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ORIGINAL PAPER

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# Neutrophil bactericidal function is defective in patients with recurrent urinary tract infections

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**Abstract** Urinary tract infection (UTI), most frequently caused by *Escherichia coli*, is one of the most common bacterial infections in humans. It is a host inflammatory response to bacterial invasion in which large numbers of neutrophils are recruited into the bladder in response to cytokines secreted by the infected bladder epithelium. We hypothesise that an impaired host neutrophil response leads to susceptibility to recurrent UTI (rUTI). Therefore, the neutrophil inflammatory response of patients with a history of rUTI was compared to healthy controls. No difference in neutrophil adhesion receptor expression or complement receptor expression following bacterial stimulus was detected between the two groups. However, the expression of the IgG receptor CD16, bacterial phagocytosis and reactive oxygen intermediate (ROI) production were significantly reduced in UTI patient neutrophils compared to controls. Neutrophils from rUTI patients have a significantly reduced bactericidal function when compared to healthy controls, with the former showing a reduced capacity for activation. This reduced neutrophil function may result in defective bacterial clearance and lead to susceptibility to recurrent infection.

**Keywords** Neutrophil · Respiratory burst · Phagocytosis

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## Introduction

Recurrent urinary tract infection (UTI) in otherwise healthy people remains an exceedingly common and poorly understood problem. Worldwide, an estimated 150 million people are diagnosed with this infection each year [1]. Studies suggest that 20–40% of women who experience an initial infection will develop recurrent infections [4, 19]. Susceptibility to recurrence appears to be related to intrinsic host-bacterial interactions, and it has been proposed that women with recurrent infection have a predisposition to UTI [23, 24, 26]. Uropathogenic *Escherichia coli* (UPEC) is the causative agent in 70–95% of community acquired UTIs [29]. Adhesion of these bacteria to the urinary tract epithelium triggers an innate immune response characterised by the production interleukin 8 (IL-8) and other chemokines, which leads to neutrophil (PMN) recruitment into the bladder [14]. Indeed, pyuria, the presence of neutrophils in the urine, is a hallmark of UTI. Neutrophil recruitment is the pivotal pathophysiological event underlying the tissue damage and renal scarring associated with UTI [30]. The ability to mount such a response is, however, vital to the body's defences against infection, and is fundamental to the repair process.

Neutrophils dominate the inflammatory infiltrate of the urinary tract and infiltration of the urothelium and bladder lumen by neutrophils has been demonstrated as early as 6 h post transurethral inoculation with UPEC [21]. Neutrophil-dependent "innate" defence mechanisms are more important than specific immunity for resistance to UTI [9]. Mice with genetic defects in specific immunity mediated by T and B lymphocytes are as resistant to UTI as immunocompetent animals; however, mice with a neutrophil recruitment deficiency are highly susceptible [31]. Normal mice treated with monoclonal antibodies that specifically block neutrophil recruitment showed a significant reduction in bacterial clearance from the urinary tract. In the absence of neutrophils, other antibacterial defence mechanisms

were inefficient in killing the bacterial inoculum [13]. Therefore, susceptibility to recurrent infection may, in part, be due to impaired neutrophil function. The potential areas of this defective function may be grouped into three distinct processes: (1) chemotaxis and extravasation mediated by cell surface adhesion receptors, (2) recognition and phagocytosis of bacteria, and (3) bactericidal killing by reactive oxygen intermediate (ROI) production.

In the circulating state, neutrophils travel at 1,000 $\mu$m/s in the centre of the lumen post capillary venule and the extravasation of neutrophils under physiological sheer stress is a sequential process involving multiple specific molecular interactions [3]. Following a chemotactic signal, the first step in adhesion is transition to the rolling state in which neutrophils roll along the endothelium of the vessel wall at a speed of 30 $\mu$m/s facilitated by lectin-carbohydrate interactions of the selectin family of adhesion receptors [12], which include L selectin (CD62L), the ligand for P selectin (CD62P) and Sialyl Lewis×(CD15), the ligand for E selectin (CD62E) on the endothelial cell [16]. L-selectin is responsible for the initial capture of neutrophils, and is shed following activation and the up-regulation of CD11b on the neutrophil [17, 34]. LFA-1, /CD11a, MAC-1/CD11B and P150/95/CD11C are members of the integrin-type cellular adhesion molecule (CAM) family which mediate neutrophil adhesion to endothelium and facilitate transmigration in association with their ligand ICAM-1 on endothelial cells [20]. Thus, CAMs play a key role in the inflammatory processes and expression of these receptors has been shown to reflect the activation state of the neutrophils [6].

