Platelet activation and biofilm formation by *Aerococcus urinae*, an endocarditis causing pathogen

By: Oonagh Shannon, Matthias Mörgelin, and Magnus Rasmussen*

From the Department of Clinical Sciences, Division of Infection Medicine, Lund University, Lund, Sweden.

* Corresponding author

Magnus Rasmussen, M.D., Ph.D.
Department of Clinical Sciences, Division of Infection Medicine
BMC B14
Tornavägen 10
221 84 Lund
Sweden
Magnus.Rasmussen@med.lu.se
Telephone: +46-462220720
Fax: +46-46157756

Running title: *Aerococcus urinae* virulence strategies
The Gram-positive bacterium *Aerococcus urinae* can cause infectious endocarditis (IE) in older persons. Biofilm formation and platelet aggregation is believed to contribute to bacterial virulence in IE. Five *A. urinae* isolates from human blood were shown to form biofilms *in vitro* and biofilm formation was enhanced by the presence of human plasma. Four of the *A. urinae* isolates caused platelet aggregation in platelet rich plasma from healthy donors. The Au3 isolate, which induced platelet aggregation in all donors, also activated platelets as determined by flow cytometry. Platelet aggregation was dependent on bacterial protein structures and on platelet activation since it was sensitive to both trypsin and Prostaglandin E1. Plasma proteins at the bacterial surface were needed for platelet aggregation and a role for the complement system, fibrinogen, and immunoglobulin G was demonstrated. Complement depleted serum was unable to support platelet aggregation by Au3 and complement blockade using compstatin inhibited platelet activation. Platelet activation by Au3 was inhibited by blocking of the platelet fibrinogen receptor and this isolate was also shown to bind radiolabeled fibrinogen. Removal of IgG from platelet rich plasma by a specific protease inhibited the platelet aggregation induced by *A. urinae* and blockade of the platelet FcRγIIa hindered platelet activation induced by Au3. Convalescent serum from a patient with *A. urinae* IE transferred the ability of the bacterium to aggregate platelets in an otherwise non-responsive donor. Our results show that *A. urinae* exhibits virulence strategies of importance for IE.
INTRODUCTION

*Aerococcus urinae* is a Gram-positive coccus with the capacity to cause urinary tract infections (7), infectious endocarditis (IE) (6), and spondylodiscitis (3). *A. urinae* was recognized as a distinct species in 1992 (1) and can be misreported as an α-hemolytic streptococcus due to its growth characteristics and hemolysis pattern. Correct classification is based on sequencing of the 16S rRNA gene. The bacterium is generally susceptible to penicillin, vancomycin, and cephalosporins whereas it is resistant to sulfonamide (8, 28). Patients who present with severe infection are typically elderly men with underlying urinary tract abnormalities. Twenty cases of *A. urinae* IE have been described in the literature and the case fatality is around 50% (10, 15). Despite reports of invasive disease caused by *A. urinae*, including IE, nothing is known about potential virulence strategies of the bacterium.

Biofilm formation is believed to contribute to the ability of a bacterium to colonize foreign materials such as urinary tract catheters. In the biofilm, the bacteria are protected against host defenses and against antibiotics. The first step in the pathogenesis of IE is believed to be bacterial adherence to damaged heart valves. This is followed by bacterial accumulation and probably also formation of a bacterial biofilm (9). In addition, fibrin, neutrophils, and platelets are deposited on the infected heart valve leading to the formation of a typical IE vegetation.

