the nonenzymatic cofactor high molecular weight kininogen (HK). In plasma, HK circulates in complexes with factor XI or plasma kallikrein. The system is activated when these complexes (via HK) and factor XII interact with negatively charged surfaces. As a consequence, plasma kallikrein is activated in the HK complex, and HK is cleaved to release the proinflammatory peptide bradykinin (BK). Factor XI is also activated in its complex with HK, whereby the intrinsic pathway of coagulation is initiated. HK consists of six domains: domains 1–3 are cystatin-like, D4 contains the BK peptide, D5 mediates binding to negatively charged surfaces, and D6 is responsible for the binding of plasma kallikrein and factor XI (Colman and Schmaier, 1997). There are two forms of kininogen, HK and low molecular weight kininogen (LK), resulting from alternative splicing of a single gene (Kitamura *et al*, 1985). The two forms have an identical heavy chain consisting of domains D1–D3, which through the BK-containing D4 domain is connected with the light chains that are unique for HK and LK, respectively (Müller-Esterl *et al*, 1988). In contrast to HK, LK is not cleaved as a result of contact activation.

Previous work has demonstrated that HK and the other components of the contact system can bind to the surface of several important bacterial pathogens, leading to the assembly and activation of the system (Ben Nasr *et al*, 1996; Herwald *et al*, 1998; Persson *et al*, 2000; Mattsson *et al*, 2001). In addition, the BK peptide and domain D5 of HK were reported to have antimicrobial activity (Kowalska *et al*, 2002; Brogden *et al*, 2005; Nordahl *et al*, 2005), and a number of other human proteins also contain sequences that are antimicrobial when tested as synthetic peptides (Andersson *et al*, 2004). Finally, work in many laboratories has demonstrated that proteolytic activity at the site of infection, degrades bacterial surface proteins and human proteins associated with the bacterial surface (for references, see Rasmussen and Björck, 2002). This information raised the question whether HK itself, or fragments of HK generated at bacterial surfaces through contact activation or through degradation by proteinases released at the site of infection, could have antibacterial activity. The results identify a previously unknown role for the contact system in innate immunity.

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Results

Domain D3 of kininogens is antibacterial

Purified intact HK at a concentration of 40 µg/ml and LK at 80 µg/ml (corresponds to half of their concentrations in human plasma) showed no activity when tested against *Streptococcus pyogenes* in a bactericidal assay. Earlier studies have shown that neutrophil elastase acts on proteinase-sensitive regions in kininogens, whereby smaller fragments, such as the D3 and D5 domains, are generated (Vogel *et al*, 1988; Nordahl *et al*, 2005). HK was therefore incubated with supernatants from activated neutrophils or with purified human elastase, and cleavage products were separated by Tricine-SDS-PAGE for the detection of degradation products in the low molecular range. In both cases, HK was degraded into several fragments (Figure 1A, stain), and in Western blots a prominent band of approximately 15 kDa reacted with antibodies raised against a peptide sequence (NAT26) within the D3 domain of HK (Figure 1A, blot). Peptide sequencing by MALDI-TOF/TOF identified the 15 kDa band within the D3 domain, with a COOH-terminal extension containing the BK sequence. The peptides used for identification of the 15 kDa band are indicated in the schematic representation of the heavy chain of HK (D1–D3) shown in Figure 1B. In contrast to intact HK, the elastase-cleaved HK killed *S. pyogenes*, whereas elastase alone had no effect (data not shown).

The D3 domain was recombinantly expressed in *Escherichia coli* and affinity purified using antibodies against a peptide (NAT26) sequence in the central part of D3. On SDS-PAGE purified D3 migrates as a 15 kDa band (Figure 1C), similar to the size of the HK fragment generated by incubation with supernatants from activated neutrophils or by treatment with purified elastase (Figure 1A, blot). The purified D3 was tested against *S. pyogenes* strain AP1 in a bactericidal assay, and a dose-dependent killing of the target bacteria was obtained (Figure 1D). The results show that the cleavage of HK by neutrophil proteinase(s) generates a D3-related peptide, and that D3 has antibacterial activity.

Contact activation at bacterial surfaces generates antibacterial domain D3-related fragments of HK

Several pathogenic bacteria such as *S. pyogenes*, *Staphylococcus aureus*, and *Salmonella* activate the contact system, resulting in the proteolytic cleavage of HK and the release of BK (for references, see Herwald *et al*, 2003). To investigate whether HK is further degraded at the bacterial surface, isolates of *S. pyogenes* (AP1), *S. aureus* (Cowan I) and *Salmonella* (SR11B) were grown to mid exponential phase, and separately incubated with human plasma for 1 h at 37°C. Proteins bound to the bacteria were eluted by low pH, followed by Western blot analysis using antibodies against the NAT26 peptide from domain D3. Multiple immuno-reactive fragments, including a peptide of approximately 14–15 kDa, were released from the surface of all strains (Figure 2A). When the contact system is activated, native HK (120 kDa) is cleaved by plasma kallikrein into a heavy chain of approximately 65 kDa, containing the D3 domain, and a light chain of approximately 55 kDa. HK eluted from the surface of AP1 was fully cleaved into its heavy and light chain. In contrast, a portion of HK bound to the surface of Cowan I and SR11B remained uncleaved (Figure 2A). Moreover, as judged from the presence of immuno-reactive