Neutrophils mediate the immune adherence phenomenon, a fundamental event for effecting the internalisation and killing of opsonised bacteria by the complement receptors CD35/CR1 and CD11b/CR3 which identify C3b and C3bi on the surface of the opsonised microbe [22]. The key receptors for inducing neutrophil phagocytosis of captured bacteria are those for the Fc region of immunoglobulin G (IgG) Fc$\gamma$RIII/CD16 and Fc$\gamma$RII/CD32 [7]. Finally, a PMN respiratory burst is triggered upon phagocytosis and results in the production of microbicidal ROI such as superoxide, hydrogen peroxide and the most powerful antimicrobial agent, hypochlorous acid [8].

We hypothesized that an impaired host neutrophil response leads to susceptibility to recurrent UTI (rUTI). To test this hypothesis we compared neutrophil bactericidal function from patients with a history of rUTI to healthy control volunteers.

## Materials and methods

The present study was conducted with the approval of the local Ethics Committee and the informed consent of all participants. Blood was collected in heparinised tubes from otherwise healthy premenopausal female patients ($n=22$, age 31 $\pm 2.2$ years) with a history of recurrent UTI infections attending urology out patient clinics, and from age matched healthy female controls ($n=10$; age $28 \pm 1.6$ years) by venupuncture. Blood was analysed within 1 h of venesection.

### Reagents

Orpengen BurstTest and PhagoTest, FACS lysing solution, dihydrorhodamine 123, *E. coli* and falcon tubes, fluorescent labelled Pharmingen antibodies, fluorescent isothiocyanate (FITC) or phycoerithin (PE) to CD11a PE, CD11bPE, CD11cPE, CD14PE, CD15PE, CD16 PE, CD32 FITC, CD35 FITC, CD62L PE were obtained from Becton Dickinson (Mountain View, Calif. USA)

### Flow cytometry

Blood cells were acquired on a FACScan (Becton Dickinson) flow cytometer equipped with an argon laser excitation wavelength of $\lambda_{ex}=488$ nm. Fluorescence signals at $\lambda_{em}=520$ nm (green fluorescence) and 580 nm (orange fluorescence) together with forward light scatter (FLS) and side light scatter (SLS) were recorded. A total of 10,000 events were recorded per sample. The data were registered on a logarithmic scale and analysed on CellQuest software. The instrument was calibrated daily. Neutrophils were gated on a dual parameter dot plot of FLS and SLS and this region was selected for analysis.

### Reactive oxygen intermediate generation

The intracellular ROI generation by PMN was assessed in lysed whole blood using the BurstTest kit. Briefly, 100 $\mu$l of blood was incubated with 20 $\mu$l of $1\times10^9$ human oponised *E. coli* at 37°C for 10 min. The fluorogenic probe dihydrorhodamine 123 was added for a further 10 min incubation at 37°C. Red blood cells were hypotonically lysed for 20 min and the samples centrifuged, washed and analysed flow cytometrically with $\lambda_{em}=520$ nm. ROI production is expressed as mean channel fluorescence emitted by the cells.

### Phagocytosis

Bacterial phagocytosis was assayed in whole blood using the PhagoTest kit. Briefly, 100 $\mu$l of blood was incubated with 20 $\mu$l of $1\times10^9$ fluorescently labelled *E. coli* at 37°C for 10 min. Non-cell associated bacterial fluorescence was quenched and the samples were washed. Red blood cells were lysed for 20 min and samples were centrifuged, washed and analysed flow cytometrically with $\lambda_{em}=520$ nm. Phagocytosis was expressed as the mean channel fluorescence (MCF) emitted by the cells which is proportional to the number of phagocytosed bacteria.