The majority of Gram-positive pathogens that cause IE interact with and activate human platelets and this has been proposed to contribute to virulence. The molecular mechanisms leading to platelet activation have been described in detail for many pathogens (recently reviewed in (11, 16)). Many pathogens activate platelets through bacterial bound fibrinogen in combination with specific immunoglobulin G (IgG) bound to the bacterial surface. This mechanism has been described for...
Staphylococcus aureus ClfA (19) and FNBPA (12), Streptococcus pyogenes (27), and Streptococcus agalactiae (23). Direct interactions between bacteria and platelets can also lead to platelet activation (5, 17, 21). In addition, both Streptococcus sanguis, S. aureus, and Enterococcus faecalis can activate platelets in the absence of bacterial bound fibrinogen through an IgG and sometimes complement dependent mechanism with a longer lag-time (13, 19, 20, 24). Upon activation, platelets release antibacterial peptides and resistance to such substances has been linked to the capacity of S. aureus to cause IE (4, 32). Thus platelets activation may on the one hand contribute to host defense against invading bacteria and on the other hand contribute to the pathogenesis of IE (31).

Given the potential importance of biofilm formation and platelet activation in the pathogenesis of IE and the ability of A. urinae to cause this condition, we investigated the capacity of the bacteria to form biofilm and to activate human platelets.
MATERIALS AND METHODS

Bacteria and culture conditions. Five isolates of *Aerococcus urinae* from human blood cultures were obtained from the accredited routine diagnostic laboratory for Clinical Microbiology, Skåne University Hospital, Lund, Sweden. The isolates were collected from 2005 to 2008 and were from five distinct patients. The isolates had been subjected to PCR and sequencing of the 16S rRNA gene to confirm their identity. *A. urinae* was cultivated on blood agar or in tryptic soy broth (Difco) with the addition of 0.5% glucose (TSBG) at 37°C with 5% CO₂. *Streptococcus pyogenes* strain API1 was grown as described previously (25).

Biofilm assay. Quantification of biofilm formation was performed using a previously described microtiter plate assay (29), with modifications. Briefly, 20 μl of precultured bacteria was added to wells of a sterile 96-well flat-bottomed plastic culture plate (Nunclon Surface F, Nunc A/S, Denmark) containing 180 μl of TSBG medium and incubated at 37°C for different time periods. The medium alone served as a negative control. The medium was removed and the wells were gently washed three times with 200 μl PBS. The biofilm was fixed in 200 μl methanol for 10 min, air dried for 15 minutes, and stained with 160 μl crystal violet (1% w/v in water, Diagnostica Erlich, Darmstadt, Germany) for 5 min. The staining solution was removed and the plate was washed three times with 200 μl PBS per well, prior to extraction of the dye with 200 μl acetone-ethanol (20/80 v/v). 100 μl of this solution was transferred to a new plate and diluted in an equal volume of acetone-ethanol. The absorbance was measured at 550 nm in a Victor³ 1420 multilabel counter (Perkin Elmer Precisely). If absorbance was above 2.5, the sample was diluted in an equal volume of acetone-ethanol and the absorbance was measured again. The obtained value was multiplied by the number of times the sample had been diluted.
and the absorbance of the negative control was subtracted. Each isolate was tested in duplicate at least three times.

**Platelet preparation.** Blood samples were collected from five healthy donors who had not taken antiplatelet medication in the previous ten days. Five ml of blood was collected into citrated vacuum tubes. Centrifugation at 150g for 10 minutes produced an upper platelet rich plasma (PRP), which was removed. Subsequent centrifugation at 1500g for 10 minutes produced an upper platelet poor plasma (PPP). Washed platelets (WP) were obtained from 5 ml of PRP, which was supplemented with 1 µM Prostaglandin E\(_1\) (PGE\(_1\), Sigma) followed by centrifugation at 1000g for 10 minutes. The soft pellet was washed once in 5 ml washing buffer (26) followed by centrifugation at 800g for 15 minutes and gentle resuspension in 1 ml HEPES buffer pH 7.4. Blood sampling of healthy volunteers had been approved by the local ethics committee (2008/657).