Figure 1 Proteolytic cleavage of HK *in vitro* generates antibacterial peptides. (A) HK (6 µg) was digested with supernatant from activated human neutrophils (1 µl) or purified human elastase (2.5 mU) for 1.5 h at 37°C, and subjected to Tricine-SDS/PAGE. One gel was stained with Coomassie blue (stain), and one was transferred to a PVDF membrane and probed with polyclonal antibodies raised against the NAT26 peptide derived from domain D3 of HK (blot). Lane 1: native HK (5 µg); lane 2: HK incubated with supernatant from activated neutrophils; lane 3: HK cleaved by elastase. The band at 15 kDa, marked with an arrow, was cut out and subjected to MALDI-TOF/TOF peptide sequencing. (B) Schematic representation of the heavy chain of HK (D1–D3). NAT26, BK and the peptides used for identification of the 15 kDa band by MALDI-TOF/TOF are indicated. Numbers refer to amino-acid residue positions (bars are not in scale). (C) Purified D3 domain (1 µg) was subjected to Tricine-SDS/PAGE analysis. One gel was stained with Coomassie Blue (stain), and one was transferred to a PVDF membrane and probed with antibodies against NAT26 (blot). (D) *S. pyogenes* AP1 bacteria (2×10^6 CFU/ml) were incubated for 1 h at 37°C in buffer with various concentrations of recombinant D3. Following incubation, appropriate dilutions of the mixtures were plated on TH agar plates. Plates were incubated at 37°C over night and CFUs were determined. The bars represent the mean $\pm$ s.e.m. of at least three experiments.

bands in the high molecular weight range (45–66 kDa), the heavy chain of HK was not completely processed at the bacterial surface of the investigated strains (Figure 2A). The smaller bands (approximately 13–17 kDa) are D3-related fragments containing NAT26 epitopes, produced by further cleavage of HK (Figure 2A). The band pattern in the 13–17 kDa range differs between the three species, indicating variations in HK degradation and/or in the interactions between the generated fragments and the bacterial surfaces. Contact activation on bacterial surfaces is an immediate event as judged from incubations of AP1 bacteria in plasma for various time points (5–60 min). Already after 5 min of

Figure 2 Domain D3-related antibacterial fragments of HK are generated at bacterial surfaces in plasma environment. (A) Suspensions of *S. pyogenes* strain AP1, *S. aureus* strain Cowan I and *Salmonella* strain SR11B in 1 ml of PBS (2×10^9 CFU/ml) were separately incubated with undiluted human plasma (1:1) for 1 h at 37°C. Bacteria were pelleted and washed with PBS. Plasma proteins bound to the bacteria were eluted with 0.1 M glycine buffer, pH 2.0, followed by TCA precipitation. This material was subjected to Tricine-SDS/PAGE followed by Western blot. Plasma diluted 1:25 and eluted material from the three bacterial strains was probed with anti-NAT26 antiserum. (B) Fluid from a wound infected with *S. aureus* was subjected to Tricine-SDS/PAGE followed by Western blot, and probed with anti-NAT26 antiserum. (C) Plasma preincubated with AP1 bacteria for indicated time points was subjected to anti-NAT26 Sepharose, and bound proteins were eluted with glycine buffer, pH 2.0. The samples were neutralized (pH 7.5) with 1 M Tris. *S. pyogenes* AP1 bacteria (2×10^6 CFU/ml) were incubated with equal volumes of eluates from the various time points for 1 h at 37°C, and CFUs were determined. Neutralized glycine buffer was used as a control. The bars represent the mean $\pm$ s.e.m. of at least three experiments. Data representing the bactericidal activity of the eluates were statistically analysed using the Mann–Whitney *U*-test. (D) Plasma was preincubated with AP1 bacteria for 60 min at 37°C and subjected to anti-NAT26 affinity chromatography as described in (C). The protein content of the affinity purified material was determined by Bradford analysis and different amounts were tested for killing of AP1 bacteria. The bactericidal activity of LL-37 was used as a positive control. The bars represent the mean $\pm$ s.e.m. of at least three experiments. Data were statistically analysed using the Mann–Whitney *U*-test.

incubation, native HK was fully cleaved generating the fragments of 45–66 and 22 kDa (data not shown). Over time the smaller D3-related fragments (22 and 13–17 kDa) accumulated at the bacterial surface, as a result of further cleavage of HK. The band of 66 kDa in the plasma sample (Figure 2A, far left lane) represents LK. LK has the same heavy chain as HK and contains the D3 domain. However, LK is not part of the contact system and is not cleaved when the system is activated. Furthermore, Western blot analysis of wound fluid from a patient with a leg ulcer infected with *S. aureus*, showed bands in the low molecular range reacting with antibodies against NAT26, demonstrating that D3-related fragments are generated *in vivo* (Figure 2B).

In plasma, contact activation on bacterial surfaces could also generate soluble HK fragments not bound to the bacteria.

Plasma samples that had been incubated with AP1 bacteria for various time points were therefore subjected to affinity chromatography on anti-NAT26-Sepharose. When tested in the bactericidal assay, this purified material had antibacterial activity (Figure 2C). Maximum effect was obtained with material purified after 60 min of bacterial incubation with plasma, emphasizing the importance of the smaller D3-related fragments in bacterial killing. Plasma was again preincubated with AP1 bacteria for 1 h at 37°C, and subjected to affinity purification on anti-NAT26 Sepharose. This material contained HK fragments similar to those eluted from the surface of AP1 bacteria (see Figure 2A), including the 13–17 kDa D3-related peptides, and the material showed a dose-dependent killing of AP1 bacteria (Figure 2D). The data demonstrate that contact activation at the surface of pathogenic bacteria in plasma, results in the production of domain D3-related peptides that are antibacterial, suggesting that the contact system plays a role in the defence against invasive bacteria.

