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Uropathogenic *Escherichia coli*-induced neutrophil adhesion to urinary epithelium is strain-specific and mediated by CD11b/CD18

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Abstract Adherence of *Escherichia coli* to urinary tract epithelium induces neutrophil migration across the uroepithelium to combat bacterial infection. Neutrophil adherence to the apical membrane of uroepithelial cells may be an important factor for bacterial clearance. We used an in vitro model of urinary tract infection to examine the effects of uropathogenic *E. coli* on neutrophil adhesion to the uroepithelial cell line RT4. We found that distinct clinical isolates caused different levels of neutrophil adherence. One particular isolate caused significant neutrophil adhesion in a dose- and time-dependent manner. The neutrophil adhesion-promoting effect induced by this isolate was not caused by bacterial secreted products, suggesting that contact between intact *E. coli* and uroepithelial cells is required for promoting neutrophil adhesion. This adhesion was almost exclusively mediated by CD11b/CD18, suggesting that *E. coli* upregulates CD11b/CD18 counterligands on the uroepithelial surface. These data suggest that certain uropathogenic *E. coli* selectively promote adhesion of neutrophils to ligands on uroepithelial cells by a CD11b/CD18-dependent mechanism.

Key words *Escherichia coli* · Neutrophil · Adhesion · Urinary tract infection · Epithelium

Introduction

Escherichia coli is the most commonly isolated microorganism from human urinary tract infections (UTIs). The pathogenesis of UTI involves localisation of bacteria to the infection site, induction of localised inflammation, and systemic responses. During UTIs, neutrophils traverse the urinary epithelium in response to chemoattractants released by the uroepithelium [2,11] and enter the urine. The mechanisms by which neutrophils adhere to and traverse the vascular endothelium has been extensively studied and is well understood [24], but there are comparatively few data on neutrophil adhesion to epithelium. Such studies have focused on neutrophil interactions with intestinal epithelium [19] and respiratory epithelium [4,23], and data on the regulation of neutrophil migration through uroepithelium during the pathogenesis of *E. coli* infection has been recently generated [3,11]. It appears that neutrophils are essential for bacterial clearance from *E. coli* urinary tract infections [12], and neutrophil adherence to the apical membrane of uroepithelial cells might be an important factor in neutrophil-mediated clearance. However, it is not clear to what extent neutrophils adhere to the luminal uroepithelial surface following transepithelial migration.

In this study we used an in vitro model of urinary tract infection to examine the effects of several uropathogenic *E.coli* isolates on the neutrophil adhesive properties of uroepithelial cells. In addition, we examined the role of neutrophil-expressed β_2 integrins in neutrophil-uroepithelial adhesion.

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Materials and methods

Cell lines and culture

The well-differentiated and phenotypically transitional urinary bladder epithelial cell line RT4 [21] was purchased from the European Collection of Animal Cell Cultures, ECACC 91091914 (Porton Down, U.K.) and routinely cultured in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 25 mg/ml streptomycin, and 10% foetal calf serum (all from Sigma, Poole, U.K.) in 75-cm^2 tissue culture flasks (Corning Costar, Acton, Mass., USA) and incubated at 37 °C with 5% CO_2. Cells were passaged using 0.05% trypsin and 0.02% ethylenediaminetetra-acetate (EDTA) (Sigma) after washing

twice with calcium and magnesium-free Hank's balanced salt solution (HBSS). Experiments were performed in 96-well, flat-based plates (Corning Costar) seeded with 1×10^5 RT4 cells/well and incubated overnight with antibiotic-free tissue culture medium at 37 °C in a 5% CO_2 atmosphere to obtain a confluent monolayer.

Escherichia coli

Clinical *E. coli* isolates were anonymously obtained from urine specimens of adult patients with symptomatic urinary tract infection at the Royal Hallamshire Hospital, Sheffield, UK. All isolates were derived from specimens associated with microscopic pyuria (> 10 white blood cells per high power field). Isolates were originally obtained as pure growths of $\geq 10^5$ organisms/ml and routinely cultured in brain-heart infusion broth (Oxoid, Basingstoke, U.K.) at 37 °C, and aliquots were stored at −70 °C until use. Viable counts were performed using standard serial dilution techniques.