**Aggregometry.** Bacteria from 10 ml of TSBG grown for 16 hours were harvested by centrifugation at 2500g for 10 minutes, washed in phosphate buffered saline (PBS), and resuspended in 500 µl of the same buffer. Absorbance at 620 nm was determined for a ten-fold diluted bacterial suspension and bacterial concentration was adjusted so that the diluted suspension had an OD\(_{620}\) of 0.59, which corresponds to 2.6x10\(^8\) cfu/ml of isolate Au3 and 2.6x10\(^9\) cfu/ml in the un-diluted suspension after the corresponding adjustment. Forty µl of the latter bacterial solution was added to 450 µl of PRP and the platelet response was monitored in a Chrono-Log aggregometer for a maximum of 25 minutes. As a positive control, 1 µl of soluble collagen I (Triolab, Sweden) was added to 450 µl of PRP. To inactivate IgG in PRP, 5
µg of purified IdeS was added to 450 µl PRP and incubated for 15 minutes at 37°C.

Alternatively, 25 µl of serum was treated with 0.25 µg IdeS for 15 minutes at 37°C.

IdeS was purified as a fusion to GST as previously described (30). For some experiments, 0.1 mg of trypsin from bovine pancreas (Sigma) was added to 100 µl of bacterial suspension (2.6x10^9/ml in PBS) followed by incubation at 37°C for 30 minutes. Trypsin was blocked by the addition of benzamidine hydrochloride (Sigma) to a final concentration of 6 mM. After incubation for 5 minutes the sample was centrifuged at 3000g for 5 minutes and the bacterial pellet was resuspended in 100 µl of PBS. For some experiments, PGE_1 was added to a final concentration of 1 µM to PRP and incubated for 5 minutes before addition of agonist. Serum was prepared from blood that was allowed to coagulate in glass tubes for one hour followed by centrifugation at 1500g for 10 minutes. For inactivation of complement proteins, serum was heated to 56°C for 15 minutes. Alternatively, serum was treated with 50 mg/ml of zymosan A (Sigma) at 37°C for 20 minutes followed by centrifugation at 10000g for 2 minutes (14). To reconstitute serum with fibrinogen, 50 µl of a solution containing 5 mg of fibrinogen/ml (ICN biochemicals, Aurora, Ohio) in PBS was added to 350 µl of serum. Results were analyzed using the Aggrolink, version 5.2.1 software.

Determination of platelet activation by *A. urinae*. Twenty µl of PRP was incubated for 25 minutes at room temperature with 40 µl of HEPES buffer pH 7.4, either in the presence or absence of washed bacteria (approximately 1.2x10^7 bacteria). After 25 minutes, 5 µl fluorochrome conjugated antibody (CD42PerCP, CD62PE, and PAC-1FITC) (all from BD Biosciences) was added and after 10 minutes the incubation was stopped by addition of 500 µl of 0.5 % formaldehyde in ice cold PBS.
Samples were analyzed using a FACSCalibur flow cytometer in logarithmic mode with a gate setting for the CD42 positive platelet population. 50,000 cells were acquired and analyzed using Cell Quest software (Becton Dickinson). In order to assess the role of plasma factors for platelet activation, PRP was treated with specific blockers, 20 µg/ml ReoPro (Eli Lily), 50 µg/ml AT10 (Serotec), or 70 µg/ml compstatin (a kind gift from Dr Ulf Sjöbring described in (22)) for 30 minutes at room temperature.

**Electron microscopy.** For scanning electron microscopy (SEM), 300 µl of bacterial culture was added to 2.7 ml of TSBG and bacteria were allowed to form a biofilm on a heat sterilized glass cover slip for 72 hours in a twelve well cell culture cluster (Corning Incorporated, Costar). The glass was carefully washed three times in PBS followed by incubation in 3 ml of fixation solution (4% formaldehyde, 2.5% glutaraldehyde in PBS) at room temperature for 12 hours. Fixed specimens were dehydrated for 10 min at each step of an ascending ethanol series and critical point dried in a Balzers critical point dryer in liquid carbon dioxide using absolute ethanol as an intermediate solvent. Samples were examined in a Jeol J-330 scanning electron microscope at an acceleration voltage of 5 kV and a working distance of 10 mm.

