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Escherichia coli Induces Transuroepithelial Neutrophil Migration by an Intercellular Adhesion Molecule-1-Dependent Mechanism

WILLIAM W. AGACE,[1]* MANUEL PATARROYO,[2] MAJLIS SVENSSON,[1] ERIC CARLEMALM,[3] AND CATHARINA SVANBORG[1]

Department of Medical Microbiology, Division of Clinical Immunology,[1] and Department of Pathology,[3] Lund University, Lund, and Microbiology and Tumor Biology Center, Karolinska Institute, Stockholm,[2] Sweden

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During bacterial infections at mucosal sites, neutrophils migrate to the mucosa and cross the epithelial barrier. We have examined neutrophil migration across *Escherichia coli*-stimulated uroepithelial cell layers in an attempt to more fully understand this process. Stimulation of uroepithelial cells with *E. coli* or interleukin-1α (IL-1α) induced transepithelial neutrophil migration in a time- and stimulant dose-dependent manner. Uroepithelial cell lines and nontransformed uroepithelial cells expressed intercellular adhesion molecule-1 (ICAM-1) but not ICAM-2, E-selectin, or P-selectin. Epithelial ICAM-1 expression was enhanced after stimulation with *E. coli* or IL-1α. Anti-ICAM-1 antibody reduced transepithelial neutrophil migration by 61 to 85%, indicating that neutrophils bound ICAM-1 on the epithelial surface. Antibodies to CD18 and CD11b reduced migration by 70 to 79%, suggesting that CD11b/CD18 (Mac-1) was acting as the neutrophil receptor for ICAM-1 in this process. Anti-CD11a antibodies had no effect on neutrophil migration. In conclusion, *E. coli* induced ICAM-1- and Mac-1-dependent transepithelial neutrophil migration. Previous studies have shown that urinary tract epithelial cells secrete IL-8 when exposed to *E. coli* or IL-1α. These observations suggest that epithelial cells play an active role in neutrophil migration during urinary tract infections.

Neutrophils migrate to mucosal sites in response to bacterial infection. This process involves the generation of a chemotactic gradient from the site of infection, the adherence of neutrophils to the endothelial vessel wall, and their extravasation into the lamina propria. During infections of the lung, the intestine, and the urinary tract, neutrophils migrate across an epithelial lining into the mucosal space (4, 37, 38).

Rapid advances have been made in characterizing the molecules involved in neutrophil adherence to and migration across endothelial cell layers. E-selectin (ELAM-1, CD62E), intercellular adhesion molecule-1 (ICAM-1, CD54) and intercellular adhesion molecule-2 (ICAM-2, CD102) are expressed at the endothelial cell surface (6, 20, 34). E-selectin binds to sialylated, fucosylated lactosamine structures, notably sialyl-Lewis X, on the neutrophil surface, causing these cells to roll along the endothelial vessel wall (5, 17). ICAM-1 and ICAM-2 are ligands for the β_2 integrins CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac-1), which are expressed on the neutrophil surface (8, 21, 34). Integrin-ligand interactions mediate strong adhesion of neutrophils to vascular endothelium and transendothelial migration. The mechanisms of neutrophil migration across mucosal epithelia are less well characterized. Neutrophil migration across intestinal T_{84} epithelial monolayers required the β_2 integrin CD11b/CD18 (26). ICAM-1, a potential ligand for CD11b/CD18, was not involved in neutrophil migration across these cells, indicating the presence of a previously unrecognized ligand(s) for this integrin (7, 26).

Neutrophils migrate across urinary tract epithelium into the urine during urinary tract infections (UTI). Neutrophil chemoattractants such as interleukin-8 (IL-8) are produced by urinary tract epithelial cells in response to *Escherichia coli* stimulation (3). The interactions that occur between neutrophils and urinary tract epithelial cells, mechanisms of transuroepithelial migration, and the role of bacterial infection in these events have not been determined.

In this study, we examined the ability of *E. coli* to induce neutrophil migration across urinary tract epithelial cell layers. In addition, we examined the expression of adhesion molecules by urinary tract epithelial cells and their involvement in transepithelial neutrophil migration.