Isolation of peripheral blood neutrophils

Venous blood donated by healthy volunteers was anticoagulated with 20 IU/ml of preservative-free heparin (Sigma), layered onto endotoxin-free Histopaque-1077 (Sigma), and centrifuged at 400 *g* for 30 min. Plasma, mononuclear cell layer, and separation medium were removed and the erythrocyte/neutrophil-rich sediment transferred to a fresh tube. Erythrocytes were lysed using two 7-min incubations with a solution of endotoxin-free 0.832% ammonium chloride and 0.084% sodium bicarbonate, followed by centrifugation at 160 *g* for 8 min. The neutrophil-containing pellet was washed with endotoxin-free phosphate-buffered saline (PBS) (Biowhittaker, Walkerville, Md., USA) and resuspended at 2.5×10^6 cells/ml in serum-free, antibiotic-free DMEM. The resultant cell suspension contained > 93% neutrophils, as determined by direct microscopy of fixed cells stained with Wright's Giemsa stain, and was typically > 95% viable, as determined by propidium iodide exclusion using fluorescence microscopy.

Neutrophil adhesion assay

Prior to each experiment, *E. coli* aliquots were thawed, washed in endotoxin-free PBS, and resuspended in DMEM. RT4 cells cultured in 96-well plates were washed once with DMEM, and 10^8 colony-forming units per ml (CFU/ml) of *E. coli* were added and incubated at 37 °C with 5% CO_2 for up to 20 h. After two washes with calcium and magnesium-free HBSS, 2.5×10^5 neutrophils in 100 µl DMEM were added to each well and incubated for 10 min. Neutrophils were then activated using formyl-methionyl-leucyl-phenylalanine (fMLP) (Sigma) at a final concentration of 1 µM and incubated for a further 20 min. The plates were then inverted and washed gently three times to remove nonadherent neutrophils. Cells were fixed with 4% formaldehyde for 10 min, then washed once with PBS and left to air-dry. Frequent microscopic checks were carried out during the washing period to assess the effectiveness of the washing and confirm the structural integrity of the monolayer. Wells were stained with 5 µg/ml Hoechst 33258 (Sigma) for 5 min at room temperature and the number of adherent neutrophils per high power field (×40) counted on an inverted fluorescence microscope. For neutrophil-adhesion blocking studies, mouse antihuman monoclonal antibodies raised against CD11a (clone TS1/22, ATCC), CD11b (clone MY904, ATCC), and CD18 (clone TS1/18, ATCC) (Endogen, Cambridge, Mass., USA) were used. An isotype-matched mouse monoclonal antibody directed against *Aspergillus niger* glucose oxidase (clone DAK-G01) (Dako, Cambridge, U.K.) was used as a negative control.

Cell-free conditioned medium production

E. coli (10^8 CFU/ml) were grown in antibiotic-free DMEM at 37 °C in a 5% CO_2 atmosphere for 20 h. Bacteria were then removed by passing the conditioned medium through a 0.2 µM filter. The resultant supernatant was stored at −20 °C until required. For *E. coli*-stimulated RT4 cell conditioned medium, 10^8 CFU/ml *E. coli* were incubated with cultured monolayers of RT4 cells in antibiotic-free DMEM at 37 °C with 5% CO_2 for 20 h. The conditioned medium was then removed, passed through a 0.2-µM filter, and stored at −20 °C.

Virulence factor profiles

Genotypes for 24 putative virulence genes of extraintestinal *E. coli*, including the three alleles of the P fimbrial adhesin gene *papG*, were determined by multiplex polymerase chain reaction (PCR) as previously described [16]. Strains positive for *pap* were tested for 11 alleles of the P fimbrial structural subunit gene *papA* by multiplex PCR as previously described [18]. Mannose-resistant hemagglutination of human A_1P_1 erythrocytes, with pigeon egg white as a specific inhibitor of P fimbriae, was used to detect expression of mannose-resistant adhesins after overnight growth on agar plates at 37 °C and to differentiate between P and non-P adhesins as previously described [17]. Mannose-sensitive agglutination of *Saccharomyces cerevisiae* cells was used to detect expression of mannose-sensitive adhesins after overnight growth of bacteria in static broth at 37 °C as previously described [15].