**Other methods.** Human fibrinogen (Sigma) was radiolabeled and tested for binding to bacteria as described (25). Statistical analysis was performed using the GraphPad Prism 4 for Macintosh, version 4.0C. Handling of patient data was approved by the local ethics committee (2008/657).
RESULTS

Characterization of isolates and patients. Five isolates of *A. urinae* were identified in blood cultures from 2005-2008 at the diagnostic laboratory for Clinical Microbiology, Skåne University Hospital, Lund, Sweden. The bacterium was isolated from two or more flasks and *A. urinae* was the only bacterium isolated in all cases. The identity of the isolates was confirmed by sequencing of the 16S rRNA gene. The isolates were from male patients between 82 and 96 years of age. Three patients had received a diagnosis of infectious endocarditis (ICD10 I33.0) but in two of the patients this diagnosis was delayed by more than three weeks, as the condition was initially misinterpreted as a urinary tract infection. In one patient an anal abscess was suspected to be the cause of the bacteremia and in one patient hydronephrosis was the main diagnosis indicating that the bacteremia was judged to be secondary to the hydronephrosis.

*A. urinae* biofilm formation is stimulated by plasma. Biofilm formation was determined for each *A. urinae* isolate by measuring the amount of crystal violet absorbed by the bacteria in a biofilm formed on polystyrene plastic. Biofilm formation was determined after incubation for 24, 48, and 72 hours. At 48 and 72 hours all isolates formed a robust, macroscopically visible biofilm. Biofilm formation for all isolates at 72 hours is illustrated in figure 1A. The addition of 10% human plasma to the growth medium lead to an increased amount of dye bound to the biofilm of all isolates by 30-758% (mean 397%). To investigate if the stimulatory effect of plasma was due to increased adherence of bacteria to the surface, the wells were pretreated with 20% plasma in TSBG for 2 hours followed by removal of plasma and determination of biofilm formation. Pretreatment of wells did not increase biofilm
formation but rather decreased the biofilm formation of the five isolates with 47-66% (Fig. 1). This indicates that the stimulatory effect of plasma on biofilm formation is not explained by the presence of plasma proteins at the plastic surface. The biofilms formed by two isolates (Au1 and Au3) on glass cover slips in the presence or absence of 10% human plasma were visualized by scanning electron microscopy (SEM, Fig. 2). Both isolates formed multilayered bacterial conglomerates on the glass surface. An intercellular substance covers the bacterial aggregates present in the biofilm formed by Au1 irrespective of the presence of plasma (Fig. 2A and 2B). In the biofilm formed by Au3 intercellular substance could be seen only on bacteria grown in the presence of plasma (Fig. 2C and 2D). This likely reflects the ability of Au1 to produce biofilm in the microtiter plates irrespective of the plasma content, while formation of biofilm by Au3 in the microtiter plates was stimulated by plasma (Fig 1).

**A. urinae aggregates human platelets.** The five isolates of *A. urinae* were tested for their ability to induce aggregation of platelets from five healthy donors. Washed bacteria were added to PRP and the response was monitored for 25 minutes in an aggregometer. The positive control bacterium, the AP1 strain of *S. pyogenes* induced aggregation in all donors within 5 minutes. Platelet aggregation in response to *A. urinae* occurred in all donors and was induced by 2-4 isolates depending on the donor (Table 1). One isolate, Au3, induced aggregation in all donors, whereas one isolate (Au4) failed to induce aggregation in any donor (Table 1). Mean time to aggregation for all isolates was 13 minutes (range 6-25). Typical aggregation curves for Au3 and Au4 in donor 1 are shown in figure 3. Treatment of Au3 bacteria with trypsin abolished the ability to aggregate platelets in the three donors tested (data not shown). To exclude an effect of trypsin on platelets, the Bef5 isolate of *Enterococcus*
faecalis (24) was used as a control. Trypsin-treated Bef5 bacteria retained the ability to aggregate platelets in the same donors (data not shown). Platelet aggregation by all isolates could be blocked by PGE1, in the same three donors, indicating that passive agglutination did not explain the platelet aggregation (data not shown).

