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ANTIBACTERIAL ACTIVITY OF THE CONTACT AND COMPLEMENT SYSTEMS IS BLOCKED BY SIC, A PROTEIN SECRETED BY *STREPTOCOCCUS PYOGENES*

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Running title: SIC and innate immunity

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Recent studies have shown that activation of the complement and contact systems results in the generation of antibacterial peptides. *Streptococcus pyogenes*, a major bacterial pathogen in humans, exists in more than one hundred different serotypes due to sequence variation in the surface-associated M protein. Cases of invasive and life-threatening *S. pyogenes* infections are commonly associated with isolates of the M1 serotype, and in contrast to the large majority of M serotypes, M1 isolates all secrete the SIC protein. Here we show that SIC interferes with the activation of the contact system, and blocks the activity of antibacterial peptides generated through complement and contact activation. This effect promotes the growth of *S. pyogenes* in human plasma, and in a mouse model of *S. pyogenes* sepsis, SIC enhances bacterial dissemination, results which help to explain the high frequency of severe *S. pyogenes* infections caused by isolates of the M1 serotype.

The innate immune system provides a rapid and non-specific defence at biological boundaries prone to infection. Essential elements involved in this immediate host response are antimicrobial peptides (AMPs) and complement components (1-3). The complement system can be activated through three different pathways; the classical, alternative and lectin pathways (4). Although the initiation of these complement cascades differs, they all converge at the level of C3 with the generation of several C3 peptide fragments including the anaphylatoxin C3a. This proinflammatory peptide has a number of biological effects, such as enhancement of vascular permeability, contraction of smooth muscle cells and histamine release from mast cells (5). C3a also exerts a potent antimicrobial activity, a property that has been highly conserved from invertebrates to humans (6-8).

Another branch of innate immunity is represented by the human contact system, which generates AMPs upon activation (9). This system comprises three serine proteinases; factor XII (FXII), factor XI (FXI) and plasma kallikrein (PK), and the non-enzymatic co-factor high molecular weight kininogen (HK) (10-12). HK consists of six domains; the cystatin-like domains D1-D3, the bradykinin-containing domain D4, domain D5 mediating binding to negatively charged surfaces, and domain D6 that binds FXI and PK (HK is schematically depicted in Fig. 2A). Classical consequences of contact activation are cleavage of HK leading to the release of the potent pro-inflammatory peptide bradykinin, and the initiation of the intrinsic pathway of coagulation through FXI activation. When activated at the surface of significant human bacterial pathogens, including *Streptococcus pyogenes*, further processing of HK results in the generation of antibacterial fragments from domain D3 of HK, mainly involving a 26 amino acid sequence (9).

S. pyogenes is a common and important human bacterial pathogen causing a wide spectrum of diseases ranging from uncomplicated throat and skin infections to life-threatening invasive conditions, such as necrotizing fasciitis, septicaemia and a toxic–shock syndrome (13-15). Based on antigenic differences in the M protein, a surface protein of *S. pyogenes*, isolates are divided into more than 100 serotypes (16,17). Several studies have demonstrated that the M1 serotype is the serotype most frequently associated with invasive infections and toxic shock (18-23), and all strains of the M1 serotype secrete the SIC (Streptococcal Inhibitor of Complement) protein that inhibits the function of the membrane attack complex of complement (24). The *sic* gene shows a high degree of variation among different M1 strains and nearly 300 alleles are known (25). SIC also binds to and interferes with the

activity of several antibacterial proteins and peptides such as lysozyme, LL-37, members of the defensin family, and the chemokine MIG/CXCL9 (26-29). Recently SIC was also shown to inhibit an antibacterial peptide produced by *Streptococcus salivarius*, a bacteriocin-like inhibitory substance (BLIS) (30).

In the present work we demonstrate that SIC interferes with innate immunity functions of the complement and contact systems. As a consequence, SIC enhances the virulence of *S. pyogenes* by promoting growth in human plasma and bacterial dissemination in a mouse model of sepsis.

Experimental Procedures

Bacteria, growth conditions, plasma sources, analysis of bacterial multiplication and SIC production during growth- The *S. pyogenes* strain AP1 (40/58), was from the World Health Organization Collaborating Centre for Reference and Research on Streptococci, Prague, Czech Republic. The mutant strain SIC^- has been described previously (29). Bacteria were cultivated in Todd-Hewitt broth (TH; Difco) at 37°C. Fresh frozen plasma from healthy individuals was obtained from the blood bank at Lund University Hospital, Lund, Sweden, and kept at -80°C until use. Human plasma depleted of HK, PK, FXII, or FXI, respectively, was purchased from George King Bio-Medical, Inc. (Overland Park, Kans).

Bacterial cultivation in plasma was performed as follows: 10 µl of an overnight bacterial culture in TH was added to 250 µl plasma, and bacteria were left to grow at 37°C. At various time points, including time zero, growth was monitored by plating appropriate dilutions of the bacterial solution on TH agar plates. Plates were incubated overnight at 37°C and the number of colony forming units (cfu) were determined. The multipliction factor (MF) was calculated by dividing the number of cfu at the individual time points with the number of cfu at time point zero. Bacteria grown for 8 h in plasma (undiluted or diluted 1:1 in TH) were collected by centrifugation. Plasma supernatants were analysed for SIC content by ELISA. Supernatants were also 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 SDS-PAGE and Western blot analysis. The bacterial cells were 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 TCA precipitated and analysed with SDS-PAGE and Western blot.

Proteins, antibodies, and iodination- Human HK was purchased from Kordia. The synthetic peptides based on sequences in domain D3 of HK were described previously (31) and are shown in Figure 2A. LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) and peptide HKH20 (^{479}HKHGHGHGKHKNKGKKNGKH^{498} in domain D5 of HK) were synthesized by Innovagen AB, Lund, Sweden. The complement peptide C3a was from Calbiochem, and rabbit anti-C3 was from Serotec. Protein SIC was purified from the *S. pyogenes* strain AP1 as described (24). Polyclonal antisera to protein SIC and NAT26 were raised in rabbits. Horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG was purchased from Pierce, and HRP-conjugated protein A from Sigma. FITC-conjugated anti-rabbit IgGF(ab´)$_2$ was purchased from Sigma and anti-NAT26 F(ab´)$_2$ fragments were prepared as described (9). Polyclonal anti-SIC IgG was affinity-purified using protein A-Sepharose (GE-Healthcare) and the FabRICATOR® Kit (Genovis) was then utilized for preparation and purification of F(ab´)$_2$ antibody fragments. Proteins were radiolabelled with ^{125}I using iodobeads (Pierce) as described by the manufacturer. Binding of radiolabelled protein to bacteria was performed as described (32).