### Cell surface receptor expression

Receptor expression was assayed in whole blood by staining with fluorescently labelled antibodies. Briefly 100 $\mu$l of blood was stimulated by 20 $\mu$l of $1\times10^9$ *E. coli* at 37°C for 10 min. A baseline sample without *E. coli* and a negative control sample were also incubated at 37°C for 10 min. A total of 20 $\mu$l of PE or FITC labelled antibody was then added to the baseline and *E. coli* treated samples and 20 $\mu$l of PE or FITC labelled negative control antibody was added to the negative control sample. Samples were incubated at room temperature for a further 20 min in the dark. Red blood cells were lysed for 20 min and samples were centrifuged, washed and analysed by flow cytometry with emission $\lambda=520$ nm for FITC and 580 nm for PE using CellQuest. Receptor density on the cell surface was expressed as mean channel fluorescence intensity of the cells.

### Statistics

Data are expressed as mean ± SEM. Statistical differences between groups were determined using the *t*-test, where a *P* value of $< 0.05$ was taken as a significant. SPSS software (version 11) was used.

## Results

### Percentage of neutrophils in lysed whole blood

The neutrophil population from lysed whole blood was gated on the forward scatter and side scatter dot plot. UTI patients had significantly lower percentages of neutrophils present in their venous blood than healthy controls (62% ± 1.9 vs 55% ± 2.2, $P < 0.05$, *t*-test) (Fig. 1).

### Neutrophil phagocytosis of opsonised *E. coli*

The capacity of neutrophils to phagocytose opsonised bacteria was measured by incubating them with fluorescently labelled *E. coli* for 10 min. The amount of cell-associated bacteria is proportional to the fluorescence emitted by the neutrophil. Neutrophils from UTI patients had significantly impaired phagocytosis when compared to healthy controls (602 ± 48.5 vs 861 ± 144.6, $P < 0.05$) (Fig. 2).

### Neutrophil bactericidal capacity

Neutrophil respiratory burst was measured to assess cellular bactericidal capacity in unstimulated cells (baseline) and post *E. coli* ($1 \times 10^9$) stimulation for 10 min at 37°C. UTI patients had a significantly higher production of ROI by unstimulated neutrophils when compared to controls (17 ± 1.6 vs 9 ± 1.8, $P < 0.0001$) (Fig. 3a). However the ability of UTI patient neutrophils to produce ROI in response to an *E. coli* challenge was significantly impaired compared to healthy controls (334 ± 37.2 vs 485 ± 50.1, $P < 0.05$) (Fig. 3b).

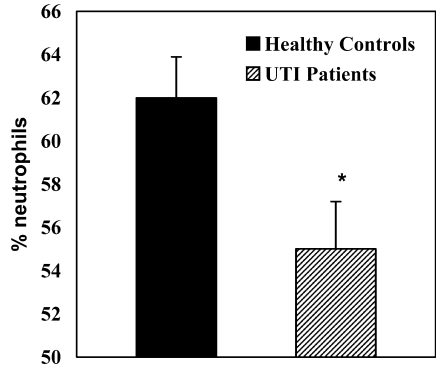


**Fig. 1** Percentage neutrophils in the circulation. Neutrophils were gated on a dual parameter dot plot of forward light scatter and side light scatter. Neutrophils are expressed as a percentage of lysed white blood cells. An *asterisk* denotes a significant difference from the control ($n = 10$ healthy volunteers)

### Cell surface adhesion receptor expression

There were no significant differences between control and UTI expression of CD11a baseline or post *E. coli* stimulation (186 ± 20.5 control baseline vs 189 ± 15.6 UTI baseline, $P = 0.3$ and 227 ± 29.7 control *E. coli* vs 222 ± 21.6 UTI *E. coli*, $P = 0.12$). There was a