MATERIALS AND METHODS

Stimulants. *E. coli* Hu734, a *lac* mutant of the wild-type pyelonephritis strain GR12 (35), serotype O75:K5:H$^-$, *hly*$^+$ ColV$^+$, is phenotypically positive for type 1 and P fimbriae. The strain was maintained on tryptic soy agar (Difco, Detroit, Mich.) plates. For experiments, Luria broth was inoculated with colonies from tryptic soy agar plates and incubated overnight at 37°C. Fimbrial expression was tested at regular intervals during the course of the study. *E. coli* Hu734 agglutinated Galα1-4Galβ latex beads and caused mannose-sensitive agglutination of guinea pig erythrocytes. Lipopolysaccharide (LPS; *E. coli* serotype O111:B4) was purchased from Sigma Chemical Co., St. Louis, Mo. Human recombinant IL-1α was purchased from Genzyme Corporation, Boston, Mass., and diluted as described in the manufacturer's instructions.

Cells. The A-498 kidney (ATCC HTB44) and J82 bladder (ATCC HTB1) epithelial cell lines were grown in culture medium (RPMI 1640; Flow, Gothenburg, Sweden) supplemented with gentamicin (0.05 mg/ml; Flow), glutamine (2 mM), and fetal calf serum (5%; Gibco Ltd., Paisley, Scotland) as described previously (8).

Urinary tract epithelial cells were isolated from the urine of three healthy female volunteers. Freshly voided urine was poured into 50-ml conical tubes (Falcon, Lincoln Park, N.J.) and centrifuged for 15 min at 1,000 rpm (RP centrifuge; Hettich Rotanta, Malmö, Sweden). The supernatants were removed, and the cell pellets were pooled into a 1-ml plastic V-bottom tube (Eppendorf, Hamburg, Germany). The cells were washed twice in culture medium and resuspended in a 10-ml round-bottom tube (Falcon) to a concentration of $\approx 10^4$ cells per ml. One milliliter of cell suspension was then seeded onto each well of a six-well plate (Falcon).

Neutrophils were isolated from peripheral blood obtained from healthy human volunteers. Briefly, heparinized blood was run on a Polymorphprep density gradient (Nycomed Pharma AS, Oslo, Norway) as described in the manufacturer's instructions. Osmolarity was restored to the neutrophil suspension by the addition of an equal volume of 0.45% NaCl, and remaining erythrocytes were removed by hypotonic lysis. The resulting cell suspension contained >97% neu-

* Corresponding author. Mailing address. Department of Clinical Immunology, Lund University, Lund, Sweden. Phone: 46 46 173282. Fax: 46 46 137468.