Elisa- Microtiter plates (Maxisorb, NUNC, Denmark) were coated with peptides spanning the HK domain D3 (0.5 µg/ml). After washing with PBST (PBS containing 0.05% Tween-20) the plates were incubated with protein SIC in a serial dilution (2^n, starting concentration 5 µg/ml). Bound protein was detected with a polyclonal rabbit antibody against protein SIC (1:500), followed by HRP-conjugated goat anti-rabbit IgG (1:5000). All incubations were done at 37°C for 1 h and followed by a washing step. Substrate solution, 0.1% diammonium-2, 2-azino-bis- (3-ethyl-2,3-dihydrobenzthiazoline)-6-sulfonate (ABTS), 0.012% H_2O_2 in 100 mM citric acid, 100 mM NaH_2PO_4, pH 4.5, was

added and the change in absorbance at 405 nm was determined after 30 min. Plates were also coated with plasma supernatants from bacterial growth and the content of SIC in the samples was determined as above.

SDS-PAGE, Western blot and Slot-binding analysis- SDS-PAGE was performed as described by Laemmli (33) using a polyacrylamide concentration of 10% and 3.3% cross-linking or a Tricine-SDS/PAGE system was used (34). Separated proteins were transferred to PVDF membranes (Amersham Biosciences). Membranes were blocked with PBST containing 5% dry milk powder (blocking buffer), incubated with primary antibody (rabbit anti-SIC 1:1000) in blocking buffer for 30 min at 37°C. Following a washing step with PBST the membranes were incubated with secondary antibodies (HRP-conjugated protein A 1:5000) in blocking buffer for 30 min at 37°C. The membranes were washed and bound antibodies were detected by chemiluminescence. When radiolabelled SIC was analysed gels were dried and the radioactivity was visualized by autoradiography using Kodak x-Omat AR films and regular intensifying screens. Peptides were also directly applied to PVDF membranes using a Milliblot-D system (Millipore). Membranes were blocked in TBS (0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl) containing 3% bovine serum albumin, incubated with ^{125}I-labelled protein SIC for 3 h, and washed with TBS containing 0.05% Tween-20. Autoradiography was carried out as described above.

Antimicrobial assay- AP1 bacteria were grown to mid-log phase in TH, washed and diluted in 10 mM Tris-HCl, pH 7.5, containing 5 mM glucose. 50 µl bacterial solution (2 x 10^6 cfu/ml) was incubated with NAT26, LL-37, HKH20 or C3a at various concentrations for 1 h at 37°C. In subsequent experiments bacteria were incubated with bactericidal concentrations of the peptides together with various concentrations of protein SIC. The bactericidal activity was quantified by plating serial dilutions of the incubation mixtures on TH agar, incubated over night at 37°C, and the number of cfu were determined.

Flow cytometry- S. pyogenes AP1 bacteria were cultivated overnight at 37°C, washed and resuspended in PBS (final concentration of 2 x 10^8 cfu/ml). 400 µl of the bacterial solution (8 x 10^7 cfu) was incubated with 400 µl PBS or plasma together with various concentrations of protein SIC for 1 h at 37°C. The bacterial cells were collected, washed with PBS and resuspended in 75 µl PBS. Then, 25 µl bacterial solution (2.6 x 10^7 cfu) was incubated with rabbit anti-NAT26 F(ab´)$_2$-fragments (0.2 mg/ml) or rabbit anti-C3 (0.1 mg/ml) for 30 min at room temperature. Following a washing step with PBS the bacterial cells were incubated with FITC-conjugated goat anti-rabbit IgGF(ab´)$_2$-fragments (11 µg/ml) for 30 min at room temperature. After washing with PBS, bacteria were resuspended in PBS and samples were analysed on a FACSCalibur (Becton Dickinson) and 100000 events were acquired using logarithmic settings. Data were analysed using histogram plots of FITC fluorescence intensity acquired in the Cell Quest Pro software.

Animal experiments and clotting assay- AP1 bacteria were grown to early-logarithmic phase (OD620 nm, approximately 0.5), washed and diluted in PBS to 1 x 10^8 cfu/ml. Female Balb/c mice, 10 weeks old, were anesthetized with isofluorane and injected subcutaneously, in an air pouch on the neck, with 200 µl of the bacterial solution (2 x 10^7 cfu/animal) or with 200 µl PBS (control group). At 8 h and 20 h post infection 200 µl protein SIC (200 µg/dose) or PBS was injected intraperitoneally in infected animals (mice in the control group were injected with PBS). At 30 h post infection animals were terminally anesthetized with isofluorane, and blood was drawn by cardiac puncture into polypropylene tubes containing one-tenth volume of 3.8% trisodium citrate. Following centrifugation the separated plasma was collected. The concentration of IL-6 in the plasma was determined using an ELISA kit (Invitrogen), and clotting times were measured in a coagulometer (Amelung). For the activated partial thromboplastin time (aPTT) 50 µl plasma was prewarmed at 37°C for 60 seconds, incubated with 50 µl DAPTTIN (Technoclone) for 60 seconds, followed by the addition of 50 µl 30 mM $CaCl_2$ and the time to form a clot was measured. For determination of bacterial dissemination the spleens were removed, kept on ice, homogenized in PBS, and the materials were plated on TH agar, incubated overnight at 37°C and the number of cfu were determined. P-values were determined by using the Mann-Whitney U-test. The animal experiments were approved by

the regional ethic committee for animal experimentation (permit M220-08).

Immunofluorescence Microscopy- The *S. pyogenes* strains AP1 and SIC^- were grown overnight at 37°C. Ten µl from these cultures were inoculated into 250 µl citrated plasma (1:1 in TH) and bacteria were allowed to grow at 37°C for 20 h in eppendorf tubes. The bacterial cells were washed twice in PBS supplemented with goat serum to a final concentration of 1% (wash buffer). Bacteria were then incubated for 30 min on ice with polyclonal rabbit anti-SIC IgG $F(ab')_2$ fragments (0.1 mg/ml), washed with wash-buffer, and incubated with secondary fluorochrome-conjugated goat anti-rabbit $F(ab')_2$ fragments (Alexa-Fluor-594; Invitrogen) (1:600), for 30 min on ice. The bacterial cells were washed with wash-buffer and bound antibodies were visualized in a fluorescence microscope (Nikon Eclipse TE300 inverted fluorescence microscope equipped with a Hamamatsu C4742-95 cooled CCD camera, using a Plan Apochromat 100x objective with NA 1.4 (Nikon)). The software used for acquisition was NIS-Elements 3 (Nikon). The same exposure time was used for all images and during post-processing all images were treated identically.

Negative staining and transmission electron microscopy- Negative staining of AP1 bacteria, incubated with PBS, plasma, or plasma with protein SIC (see above), was performed with 0.75% uranyl formate as described (35). 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.

Homology modelling of the D3 domain of H-kininogen- Amino acids 261-370 in the D3 domain of H-kininogen was aligned with human cystatin F (36) and subjected to Swiss model (37) automated protein homology modelling using cystatin F (PDB 2CH9 chain A) as the template. Model was edited and visualized using VMD 1.8.7 (38) and Tachyon Ray tracer (39).

