Abstract

Urinary tract infection due to uropathogenic *E. coli* is a common clinical problem. The innate immune system and the uroepithelium are critical in defence against infection. The complement system is both part of the innate immune system and influences the interaction between epithelium and pathogen. We have therefore investigated the mechanism by which uropathogenic *E. coli* activate complement and the potential for this to occur during clinical infection. The classical pathway is responsible for bacterial opsonisation when complement proteins are present at low concentrations. At higher concentrations the alternative pathway predominates but still requires the classical pathway for its initiation. In contrast the mannose binding lectin pathway is not involved. Early classical pathway components are present in the urine during infection and actively contribute to bacterial opsonisation. The classical pathway could be initiated by anti-*E. coli* antibodies of IgG or IgM subclasses that are present in urine during infection. Additionally immunoglobulin-independent mechanisms, such as direct C1q binding to bacteria, may be involved. In conclusion uropathogenic *E. coli* are readily opsonised by complement in a classical pathway dependent manner. This can occur within the urinary tract during the development of clinical infection.
The classical complement pathway plays a critical role in the opsonisation of uropathogenic *Escherichia coli*

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Abstract
Urinary tract infection due to uropathogenic *E. coli* is a common clinical problem. The innate immune system and the uroepithelium are critical in defence against infection. The complement system is both part of the innate immune system and influences the interaction between epithelium and pathogen. We have therefore investigated the mechanism by which uropathogenic *E. coli* activate complement and the potential for this to occur during clinical infection. The classical pathway is responsible for bacterial opsonisation when complement proteins are present at low concentrations. At higher concentrations the alternative pathway predominates but still requires the classical pathway for its initiation. In contrast the mannose binding lectin pathway is not involved. Early classical pathway components are present in the urine during infection and actively contribute to bacterial opsonisation. The classical pathway could be initiated by anti-*E. coli* antibodies of IgG or IgM subclasses that are present in urine during infection. Additionally immunoglobulin-independent mechanisms, such as direct C1q binding to bacteria, may be involved. In conclusion uropathogenic *E. coli* are readily opsonised by complement in a classical pathway dependent manner. This can occur within the urinary tract during the development of clinical infection.

*Keywords: Complement, Escherichia coli, urinary tract infection, innate immunity.*
Introduction

Bacterial infection of the urinary tract is a common clinical problem, estimated to affect 40-50% of women at least once in their life time (Foxman et al., 2000). Frequent recurrence is an important characteristic of these infections, as up to 25% of women who experience a first urinary tract infection (UTI) will have a second infection within 6 months (Foxman, 1990). Recent research indicates that *E. coli* can establish long-lived intracellular reservoirs within the bladder mucosa of mice without bacteriuria (Mulvey et al., 2001; Mysorekar and Hultgren, 2006; Schilling et al., 2002). These persistent intracellular reservoirs may explain the recurrent nature of UTI in humans. Protection against microbial infection is usually achieved by the cooperation between adaptive and innate immune defences. However, adaptive immunity does not play an important role in the early clearance of bacteria from the urinary tract, as immunodeficient mice lacking B or T lymphocytes do not show an enhanced UTI susceptibility (Jones-Carson et al., 1999). Therefore, understanding the interactions between *E. coli* and the host’s innate immune response is critical in understanding the pathogenesis of this important infection.

An important component of innate immunity against many microorganisms is the complement system. Bacterial and viral pathogens can active the complement system via three distinct pathways: the classical, mannose binding lectin (MBL) and alternative pathways. The classical pathway is initiated by the binding of C1q either directly to bacteria (Alberti et al., 1993; Butko et al., 1999; Clas and Loos, 1981), indirectly via the Fc-portion of antibody (IgG or IgM) -antigen complexes on the bacterial surface or to the acute-phase reactant C reactive protein (CRP) bound to phosphorylcholine on the bacterial surface (Mold et al., 2002; Yother et al., 1982). The alternative and MBL pathways are activated by direct binding of complement
proteins to bacterial cell surface components (Walport, 2001). The activation of C3 is the convergence point of the three pathways of complement activation. C3 is cleaved into C3a and C3b, exposing an internal thiolester bond allowing the C3b to bind covalently to hydroxyl groups (carbohydrates) or amine groups (proteins) on the pathogen surface. C3b tags a pathogen as foreign and the bound C3 acts as a focus for further complement activation on and around the microbe.