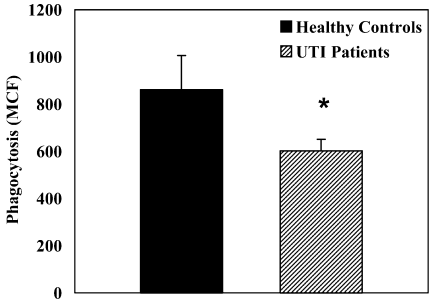


**Fig. 2** PMN bactericidal capacity. Whole blood was incubated with fluorescent *Escherichia coli* for 10 min at 37°C. The data are recorded as log mean channel fluorescence and expressed as mean ± SEM. An *asterisk* denotes a significant difference from the control ($n = 10$ healthy volunteers). PMN phagocytosis of *E. coli* is significantly reduced in the UTI patient group ($n = 22$)

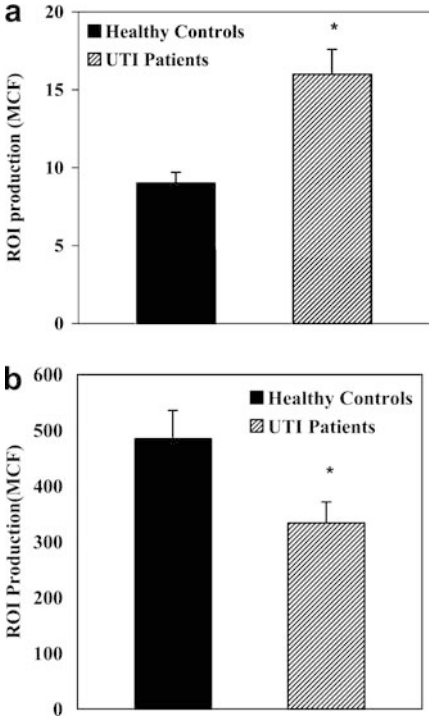


**Fig. 3** Neutrophil reactive oxygen intermediate (ROI) production. (**a**) Neutrophil ROI production at baseline is significantly upregulated in the UTI patient group compared to controls. (**b**) However, UTI patient ROI production in response to *E. coli* is impaired. An *asterisk* denotes a significant difference from the control

significantly higher baseline expression of CD11b on the neutrophils of UTI patients compared to the control group (437 ± 61.3 vs 238 ± 47.9, $P < 0.05$). However, both groups of neutrophils responded to an *E. coli* challenge with significant upregulation of the CD11b receptor to similar levels (1,773 ± 206.3 vs 1,595 ± 344.0, $P = 0.6$) (Fig. 4a). There was a significantly higher baseline expression of CD11c on neutrophils of UTI patients compared to the control group (38 ± 6.9 vs 68 ± 10.6 $P < 0.05$). Again both groups of neutrophils responded to an *E. coli* challenge with an upregulation of the CD11c receptor to a similar level (210 ± 42.9 vs 185 ± 23.7 $P = 0.62$) (Fig. 4b).