RESULTS

Multiplication of S. pyogenes in human plasma is controlled by the complement and contact systems. To investigate the importance of contact activation in controlling bacterial growth, the *S. pyogenes* isolate AP1 (a strain of the M1 serotype used throughout this study) was cultivated in human plasma deficient of the various contact factors. As compared to normal plasma, the growth rate was enhanced in plasma that was deficient of PK, FXII or HK (Fig. 1A). After eight hours of growth, the bacterial numbers were significantly higher in plasma lacking either of these contact factors (Fig. 1A, insert), suggesting that the generation of antibacterial peptide fragments from HK was affected. Factor XI triggers the intrinsic pathway of coagulation but does not participate in the cleavage of HK, and the growth of AP1 bacteria in FXI deficient plasma was the same as in normal plasma (Fig. 1A).

The complement cascade is another proteolytic system that is activated at sites of infection. Therefore, the contribution of this system in reducing the multiplication of *S. pyogenes* was examined by performing growth experiments in human serum. Compared to plasma, AP1 bacteria grew better in serum, and in complement inactivated serum (heated to 56°C) a further increase of bacterial multiplication was recorded (Fig. 1B). Taken together, the results suggest that the contact and complement systems both interfere with *S. pyogenes* growth in human blood.

SIC interferes with the antibacterial activity of HK-derived peptides. Following assembly of the contact factors on the bacterial surface, activation of the contact system results in the generation of antibacterial peptides derived from the D3 domain of HK (9). SIC is secreted by some *S. pyogenes* strains, including all isolates of the M1 serotype, and an interaction between SIC and HK mediated through domains D3 and D5, was recently demonstrated (40). Here synthetic peptides spanning the D3 domain (Fig. 2A) were analysed by indirect ELISA for binding to SIC. As shown in Figure 2B, a strong binding was obtained with the NAT26 peptide. Also peptide LDC27 in the COOH-terminus of D3 interacted with SIC, whereas the other peptides showed no affinity (Fig. 2B). The results were in full agreement with experiments where the peptides were applied to PVDF filters and probed with ^{125}I-SIC (Fig. 2C). A homology model of the cystatin-like D3 domain of HK was generated by aligning amino acids 261-370 of HK with human cystatin F (E-value 4.33×10^{-10}) (36). The Swiss-

model automated homology modelling server (37) revealed a typical cystatin-like structure with one α-helix and four anti-parallel β-strands (Fig. 2D). Interestingly, the SIC-binding peptides NAT26 (red) and LDC27 (green) are predicted to be located in two separate intersecting anti-parallel β strands forming surface-exposed epitopes within the D3 domain (Fig. 2D). Intact domain D3 is antibacterial and this activity is mainly located in the NAT26 sequence (9). When tested at concentrations of 12-13 μM the peptide LDC27 reduced the bacterial number with approximately 8% as compared to 100% cfu reduction obtained with the peptide NAT26 (9), suggesting that the antibacterial activity of the NAT26 peptide could be blocked by SIC. Indeed, at a bactericidal concentration of NAT26 (0.642 μM), SIC blocked the killing of AP1 bacteria. In these experiments the inhibitory effect of SIC on the classical antibacterial peptide LL-37 (0.446 μM) was included as a positive control (Fig. 2E). Fragments of domain D5 of HK, including the sequence HKH20, are also antibacterial (41). Such fragments are however not generated as a result of contact activation (9), but at the site of infection they are produced through proteolytic cleavage of HK by enzymes released by activated neutrophils (41). Also the antibacterial activity of HKH20 against AP1 was blocked by SIC (data not shown).

SIC blocks the antibacterial activity of complement C3a. Complement activation leads to cleavage of C3 and generation of the inflammatory mediator C3a. Previous studies have demonstrated that this peptide is capable of killing various microorganisms (6-8). Here C3a was found to kill also AP1 bacteria (Fig. 3A), and at an antibacterial concentration of the peptide (0.22 μM) SIC inhibited this activity and promoted bacterial growth (Fig. 3B). However, higher molar concentrations of SIC were required to efficiently block C3a activity as compared to peptides NAT26 and LL-37. Whereas SIC at a concentration of 60 nM inhibited the activity of these peptides to 80-90% (Fig. 2E), C3a still killed approximately 60% of the bacteria (Fig. 3B).

The antibacterial action of the contact system is inhibited by SIC. The activation of the contact system at bacterial surfaces requires initial binding of the contact factors. HK has been demonstrated to bind to the surface of *S. pyogenes* (42), and therefore we tested whether SIC could interfere with this binding. Binding of radiolabelled HK to AP1 bacteria (Fig. 4A) was efficiently blocked by SIC (Fig. 4B), indicating that SIC not only blocks the action of antibacterial peptides generated through contact activation, but also interferes with the activation process itself. We therefore investigated whether SIC influences bacterial growth in human plasma, and exogenously added SIC was found to enhance the multiplication of AP1 bacteria (Fig. 5A). This finding suggests that antibacterial HK-fragments are bound and inactivated by SIC, and/or that SIC blocks binding of HK to the bacterial surface and thereby reduces the generation of antibacterial D3-fragments.

Next AP1 bacteria were incubated with human plasma, extensively washed and then incubated with antibodies against NAT26, followed by FITC-labelled secondary antibodies. Flow cytometry was then used to analyse bacterial cells interacting with anti-NAT26. The background signal was defined as the fluorescence signal in the presence of secondary antibodies alone (Fig. 5B, blue line). The increase in the fluorescence signal obtained in the presence of both primary and secondary antibodies (Fig. 5B, green line) represents that proportion of the cell population that have specifically bound the primary antibodies. In Fig 5B, binding of HK or NAT26-containing HK-fragments were detected in 35% of the population as these cells have a fluorescent intensity greater than background as calculated using Cell Quest Software. For bacteria preincubated with SIC (Fig. 5B, red line) only 13% of the population have a fluorescent intensity greater than background, corresponding to approximately 60% inhibition. The bacterial cells were also analysed by electron microscopy following negative staining. Contrary to bacteria incubated with PBS (Fig. 5C), the cell walls of bacteria exposed to plasma were disintegrated resulting in the leakage of cytoplasmic material (Fig. 5D). This effect was much less pronounced when excess amount of SIC was present during plasma incubation (Fig. 5E), suggesting that SIC interferes with the antibacterial activity of peptides generated through contact activation.

Multiplication of S. pyogenes in plasma is enhanced by SIC. When AP1 bacteria were grown in human plasma, only trace amounts of SIC could be detected in the plasma by Western

blot or ELISA following eight hours of growth. This observation indicated that the SIC secreted by the bacteria in plasma environment, could be bound to the bacterial surface where it blocks binding of HK or the activity of generated antibacterial HK-fragments. The SIC deficient isogenic mutant strain SIC$^-$ derived from AP1 (29) was included as a negative control in these experiments. Due to the poor growth of *S. pyogenes* in 100% plasma, the bacteria were cultivated in 50% plasma to enhance the number of bacterial cells. Following growth, bacteria were carefully washed and proteins associated with the surface were released by low pH and subjected to Western blot analysis using antibodies against SIC. A prominent band of 35 kDa, released from AP1 bacteria, reacted with the SIC antibody (Fig. 6A, lane 1). No such band was identified in the material eluted from the SIC$^-$ mutant (Fig. 6A, lane 2). SIC eluted from AP1 bacteria appeared slightly smaller than the SIC control isolated from AP1 growth medium (Fig. 6A, lane 3), indicating that the surface-bound SIC represents a processed form of the protein. In addition, immunoreactive bands of approximately 31, 50 and 55 kDa, respectively, were present in the material eluted from both strains. AP1 bacteria bind several abundant plasma proteins, including albumin, fibrinogen and IgG, via the surface proteins H and M1 (43,44). Thus, the band migrating at 55 kDa corresponds to IgG heavy chains interacting with the HRP-conjugated protein A used in the assay. M1 protein is released from the bacterial surface (44), and the 50 kDa band represents M1 protein interacting with the rabbit antibody against SIC through non-immune IgGFc-binding (44). AP1 bacteria secretes IdeS, a proteolytic enzyme that cleaves IgG heavy chains in the hinge region (45). Surface-bound IgG is cleaved by IdeS, and under reducing conditions heavy chain fragments of 31 kDa are generated which bind the secondary protein A reagent in the Western blot assay. NH_2-terminal sequencing of this band confirmed it to be the 31 kDa IgG fragment mentioned above.