Complement activation protects against infection in several ways. Firstly, complement activation leads to opsonisation of pathogens, thereby enhancing phagocytosis by macrophages and neutrophils. Secondly, activation releases the small complement cleavage fragments C3a and C5a, which act as chemoattractants to recruit more phagocytes to the site of infection and also active these phagocytes. Thirdly, formation of the membrane attack complex (C5b-9) on the surface of certain pathogens can result in their direct lysis. Finally, the complement system has an important role in augmenting the adaptive immune response (Carroll, 2004; Dempsey et al., 1996).

Several observations suggest that complement plays an important role in the pathogenesis of ascending UTI. First, production of complement proteins within the kidney is increased in response to cytokines associated with acute infection (Brooimans et al., 1991; Seelen et al., 1993). Second, most uropathogens are resistant to killing by complement suggesting evolutionary pressure on bacteria to develop resistance. Third, previous studies have shown that decomplementation by cobra venom factor (CVF) decreases the degree of tissue damage during renal infection (Glauser et al., 1978; Roberts et al., 1983; Shimamura, 1981). Furthermore, studies from our group have shown that mice deficient in C3 are resistant to ascending infection and complement can alter bacterial uptake by mouse uroepithelial cells.
(Springall et al., 2001). Recently we reported that the C3 concentration in the urine of patients rises sufficiently during infection to allow bacterial opsonisation and that opsonised \textit{E. coli} invade human uroepithelial cells via CD46, a complement regulatory protein expressed on host cell membranes (Li et al., 2006).

In the present study, we defined the mechanism by which C3 opsonises uropathogenic \textit{E. coli} in human serum and infected urine. We evaluated the role of each complement pathway, the source of urinary complement proteins and assessed how complement activation is initiated.
Materials and methods

Bacterial strains

The uropathogenic *E. coli* strain J96 (serotype O4: K6) was provided by Dr. R. Welch, (University of Wisconsin, Madison, USA). It is a serum resistant, haemolysin secreting *E. coli* strain that expresses both Type 1 and P fimbriae (O'Hanley et al., 1985). GR12 (O75: K5), a non-haemolytic human pyelonephritis isolate was provided by Dr. R. Hull (Baylor College of Medicine, Texas, USA) (Svanborg Eden et al., 1983). Bacteria were grown in 5 ml of static Luria-Bertani (LB) broth at 37°C for 16 hours to induce fimbral expression prior to use in experiments. For each experiment bacterial concentration was standardised by photospectrometry at 600nm, and colony number confirmed using serial dilutions and plating to agar plates.

Sera, complement proteins and buffers

Normal human serum (NHS) was obtained from 5 healthy volunteers. After collection, samples were pooled and stored at -70°C for up to 3 months. C1q depleted serum and purified C1q were purchased from Quidel (San Diego, USA). MBL deficient and sufficient sera were purchased from Antibody Shop (Gentofte, Denmark). Complement activation was assessed in isotonic VBS. In GVBS++ buffer (VBS with 0.1% gelatin, 0.15mM CaCl$_2$, and 1.0mM MgCl$_2$), all complement pathways are active. In Mg-EGTA-GVBS buffer (VBS with 5mM MgCl$_2$ and 8mM EGTA, pH 7.5) only the alternative complement pathway is active. EDTA-GVBS- buffer (GVBS with 10mM EDTA) inhibits all complement pathways. High ionic strength buffer (GVBS++ buffer containing 1M NaCl) inhibits the binding of C1q to immune complexes and disrupts the C1 complex (Burton et al., 1980), whereas the MBL pathway is unaffected. In contrast, buffer containing mannose (GVBS++ buffer with 100nM mannose) inhibits the MBL pathway specifically (Petersen et al., 2000).
Urine samples

Urine samples were collected from patients with *E. coli* acute lower urinary tract infection (20 samples, provided by the Department of Microbiology, Guy’s and St Thomas’ National Health Service Foundation Trust) and healthy volunteer controls (6 samples). Each urine sample was spun at 2000 rpm for 15 minutes at 4°C and passed through a 0.2 μm filter to remove cell debris and bacteria. Samples were aliquoted and immediately stored at -70°C for future analysis.