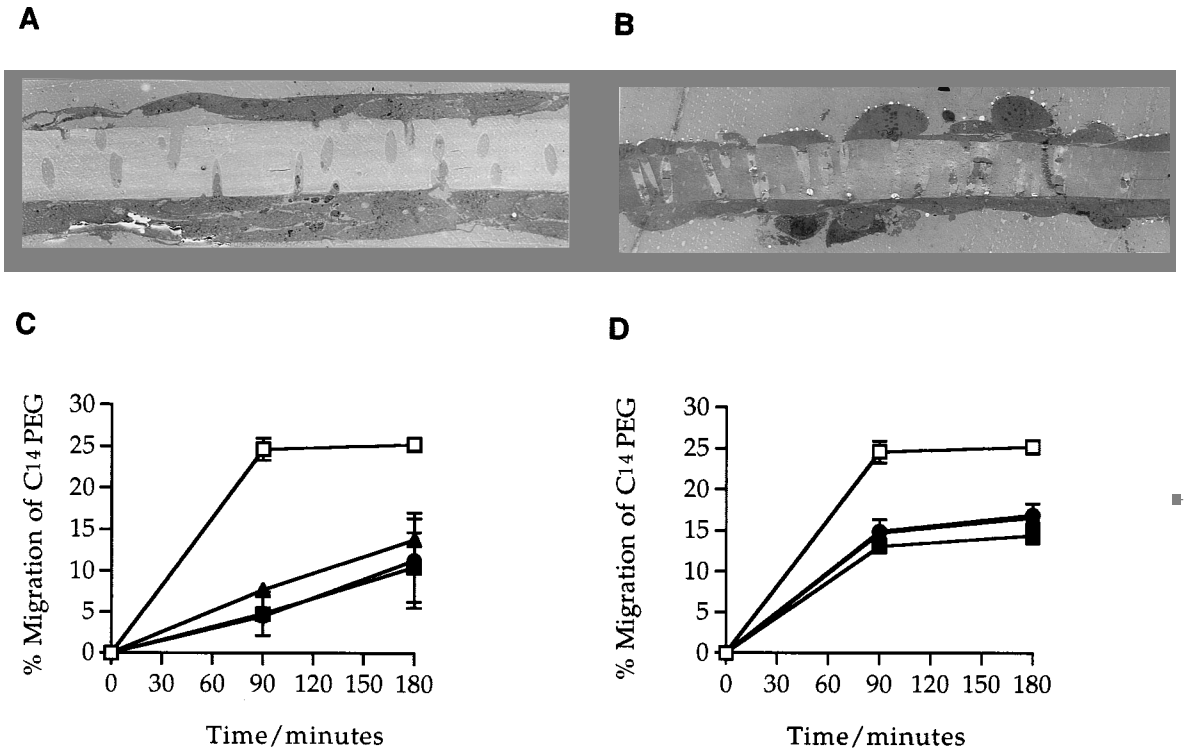

FIG. 1. Morphology of epithelial cell layers on transwell membranes. (A and B) A-498 kidney cell line (A) and J82 bladder cell line (B) after 14 days of growth at 37°C in 5% CO_2; (C and D) migration of ^{14}C-labelled PEG (C14 PEG) through confluent A-498 epithelial layers (C) and confluent J82 epithelial layers (D). Symbols: □, filters; solid symbols, confluent cell layers pretreated for 24 h with medium (■), *E. coli* (▲), or IL-1α (●). Results are the means (± standard errors) of two experiments.

trophils as determined by Wright's Giemsa stain and was 98% viable as determined by trypan blue exclusion.

Preparation of epithelial cell layers. A-498 or J82 epithelial cells were suspended in culture medium at a concentration of 3×10^5 cells per ml, and 1 ml was used to seed individual inverted transwell inserts (Costar, Cambridge, Mass.; 24.5-mm-diameter polycarbonate membrane with 3-μm pores). Cells were allowed to settle onto the transwell membranes for 6 h at 37°C in a 5% CO_2 atmosphere. The remaining medium was then removed, and the wells were placed into cluster plates in fresh medium. The plates were incubated at 37°C in a 5% CO_2 atmosphere until the cell layers were fully confluent. Analysis of Giemsa-stained filters showed confluent cell layers at 14 days.

For electron microscopy studies, the filters were fixed with 2.5% glutaraldehyde in phosphate-buffered saline for 15 min and postfixed in 1% OsO_4 for 30 min. After successive dehydration in alcohol, the filters were embedded in Agar 100 (Agar Sci. Ltd., Essex, England), cut with a diamond knife (Reichert Jung Super nova), placed on copper grids, and stained with 4% uranyl–0.5% lead citrate. The filters were examined in a Geol 100C electron microscope at an ×700 magnification. Figure 1A and B show cross-sections of A-498 and J82 epithelial cell layers on polycarbonate filters. A-498 cells typically formed multilayers on the seeded sides of the filters and monolayers on the upper sides of the filters. J82 cells formed monolayers on both sides of the filters.

For studies of filter permeability by ^{14}C-labelled polyethylene glycol (PEG; molecular weight, 4,000; Amersham, Solna, Sweden), transwell inserts were transferred to new cluster plates. A 1.5-ml volume of PEG diluted in RPMI 1640 was added to the upper compartments, and 2.5 ml of RPMI 1640 was added to the lower compartments. Samples (100 μl) were removed from the lower compartments after 90 and 180 min, and their radioactivity was measured in a beta counter (Beckman LS1800). The migration of PEG across A-498 and J82 epithelial layers is shown in Fig. 1C and D. The presence of *E. coli* or IL-1α in the transwell chambers for 24 h had no effect on cell layer permeability by PEG. The presence of gentamicin in the medium maintained epithelial cell layer integrity by preventing bacterial growth. *E. coli* in the presence of gentamicin is known to bind to uroepithelial cells and induce epithelial cytokine production (3).