To further demonstrate an association of SIC with the bacterial surface, AP1 bacteria were incubated with radiolabelled SIC. As shown in Figure 6B, SIC is bound to AP1 also when there are no plasma proteins present. Next, we used fluorescence microscopy in order to investigate whether some of the SIC produced by AP1 is retained at the bacterial surface. In these experiments the AP1 and SIC$^-$ strains were grown in plasma over night. Bacteria were then carefully washed and incubated with anti-SIC followed by a secondary fluorophore-conjugated antibody. The results show SIC associated with AP1 bacteria and a low background binding of the antibody to the SIC$^-$ mutant (Fig. 6C-E). This finding is in agreement with the Western blot data above, and the combined data suggest that a fraction of secreted SIC associates with the bacterial surface during growth. Moreover, analysis of the *S. pyogenes* surface proteome identified SIC at the surface of the M1-SF370 strain, further supporting our finding (46).

In order to examine whether SIC provides a selective advantage, AP1 bacteria and the mutant strain SIC$^-$ were grown in human citrated plasma. At various time-points bacteria were plated, cfu was determined and the multiplication factor (MF) was calculated. For comparison the MF of AP1 was set to 1 in each individual experiment (ten in total), and the MF of the mutant SIC$^-$ analyzed in parallel experiments was related to this (Fig. 6F). After six and eight hours, the Mann-Whitney U-test showed a highly significant growth advantage for wild-type AP1 as compared to the SIC mutant, data supporting the notion that SIC promotes bacterial survival in human plasma.

SIC promotes bacterial growth in vivo. The AP1 strain is of the M1 serotype and produces SIC, and this strain is also highly virulent to mice. Previous studies have demonstrated that compared to the wild-type AP1 strain, the isogenic mutant strain SIC$^-$ is attenuated in virulence (29). To study whether blocking of the antibacterial activity of the contact system *in vivo* by SIC influences the virulence of AP1 bacteria, we used a mouse model of *S. pyogenes* infection (47). Mice were injected subcutaneously with AP1 bacteria (2×10^7 cfu/mouse), followed by an intraperitoneal injection of PBS or SIC (200 μg/mouse) after eight and twenty hours. Mice injected subcutaneously and intraperitoneally with PBS served as a healthy control group. After thirty hours the dissemination of bacteria to the spleen and the concentration of the proinflammatory marker IL-6 in plasma were determined. Statistical analysis revealed that significantly higher numbers of bacteria were detected in animals treated with SIC as compared to the animals treated with PBS ($P = 0.042$) (Fig. 7). Also, the concentration of IL-6 was higher in the group given SIC, but this was not statistically

significant (P = 0.055). In plasma from the control group the concentration of IL-6 was below the detection level and no bacteria were found in the spleens.

To determine whether the contact system is activated in *S. pyogenes* infected animals, clotting assays were performed. In plasma from infected animals activation of the intrinsic pathway of coagulation was impaired as judged by the prolonged activated partial thromboplastin time (aPTT) (Fig. 7). This increase of the aPTT was statistically significant compared to the healthy control group, demonstrating that activation of the contact system in infected animals results in a consumption of contact factors. There was no statistical difference of the aPTT between the groups of infected animals treated with PBS or SIC, which does not exclude that SIC interferes with HK-binding and contact activation at the bacterial surface *in vivo*. These results demonstrate that invading AP1 bacteria activate the contact system, whereas the enhanced dissemination of bacteria to the spleen in mice treated with SIC suggests that antibacterial peptides generated through contact activation are inhibited by SIC.

DISCUSSION

The contact and complement systems constitute important components of innate immunity that are activated locally at the site of infection to prevent bacterial invasion and dissemination. Upon activation of these proteolytic cascades, antibacterial peptide fragments are generated which efficiently kill several bacterial pathogens, including *S. pyogenes*. This common and significant human pathogen is responsible for a number of diseases, including both localized and systemic infections (13-15). In the present investigation the SIC protein, secreted by *S. pyogenes* of the M1 serotype, was found to interfere with the antibacterial function of the contact and the complement systems, both *in vitro* and *in vivo*.

S. pyogenes is known to induce powerful local inflammatory responses causing vascular leakage and the emergence of plasma proteins at the infectious site, leading to the activation of the contact and the complement systems and the generation of antibacterial peptides. The importance of a functional contact system in bacterial surveillance was previously demonstrated in an *in vivo* mouse model, where blocking of the contact system promoted bacterial dissemination and growth (9). The finding here that the growth of *S. pyogenes* is enhanced in human plasma deficient of the contact components FXII, PK or HK (see Fig. 1A), further emphasizes the role of contact activation in controlling bacterial numbers. In addition, both constitutively expressed AMPs and AMPs produced in response to inflammation at the site of infection, contribute to a rapid and unspecific defence against invasive bacteria (1). *In vitro* the *sic* gene is expressed already during early growth phase (24), suggesting that SIC is produced immediately when *S. pyogenes* starts to multiply after colonization. At this early and crucial stage of infection, *S. pyogenes* strains expressing SIC that inactivates AMPs should have a selective advantage. Previous work has demonstrated that isogenic M1 mutant strains lacking SIC, are significantly attenuated in virulence (29,48). The significance of SIC as a virulence determinant is further supported by the present study, where SIC enhanced the bacterial dissemination in a mouse model of infection. Moreover, in a recent report by Pence and co-workers it was shown that SIC bound both human and murine cathelicidins thereby promoting resistance to LL-37 and virulence in mice (49). In relation to the present study, their finding that SIC enhanced *S. pyogenes* growth in human whole blood and serum is of particular interest.

Another host defence mechanism considered as part of innate immunity, is the formation of NETs (neutrophil extracellular traps) containing bactericidal molecules like AMPs, proteinases and histones (50). Interestingly, the contact system was recently demonstrated to be activated on NETs (51), but it was not shown whether HK-derived antibacterial peptides are generated. However, it can be hypothesized that HK fragments constitute an additional factor promoting bacterial killing by NETs. In addition, M1 protein has been identified as a strong inducer of NET formation, but surprisingly also important for promoting *S. pyogenes* survival through inhibition of LL-37 (52). The results of this study suggest that SIC bound to the bacterial surface, or secreted into the microenvironment and associated with NETs, contribute to bacterial survival.