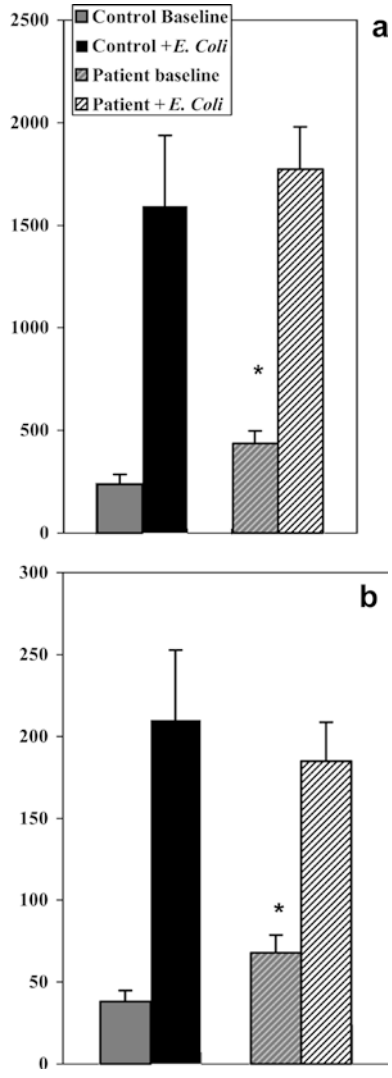


**Fig. 4** PMN cell surface adhesion receptor expression. Expression of surface receptors was determined by direct immunofluorescence on flow cytometry. The data are recorded as log mean channel fluorescence and expressed as mean ± SEM. An *asterisk* denotes a significant difference from control ($n = 10$ healthy volunteers). PMN surface expression of the CD11b adhesion receptor was significantly upregulated at the baseline in UTI patients compared to control PMN (**a**). Both groups of PMN responded to the *E. coli* challenge with an upregulation of expression. PMN surface expression of the CD11c adhesion receptor was also significantly upregulated at baseline in UTI patients compared to control PMN (**b**). Both groups of PMN responded to the *E. coli* challenge with an upregulation of expression

## Bacterial phagocytosis receptors

We found no difference in Fc$\gamma$RII CD32 receptor expression at baseline or following stimulus between control neutrophils and those of UTI patients (baseline: 146 ± 13.8 control vs 143 ± 14.8, $P =$ 0.6; *E. coli*: 159 ± 10.8 vs 159 ± 13.2, $P = 0.9$). There was a large interpersonal difference in the expression of Fc$\gamma$RIII CD16 in both groups at baseline (CD16: 2,975 ± 269.9 control vs 3,431 ± 373.0 UTI, $P = 0.4$) and stimulation with *E. coli* upregulated CD16 expression for both groups (3,915 ± 303.9 vs 3,874 ± 389.6, $P = 0.9$). However, when the percentage change from baseline to stimulated was analysed, the UTI patients had a reduced response to *E. coli* (17 ± 8% vs 44 ± 11% $P < 0.05$) (Fig. 5).

## Complement receptors

There was a significantly higher expression of CD 35 by UTI neutrophils compared to control at baseline (46 ± 10.6 vs 21 ± 0.3, $P < 0.05$). Stimulation with *E. coli* resulted in a similar CD35 expression by both groups (124 ± 13.0 vs 113 ± 21.0 $P = 0.6$) (Fig. 6).

## Selectins

There was no difference between control or UTI neutrophils selectin expression before or following stimulus (baseline CD15: 98 ± 15.4 UTI vs 58 ± 12.3 control, $P = 0.1$, *E. coli*: 267 ± 159.1 UTI vs 100 ± 24.6 UTI, $P = 0.3$) (baseline CD 62L: 421. ± 43.9 UTI vs 364 ± 54.5 control, $P = 0.1$, *E. coli*: 43 ± 6.2 UTI vs 36 ± 10.8 UTI, $P = 0.6$).

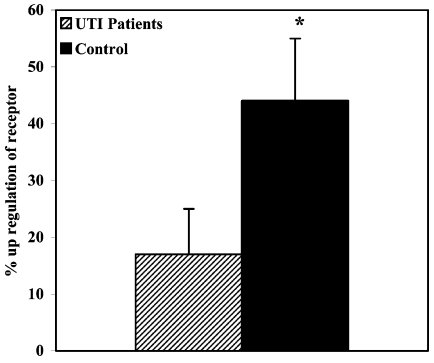


**Fig. 5** Cell surface FC$\gamma$R111 receptor expression. Expression of cell surface CD16 was determined by direct immunofluorescence on flow cytometry. The data are recorded as log mean channel fluorescence and expressed as mean percentage up regulation from control ± SEM. An *asterisk* denotes a significant difference from the control

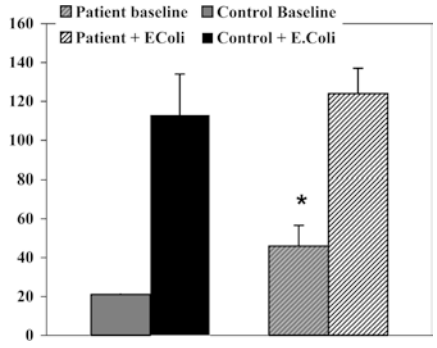


**Fig. 6** Cell surface CD 35 complement receptor expression. CD 35 expression was assessed by flow cytometry. The data are recorded as log mean channel fluorescence and expressed as mean ± SEM. An *asterisk* denotes a significant difference from the control