Neutrophil transepithelial migration assay. At the start of each experiment, transwell inserts were transferred to new cluster plates. Fresh medium was added to the upper compartment (1.5 ml), and fresh medium or medium containing *E. coli* or IL-1α (2.7 ml) was added to the lower compartment. The medium in the upper compartment was removed after 0, 4, or 24 h and replaced by fresh medium containing neutrophils (1.5 ml; 1×10^6 to 2×10^6 neutrophils per ml). Samples of 100 μl were collected from the lower wells at 1, 2, and 3 h after thorough mixing of the culture medium. Removal of the neutrophils from the lower well surface was confirmed by light microscopy. Neutrophil numbers in the samples were counted in a Bürker chamber and expressed as a percentage of the neutrophils added to the upper compartment.

Expression of adhesion molecules by urinary tract epithelial cells. A-498 and J82 epithelial cells were grown to confluency in 25-ml culture flasks (Falcon). Medium was removed from the flasks and replaced with fresh medium or medium containing *E. coli* (10^8 bacteria per ml), LPS (0.1 to 10 μg/ml), or IL-1α (1 ng/ml). The cells were incubated at 37°C in a 5% CO_2 atmosphere for 0, 4, 24, or 48 h and detached from the flasks by incubation with 10 mM EDTA in RPMI 1640 for 20 min at 37°C in a 5% CO_2 atmosphere. A cell pellet was obtained by spinning the cell suspension at 1,000 rpm (Hettich Rotanta RP centrifuge) for 10 min. Cells were then resuspended in ice-cold RPMI 1640 containing 0.3% bovine serum albumin (RPMI-BSA).

Urinary tract epithelial cells isolated from freshly voided urine were incubated at 37°C in a 5% CO_2 atmosphere with medium or medium containing *E. coli* (10^8 bacteria per ml) or IL-1α (1 ng/ml). The cells remained in suspension during the course of the incubation. Cells were collected after 0 and 4 h of stimulation, centrifuged at 1,000 rpm (Hettich Rotanta RP centrifuge) for 10 min, and resuspended in ice-cold RPMI-BSA.

Immunofluorescence staining. Anti-ICAM-1 (anti-CD54; monoclonal antibody [MAb] LB-2 [27]), anti-ICAM-2 (anti-CD102; MAb IC2/2; Bender Medsystems, Vienna, Austria [11, 31]), anti-E-selectin and -P-selectin (MAb BBA 1; British Biotechnology, Oxon, England), and mouse immunoglobulin G (IgG) negative control (MAb DAK-GO1; Dakopatts AB, Älvsjö, Sweden) antibodies were used for immunofluorescence staining. Cells were suspended in 10 μl of RPMI-BSA containing primary antibody and incubated for 30 min on ice. Primary antibodies were used at saturating concentrations for the adhesion molecules. After two washes with ice-cold RPMI-BSA, the cells were reincubated for 30 min on ice in 50 μl of RPMI-BSA containing fluorescein-conjugated goat $F(ab')_2$ antibody (anti-mouse Ig) (Dakopatts) at a 1/10 dilution.

The percentage of epithelial cells, obtained from freshly voided urine, was determined by staining with anticytokeratin (MAb MNF116; Dakopatts) antibody. Briefly, cells were spun onto glass slides at 300 rpm in a cytocentrifuge (Cytospin 2; Shandon Southern, Shandon, England), fixed with 4% paraformaldehyde, and incubated with primary anticytokeratin antibody and secondary fluorescein-conjugated goat $F(ab')_2$ antibody (anti-mouse Ig) (Dakopatts) in the presence of 0.1% saponin (Riedal de Haen AG, Seelze, Germany).