Originally the *sic* gene was identified in *S. pyogenes* strains of the M1 and M57 serotypes (24). Subsequent work demonstrated that distantly related *sic* variants (*drs*) are also

present in strains of the M12 and M55 serotypes (53). SIC binds to and interferes with the function of the membrane-attack complex (MAC) of complement (24,54), and subsequently a number of investigations have demonstrated the ability of SIC and SIC variants to block the activity of several antibacterial proteins and peptides (26-29,55). In this context it is interesting that HRG, a plasma protein that interacts with SIC (24) and the MAC (56) exerts antibacterial activity against various bacterial species (57). Indeed, SIC was recently found to block the antibacterial activity of HRG against AP1 bacteria (58). When *S. pyogenes* bacteria of the M1 serotype are exposed to saliva or blood, *sic* is upregulated (59,60). Surprisingly, we could not detect SIC in the supernatant but only at the bacterial surface when AP1 bacteria were grown in human plasma. This could be explained if secreted SIC is bound to SIC-binding plasma proteins (HRG, clusterin and/or HK). Thus, in the ELISA the antibodies may not detect SIC epitopes hidden in these protein complexes.

There is a remarkable variation among *sic* genes from different isolates of the M1 serotype (25) due to natural selection on mucosal surfaces (61), which may facilitate adaptation to changes in the antibacterial activity at the local site of infection and promote bacterial survival. Strains of the M1 serotype are common and are frequently associated with invasive infections (18-23), and the interference of SIC with antibacterial activities of the contact and complement systems may facilitate *S. pyogenes* invasion. Virulence is of course a polygenic property, but the results of the present work could in part explain the evolutionary success of the M1 serotype and the severity of infections caused by M1 strains.

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FOOTNOTES

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The abbreviations used are: AMPs, antimicrobial peptides; FXII, factor XII; FXI, factor XI; PK, plasma kallikrein; HK, high molecular weight kininogen; *S. pyogenes*, *Streptococcus pyogenes*; SIC, Streptococcal Inhibitor of Complement; TH, Todd-Hewitt broth; apt, activated partial thromboplastin time; MF, multiplication factor; NETs, neutrophil extracellular traps; MAC, membrane attack complex

FIGURE LEGENDS

Fig. 1. Growth of *S. pyogenes* in human plasma is controlled by the contact and complement systems. *A* AP1 bacteria were grown in human citrated plasma samples; normal plasma (●), PK deficient plasma (◇), FXII deficient plasma (△), HK deficient plasma (○) or FXI deficient plasma (□). At indicated time points bacteria were plated on TH-agar plates. Plates were incubated overnight at 37°C, the number of cfu was determined and the multiplication factor (MF) was calculated by dividing the number of cfu at each time point with the number of cfu at time zero. Mean values of at least four experiments are shown. The bars in the insert represent the mean values ± SEM after eight hours of growth. P-values were determined by using the Mann-Whitney U-test. *B* AP1 bacteria were cultivated in citrated plasma (black bars), serum (grey bars), and heat-inactivated serum (white bars). Bacterial growth was followed as above. Mean values of three experiments ± SEM are shown.

Fig. 2. SIC binds to the NAT26 peptide sequence of HK and inhibits its antibacterial activity.

A Schematic representation of HK. S: signal sequence, D1-D3: cystatin-like domains, D4: bradykinin-containing domain, D5: domain binding to negatively charged surfaces, D6: domain binding to FXI and plasma kallikrein. Peptides derived from the D3 sequence and used in this study are shown. Numbers refer to amino acid residue positions (bars are not in scale), and the letter code indicates their three NH_2-terminal amino acids followed by the total number of residues. The position of the peptides in the D3 domain is also indicated. *B* Microtiter plates were coated with 0.5 μg/ml of the synthetic peptides from domain D3 of HK: GKD25 (○), GCP28 (Δ), NAT26 (▼), KKY30 (▽), RET27 (⊕) and LDC27 (■). After incubation with SIC in a serial dilution, bound protein was detected with a specific antiserum against SIC and a peroxidase-labelled secondary antibody. *C* Various amounts of the D3-derived peptides were applied to a PVDF membrane. The membrane was incubated with radiolabelled SIC and autoradiographed. *D* Homology model of the D3 domain of HK. Peptides NAT26 (red) and LDC27 (green) are located in two separate intersecting anti-parallel β-strands. *E* AP1 bacteria were incubated with NAT26 (black bars) or LL-37 (grey bars) at 0.642 μM and 0.446 μM, respectively. At these concentrations no cfu were detected. The bactericidal effect of the peptides was inhibited with indicated concentrations of SIC. Bars represent the mean ± SEM of at least three experiments.

Fig. 3. SIC inhibits the bactericidal activity of C3a. *A S. pyogenes* strain AP1 (2 x 10^6 cfu/ml) was incubated with C3a at indicated concentrations and cfu were determined. *B* The bactericidal effect of C3a at 0.22 μM was inhibited with various concentrations of SIC. Experiments were repeated at least three times, and mean values ± SEM are shown.

Fig. 4. Binding of HK to *S. pyogenes* is inhibited by SIC. *A* AP1 bacteria at indicated concentrations were tested for binding of radiolabelled HK. *B* The binding of radiolabelled HK to AP1 bacteria (2 x 10^9 cfu/ml) was inhibited with various amounts of SIC. The bars represent the mean ± SEM of at least three experiments.

Fig. 5. SIC protects *S. pyogenes* bacteria from killing in plasma. *A* AP1 bacteria were cultivated in human citrated plasma with various concentrations of SIC; plasma alone (●), plasma + SIC 7.65 μM (□), plasma + SIC 15.3 μM (△), plasma + SIC 23 μM (○). At indicated time points bacteria were plated on TH-agar plates. Plates were incubated overnight at 37°C, the number of cfu was determined and the MF was calculated by dividing the number of cfu at each time point with the number of cfu at time zero. The bars in the insert represent the mean values ± SEM of at least three experiments following eight hours of growth. *B* AP1 bacteria (8 x 10^7 cfu) were incubated with human citrated plasma at 37°C for 1 h ± SIC. Bacteria were washed with PBS and the binding of HK or HK fragments was analysed by flow cytometry. Rabbit anti-NAT26 $F(ab')_2$-fragments were used as primary antibodies and FITC-conjugated goat anti-rabbit $F(ab')_2$-fragments were employed in the secondary step. Blue line: background fluorescence obtained with secondary antibodies; Green line: detection of HK/D3-containing fragments with primary and secondary antibodies; Red line: detection of HK/D3-containing fragments in the presence of SIC (13.5 μM). *C-E* An aliquot of AP1 bacteria incubated with human plasma (see *B*) was analysed by electron microscopy following negative staining. *C* AP1 bacteria incubated with PBS; *D* AP1 bacteria incubated with plasma; *E* AP1 bacteria incubated with plasma in the presence of SIC (13.5 μM). Scale bar: 2 μm.