Several human proteins contain heparin-binding sequences, and peptides spanning these sequences are often antibacterial (Andersson *et al*, 2004). The D5 domain of HK has such a sequence and is antibacterial (Nordahl *et al*, 2005). However, there are no data published suggesting that D5 is released during contact activation, and when antibodies against D5 were used in the Western blot experiment described above (Figure 2A), only intact HK but no D5-related fragments were identified (data not shown). It was reported that BK and other endogenous peptides (substance P and neurotensin) are antimicrobial *in vitro* (Kowalska *et al*, 2002), suggesting that BK could contribute to the antibacterial effect of contact activation. Complete cleavage of HK in plasma would result in a BK concentration of approximately 1 μg/ml. However, even at 20 μg/ml, BK had no effect in the bactericidal assay, which together with the short half-life of BK (<1 min) (Decarie *et al*, 1996) makes it highly unlikely that BK is antibacterial *in vivo*. In conclusion, the results suggest that domain D3-related peptides are solely responsible for the antibacterial effect of contact activation.

Inhibition of bacterial growth by human plasma depends on contact activation

The finding that antibacterial domain D3-related HK fragments are generated in plasma indicated that bacterial growth could be inhibited by plasma. The *S. pyogenes*, *S. aureus* and *Salmonella* strains described above (AP1, Cowan I and SR11B) are known to bind HK and activate the contact system (Ben Nasr *et al*, 1996; Herwald *et al*, 1998; Mattsson *et al*, 2001). However, strains of *Enterococcus faecalis* and *E. coli* strains not expressing fibrous surface proteins called curli (Olsén *et al*, 1989) do not have this property (Ben Nasr *et al*, 1996; Herwald *et al*, 1998). The growth of the three HK-binding strains mentioned above, a strain of *E. faecalis* (2374) and a non-curliated *E. coli* strain (B1351), was therefore investigated in plasma and in conventional TH medium. Figure 3A shows that the growth of bacteria that activate the contact system is dramatically reduced in plasma as compared to TH medium. In contrast, the *E. faecalis* and *E. coli* strains showed similar growth curves in plasma and TH medium (Figure 3B). Moreover, when grown in HK-free plasma, *S. pyogenes* AP1 bacteria multiplied significantly faster than in normal plasma (Figure 3C). Activation of the

Figure 3 Contact activation in plasma inhibits bacterial growth. Various bacterial strains were grown in TH medium or in human plasma at 37°C. The optical density at 620 nm was measured at indicated time points. The multiplication factors at various time points are given. (A) Bacteria known to activate the contact system in plasma: *S. pyogenes* strain AP1 (□, ■), *S. aureus* strain Cowan I (△, ▲), and *Salmonella* strain SR11B (○, ●). White symbols represent growth in TH medium and filled symbols represent growth in plasma. (B) Bacteria that do not activate the contact system in plasma: *E. coli* strain B1351 (□, ■) and *E. faecalis* strain 2374 (○, ●). White symbols represent growth in TH medium and filled symbols represent growth in plasma. (C) *S. pyogenes* AP1 bacteria were cultivated in undiluted human plasma (□), in human kininogen-free plasma (■), or in human plasma supplemented with FXII/kallikrein inhibitor (100 µg/ml) (●), at 37°C. Bacterial growth was followed by plating appropriate dilutions of the bacterial cultures, at the indicated time points, on TH agar plates. Plates were incubated at 37°C over night, CFUs were determined, and the multiplication factors were calculated. Experiments were repeated three times and a representative experiment is shown.

contact system is blocked by the synthetic peptide H-D-Pro-Phe-Arg-CMK, which is a specific inhibitor of FXII and plasma kallikrein (Ghebrehiwet *et al*, 1983). Addition of this inhibitor to human plasma, at concentrations known to block contact activation (Persson *et al*, 2000), enhanced the growth of AP1 bacteria (Figure 3C). The inhibitor also stimulated growth of *Salmonella* and *S. aureus* activating the contact system (data not shown). Taken together, the results demonstrate that contact activation at bacterial

Figure 4 Inhibition of the contact system promotes bacterial growth *in vivo*. Female Balb/c mice were injected i.p. with PBS or FXII/kallikrein inhibitor (1 mg/animal), followed by i.p. injection of *S. pyogenes* AP1 bacteria (450 000 CFU). The mice were killed 18 h after injection and the total number of CFUs in the spleens was determined. The number of CFUs was significantly higher in animals treated with the inhibitor, compared to the control group ($P = 0.024$, $n = 12$). The P -value was determined by using the Mann-Whitney U -test. Bars represent the median value in each group.

surfaces inhibits bacterial growth, and that the contact system is part of innate immunity.

Contact activation inhibits bacterial growth *in vivo*

To investigate the role of contact activation *in vivo*, we used a mouse model of *S. pyogenes* infection (Nordahl *et al*, 2004). In these experiments, mice were intraperitoneally injected with PBS or the FXII/kallikrein inhibitor mentioned above, at a dose where contact activation is completely blocked in mouse plasma, as judged by pronounced prolongation of the activated partial thromboplastin time. This was followed by intraperitoneal injection of *S. pyogenes* bacteria of the AP1 strain, and the dissemination of bacteria to the spleen after 18 h was determined. To demonstrate the effect of the inhibitor the bacterial load had to be carefully titrated. Thus, when small doses (50×10^3 colony-forming unit (CFU)/mouse) of bacteria were administered, no or very few bacteria were detected in the spleens, whereas high doses of bacteria (850×10^3 CFU/mouse) resulted in a massive spread to the spleens in both groups. However, at a bacterial load of 450×10^3 CFU/mouse, significantly higher number of bacteria were detected in the animals treated with the FXII/kallikrein inhibitor, as compared to the PBS control animals ($P = 0.024$) (Figure 4). These results show that a functional contact system contributes to the defence against invasive bacteria *in vivo*.