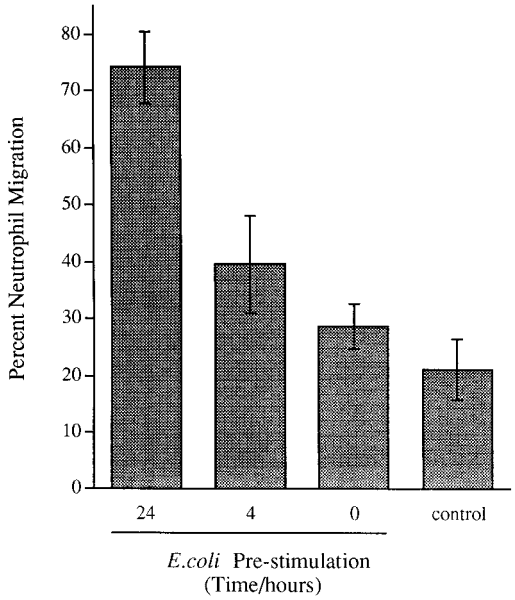

FIG. 2. Role of epithelial cell prestimulation in transepithelial neutrophil migration. Neutrophil migration (3 h) was measured across A-498 kidney epithelial cell layers stimulated with *E. coli* for 0, 4, and 24 h. Results are the means (± standard errors) of four separate experiments.

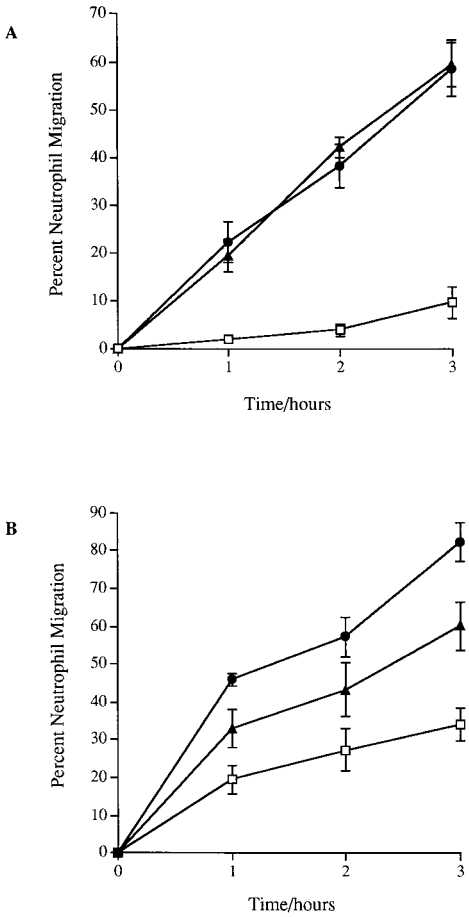

FIG. 3. Kinetics of neutrophil migration across A-498 kidney epithelial cell layers (A) and J82 bladder epithelial cell layers (B). Cells were prestimulated for 24 h with medium (□), *E. coli* (10^8 bacteria per ml) (▲), or IL-1α (1 ng/ml) (●). Results are the means (± standard errors) of five or six separate experiments.

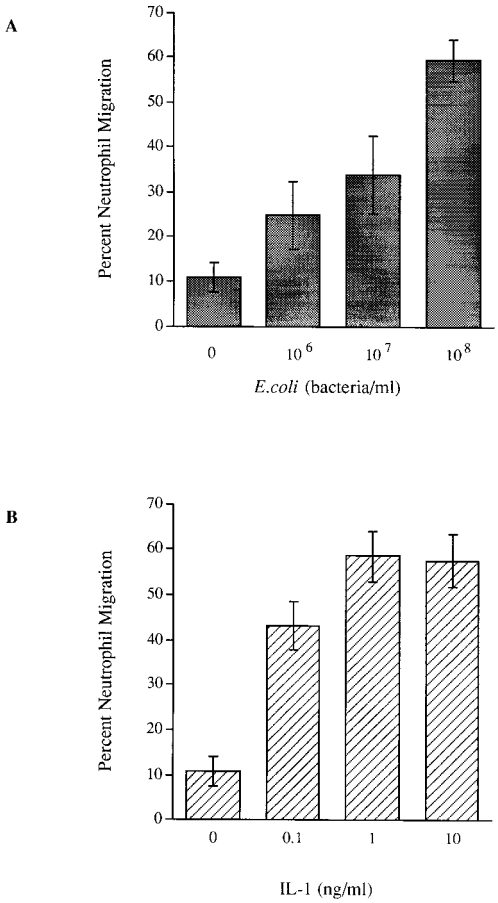

FIG. 4. Stimulant dose-dependent neutrophil migration (3 h) across A-498 kidney epithelial cell layers prestimulated for 24 h with *E. coli* or IL-1α. Results are the means (± standard errors) of five separate experiments.