Fig. 6. SIC associated with the surface of *S. pyogenes* enhances the bacterial multiplication in human plasma. *A* The AP1 strain and the isogenic AP1 SIC deficient mutant strain SIC^- were cultivated in human plasma (diluted in TH 1:1) for 8 h at 37°C. Bacteria were washed with PBS and bound proteins were eluted at pH 2.0 and subjected to SDS-PAGE followed by Western blotting. Lane 1: Proteins eluted from AP1 bacteria. Lane 2: proteins eluted from the AP1 mutant SIC^-. Lane 3 SIC, 0.5 μg. *B* AP1 bacteria (1 x 10^8 cfu) were incubated with radiolabelled protein SIC (750 000 cpm) in TH or in plasma (diluted 1:5 with TH) at 37°C for 1 h. The bacteria were washed with PBS and resuspended in SDS sample buffer. Following separation by Tricine-SDS/PAGE the gel was dried and subjected to autoradiography. Lane 1: AP1 in TH. Lane 2: AP1 in plasma (1:5 with TH). *C-E* Wild-type AP1 bacteria and the isogenic mutant SIC^- were grown in human plasma (diluted in TH 1:1) overnight at

37°C. The presence of SIC at the bacterial surface was demonstrated by fluorescence microscopy (right panel) using rabbit anti-SIC $F(ab')_2$-fragments, followed by Alexa-Fluor-594-conjugated goat anti-rabbit $F(ab')_2$-fragments. Scale bar represents 10 μm. *C* AP1 bacteria. *D* SIC^- bacteria. *E* AP1 background fluorescence with the secondary goat $F(ab')_2$-fragments only. *F* Wild-type AP1 bacteria (black bars) and the isogenic mutant SIC^- (grey bars) were grown in human plasma. At indicated time points bacteria were plated on TH-agar plates. Plates were incubated overnight at 37°C, the number of cfu was determined and the MF was calculated. The MF of AP1 was set to 1 at each time point in each individual experiment and the MF of the mutant SIC^- was related to this. Mean values ± SEM of ten experiments are shown. P-values were determined by using the Mann-Whitney U-test.

Fig. 7. Inhibition of the antibacterial activity of the contact system by SIC promotes bacterial growth *in vivo*. Female Balb/c mice were injected subcutaneously with AP1 bacteria (2×10^7 cfu/animal), and at eight and twenty hours post infection PBS or SIC was injected i.p. (200 μg SIC/dose). Thirty hours after infection, blood was drawn by cardiac puncture and the concentration of IL-6 in the plasma was determined. Activation of the intrinsic pathway of coagulation was measured as the activated partial thromboplastin time (aPTT) in these samples, and the number of cfu in the spleens of the sacrificed mice was determined. In the group treated with SIC ($n = 8$) the number of cfu/ml spleen homogenate was significantly higher as compared to the group treated with PBS alone ($n = 7$; $P = 0.042$). The increased concentration of IL-6 in the SIC-treated group was not statistically significant ($P = 0.055$). Compared to the healthy control group ($n = 12$), the increase in aPTT was significantly prolonged in the groups treated with PBS ($P = 0.022$) or SIC ($P = 0.0073$). There was no statistical difference between the PBS and SIC treated groups ($P = 0.61$). The Mann-Whitney U-test was used for the determination of P-values. Bars represent the median value in each group.