A central region of domain D3 is antibacterial

In order to localize the antibacterial region of domain D3, six peptides spanning the domain were synthesized (Table I) and analysed in the bactericidal assay. Initial testing with

S. pyogenes AP1 bacteria revealed that only the NAT26 peptide from the central part of D3 was bactericidal (Figure 5A) at the concentration used (12–13 μ M). A partial

Table I Synthetic peptides derived from the D3 domain used in this study

Peptide ^a	Sequence ^b	Position ^c
GKD25	GKDFVQPPTKICVGCPRDIPTNSPE	253–277
GCP28	GCPRDIPTNSPEELTLHTITKLNAEN	266–293
NAT26	NATFYFKIDNVKKARVQVAGKKYFI	294–319
KKY30	KKYFIDFVARETTCSKESNEELTESCETKK	315–344
RET27	RETTCSKESNEELTESCETKKLGQSLD	324–350
LDC27	LDCNAEYVYVPEWKIYPTVNCQPLGM	349–375

^aPeptides are identified by their first three NH₂-terminal residues using the one-letter code, followed by the total number of residues constituting the peptide.

^bThe one-letter code for amino-acid residues is used.

^cThe amino-acid positions in the HK sequence.

Figure 5 Mapping of the bactericidal region in domain D3. (A) *S. pyogenes* AP1 bacteria (2×10^6 CFU/ml) were incubated for 1 h at 37°C in buffer with synthetic peptides, covering various regions of the D3 domain (Table I), at a concentration of 12–13 μ M. Following incubation, appropriate dilutions of the mixtures were plated on TH agar plates. Plates were incubated at 37°C over night and CFUs were determined. The bars represent the mean $\pm$ s.e.m. of at least three experiments. (B) The bactericidal effect against AP1 bacteria of NAT26 (□) and LL-37 (○) at indicated concentrations in buffer without (left) or with 0.15 M NaCl (right). Experiments were repeated at least three times, and representative experiments are shown.

antibacterial activity was recorded for the other peptides, except for KKY30 that had no effect (Figure 5A). NAT26 has physico-chemical properties typical of many well-characterized AMPs; small size, cationic charge (pI of 10.1 and net positive charge of +5), and a relatively high predicted helical content. When tested against the strains used previously in this study, plus a group G streptococcal strain (G41), NAT26 was bactericidal at 12.8 μ M for all strains except *Salmonella* SR11B (Table II). However, also this strain was partially killed. LL-37 is a classical and significant human antibacterial peptide (Bals and Wilson, 2003). Notably, the bactericidal activity of NAT26 against *S. pyogenes* bacteria was comparable to LL-37 at low salt concentration, but superior to LL-37 at physiological salt concentration (Figure 5B).

The effect of NAT26 on *S. pyogenes* was analysed also by electron microscopy. AP1 bacteria grown in TH or in plasma over night were incubated with F(ab')₂ fragments of IgG antibodies against NAT26, labelled with colloidal gold. Bacterial cells grown in TH were also incubated with the NAT26 peptide for 1 h prior to the addition of the antibody fragments. Following negative staining, bacteria were analysed by electron microscopy. In contrast to bacteria grown in TH (Figure 6A and B), the cell wall architecture of bacteria grown in plasma (Figure 6C and D), or incubated with the NAT26 peptide (Figure 6E and F), was disintegrated as shown by ejected cytoplasmic material and membrane blebs. Furthermore, NAT26-containing peptides were detected at the bacterial surface by gold-labelled F(ab')₂ antibody fragments against NAT26 (Figure 6D and F, insets).

A liposome model was used to further investigate the effect of NAT26 on membranes. NAT26 displayed a high capacity to induce leakage in anionic liposomes at moderate ionic strength (Figure 7A), and leakage was also very fast ($\approx$ minutes; Figure 7B). At these conditions, the membrane-disruptive effect of NAT26 was quantitatively comparable to that of LL37 (Figure 7A and B). At higher ionic strength, NAT26 loses part of its membrane-disruptive effect, although the peptide is still quite potent against the negatively charged liposomes. This partial salt inactivation suggests that electrostatically driven binding of the peptide to the liposome surface is crucial to the membrane-disruptive effect of NAT26. Since the charge of the anionic liposomes (≈ -40 mV) is high, electrostatically dominated binding may still be possible at high ionic strength. It should also be noted that NAT26 is comparable in its membrane-disruptive effect to that of LL37 at high ionic strength. However, mechanistically, there might be differences in the action of the two peptides, since LL37 displays a quite high helical content when incorporated into liposomes (Oren *et al*, 1999), while this is not the case for NAT26. Thus, the helical content

Table II Antibacterial activity of NAT26 against different bacterial species^a

Peptide (μ M)	Killing of bacteria (%)					
	<i>S. pyogenes</i> AP1	GGs ^b G41	<i>S. aureus</i> Cowan I	<i>Salmonella</i> SR11B	<i>E. coli</i> B1351	<i>E. faecalis</i> 2374
12.8	100	100	100	90.1 $\pm$ 17.9	100	100
6.4	100	100	100	76.3 $\pm$ 33.0	93.3 $\pm$ 11.5	100
0.64	100	47.5 $\pm$ 30.5	87.3 $\pm$ 15.4	3.8 $\pm$ 5.3	37.6 $\pm$ 9.5	89.3 $\pm$ 7.6
0.064	6.0 $\pm$ 6.6	12.1 $\pm$ 17.5	15.6 $\pm$ 16.5	3.3 $\pm$ 5.7	2.3 $\pm$ 2.6	1.1 $\pm$ 1.8

^aBacteria (2×10^6 CFU/ml) were incubated with indicated concentrations of NAT26 for 1 h at 37°C.