A-498 and J82 cells were examined by flow cytometry in an EPICS Profile II (Coulter, Miami, Fla.). A total of 3,000 to 5,000 cells were counted for each sample. Urinary tract epithelial cells isolated from fresh urine were examined with a fluorescence microscope (Nikon) equipped with a 100-W mercury lamp (Osram, Berlin, Germany) and a Ploem-pac with filter sets for fluorescein isothiocyanate. To prevent UV light quenching, buffered glycerol (ACO, Stockholm, Sweden) containing 2% 1,4-diazabicyclo-[2.2.2]octane (Sigma) was used as a mounting medium.

Role of adhesion molecules in transepithelial neutrophil migration. The blocking antibodies, i.e., anti-CD18 (MAb IB4; provided by S. Wright [33]), anti-CD11a (MAb H12; provided by H. Wigzell [9]), anti-CD11b (MAb 60.1; provided P. Beatty [33]), anti-ICAM-1 (MAb BBA 4; British Biotechnology), anti-ICAM-1 (MAb LB-2 [27]), and anti-ICAM-1 Fab fragments (MAb LB-2), and control antibodies, i.e., anti-HLA-1 (BH-9; Serotec, Oxford, United Kingdom) and mouse IgG negative control, were used in the neutrophil migration inhibition studies. A-498 cells were cultured on inverted transwell inserts as described above and stimulated by the addition of *E. coli* (10^8 bacteria per ml) or IL-1α (1 ng/ml) to the lower well 24 h prior to the addition of neutrophils. The effect of anti-ICAM-1 antibody on neutrophil transmigration was examined by incubating the epithelial cell layers with antibody for 30 min prior to the addition of neutrophils. The medium was removed from the upper wells and replaced with 1.4 ml of fresh medium containing anti-ICAM-1 antibody, negative control antibody, or medium alone. Subsequently, 100 μl of neutrophil suspension (1.5×10^7 to 3×10^7 neutrophils per ml) was added to the upper wells, giving a final neutrophil concentration of 1×10^6 to 2×10^6 neutrophils per ml in a 1.5-ml volume. Transmigration was quantitated as described above. The effect of antibodies to CD11a, CD11b, and CD18 on transepithelial neutrophil migration was examined by incubating neutrophils with antibody for 30 min prior to their addition to the transwells. Neutrophils (1×10^6 to 2×10^6 neutrophils per ml) were incubated with antibody (10 μg/ml) diluted in medium or with medium alone. At time zero, the medium was removed from the upper wells and replaced with 1.5 ml of the neutrophil suspension. Transmigration was quantitated as described above.