C3 and C4 deposition on *E. coli*

*E. coli* (2×10⁸ c.f.u) were washed and incubated in VBS buffers containing NHS at 37°C. In the initial experiments, *E. coli* were incubated in different serum concentrations in different complement activation buffers for the times specified. At the end of the incubation period, the bacteria were washed in EDTA-GVBS to stop further complement activation. *E. coli* were then stained with rabbit anti-human C3 (1:100 dilution) (Serotec, Oxfordshire, UK) or mouse anti-human C4d (10µg/ml, Quidel) for 1 hour at 25°C. Bacteria were washed again then stained with FITC-labelled swine anti-rabbit IgG (1:100 dilution, Serotec) or goat anti-mouse IgG (1:100, Sigma-Aldrich, Dorset, UK). After washing, bacteria were fixed with 1% paraformaldehyde and analysed by flow cytometry. The intensity of staining was analysed using CellQuest software (BD Biosciences, Cowley, UK).

In some experiments, *E. coli* J96 were added to urine with either EGTA (8mM EGTA with 5mM MgCl₂) or EDTA (10mM) and incubated at 37°C for 2 hours. Urine had been concentrated 10 fold by centrifugation (Vivaspin (molecular weight cut off 5kDa), Sartorius AG, Goettingen, Germany). At the end of incubation period, bacterial-bound complement proteins were eluted with 4mM sodium carbonate, 46mM sodium bicarbonate (pH 9.2) for 2 hours at 37°C (de Astorza et al., 2004).
Bacteria were removed by centrifugation. Eluted proteins were separated by 10% SDS-PAGE under reducing conditions and transferred to a Hybond-c Extra membrane (GE Healthcare UK Limited, Bucks, UK). The membrane was sequentially incubated with blocking buffer (phosphate buffered saline (PBS)-5% milk powder) at 4°C overnight, rabbit anti-human C3c (1/1000; Dako UK Ltd, Cambridgeshire, UK), and peroxidase-conjugated goat anti-rabbit IgG (1/5000; Dako). The membrane was then developed using the ECL system (GE Healthcare UK Limited).

*Initiation of complement activation*

Binding of human Ig (IgG, IgM & IgA), CRP, C1q and MBL to *E. coli* surface was assessed by FACS staining. *E. coli* were incubated in 5% serum for 30 minutes or incubated with infected urine for 2 hours, followed by staining with specific Abs (mouse anti-human Igs and rabbit anti-human CRP are from Sigma-Aldrich, rabbit anti-human C1q from Dako, mouse anti-MBL from Antibody Shop) followed by a FITC-labelled secondary Ab. Monoclonal Abs were used at 10µg/ml and polyclonal Abs at a dilution of 1:100. To demonstrate that C1q and CRP can directly bind to *E. coli*, purified C1q (25µg/ml, Quidel) or purified CRP (160ng/ml and 1600ng/ml, Cambridge Bioscience Ltd, Cambridge, UK) were incubated with 2×10^8 c.f.u J96 for 30 minutes followed by staining as above.

*Detection of urinary complement proteins*

Urine samples were electrophoresed on 10% (C4, and C2) or 15% (C1q) SDS-PAGE gels under reducing conditions and transferred to a Hybond-c Extra membrane. Membranes were sequentially incubated with blocking buffer at 4°C overnight and then incubated with anti-C1q, C4, and C2 polyclonal Abs (1/1000, rabbit anti-human C1q from Dako, rabbit anti-human C4 from Serotec, goat anti-human C2 from Quidel), followed by peroxidase-conjugated secondary Abs (1/5000, Dako).
Membranes were developed using the ECL system. In addition, C1q concentration was measured by ELISA. In brief, 96 well plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with mouse anti-human C1q (Quidel) diluted 1/200 in PBS. After blocking with PBS containing 1% bovine serum albumin (BSA), the plate was incubated with appropriately diluted urine samples, followed by rabbit anti-human C1q (Dako) diluted 1/3000, then peroxidase conjugated goat anti-rabbit IgG (Dako) diluted 1/5000. Each antibody incubation was performed in 100µl PBS containing 1% BSA, 0.1% Tween 20 at 37°C for 1 hour and followed by washing in PBS containing 0.1% Tween 20. The enzyme activity was assessed after incubation with O-phenylenediamine by measuring absorbance at 490nm. Purified C1q (1.0mg/ml, Quidel) was used to generate a standard curve. The limit of sensitivity of this assay was 0.25ng/ml. ELISA studies were performed in triplicate. Urinary creatinine, albumin and CRP levels were measured by standard methodology.