## Discussion

The neutrophil is a potent pro-inflammatory cell and its phagocytic and cytotoxic activities are fundamental to host defence. Defects in this acute immune response have previously been shown to render animals hypersusceptible to experimental UTI. [20]. Much work has focused on neutrophil function in the urine in which harsh environmental conditions due to hyperosmolarity and high pH result in defective neutrophil function [10, 11, 25]. However, from a clinical perspective these conditions do not appear to distinguish between patients who are susceptible and those who are resistant to recurrent infection. To date, little is known about host immune status and susceptibility to rUTI, although recent in vivo experimental results indicate that an underlying defect in neutrophil function may predispose to rUTI. In this study we found that neutrophil cell motility, as assessed by adhesion receptor expression in UTI patients, is comparable to that of controls. This is not unexpected as the presence of neutrophils in the urine and the bladder tissue is a hallmark of infection. However, this study does demonstrate that the bactericidal function of neutrophils from patients with recurrent UTI is significantly impaired when compared to healthy controls. We found that neutrophils isolated from patients with a history of recurrent infection had a significantly reduced ability to phagocytose bacteria and to produce the toxic arsenal of ROI necessary to kill ingested bacteria. Our data supports a previous study that reported suppressed, rather than increased, PMN function in rUTI patients during infection when compared to healthy controls [32].

Many authors have proposed a genetic predisposition to rUTI [23, 24, 28]. It is known that women who suffer rUTIs are more likely to be non-secretors of the Lewis blood group antigen sIgA, rendering epithelial surfaces susceptible to bacterial colonisation [27]. Genetic factors may also be responsible for the reduced ability of UTI neutrophils to effectively phagocytose and kill bacteria. Efficient killing of bacteria is dependant on opsonisation of the bacteria and subsequent recognition of these opsonins by the phagocytic cell. Phagocytosis of immunoglobulin G (IgG)-opsonized bacteria via IgG Fc receptors (Fc gamma R) on PMN constitutes a central defence mechanism in innate immunity. Fc gamma RIIIb is the most abundantly expressed Fc gamma R on PMN. Functional polymorphisms exist for Fc gamma RIIIb, which result in different levels of phagocytosis of IgG2-opsonised encapsulated bacteria. These have been implicated in susceptibility to infection with encapsulated bacteria [18, 28, 33]. Our study indicates a reduced neutrophil Fc gamma RIIIb response to *E. coli* in UTI patients, which could account for the observed reduction in phagocytosis and ROI production. Further investigation into functional polymorphism of this receptor in patients with rUTI may yield insights into disease susceptibility and pathogenesis.

Our study also shows that cell surface expression of the complement receptor CD35, the cell adhesion receptors CD11b and CD11c and ROI production was significantly higher in unstimulated UTI PMN compared to controls, suggesting that the PMN of UTI patients are primed in the circulation. However, these cells demonstrated impaired anti-microbial function when stimulated which may suggest that they are somehow tolerised and are incapable of responding fully to subsequent bacterial challenge. We previously identified two distinct patterns of neutrophil response to surgery, which are dependent on the pre-operative activation state of this cell. Patients with neutrophils that are activated prior to a surgical insult fail to respond to the injury as competently as patients whose cells are not activated prior to surgery [2].

This study demonstrates that, rather than environmentally stimulated neutrophil dysfunction underlying the etiology of rUTI, the pathogenesis of this disease is mediated by an intrinsic defect in neutrophil bactericidal action. Identification of at risk groups will create an opportunity for the therapeutic manipulation and augmentation of neutrophil function. We have previously demonstrated that taurine, the most abundant amino acid in the cytosol of neutrophils, plays an important physiological role in the intracellular signalling of this cell by upregulating ROI activity and preventing cell death [5, 35, 36]. Currently, we are investigating the potential therapeutic role of this amino acid in rUTI.

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