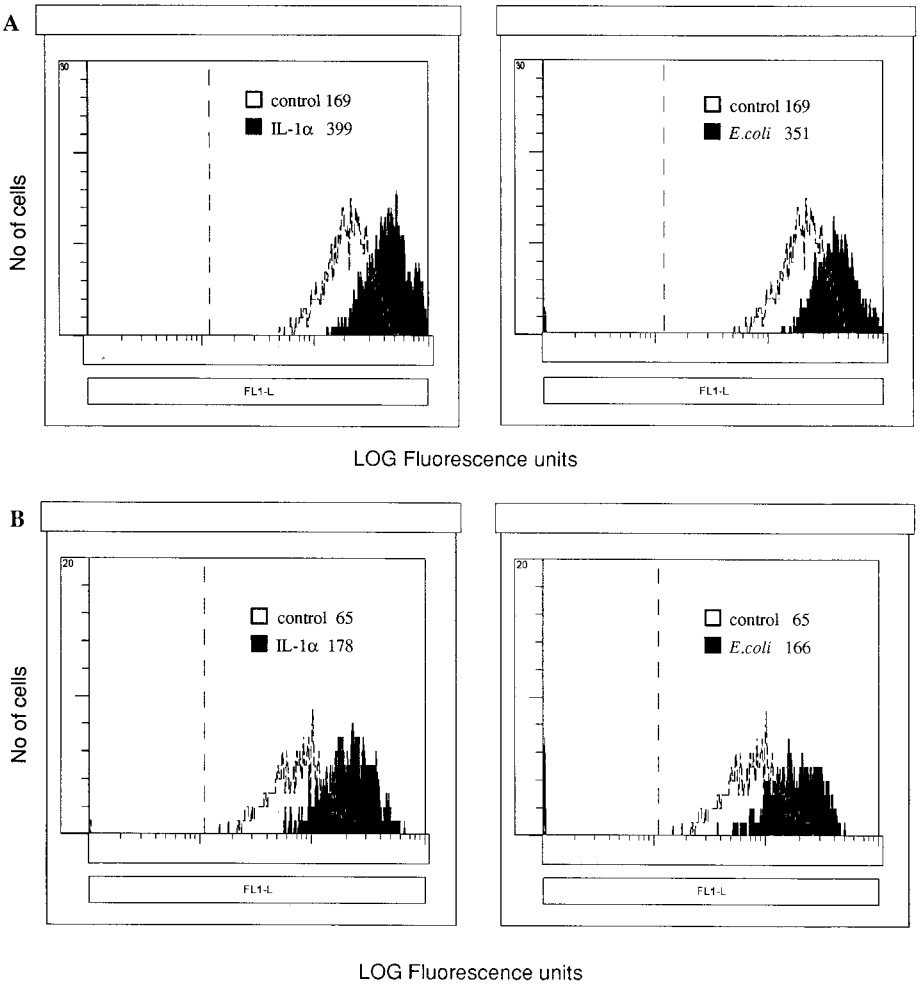

FIG. 5. ICAM-1 expression by the A-498 kidney epithelial cell line (A) and the J82 bladder epithelial cell line (B) as determined by flow cytometry analysis. Cells were stimulated for 24 h with *E. coli* (10^8 bacteria per ml) or IL-1α (1 ng/ml). The numbers depict the mean fluorescence (units) of the cells from one representative experiment of six. Of cells incubated with control antibody, 98% fell to the left of the dotted line.

RESULTS

Stimulation of epithelial cell layers induces transepithelial neutrophil migration. Transepithelial neutrophil migration was examined by use of a modified transwell system. Epithelial cells were grown to confluency on the underside of a transwell membrane. Epithelial layers were stimulated by the addition of *E. coli* or IL-1α to the lower compartment of the transwells. Neutrophils were added to the upper compartment, and migration was recorded as the percentage of neutrophils passing from the upper to the lower compartment.

Epithelial cell layers were exposed to *E. coli* (10^8 bacteria per ml) for 0, 4, and 24 h prior to the addition of neutrophils. Neutrophil migration across epithelial layers prestimulated for 24 h with *E. coli* was higher than migration across unstimulated epithelial cells or migration across cells prestimulated for 4 h (Fig. 2). Migration across epithelial layers prestimulated for 4 h was higher than that across unstimulated epithelial cells. The addition of *E. coli* at the same time as that of neutrophils did not induce neutrophil migration. This suggested that prestimulation of the epithelial cells was required for neutrophil migration to occur. Subsequent experiments used epithelial layers prestimulated for 24 h.

Kinetics of transepithelial neutrophil migration. The kinetics of neutrophil migration across A-498 epithelial layers is shown in Fig. 3A. Neutrophil migration across *E. coli*-stimulated epithelial layers reached 20% by 1 h, 42% by 2 h, and 59% by 3 h. Neutrophil migration across IL-1α (1 ng/ml)-stimulated epithelial layers reached 22% by 1 h, 38% by 2 h, and 59% by 3 h. Ten percent of neutrophils moved across unstimulated epithelial layers by 3 h. *E. coli* and IL-1α also induced neutrophil migration across J82 bladder epithelial layers (Fig. 3B). Neutrophil migration across *E. coli*-stimulated J82 epithelial layers reached 46% by 1 h, 57% by 2 h, and 82% by 3 h. Neutrophil migration across IL-1α (1 ng/ml)-stimulated J82 epithelial layers reached 33% by 1 h, 43% by 2 h, and 60% by 3 h. In comparison, 33% of neutrophils moved across unstimulated J82 epithelial layers by 3 h. Neutrophil migration across cell-free filters reached 10% by 1 h, 24% by 2 h, and 31% by 3 h. The presence of *E. coli* or IL-1α in the lower wells had no effect on neutrophil migration across cell-free filters (data not shown).