Statistical analysis

Data were analysed using the Kruskal-Wallis test (nonparametric one-way analysis of variance) for significant differences within experiments, and two-group comparisons were made with Mann-Whitney *U* tests. A Bonferroni-type correction was applied to final *P* values to compensate for multiple comparisons. Differences were deemed significant if $P \leq 0.05$ after corrections.

Results

Neutrophil-uroepithelial adhesion mediated by uropathogenic *E. coli* is dose- and time-dependent

To investigate whether uropathogenic *E. coli* induce neutrophil adherence to uroepithelial cells, neutrophil adhesion assays were conducted with cultured RT4 epithelial cells which had been prestimulated for 20 h with clinical isolates of uropathogenic *E. coli*. There were marked and consistent differences in the neutrophil adhesion caused by the six uropathogenic *E. coli* isolates tested ($P < 0.001$). Isolates 5 and 6 caused only weak neutrophil adherence, while isolates 1 to 3 showed no *E. coli*-mediated neutrophil adherence. Isolate 4 caused significantly greater adhesion than any of the other strains (all $P < 0.01$) (Fig. 1). The effect of isolate 4 on neutrophil adhesion was dose-dependent over a bacterial concentration range of 10^2–10^9 CFU/ml (Fig. 2), with maximal adherence occurring at 10^8 CFU/ml. The adhesion-promoting effect was also time-dependent, with progressive adherence being seen from 5 h of bacterial incubation onwards and maximal adhesion occurring after 20 h (data not shown). The isolate-dependent differences seen in Fig. 1 were not due to differences in the proliferation rates of individual isolates, since no significant differences were observed between the rates of change in viable counts over 20 h for all isolates (data not shown).

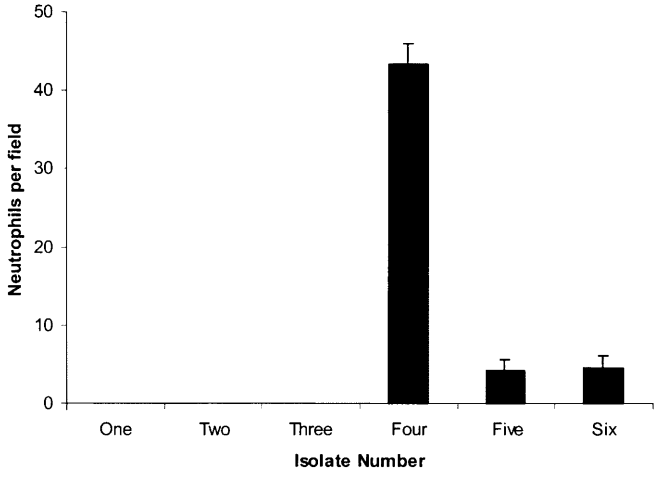

Fig. 1 Adherence of neutrophils to RT4 uroepithelial cells following preincubation with six different isolates of uropathogenic *E. coli* (10^8 CFU/ml for 20 h). Results are given as means ± SE of eight replicate experiments. Isolate 4 showed significantly greater neutrophil adhesion than any of the other uropathogenic *E. coli* isolates ($P < 0.01$)