Appropriate dilutions were plated on TH agar plates, incubated over night at 37°C, and CFUs were determined.

^bGroup G Streptococcus.

Figure 6 Visualization of the antimicrobial affect of NAT26. API bacteria were grown in TH broth (A, B), or in human plasma (C, D) over night at 37°C. Bacteria were washed and incubated with gold-labelled anti-NAT26 F(ab')₂-fragments. An aliquot of the bacteria grown in TH was incubated for 1 h at 37°C with 3.2 µM NAT26 (E, F) prior to the incubation with antibodies. Following negative staining, samples were analysed by electron microscopy. (A, C, E) Overviews at low magnification. The scale bar represents 2 µm. (B, D, F) Selected areas at higher magnification. The scale bar represents 200 nm. Arrows point to ejected cytoplasmic material due to exposure to plasma (C, D) or purified NAT26 (E, F). Note the intact bacterial membrane structure in (B) as compared to the disintegrated membranes in (D) and (F). Arrowheads in the insets point to gold-labelled F(ab')₂-fragments directed against NAT26. Scale bar, 20 nm.

of NAT26 recorded by circular dichroism at moderate and high ionic strength (15 ± 2 and $18 \pm 2\%$, respectively) did not significantly change when the peptide was incorporated in liposomes (16 ± 2 and $17 \pm 3\%$, respectively).

Discussion

The key finding presented here is that the assembly and activation of the contact system on bacterial surfaces in human plasma generates antibacterial domain D3-related fragments of HK. Innate immune responses are crucial for the initial and rapid clearing of bacteria appearing at sterile sites. Human blood is normally sterile. However, local lesions of physiological barriers in the skin, the oral cavity, the urogenital and gastrointestinal tracts, and in the airways, will allow pathogens and bacteria of the normal flora to penetrate these barriers and appear in the microcirculation. Most likely, this influx of small numbers of bacteria takes place on a regular basis. Previous reports (Ben Nasr *et al*, 1996; Herwald *et al*, 1998) have shown that the contact system is rapidly and efficiently activated on the surface of various human bacterial pathogens, and the data of the present study suggest that this represents an important surveillance and protective function. As mentioned, factor XII is part of the contact system and crucial for its activation. This factor is also called the Hageman factor, named after a

Figure 7 NAT26 causes liposome leakage. Membrane-disruptive effects of NAT26 and LL-37 were analysed by measuring the release of carboxyfluorescein from liposomes. (A) Leakage induced by various peptide concentrations in 10 mM Tris-HCl buffer containing 5 mM glucose (pH 7.4) with (+) or without (–) additional 150 mM NaCl. (B) Kinetics of peptide-induced liposome leakage. At time $t = 0$ s, 1 µM peptide was added.

patient with factor XII deficiency. In relation to the present study, it is noteworthy that Mr John Hageman did not suffer from bleeding disorders but from recurrent infections (Price *et al*, 2004). Prior to this investigation, a role for the contact system in the defence against infections has not been suggested, and apart from anecdotal cases like Mr Hageman, there are no reports in the literature linking contact protein deficiencies to bacterial infections. A lesson from complement and immunoglobulin deficiencies is that also severe homozygous cases may exhibit surprisingly light symptoms, due to overlapping immune defence systems. Clinical and subclinical infections are important in cases of pregnancy losses, and it is interesting that deficiencies in contact proteins and autoantibodies to these proteins, are found in unexplained recurrent aborters (Sugi and Makino, 2005). With the new information of the present study, analyses of these and other patients with abnormal contact system function, focused on increased susceptibility to infections, should further underline the significance of this branch of innate immunity.

Historically, the contact system has been connected with blood coagulation and inflammation, although several reports have demonstrated that the intrinsic pathway of coagulation driven by contact activation is not necessary for physiological haemostasis (for references, see Shariat-Madar and Schmaier, 2004). If the significance for haemostasis is questioned, there is no doubt that contact activation and BK release plays an important role in inflammation. BK is a powerful proinflammatory peptide, and a massive and generalized contact activation in the blood stream causes leakage of plasma into the tissue, which leads to disturbed microcirculation and fall of blood pressure, classical symptoms of severe infectious diseases such as sepsis. In patients with septic shock, plasma levels of HK, plasma kallikrein and factor XII are low (Smith-Erichsen *et al*, 1982), and in animal models of infection, large doses of bacteria activating the contact system induce severe vascular leakage, which is

prevented by the inhibitor of plasma kallikrein and factor XII (Persson *et al*, 2000) used in this study. Finally, patients with bacteraemia and hypotension, or sepsis caused by *S. aureus*, have elevated BK levels. These and other studies (for references, see Bhoola *et al*, 1992; Herwald *et al*, 2003; Joseph and Kaplan, 2005) suggest that widespread and uncontrolled contact activation contributes to the non-beneficial inflammatory response seen in sepsis. In such cases, the antibacterial effect of contact activation is probably of limited value, underlining that over-exploited defence mechanisms often are deleterious to the infected human host (Cohen, 2002). Several reports from Colman and co-workers have underlined the significance of the contact system in inflammation. In relation to this study, their finding that cell wall preparations from *S. pyogenes* activate the contact system and induce local and systemic inflammation in rats (for references see Isordia-Salas *et al*, 2005), is particularly relevant and interesting.