A. urinae activates human platelets. Platelet activation in PRP was determined using three-color flow cytometry following stimulation with washed bacteria or ADP as a positive control. Platelets were identified using a CD42 specific antibody, the activation status of the fibrinogen receptor (GPIIb/IIIa) was determined using an antibody specific for the active conformation (PAC-1), and release of the alpha granules was determined using a CD62 specific antibody. As expected, platelets from all donors were negative for CD62 and PAC-1 in the absence of an agonist and became positive only after activation. After treatment with ADP a median of 78% (range 63-83%) and 68% (range 57-76%) of the platelet population were positive for CD62 or PAC-1, respectively. Au3 mediated platelet activation occurred in all five donors rendering a median of 35% (range 27-48%) of platelets positive for CD62 and a median of 31% (range 21-41%) of platelets positive for PAC-1.

Platelet aggregation and activation by Au3 involves the complement system. Some mechanisms described for platelet aggregation with a long lag-time to aggregation involve activation of the complement system. To determine if this was the case for A. urinae, Au3 bacteria were pretreated with 100 µl of PPP for 15 minutes followed by addition of 400 µl of PRP from donor 1 (Fig 4A). As a control Au3 bacteria were added to a mixture of 100 µl of PPP and 400 µl of PRP from the same donor. As can be seen in figure 4A, pretreatment of bacteria with plasma...
shortens the lag-time to aggregation significantly, suggesting that Au3 accumulates components, which participate in platelet aggregation.

Next, 100 µl of washed platelets was added to 350 µl of plasma or serum followed by the addition of Au3 bacteria (Fig 4B). As expected, platelets aggregated in plasma but not in serum, which contains only small amounts of fibrinogen. When fibrinogen was added to serum, Au3 was able to induce aggregation of platelets (Fig 4B). In contrast, in serum pretreated at 56°C with added fibrinogen, Au3 was unable to induce platelet aggregation indicating a role for the complement system in platelet aggregation.

Importantly, the AP1 strain of *S. pyogenes* could aggregate washed platelets suspended in heat-treated serum with added fibrinogen (Fig 4B). In similar experiments, zymosan was used to deplete serum of complement proteins (14). When washed platelets were added to zymosan-depleted serum reconstituted with fibrinogen aggregation by Au3 still occurred. However, the lag-time to aggregation was increased (38, 41, and 78 % in three separate experiments) as compared to the lag-time in normal serum reconstituted with fibrinogen (data not shown).

PRP was preincubated with buffer or compstatin, a peptide inhibitor of the complement system, for 15 minutes followed by the addition of Au3 bacteria and determination of platelet activation by FACS. Compstatin decreased the number of platelets presenting PAC-1 and CD62 in response to Au3 by a mean of 59% and 63% respectively (n=3).

**Platelet activation by Au3 is dependent on fibrinogen.** To assess the role of plasma fibrinogen in mediating platelet activation, the platelet receptor for fibrinogen was blocked using a monoclonal antibody (ReoPro). Since platelet aggregation depends on fibrinogen irrespective of which agonist used, we instead used flow
cytometry to monitor activation. In the presence of ReoPro, the PAC-1 antibody can no longer bind to the platelets (Fig 5B), but platelet activation in response to ADP still occurred and as demonstrated by CD62 presentation (Fig 5B). When the fibrinogen receptor was blocked, activation of platelets by Au3 was significantly decreased in all donors (Fig 5A and 5B). This indicates that the fibrinogen receptor at the platelet surface is involved in platelet activation by Au3.