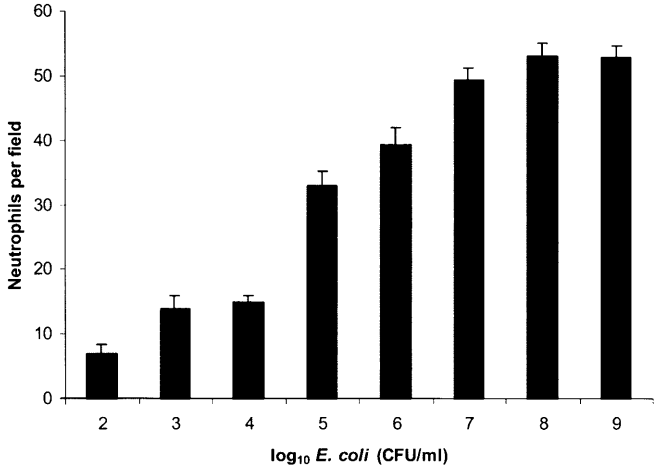

Fig. 2 Dose-dependent effect on neutrophil adhesion to RT4 cells induced by uropathogenic *E. coli* isolate 4 (10^2–10^9 CFU/ml for 20 h). Results given are means ± SE of eight replicate experiments. Stimulation of RT4 uroepithelial cells with 1×10^8 isolate 4 *E. coli* resulted in maximal neutrophil adherence after a 20-h incubation period

Neutrophil adhesion is caused by direct *E. coli* interaction and not by bacterial or cellular released products

The neutrophil adhesion-promoting effect induced by isolate 4 might be due either to soluble products released directly by the bacteria or to products released by uroepithelial cells in response to stimulation by these bacteria. Cell-free conditioned media were prepared from 20-h cultures of isolate 4 and from RT4 cells stimulated with isolate 4 for 20 h. These were then used to stimulate RT4 cells in neutrophil adhesion assays. Both supernatants failed to induce detectable neutrophil adhesion

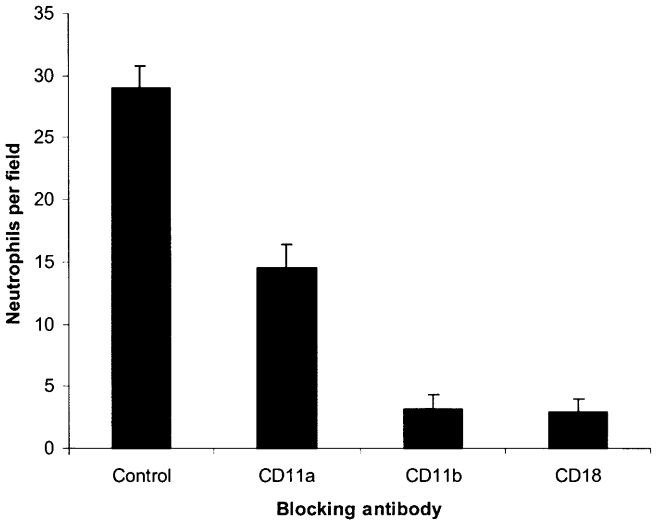

Fig. 3 Blocking effect of anti-β_2-integrin monoclonal antibodies on neutrophil adhesion to RT4 uroepithelial cells preincubated with isolate 4 *E. coli* (10^8 CFU/ml for 20 h). Anti-CD11a antibodies significantly reduced neutrophil adherence by approximately 50% ($P < 0.05$), while antibodies raised to CD11b and CD18 reduced adhesion by almost 90% (both $P < 0.01$)

over a 20-h period, whereas RT4 cells incubated with 10^8 CFU/ml *E. coli* promoted significant neutrophil adhesion as described previously. Similarly, suspensions of either paraformaldehyde or antibiotic-killed isolate 4 *E. coli* did not induce neutrophil adhesion (data not shown).

Neutrophil adhesion to RT4 uroepithelial cells is mediated by β_2 integrins expressed by neutrophils

Neutrophil adhesion assays were performed using isolate 4-stimulated RT4 monolayers and monoclonal antibodies raised against neutrophil integrin components CD11a, CD11b, and CD18 to determine if these molecules are utilised in neutrophil-uroepithelial adhesion. Anti-CD11a reduced the number of neutrophils adherent to RT4 cells by approximately 50% ($P < 0.05$). However, blocking antibodies raised against CD11b and CD18 resulted in almost complete (90%) abrogation of neutrophil adhesion to RT4 cells by isolate 4 *E. coli* (Fig. 3) (both $P < 0.01$).