Under noninflammatory conditions, the components of the contact system will not be present in tissues or on mucosal linings. However, when an infection induces local inflammatory responses, vascular permeability increases and plasma containing the contact factors will appear at the site of infection. At this point, contact activation at bacterial surfaces generates antibacterial domain D3-related fragments, and the simultaneous release of BK will further increase inflammation and plasma leakage. LK also contains a D3 domain, but is not cleaved as a result of contact activation (see above). However, domain D3-related fragments are generated following LK degradation by elastase (Vogel *et al*, 1988). When an inflammatory response is induced at the infectious site, activated neutrophils will release elastase into the inflammatory exudate containing LK and other plasma proteins. Therefore, degradation of LK (and HK) by elastase and/or other proteinases could produce domain D3-related antibacterial peptides. Generation of such peptides *in vivo* is supported by the finding that D3-related fragments are present in wound fluid from a patient infected with *S. aureus*. The proteolytic degradation of HK may also generate domain D5-related antibacterial peptides *in vivo* (Nordahl *et al*, 2005).

The bactericidal effect of D3-related peptides mainly resides in the central region of domain D3 containing the NAT26 sequence. NAT26 efficiently kills several bacterial species and induces rapid and efficient disruption of membranes. Whether exactly this peptide is generated during contact activation at bacterial surfaces is unclear. However, the lack of immunoreactive bands corresponding to the size of NAT26 (Figure 2A) does not exclude that such peptides are generated during proteolytic processing of HK at the bacterial surface. Indeed, NAT26-containing peptides were detected at the bacterial surface using gold-labelled antibodies, suggesting that small peptides like NAT26 may be stuck in the membrane.

Host–parasite relationships are mostly well-balanced, and a host defence mechanism is regularly counteracted by the parasite, and vice versa. Many bacterial species secrete proteolytic enzymes that cleave HK (for references, see Imamura *et al*, 2004), which could provide protection against killing by the contact system. Interestingly, the cleavage of HK by these bacterial proteinases generates BK, suggesting that the induction of increased vascular permeability via BK generation is favourable for the bacterium under certain conditions. Thus, apart from antibacterial contact factors,

plasma also contains valuable nutrients for growing bacteria. During starvation, the acquisition of nutrients should be a first priority also when connected with risks, and it is notable that the bacterial BK-releasing proteinases mentioned above, are not being produced until the growth medium is running out of nutrients and the culture has reached stationary growth phase (Chaussee *et al*, 1997; Tokuda *et al*, 1998).

The significance of innate immunity for microbial surveillance and the rapid elimination of microorganisms that cross biological boundaries is well established. Previous studies in this field have also emphasized that innate immunity has several and partly overlapping branches. Such redundancy is not surprising for a fundamental biological system, to which this work adds a novel component: the contact system.

Materials and methods

Bacteria, growth conditions, plasma sources and analysis of bacterial multiplication rate

The *S. pyogenes* strain AP1 (40/58) of serotype M1 was from the World Health Organization Collaborating Centre for Reference and Research on Streptococci, Prague, Czech Republic. The group G streptococcal strain G41, *S. aureus* strain Cowan I, *E. faecalis* strain 2374, *E. coli* strain B1351 and *Salmonella typhimurium* strain SR11B were collected at the Department of Clinical Microbiology, Lund University Hospital, Sweden. Bacteria were grown in Todd-Hewitt broth (TH; Difco) at 37°C. Fresh frozen plasma from healthy individuals were obtained from the blood bank at Lund University Hospital, Lund, Sweden, and kept frozen at –80°C until use. Human kininogen depleted plasma was purchased from George King Bio-Medical, Inc. (Overland Park, Kans). The FXII/plasma kallikrein inhibitor H-D-Pro-Phe-Arg-chloromethylketone (CMK) peptide was from Bachem Feinchemikalien AG. Bacteria were cultivated in TH, normal plasma, kininogen-free plasma or plasma + FXII/kallikrein inhibitor (final concentration; 100 µg/ml) at 37°C. At various time points the optical density (A_{620}) was monitored or growth was measured by plating appropriate dilutions of the bacterial solution on TH agar plates. Plates were incubated overnight at 37°C and the number of CFU was determined. Wound fluid collected from a chronic leg ulcer infected with *S. aureus* was a kind gift from Dr Artur Schmidtchen and was collected as described (Lundqvist *et al*, 2004).

Proteins, antibodies, reagents and iodination

Human HK was purchased from Kordia, LK was purified from human plasma as described (Müller-Esterl *et al*, 1988), and BK was purchased from Bachem. The synthetic peptides based on sequences in kininogen were described previously (Herwald *et al*, 1995), and are shown in Table 1. LL-37 (LLGDFFRKSKEKIGKEFK RIVQRIKDFLRNLPRTES) was synthesized by Innovagen AB, Lund, Sweden. Antibodies against NAT26 and domain D5 of HK were raised in rabbits, horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG was purchased from Pierce, and HRP-conjugated protein A from Sigma.

Coupling of anti-NAT26 IgG to Sepharose and preparation of anti-NAT26 F(ab')₂ fragments

Anti-NAT26 antiserum was applied to a protein G-Sepharose column (Amersham Bioscience). The column was extensively washed with PBS, and bound IgG was eluted with 0.1 M glycine–HCl, pH 2.0. Eluted IgG was dialysed against coupling buffer (0.1 M NaHCO₃, 0.5 M NaCl, pH 8.3) and coupled to CNBr-activated Sepharose 4B (Amersham Bioscience), with a final concentration of 0.85 mg/ml Sepharose, according to the manufacturer's protocol. For preparation of F(ab')₂ fragments eluted IgG was dialysed against acetate buffer, pH 4.5 (70 mM CH₃COONa, 50 mM HCl), followed by proteolytic cleavage with pepsin (enzyme–protein ratio 1:100) for 21 h at 37°C. The reaction was terminated by raising the pH of the solution to 7.5 with 1 M Tris. Uncleaved IgG was removed by affinity chromatography using protein G-Sepharose. Unbound material containing polyclonal anti-NAT26 F(ab')₂ fragments was collected.