Statistical analysis
Data analysis was performed using the statistical analysis option in the Prism graph pad program. P values were determined by using the unpaired two-tailed Student t test on logarithmically transformed data.
Results

We have shown previously that C3 deposition on uropathogenic *E. coli* was detectable after incubation in serum concentrations as low as 1%, and was maximal in serum concentrations above 10% (Li *et al.*, 2006). To define which complement activation pathway has the major role in opsonisation of *E. coli*, we further assessed the relative contribution of each complement activation pathway.

*The contribution of the alternative pathway to C3 opsonisation*

Mg-EGTA buffer was used to dissect whether the alternative pathway or classical/MBL pathways were responsible for bacterial opsonisation. Mg-EGTA buffer disrupts the Ca$^{2+}$-dependent C1 and MBL-MASP complexes and blocks the ligand binding of MBL, therefore inhibiting activation of the classical and MBL pathways. In this buffer the alternative pathway alone contributes to C3 opsonisation of *E. coli*. At a higher complement protein concentration (above 10% NHS) the alternative pathway predominated, accounting for 90% of total C3 deposition on J96 (Fig.1A and C). In contrast at lower protein concentrations, (from 1% to 5% NHS) the classical/MBL pathways were predominately responsible for C3 opsonisation (Fig.1A and B). To investigate whether this mechanism of C3 opsonisation was exclusive to *E. coli* J96 or was also seen in other pyelonephritic strains, C3 opsonisation of a second pyelonephritic strain, GR-12, was assessed. This strain also activated complement through the classical/MBL pathways in lower serum concentrations (Fig.1D). Therefore the relative contribution of each pathway is dependent on complement protein concentration with the activity of the alternative pathway limited to higher concentrations.

*Kinetics of C3 deposition by the alternative pathway*
Although, at higher complement protein concentrations, the alternative pathway is responsible for most of the C3 deposited on the surface of bacteria the classical/MBL pathways may be involved in initiating complement activation. We examined C3 deposition over time at different serum concentrations. As shown in Fig. 2 (A and B), C3 deposition on *E. coli* was rapid when all complement pathways were active (maximal at 5 minutes), in either lower (2%) or higher (10%) serum concentrations. C3 deposition by the alternative pathway alone was only observed at a higher serum concentration (10%) and even then maximal C3 deposition was achieved only after 30 minutes (Fig. 2B). Similar results were seen with serum concentrations up to 50% (data not shown). Therefore, the classical and/or MBL pathways are important in the initiation of complement activation at higher concentrations of complement proteins. In contrast, the alternative pathway is important in amplification to obtain maximal bacterial opsonisation.

*Classical and MBL pathway opsonisation of E. coli*

At low complement protein concentration (up to 5% NHS) pathways other than the alternative pathway were mainly responsible for complement activation. We further dissected the relative contribution of the classical and MBL pathways. To exclude an effect of the alternative pathway we measured C4 deposition on the surface of *E. coli* as a marker of complement activation. To study the contribution by the MBL pathway, the classical pathway was specifically inhibited by using either C1q depleted serum or normal serum diluted in high ionic strength buffer (buffer containing 1M NaCl) (Burton et al., 1980). C4 deposition did not occur in the either 5% C1q depleted serum or normal serum diluted in high ionic strength buffer (Fig. 3A and B), suggesting that the MBL pathway contributed little to complement opsonisation of *E. coli*. To confirm this, C4 deposition was assessed after incubation of E coli in MBL
deficient serum or normal serum diluted in mannose-containing buffer (100mM mannose). As predicted, C4 deposition was not reduced by absence or inhibition of MBL activity (Fig 3C and D). This confirms that the classical pathway plays a critical role in complement activation and bacterial opsonisation at lower complement protein concentrations.

_E. coli activates the classical pathway and fixes C3 protein in infected urine_

Our previous study demonstrated that during infection the concentrations of C3 in the urine were significantly increased (healthy controls C3: 0.5-92ng/ml, patients with infection C3: 12-14,400ng/ml) and C3 could opsonise bacteria incubated in infected urine (Li et al., 2006). C3 opsonisation of bacteria in urine is reduced in the presence of Mg-EGTA, providing direct evidence that C3 binding is due mainly to complement activation via the classical pathway (Fig. 4).