Neutrophil adhesion is not predicted by virulence profiles of *E. coli* isolates

Several of the putative virulence factor genes for which we tested were present in one or more of the six *E. coli* isolates, in various combinations (Table 1). The three strains containing *pap* elements all expressed P-pattern hemagglutination, whereas despite the ubiquity of *fimH*, only three strains expressed mannose-sensitive agglutination. Strain 4 was negative for all of the virulence factor genes assessed except *fimH* and *fyuA*, both of which were present in all other strains and expressed only weak mannose-sensitive agglutination.

Table 1 Virulence characteristics of six UTI isolates of *E. coli*. All strains were negative for *sfaS* (S fimbrial adhesins), *kpsMT*III (group 3 capsule synthesis), *ibeA* (invasion of brain endothelium), and *cvaC* (colicin V). Other virulence factors were: *sfa/foc* (common to S and F1C fimbrial operons), *focG* (F1C fimbrial adhesin), *bma* (M adhesins), *fimH* (type 1 fimbrial adhesin), *hlyA* (hemolysin), *cnf1* (cytotoxic necrotising factor), *fyuA* (yersiniabactin), *iuc* (aerobactin synthesis), *iutA* (aerobactin receptor), *kpsMT* II (group 2 capsule synthesis), *traT* (serum-resistance associated), PAI (pathogenicity-associated island marker *malX* from strain CFT073. *MR* mannose-resistant hemagglutination of A_1P_1 human erythrocytes, *MS* mannose-sensitive yeast agglutination, *P* P-pattern hemagglutination (inhibited by pigeon egg white), *n.a.* not applicable

Strain	Virulence genotype															Agglutination	
	pap[a]	*papA* allele (s)[b]	*papG* allele[b]	*sfa/foc*	*focG*	*afa/dra*	*fimH*	*hlyA*	*cnf1*	*cdtB*	*fyuA*	*iuc iutA*	*kpsMT* II	traT	PAI	MR	MS
1	–	n.a.	n.a.	–	–	–	+	–	–	–	+	+	–	+	+	–	+
2	+	F8, F9	II	–	–	–	+	–	–	–	+	+	+	+	+	P	–
3	+	F12	III	+	–	–	+	+	+	+	+	–	–	–	+	P	+
4	–	n.a.	n.a.	–	–	–	+	–	–	–	+	–	–	–	–	–	+/–
5	–	n.a.	n.a.	–	–	+	+	–	–	–	+	–	–	+	+	–	–
6	+	F7–1, F10	II	+	+	–	+	–	–	+	+	+	+	–	+	P	+

[a] Including *papA*, *papC*, *papEF*, and *papG*
[b] Not applicable for *pap*− negative strains

Discussion

Uroepithelial cells represent an important cellular barrier between tissues of the genitourinary tract and the external environment and are situated at sites prone to infection from invading pathogens. The data presented in this study suggest that certain uropathogenic isolates of *E. coli* can specifically induce adhesion of peripheral blood neutrophils to the uroepithelial cell line RT4 in a dose- and time-dependent fashion. This neutrophil adhesion-inducing potential appears to be specifically mediated by live *E. coli*, as suspensions of bacteria killed with either paraformaldehyde or antibiotics did not induce neutrophil adhesion under the same experimental conditions. Furthermore, soluble factors released by neither live *E. coli* nor RT4 cells preincubated with *E. coli* caused neutrophil adherence to uroepithelial cells. These data suggest that intimate contact between live *E. coli* and the uroepithelial cell surface is required to activate uroepithelium. This event may be essential for adhesion of neutrophils that have traversed the uroepithelium.

Three uropathogenic *E. coli* isolated from patients with urinary tract infections did not cause neutrophil adherence to RT4 cells (isolates 1–3), even though bacterial adherence to these epithelial cells was confirmed by microscopy (data not shown). It is not known why these isolates did not promote neutrophil adherence when other isolates did, albeit to varying degrees. The attachment of *E. coli* P or type-1 fimbriae to uroepithelial cells not only mediates bacterial adherence but also stimulates epithelial release of chemotactic and immunomodulatory cytokines and triggers neutrophil transepithelial migration [5, 10, 14, 25]. Thus, the observed interisolate differences in induction of neutrophil adhesion could be due to the expression of certain types of fimbriae.