We next tested the ability of the A. urinae isolates to bind radiolabeled fibrinogen. The AP1 strain of S. pyogenes, which binds fibrinogen with high affinity via the M1 protein (2), was used as a positive control. The ability of $10^9$ bacteria/ml to bind fibrinogen is shown in figure 5C. Au3 absorbed a higher proportion of added fibrinogen as compared to the other isolates. Competitive binding studies were carried out where unlabelled fibrinogen was allowed to compete with radiolabeled fibrinogen for binding to $10^8$ Au3 bacteria or to $2 \times 10^7$ AP1 bacteria (Fig. 5D). In both isolates, the unlabelled fibrinogen could compete with the labeled fibrinogen for binding indicating that binding was specific.

**A. urinae platelet aggregation is dependent on IgG.** To determine the role of plasma IgG in mediating platelet activation, the platelet receptor for the Fc part of IgG was blocked using a monoclonal antibody (AT10). As expected, platelet activation in response to ADP was unaffected by blockade of the platelet IgG receptor but AT10 diminished activation in response to Au3 in all donors (Fig. 6A and 6B). This indicates that plasma IgG is involved in mediating platelet activation in response to Au3.

The highly specific IgG-degrading enzyme IdeS of S. pyogenes (30) was used to degrade all IgG in PRP. In samples pretreated with buffer, collagen and Au3
bacteria could induce platelet aggregation. As expected, collagen retained the ability
to aggregate platelets in IdeS-treated PRP whereas Au3 failed to induce aggregation
of platelets in IdeS-treated plasma. Identical results were obtained in donors 1 and 2
and representative results from an experiment with donor 1 are shown in figure 6C.

Convalescent patient serum from *A. urinae* infection transfers the ability
to aggregate platelets. Serum from the patient infected with isolate Au1 was
obtained 4 weeks after the appearance of the first symptoms of IE. Twenty-five µl of
this serum or serum from donor 1 was added to PRP from donor 1 and platelet
aggregation in response to Au1 was monitored. In PRP from donor 1, Au1 does not
cause platelet aggregation and the addition of serum from the same donor did not
stimulate platelet aggregation (Fig 6D). As expected, Au3 induced aggregation of
platelets in PRP from donor 1 also after the addition of control serum. Importantly,
the addition of patient serum induced platelet aggregation in PRP from donor 1 in
response to Au1 (Fig. 6D). In three separate experiments the lag time to aggregation
was 12, 15, and 16 minutes. Results from one representative experiment are shown in
figure 6C. When pretreated with IdeS, the patient serum could not support Au1
mediated aggregation of platelets from donor 1, again suggesting that IgG is the
serum factor that transfers the ability to aggregate platelets.
In this work we demonstrate that *A. urinae* can form biofilms and that the bacteria can aggregate human platelets. It is the first report of potential virulence mechanisms for this pathogen and both mechanisms may contribute to the ability of *A. urinae* to cause IE. *A. urinae* is well documented as a pathogen in IE though the number of reported cases is low (10, 15). We identified three cases of IE and two additional cases of invasive infection with *A. urinae* during a four year period in a population of around 400,000 inhabitants. This indicates that the importance of *A. urinae* as a pathogen in IE and other invasive diseases has previously been underestimated. In accordance with previous reports, the patients in this study were all older males and though the material is too small to draw any definite conclusions, *A. urinae* may be a significant cause of IE among older men.

The ability of *A. urinae* to form biofilms may play a role during IE caused by this organism, especially since the biofilm formation of many isolates was stimulated by plasma. Importantly, bacteria in biofilms are partially protected against the action of antibiotics (18) so biofilm formation by *A. urinae* may hinder the eradication of the organism with antibiotics. Biofilm formation may also play a role during earlier stages of an *A. urinae* infection. Many patients with severe *A. urinae* infection have indwelling urinary catheters, and biofilm formation on the catheter may increase the likelihood of *A. urinae* bacteremia, which in turn increases the risk for colonization of heart valves.