Next, we assessed whether other classical pathway components are present in the urine. The early components of the classical pathway (C1q, C4 and C2) were detected in the urine of infected patients (Fig. 5). They were not detectable in the urine of healthy volunteers. To confirm the increase concentration of complement protein in the urine during infection, the C1q concentration was measured by ELISA. Up to 3.5µg/ml of C1q (equivalent to approximately 4.4% of the normal serum concentration) was detected in the urine of patients with UTI. Because all the samples are single spot urine samples the level of C1q detected may not reflect the total amount of C1q in urine due to differences in urine flow rate. Therefore, urine creatinine concentration was used to correct for urine volume variation (Fig. 6). There was a significant increase (approximately 150 fold) in the C1q/Cr ratio during infection (median 6.8 and 976mg/mmol in control and infected urine respectively, P<0.01, Fig 6A). These complement components could be derived from local
synthesis within the urinary tract or from leakage of plasma proteins into the urine across inflamed epithelial surfaces. After correcting the C1q concentration for urinary albumin concentration an increase in C1q/albumin ratio was evident during infection (C1q/Albumin ratio median 1.52 and 20.57ng/mg in control and infected urine, P<0.02, Fig 6B). This suggests that, during infection, local synthesis contributes to the increase in C1q concentration in the urine.

*Initiation of complement activation on E. coli*

Classical pathway activation is predominantly initiated by C1q binding to the Fc portion of immune complexes (containing IgM and IgG) on the bacterial surface (Walport, 2001). To determine whether immunoglobulins in serum and infected urine could bind to *E. coli*, we incubated J96 in NHS or urine and then assessed IgG, IgM and IgA binding by FACS. In both infected urine and serum from healthy volunteers, IgG, IgM and, at a lower level, IgA antibodies are present that bind to *E. coli* J96 and therefore could potentially contribute to complement activation.

However, the classical pathway may also be activated by immunoglobulin-independent mechanisms. C1q can bind directly to some pathogens or interact with other non-immunoglobulin proteins (e.g. CRP) which bind bacteria. The potential for these mechanisms to initiate complement activation on uropathogenic *E. coli* was therefore investigated. C1q was detected bound to the bacterial surface by FACS 30 minutes after incubation with purified C1q (25μg/ml). In response to infection, CRP was detectable in the urine of concentrations up to 300ng/ml (n=6, 4-300ng/ml). This was significantly higher than seen in samples from healthy volunteers, in whom CRP was undetectable (n=4). The ability of CRP to bind to phosphocholine(PCh)–containing bacteria (mostly Gram-positive) and activate human complement is well established. Whether CRP can bind to the Gram-negative pathogen *E. coli* is still
unknown. To investigate this, *E. coli* J96 were pre-incubated with purified human CRP for 30 minutes, then deposition of CRP on the bacterial surface detected by FACS staining using rabbit anti-human CRP. As shown in Fig. 8B CRP binding to *E. coli* could be demonstrated at CRP concentrations equivalent to those seen in infected urine. Therefore, the initiation of classical complement pathway activation could be through immune complex dependent or independent mechanisms.
**Discussion**

Uropathogenic *E. coli* is the most common pathogen causing acute pyelonephritis, the severest form of UTI. However, within the urinary tract, the presence of bacteria alone is not sufficient to cause kidney infection as asymptomatic bacteruria has been shown to occur in up to 7% of adult women (Kunin and McCormack, 1968). Host factors, in particular the innate immune response, may influence whether bacterial exposure leads to infection. Complement is an important element in innate immune response and previous investigations have suggested C3 opsonisation can augment *E. coli* internalisation into uroepithelial cells, an increasingly recognised feature of bacterial infection of the renal tract. In the current study, we described how complement is activated in response to infection with uropathogen *E. coli*.