Figure 1

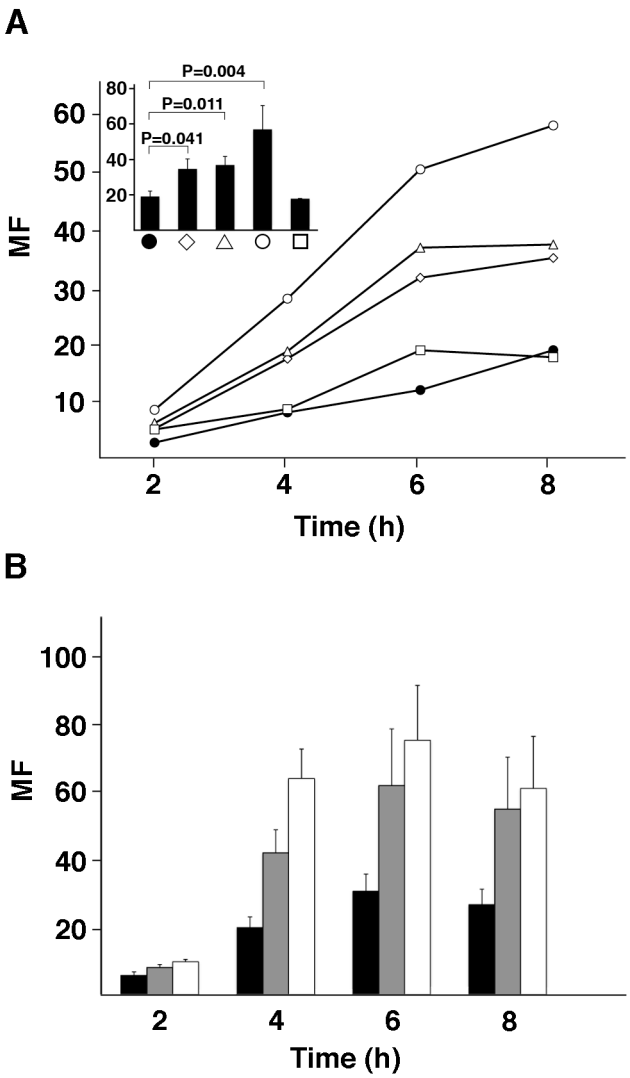


Figure 2

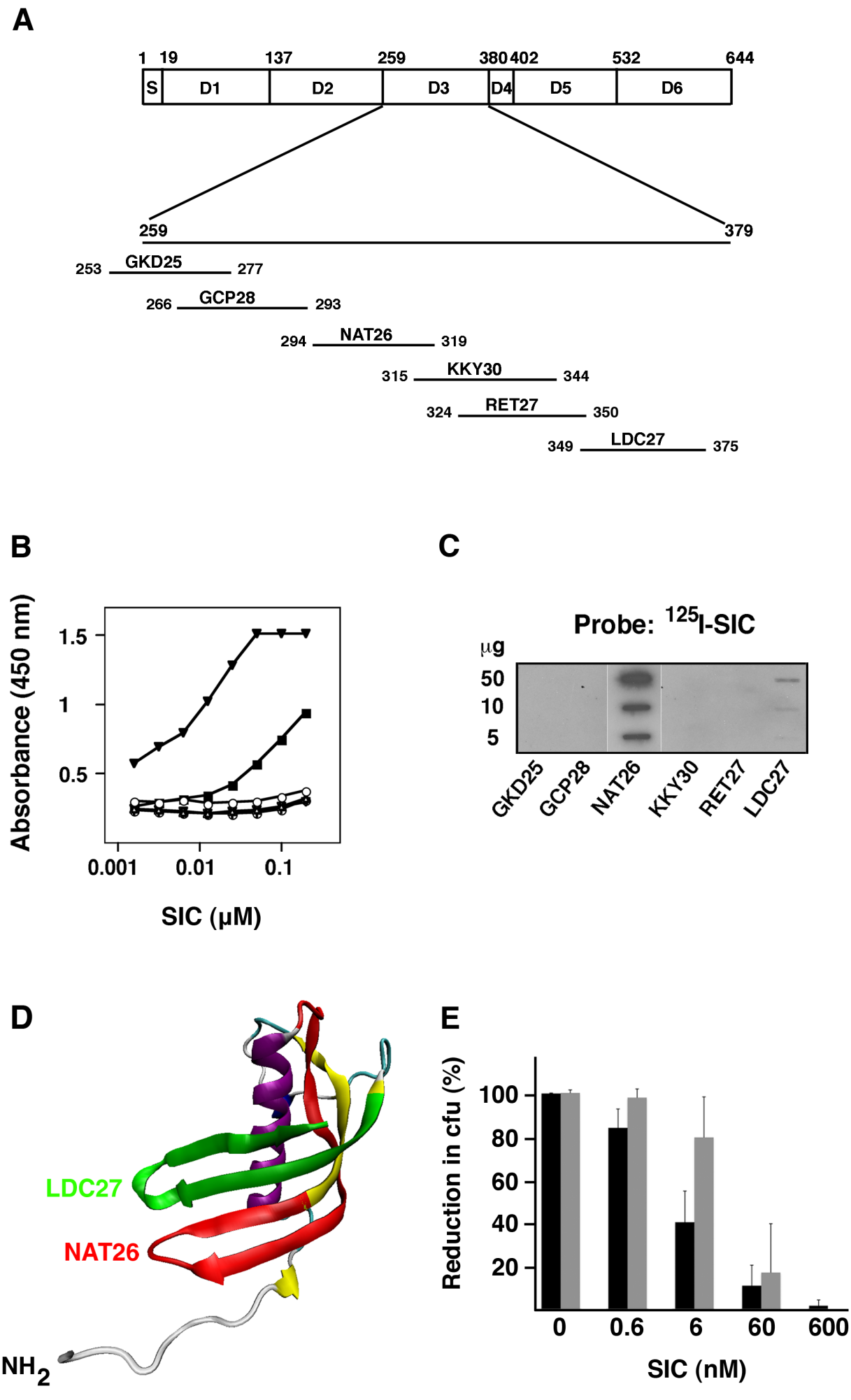

A
1 19 137 259 380 402 532 644
S D1 D2 D3 D4 D5 D6
259 379
253 GKD25 277
266 GCP28 293
294 NAT26 319
315 KKY30 344
324 RET27 350
349 LDC27 375
B
Absorbance (450 nm)
1.5
1
0.5
0.001 0.01 0.1
SIC (µM)
C
Probe: 125I-SIC
µg
50
10
5
GKD25 GCP28 NAT26 KKY30 RET27 LDC27
D
LDC27
NAT26
NH2
E
Reduction in cfu (%)
100
80
60
40
20
0 0.6 6 60 600
SIC (nM)

Figure 3

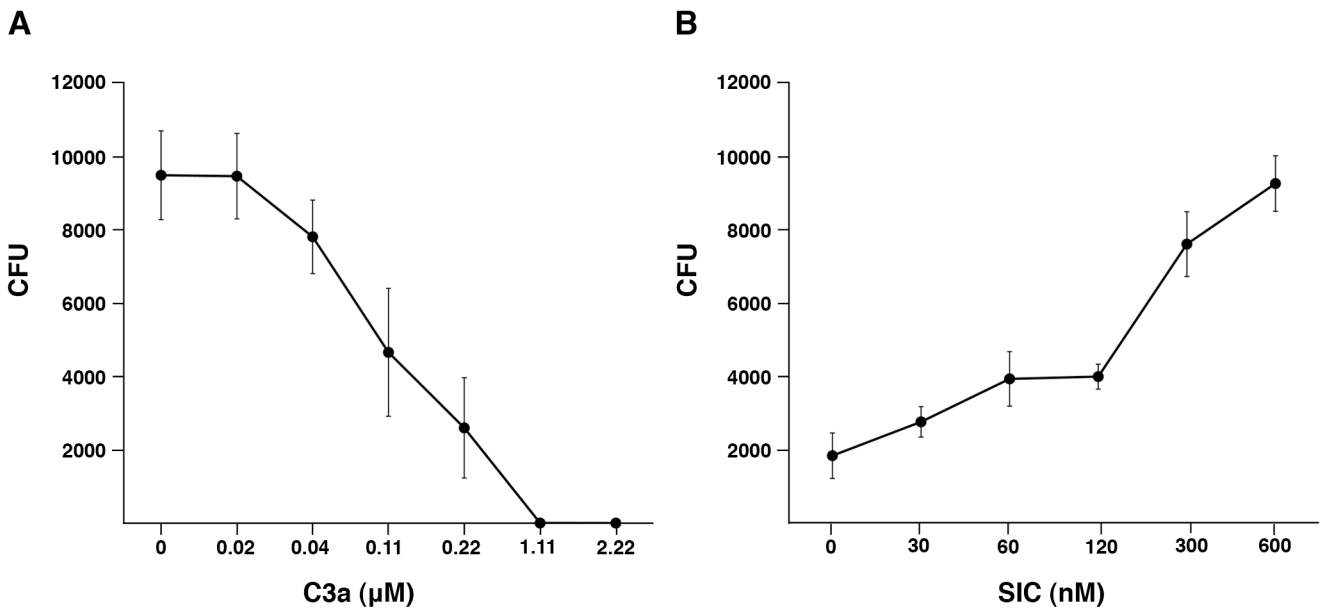

A
12000
10000
8000
6000
4000
2000
CFU
0
0.02
0.04
0.11
0.22
1.11
2.22
C3a (μM)
B
12000
10000
8000
6000
4000
2000
CFU
0
30
60
120
300
600
SIC (nM)