The interaction between Gram-positive bacteria and platelets has been extensively studied, though the importance of this interaction for the pathogenesis of IE is less clear. We show that many isolates of *A. urinae* induce aggregation and activation of human platelets and this further underlines the importance of platelet
interactions in invasive bacterial infections. The mechanism of platelet aggregation by

Au3 was studied in detail since this isolate induced aggregation of platelets from all

of the donors tested. The mechanism employed by Au3 is similar to that utilized by

many Gram-positive pathogens and was dependent on complement activation, on

fibrinogen, and on IgG.

Four of the other five A. urinae isolates also induced platelet aggregation in some

donors. In all cases, biochemical inactivation of platelets with PGE₁ abolished

aggregation, demonstrating an active role for platelets in this process. The aggregation

of platelets by the other isolates could be inhibited by removal of IgG with IdeS in the

two donors tested, supporting the role of IgG for platelet aggregation. Significantly,

serum from a patient infected with Au1 induced platelet aggregation in response to

Au1 in a previously non-responsive donor. The stimulatory effect of this serum was

lost after proteolytic cleavage of IgG. Taken together with the results obtained for

Au3, our findings imply an essential role for IgG in platelet aggregation by A. urinae.

GPIIb/IIIa activation, perhaps through a fibrinogen bridge, is not necessarily needed

for platelet aggregation by all isolates of A. urinae since most isolates bind poorly to

this plasma protein. In addition, we cannot exclude that a direct interaction between

Au3 and platelet GPIIb/IIIa is involved in platelet activation by this isolate.

Complement activation may also be important for the ability of the other A. urinae

isolates to induce platelet aggregation since a long lag-time was observed for all

isolates. In addition to these host proteins, a bacterial protein structure seems to be

involved because platelet aggregation was abolished by treatment of the bacteria with

trypsin.

Many molecular mechanisms at the bacterial surface seem to occur when A.

urinae enters the bloodstream and the outcome of these interactions will likely
determine if IE is established. We believe that both platelet activation and biofilm formation participate in the pathogenesis of IE caused by A. urinae. However, further studies on the role of platelet activation by A. urinae and other bacterial species during invasive infection are needed. A better understanding of the molecular interactions between invading bacteria and human platelets may stimulate the development of novel therapeutic strategies against invasive bacterial disease such as IE.

ACKNOWLEDGEMENTS

This work was financed by the Swedish Government Funds for Clinical Research (ALF), the Swedish Research Council (Project 21112 and 7480), the Swedish Society of Medicine, the Royal Physiographic Society in Lund, and the foundations of Österlund and Crafoord.

We acknowledge Dr Sara K Söbirk and Daniel Johansson for important help and Ingbritt Gustafsson and Maria Baumgarten for excellent technical assistance. We thank Rita Wallén from the Department of Cell and Organism Biology, Lund University, for help with electron microscopy.
REFERENCES


FIG. 1. *A. urinae* biofilm formation is stimulated by plasma. A. Biofilm formation by *A. urinae* isolates after 72 hours incubation on a plastic surface was determined as absorbance at 550 nm in the presence of medium (black bars) or medium containing with 10% human plasma (grey bars). Biofilm formation was also determined in wells pretreated with human plasma (white bars). The error bars represent SD from three independent experiments, carried out in triplicate.

FIG. 2. Morphology of the *A. urinae* biofilm. Scanning electron microscopy was used to visualize biofilms formed by Au1 (A and B) and Au3 (C and D) on glass cover slips in medium (A and C) or in medium containing 10% human plasma (B and D). The scale bar represents 10 µm.

FIG. 3. *A. urinae* stimulates platelet aggregation. A. Platelet aggregation in PRP from donor 1 in response to *A. urinae* isolates Au3 and Au4 was determined using a Chrono Log aggregometer. The relative light absorption of the sample is shown on the Y-axis where 100 represent the PRP after addition of bacteria and 0 the absorption of PPP. When platelets aggregate the opacity and light absorbance of the sample decreases.