Purification of recombinant D3 domain of kininogen

The D3 domain of human kininogen was recombinantly expressed as described (Auerswald *et al*, 1993). Initially, the *E. coli* strain JM83 was used for expression of the D3 domain; however, the *E. coli* strain TG1 is now used due to higher expression level. Bacteria were grown in LB containing ampicillin (200 µg/ml) at 37°C to mid-log phase ($A_{550}=0.6-1.0$). Protein production was induced by the addition of IPTG (final concentration 1 mM) and growth was continued over night. The bacterial cells were collected, resuspended in cold sucrose buffer (0.5 M sucrose, 0.1 M Tris-HCl, 1 mM EDTA, pH 8.0), 0.04 ml/ml bacterial culture, and the suspension was incubated on ice for 10 min. A measure of 130 µl lysozyme (10 mg/ml) and 4 ml ice-cold H₂O was added, and after 5 min incubation on ice 145 µl MgSO₄ (1 M) was added and the suspension subjected to centrifugation for 30 min at 7500 g. The supernatant was applied on affinity chromatography, using anti-NAT26 antibodies coupled to Sepharose, and following extensive washing with PBS the bound D3 domain was eluted with 0.1 M glycine-HCl pH 2.0. Fractions containing D3 were combined and dialysed against PBS or 10 mM Tris-HCl, pH 7.5.

Activation of neutrophils and proteolytic cleavage of HK

Human peripheral neutrophils were isolated from heparinized human blood kindly provided by healthy volunteers. Whole blood was layered on NIM and centrifuged at 400 g for 30 min at room temperature. The neutrophil fraction was suspended in PBS and residual contaminating erythrocytes were removed by hypotonic lysis for 20 s. The cells were washed twice in PBS and resuspended in Na medium (5.6 mM glucose, 127 mM NaCl, 10.8 mM KH₂PO₄, 1.6 mM MgSO₄, 10 mM HEPES and 1.8 mM CaCl₂, pH 7.3) to a concentration of 8×10^6 cells/ml. 8×10^6 cells were incubated with 10 µM Cyto B (Sigma) for 1 min at 37°C. The cells were further activated by adding 100 nM FMLP (Sigma) and incubation was continued for another 30 min. The sample was cooled on ice, centrifuged for 20 s at 11 600 g and the resulting supernatant was stored at -20°C. HK (1.17 mg/ml) in 4 mM acetate-HCl buffer pH 5.3, containing 0.15 M NaCl, was incubated with supernatant from activated neutrophils (1 µl to 5.85 µg HK) or human elastase from Sigma (0.5 to 5.85 µg HK) for various time points at 37°C. Cleavage products were analysed with Tricine-SDS-PAGE or tested for bactericidal activity against *S. pyogenes* AP1 bacteria.

Plasma absorption experiments

Bacteria were grown to mid exponential growth phase ($A_{620}=0.5-0.6$), washed and resuspended in PBS. One milliliter bacterial solution (2×10^9 bacteria/ml) was incubated with 1 ml citrate plasma for various time points (5, 15, 30 min or 1 h) at 37°C. The bacterial cells were collected, washed with PBS and bound proteins were eluted with 0.1 M glycine-HCl, pH 2.0. The pH of the eluted material was raised to 7.5 with the addition of 1 M Tris. Eluted proteins were precipitated with 5% trichloroacetic acid (TCA) for 30 min on ice followed by centrifugation at 15 000 g (4°C for 20 min). Precipitated material was dissolved in SDS sample buffer and subjected to Tricine-SDS gel electrophoresis and Western blot analysis. NAT26-containing protein fragments, in the absorbed plasma or eluted from bacteria, were also purified by affinity chromatography using anti-NAT26 antibodies coupled to Sepharose. Following extensive washing bound proteins were eluted, TCA precipitated and analysed as above. Unprecipitated material was also tested for antibacterial activity against AP1 bacteria (see below) and the data were statistically analysed using the Mann-Whitney *U*-test. The total protein content in this material was analysed according to Bradford.

Bactericidal assay

Bacteria were grown to mid-log phase ($A_{620} \approx 0.4$) in TH broth, washed and diluted in 10 mM Tris-HCl, pH 7.5, containing 5 mM glucose. Fifty microliters of bacteria (2×10^6 CFU/ml) were incubated together with peptides at various concentrations for 1 h at 37°C. To quantitate the bactericidal activity, serial dilutions of the incubation mixtures were plated on TH agar, incubated overnight at 37°C, and the number of CFU was determined. Bactericidal activity of NAT26 and LL-37 was also carried out in the presence of 0.15 M NaCl, and plasma concentrations of Zn²⁺, Mg²⁺ and Ca²⁺ did not affect bacterial survival or the bactericidal effect of NAT26.