Figure 4

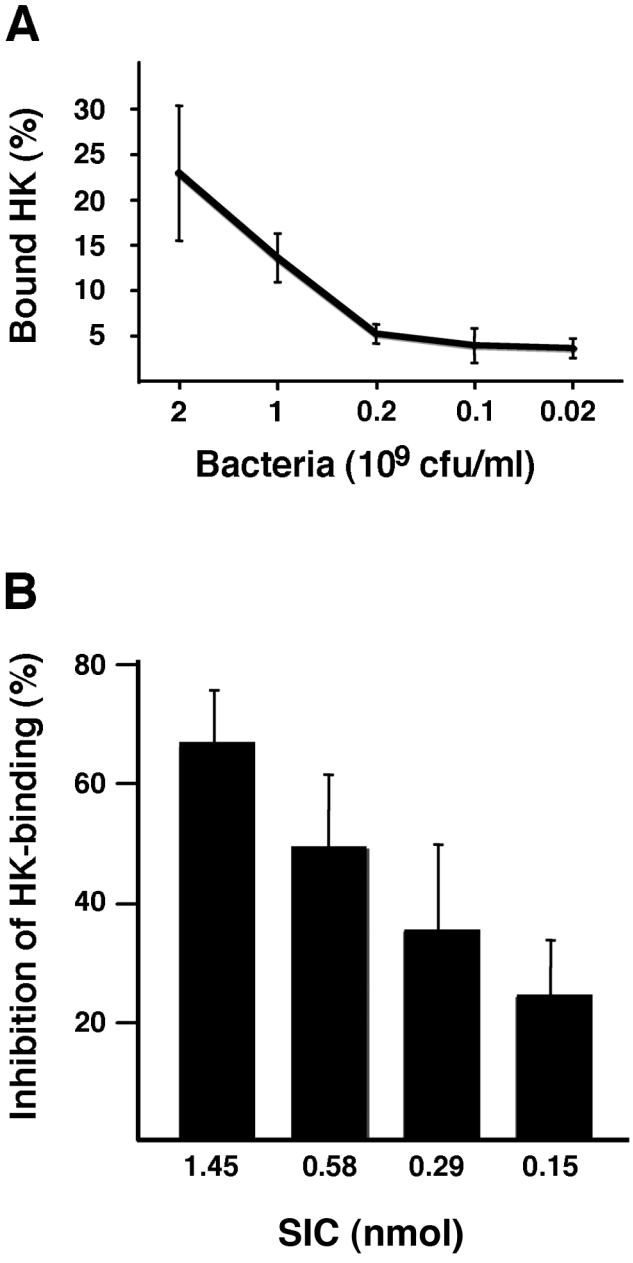

A
Bound HK (%)
30
25
20
15
10
5
2
1
0.2
0.1
0.02
Bacteria (10^9 cfu/ml)
B
Inhibition of HK-binding (%)
80
60
40
20
1.45
0.58
0.29
0.15
SIC (nmol)

Figure 5

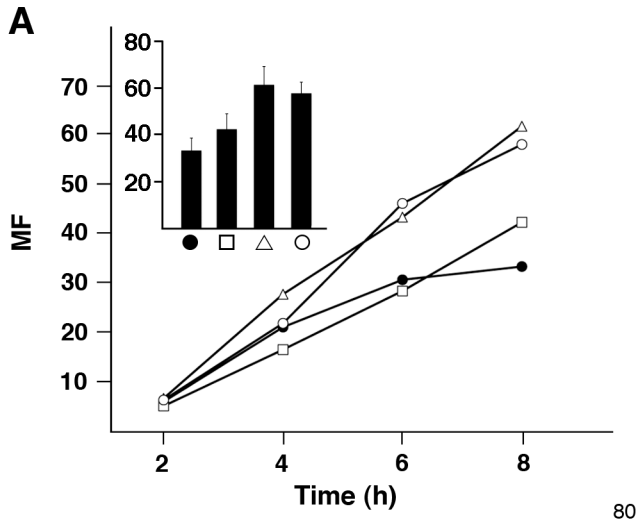


Figure 6

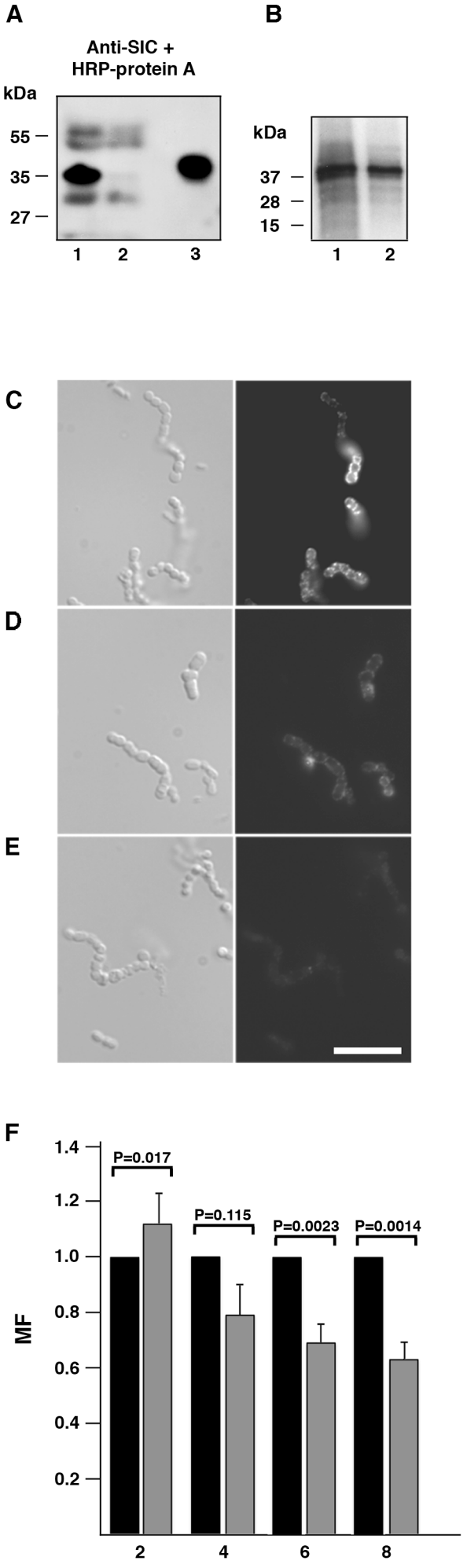
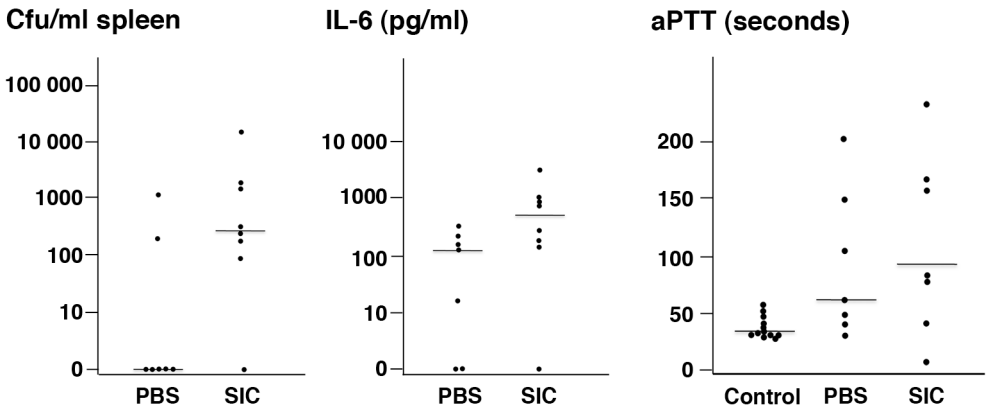

A
Anti-SIC +
HRP-protein A
kDa
55
35
27
1
2
3
B
kDa
37
28
15
1
2
C
D
E
F
MF
1.4
1.2
1.0
0.8
0.6
0.4
0.2
P=0.017
P=0.115
P=0.0023
P=0.0014
2
4
6
8

Figure 7


Cfu/ml spleen
100 000
10 000
1000
100
10
0
PBS
SIC
IL-6 (pg/ml)
10 000
1000
100
10
0
PBS
SIC
aPTT (seconds)
200
150
100
50
0
Control
PBS
SIC