FIG. 4. Platelet aggregation and activation by Au3 is dependent on the complement system. A. Au3 was either pretreated with PPP followed by addition of PRP from donor 1 (b) or added to a mixture of PPP and PRP from the same donor (a), and aggregation was determined by aggregometry. B. Washed platelets from donor 1 were added to citrated plasma from the same donor followed by addition of Au3.
bacteria (a). Washed platelets were added to serum reconstituted with fibrinogen (b) or heat-treated serum reconstituted with fibrinogen (c) followed by addition of Au3 bacteria. AP1 was able to induce aggregation of washed platelets in heat-treated serum reconstituted with fibrinogen (d).

FIG. 5. Role of platelet fibrinogen receptor and fibrinogen-binding by A. urinae.

Au3 was added to PRP from the five donors and after 25 minutes platelet activation was determined using flow cytometry. The percentage of the platelet population positive for CD62P (A) and PAC-1 (B) was determined in the presence of buffer alone (white bars), an antibody to block platelet fibrinogen-binding (ReoPro, black bars). C. The binding of radiolabeled fibrinogen to the A. urinae isolates and to the AP1 strain of S. pyogenes is expressed as the percentage of added fibrinogen associated with the bacterial pellet (n=3, bars represent SD). D. The binding of radiolabeled fibrinogen to Au3 (triangles) and to the AP1 strain of S. pyogenes (squares) was determined in the presence of unlabelled fibrinogen. Binding is expressed as a percentage of the binding of radiolabeled fibrinogen alone. Results are from three independent experiments and the bars represent SD.

FIG. 6. Role of plasma IgG in platelet activation and aggregation by A. urinae.

Au3 was added to PRP from the five donors and after 25 minutes platelet activation was determined using flow cytometry. The percentage of the platelet population positive for CD62P (A) and PAC-1 (B) was determined in the presence of buffer alone (white bars), an antibody to block platelet Ig-binding (AT10, black bars). In C PRP from donor 1 was pretreated with the IgG degrading enzyme IdeS or buffer followed by addition of collagen or Au3 bacteria. Collagen induced rapid aggregation
Au3 bacteria failed to induce aggregation in PRP pretreated with IdeS (c) whereas aggregation occurred in PRP treated with buffer alone. In D aggregation of platelets in PRP from donor 1 in response to Au1 and Au3 was determined. In the presence of 25 µl of serum from donor 1, platelets did not aggregate in response to Au1 (curve a), whereas aggregation occurred in response to Au3 (d). In the presence of 25 µl of serum from the patient infected with Au1, the platelets aggregated in response to Au1 (b). Patient serum preincubated with IdeS could not mediate platelet aggregation in response to Au1 (c).
<table>
<thead>
<tr>
<th>Isolate</th>
<th>Donors responding with aggregation</th>
<th>Lag time</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. pyogenes</em> AP1</td>
<td>5(5)</td>
<td>3 (2-5)</td>
</tr>
<tr>
<td><em>A. urinae</em> Au1</td>
<td>3(5)</td>
<td>16 (9-23)</td>
</tr>
<tr>
<td><em>A. urinae</em> Au2</td>
<td>2(5)</td>
<td>10 (7-12)</td>
</tr>
<tr>
<td><em>A. urinae</em> Au3</td>
<td>5(5)</td>
<td>12 (6-21)</td>
</tr>
<tr>
<td><em>A. urinae</em> Au4</td>
<td>0(5)</td>
<td>-</td>
</tr>
<tr>
<td><em>A. urinae</em> Au5</td>
<td>2(5)</td>
<td>19 (12-25)</td>
</tr>
</tbody>
</table>

Table 1. The number of donors in which platelets responded with aggregation to the *A. urinae* isolates is given as a ratio to the total number of donors (n=5). Mean lag time to aggregation in minutes is given in the right column with the range within parenthesis.