SDS-PAGE and Western blot analysis

A Tricine-SDS-PAGE system for separation of proteins in the range of 1-70 or 5-100 kDa was used (Schägger and von Jagow, 1987). Samples were boiled for 3 min in an equal volume of sample buffer containing 2% SDS and 5% 2-mercaptoethanol. Molecular weight markers were from Sigma. Separated proteins were stained with Coomassie blue or transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences). Membranes were blocked with PBST (PBS + 0.05% Tween-20) containing 5% dry milk powder (blocking buffer), incubated with primary antibodies (rabbit anti-NAT26 1:100, rabbit anti-D5 1:1000) in blocking buffer for 30 min at 37°C. Following a washing step with PBST membranes were incubated with HRP-conjugated secondary antibodies (protein A 1:5000 or goat anti-rabbit IgG 1:5000) in blocking buffer for 30 min at 37°C. The membranes were washed and bound antibodies were detected by the chemiluminescence method (Nesbitt and Horton, 1992).

Animal experiments

AP1 bacteria grown to early logarithmic phase ($A_{620} \approx 0.4$) were washed and diluted in PBS to a concentration of 0.9×10^6 CFU/ml. Bacteria were kept on ice until injection. Female BALB/c mice, 10-12 weeks, were injected i.p. with 500 µl FXII/kallikrein inhibitor (1 mg) diluted in PBS, or with 500 µl PBS. After 10 min 500 µl of the bacterial solution was injected i.p., and after 18 h the mice were killed and the spleens were removed and kept on ice until homogenization in PBS. The number of CFU was quantitated by plating serial dilutions of the homogenized material on blood agar plates. Plates were incubated over night at 37°C. The *P*-value was determined by using the Mann-Whitney *U*-test. The animal experiments were approved by the regional ethical committee for animal experimentation (permit M294-03).

Negative staining and transmission electron microscopy

Polyclonal anti-NAT26 F(ab')₂ fragments were labelled with 4 nm colloidal gold as described earlier (Baschong and Wrigley, 1990). AP1 bacteria grown in TH or plasma over night were washed with TBST (50 mM Tris, 150 mM NaCl, 0.05% Tween-20, 0.02% NaN₃, pH 7.4) and resuspended in the same buffer to 2×10^9 CFU/ml. A measure of 200 µl bacterial suspension (grown in TH) was incubated with NAT26 (final concentration of 3.2 µM) for 1 h at room temperature. After a washing step with TBST the bacteria were resuspended in 200 µl TBST. Each bacterial suspension was then incubated with gold-labelled anti-NAT26 F(ab')₂ fragments for 30 min at room temperature. Negative staining of the bacteria was performed with 0.75% uranyl formate as described (Roth *et al*, 1978). Specimens were examined in a Jeol JEM 1200 EX transmission electron microscope operated at 60-kV accelerating voltage. Digital images were recorded with a Gatan Multiscan 791 CCD camera.

Liposome preparation and leakage assay

1,2-Dioleoyl-*sn*-glycero-3-phosphate (DOPA) (monosodium salt) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), both of >99% purity, were from Avanti Polar Lipids (Alabaster, USA), while cholesterol (>99% purity), poly-L-lysine ($M_w=59$ and 170 kDa) and Triton X-100 were from Sigma Aldrich (St Louis, USA), and 5(6)-carboxyfluorescein (99% purity) from Acros Organics (NJ, USA). Other chemicals used were of analytical grade. Water used was of Millipore Milli-Q Plus 185 ultra-pure quality.

Liposomes investigated were anionic (DOPC/DOPA/cholesterol 30/30/40 mol/mol). Lipid films were formed on the walls of a glass flask by chloroform dissolution of the lipid mixtures, followed by evaporation, first under vacuum for 45 min at 60°C, and subsequently in a vacuum oven (Lab-line, USA) at 30 in-Hg and room temperature over night. The lipids were then resuspended in 100 mM 5(6)-carboxyfluorescein (CF) in buffer and the solution subjected to eight freeze-thawing cycles by freezing in liquid nitrogen and heating to 60°C while vortexing. Unilamellar liposomes were produced by multiple extrusions through 100 nm polycarbonate membranes mounted in a LipoFast Basic extruder (Avestin, Germany). The liposomes were separated from untrapped CF by running the sample on a Sephadex™ G-25 column (Amersham Biosciences, Sweden) with Tris buffer as eluent. The resulting liposomes were found by cryoTEM to be unilamellar, and by and photon correlation spectroscopy to have a mean diameter of 143 ± 15 nm.

Liposome leakage was studied by monitoring leakage-induced reduction of CF self-quenching. Specifically, CF release was determined by following the emitted fluorescence at 520 nm from a liposome dispersion (10 mM lipid in 10 mM Tris pH 7.4, either with or without 150 mM NaCl added), with an absolute leakage scale obtained by disrupting the liposomes at the end of the experiment through addition of 0.05 wt% Triton X-100, thereby causing 100% release and dequenching of CF. A Spex fluorolog 1680 0.22 m double spectrometer (Instruments SA Group, USA) was used and all measurements were carried out in triplicate at 37°C at pH 7.4 in 10 mM Tris-HCl buffer containing 5 mM glucose.

Circular dichroism

The effect of liposomes on the peptide secondary structure was monitored in the range 200–250 nm by a Jasco J-810 Spectropolarimeter (Jasco, Japan). The measurements were performed at 37°C in a 10 mm quartz cuvette under stirring at a peptide and lipid concentration of 10 and 100 µM, respectively. The fraction of peptide in α -helical conformation was obtained from the recorded

CD signal at 225 nm and from the corresponding data from a reference peptide in 100% α -helix and 100% random coil conformation, respectively. 100% α -helix and 100% random coil references were obtained from 0.133 mM (monomer concentration) poly-L-lysine ($M_w = 79$ kDa) in 0.1 M NaOH and 0.1 M HCl, respectively. To account for the instrumental differences between measurements the background value at 250 nm was subtracted. All measurements were performed at 37°C at pH 7.4 in 10 mM Tris-HCl buffer containing 5 mM glucose.

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