However, genotypic and phenotypic analysis of multiple adhesion and other virulence factors revealed no clear pattern with respect to neutrophil adhesion and suggests that fimbriation is not the crucial factor determining uroepithelial-mediated neutrophil adherence in this model. It is possible that the expression of virulence factors in vitro does not reflect in vivo expression. This may explain the apparent lack of association between virulence genotypes or in vitro phenotypes and neutrophil adhesion. Further studies are needed to identify the specific molecule(s) responsible for the observed neutrophil adhesion-promoting action of some *E. coli* strains.

Results showing that anti-CD11b and anti-CD18 antibodies could abrogate neutrophil adhesion by up to 90% are in accordance with previous findings showing that these antibodies reduced transuroepithelial neutrophil migration by up to 79% [3]. Taken together, these data suggest that both neutrophil adherence to and migration through uroepithelial cells are mediated almost exclusively by CD11b/CD18 and influenced by *E. coli*-

mediated upregulation of CD11b/CD18 counterligands on the uroepithelial cell surface.

CD11b/CD18 integrin has many counterligands which could serve as adhesive receptors for neutrophils. Intracellular adhesion molecules (ICAMs) are the candidates most likely to be expressed on the surface of uroepithelial cells. These are known to be upregulated by a number of proinflammatory cytokines and bacterial products on the surface of urinary tract epithelial cells [1, 3, 9], and previous studies have shown that CD11b/CD18 can bind specifically to ICAM-1, -2, and -4 but not ICAM-3 [6, 8]. Urinary epithelial cell lines and primary urinary epithelial cells express ICAM-1 but not ICAM-2 [3], suggesting that the latter is not the adhesive ligand. Expression of ICAM-1 is increased after direct stimulation with *E. coli* but not with *E. coli*-derived lipopolysaccharide (LPS) [3, 9]. In fact, previous studies have shown LPS to be a poor activator of uroepithelial cells, compared to whole bacteria [13]. These findings may explain why confluent RT4 cells treated with soluble factors released by *E. coli* did not promote neutrophil adhesion in our studies.

Blocking antibodies raised against ICAM-1 reduce transepithelial neutrophil migration by up to 85%, suggesting that ICAM-1 is the major but not only neutrophil adhesion molecule expressed by epithelial cells [3]. Other possible CD11b/CD18-binding candidates are carbohydrate membrane-bound glycoproteins such as syndecan, glycipan, and perlecan. These proteoglycans contain heparin and heparan sulphate, which can bind CD11b/CD18 expressed on neutrophils and other leucocytes [7, 20].

Previous studies using mice with inherent genetic defects have shown that neutrophils are essential for bacterial clearance in urinary tract infections. Transurethral injection of virulent *E. coli* 1177 into the bladders of C3H/HeN mice induced a rapid neutrophil influx into the urinary tract followed by bacterial clearance. In contrast, C3H/HeJ mice, which are LPS-nonresponders, showed a reduced neutrophil influx and were unable to clear the infection [12, 22]. One possible role for apically expressed ICAM-1 might be to hold neutrophils in or near sites of infection. Given urine flow within the bladder, ICAM-1 might serve as an adhesive tether to retain neutrophils at the uroepithelial surface. This would be beneficial, as uropathogenic *E. coli* would also be situated at the uroepithelial surface through fimbrial adhesive contacts [5, 25]. The close proximity of *E. coli* and adherent neutrophils would presumably aid neutrophil-mediated clearance from the mucosal surface.

In conclusion, these data suggest that certain uropathogenic *E. coli* selectively promote adhesion of neutrophils to ligands on uroepithelial cells by a CD11b/CD18-dependent mechanism. Further studies are required to determine whether the ability to stimulate neutrophil adherence to uroepithelial cells differs between UTI-associated and commensal *E. coli* or between clinical syndromes such as pyelonephritis, cystitis, and asymptomatic bacteriuria.

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