During the development of renal tract infection, *E. coli* would encounter complement at two stages. In the early stages of infection bacteria may interact with locally synthesised complement proteins within the urinary space. The kidney is an important extra-hepatic source of complement synthesis and can contribute up to 12% of the circulating pool of C3 (Tang et al., 1999b). The predominant site of C3 expression is the renal tubule, which shows both constitutive and cytokine-dependent synthesis of most complement proteins (Brooimans et al., 1991; Seelen et al., 1993). Human tubular epithelial cells secrete C3 both from the basolateral and apical surfaces and therefore C3 could be secreted into urine (Tang et al., 1999a). Increased levels of C3, both intact and activated forms, are found in the urine of patients with acute infection (Li et al., 2006; Negi et al., 2000). However, under these circumstances the concentration of complement proteins would be relatively low. The second interaction is within the interstitial compartment after bacterial invasion of the host epithelium. In this circumstance, the complement concentration may be equivalent to that in serum.
To mimic these two conditions, uropathogenic *E. coli* were incubated in different concentrations of complement proteins and the contribution of each complement activation pathway to bacterial opsonisation assessed. At higher protein concentrations (≥ 10% serum, reflecting the concentration in tissues) the alternative pathway was responsible for 90% of the C3 deposited on the bacterial surface. However, maximal activation of the alternative pathway still required the classical pathway. In its absence opsonisation was slower and therefore presumably less efficient. At lower complement protein concentrations (less than 5% serum, more representative of concentrations present in urine), *E. coli* activated complement mainly through the classical pathway. Although it has been reported that MBL can bind to the O-antigen region of LPS, a structural component of the outer membrane of Gram-negative bacteria (Selander et al., 2006; Zhao et al., 2002), the MBL pathway had little role in the opsonisation of uropathogenic *E. coli*. This was confirmed by experiments showing that either inhibition of MBL binding or use of MBL deficient serum had little effect on C4 deposition on bacteria. Our results indicate the relative contribution of each pathway is dependent on complement protein concentration. The initiation of complement activation depends mainly on the classical pathway and the major role of the alternative pathway is amplification of C3 deposition.

Similar studies have been performed on other bacterial pathogens. Cunnion et al. assessed C3 deposition on *Staphylococcus aureus* in different serum concentrations. Their results suggested the classical and alternative pathways both contribute to C3 deposition, the classical pathway predominating at a lower serum concentration (2%) and the alternative pathway at a higher serum concentration (20%) (Cunnion et al., 2001). Several studies have demonstrated a key role for the classical pathway in initiating complement opsonisation of *Streptococcus pneumoniae*. The major role of
the alternative pathway being amplification of complement activation leading to greater C3 deposition on the bacteria (Brown et al., 2002; Xu et al., 2001).

In our previous report, we demonstrated that C3 from infected urine could bind to *E. coli*. In the present study we have shown that, as would be predicted from in vitro studies, this binding is due mainly to classical pathway activation, as calcium chelation reduced C3 binding to *E. coli*. Secondly, we demonstrated that early components of the classical pathway components (C1q, C4 and C2) are present in the infected urine and their concentrations are high enough to allow activation to occur. Thirdly, we determined that local synthesis contributes to the increased concentration of complement proteins in urine. Finally, we found that the classical pathway can be activated via several routes. Antibodies against uropathogenic bacteria are present in the infected urine and could initiate complement activation. Other possible mechanisms by which bacteria could activate the classical pathway is through either direct binding of C1q to bacteria or by interaction with the acute-phase protein, CRP, bound to bacteria.

The consequence of bacterial opsonisation within the urinary space is still unclear. It could enhance phagocytosis by infiltrating neutrophils leading to the elimination of infection. However, bacteria continue to develop strategies to avoid, or even subvert, the host immune response. It has been demonstrated that C3 opsonisation markedly increases the ability of uropathogenic *E. coli* to internalise into human uroepithelial cells, perhaps as a means of evading neutrophil-mediated phagocytosis (Li et al., 2006). In addition, internalised bacteria may either proliferate leading to host cell death or remain quiescent inside vacuoles, reactivating at a later stage to cause recurrent infection (Schilling et al., 2002). In the balance between microbe and host,
the susceptibility of an individual to UTI may depend on the levels of urine C3 and/or cell surface complement receptors.

In summary, we have demonstrated that the complement classical pathway is important for efficient opsonisation of pathogenic *E. coli* and hypothesise that complement opsonisation plays a critical role during the first encounter of the pathogen with the host epithelium.

**Acknowledgements**

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References


Figure Legends

Figure 1. The contribution of the alternative pathway to C3 opsonisation

A) Flow cytometry data for C3 deposition on *E. coli* J96 after incubation in different serum concentrations for 30 minutes with all complement pathways active (black line), with only the alternative pathway active (interrupted line) and in the absence of complement activation (grey line). Each data point represents the mean of 4 assays (+/- SEM). (B and C) Representative examples of flow cytometry data for bacteria incubated with different sera concentrations (B 2.5%, C 10%). All pathways active (black line); the alternative pathway only active (interrupted line); no complement activation (grey line). (D). The *E. coli* pyelonephritis isolate GR-12 was incubated in different serum concentrations. Each data point represents the mean of 4 assays (+/- SEM). Lines are the same as panel A.

Figure 2. Time dependent C3 deposition on *E. coli*

C3 deposition on *E. coli* J96 over time in 2% (A) and 10% serum (B) with all complement pathways active, the alternative pathway only or no activation. When all pathways were active maximal opsonisation was achieved by 5 minutes. In 2% serum the alternative pathway alone was unable to opsonise bacteria. In 10% serum alternative pathway mediated opsonisation occurred but more slowly. Each data point represents the mean of four independent experiments. Error bars represent SEM.

Figure 3. Dissection of the contribution of the classical and MBL pathways

Representative flow cytometry data for C4 staining on *E. coli* J96 incubated at 37°c for 20 minutes with; (A) C1q depleted serum; (B) normal human serum diluted in
buffer containing 1M NaCl; (C) MBL depleted serum; (D) normal serum diluted in buffer containing 100mM mannose. The black line represents the C4 deposition in standard serum conditions. The interrupted line represents either the complement depleted/deficient serum or normal serum diluted in pathway inhibition buffers as stated in the title of each panel. The grey line represents an assay in which the primary antibody was omitted.

**Figure 4. E. coli J96 fixes C3 protein in infected urine**

C3 fragments deposited on J96 after incubation with the urine (concentrated 10 fold) of a patient with UTI were detected by C3 Western blot analysis. Purified C3b (0.2µg) was ran in parallel (lane 1). E. coli J96 were incubated with urine alone (lane 2); urine in the presence of Mg-EGTA (lane 3); in the presence of EDTA (lane 4). The presence of C3b is indicated by 105 kDa (α’ chain) and 75 kDa (β chain) bands, iC3b by 75 kDa (β chain), 67 kDa (α1 chain), and 40 kDa (α2 chain) bands.

**Figure 5. Identification of C1q, C2 and C4 in urine of patients with UTI**

The presence of classical pathway components C1q, C2 and C4 in the urine of a patient with UTI. (A) Three sub-units of C1q (A 29kDa, B 26kDa, and C 22 kDa) were all detected in infected urine samples (1:5 dilution). (B) The single chain of C2 was present in infected urine (1:10 dilution). (C) Intact C4 (α, β and γ chains, 95,75 and 33 kDa respectively) were present in infected urine (1:5 dilution). Human serum (1:200 dilution) was used as a positive control.

**Figure 6. Measurement of C1q concentrations in urine**
Urine C1q concentration was determined by ELISA. The urine C1q concentration was corrected for urinary creatinine (A) or urinary albumin (B). C1q in urine from acute UTI patients (n=20) were compared with the level in control, non-infected, urine samples (n=6).

**Figure 7. Detection IgM, IgG and IgA binding to E. coli**

The ability of immunoglobulins to bind to uropathogenic E. coli was determined by FACS staining. IgG, IgM and IgA binding was assessed after incubating E. coli with 5% normal serum (left panel). The solid line represents the Ig deposition. The interrupted line represents bacteria stained with detection antibody only as control. The right panel shows immunoglobulin binding after incubation with infected urine (solid black line) or control urine (interrupted black line). The interrupted gray line represents bacteria stained with detection antibody only as control. The examples are representative of at least 3 independent experiments.

**Figure 8. Detection the direct binding of C1q and CRP to E. coli**

C1q and CRP binding to E. coli J96 after incubation with purified C1q (25µg/ml) (A) or CRP (160ng/ml and 1600ng/ml) (B) for 30 minutes. The solid line represents the C1q or CRP deposition. The interrupted line represents an assay in which the primary antibody was omitted.
Figure 1. The contribution of the alternative pathway to C3 opsonisation

A

B

C

D

Figure 1
Figure 2. Time dependent C3 deposition on E. coli

A

C3 binding (MFI)

Minutes

B

C3 binding (MFI)

Minutes

- All pathways
- Alternative pathway only
- No activation
Figure 3. Dissection of the contribution of the classical and MBL pathways
Figure 4. *E.coli* activates the classical pathway in infected urine.
Figure 5. Identification of the classical pathway components in urine
Figure 6. Measurement of C1q concentrations in urine

A

B

C1q/Cr (mg/mmol)

C1q/Albumin (ng/mg)

UTI patients Healthy Controls

UTI patients Healthy Controls
**Figure 7.** Detection of human Ig on opsonised E.coli
**Figure 8.** C1q and CRP could bind directly to E. coli