Transepithelial migration is stimulant dose dependent. The influence of stimulant concentration on neutrophil migration is shown in Fig. 4. A-498 epithelial layers were exposed to 10^8,

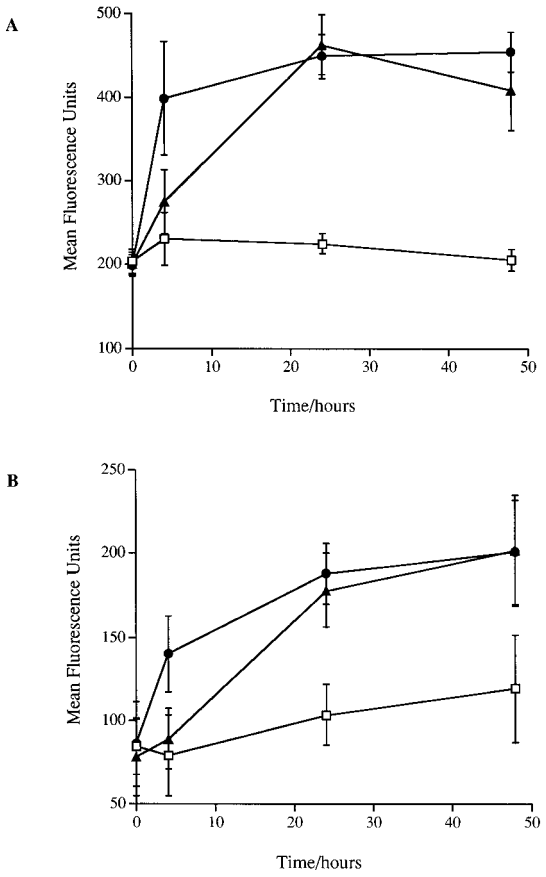

FIG. 6. Kinetics of ICAM-1 expression by A-498 kidney epithelial cell lines (A) and J82 bladder epithelial cell lines (B). Cells were stimulated with medium (□), *E. coli* (10^8 bacteria per ml) (▲), or IL-1α (1 ng/ml) (●) for the period of time depicted on the *x* axis. Results are the mean fluorescence (± standard error) of the cells from flow cytometry analysis of four to six separate experiments.

10^7, and 10^6 bacteria per ml. Neutrophil migration across the epithelial layers reached 59, 34, and 25%, respectively. A-498 epithelial layers exposed to IL-1α at 10, 1, or 0.1 ng/ml induced 58, 59, and 43% of neutrophils to migrate, respectively.

Expression of adhesion molecules by urinary tract epithelial cell lines. ICAM-1, ICAM-2, E-selectin, and P-selectin expression by A-498 and J82 epithelial cells was examined by indirect immunofluorescence. The two epithelial cell lines constitutively expressed ICAM-1 (99% positive cells) but not ICAM-2, E-selectin, or P-selectin (data not shown). A-498 epithelial cells expressed higher levels of ICAM-1 than J82 epithelial cells (Fig. 5). The expression of ICAM-1 by the epithelial cell lines increased within 4 h of stimulation with *E. coli* (10^8 bacteria per ml) or IL-1α (1 ng/ml) (Fig. 6). A-498 cells expressed the highest levels of ICAM-1 after 24 h of stimulation, and levels remained high at 48 h (Fig. 6A). J82 cells expressed the highest levels of ICAM-1 at 24 and 48 h of stimulation (Fig. 6B). ICAM-2, E-selectin, and P-selectin expression was not induced by either stimulant (data not shown).

Expression of adhesion molecules by urinary tract epithelial cells. Epithelial cells isolated from freshly voided urine were examined for ICAM-1, ICAM-2, E-selectin, and P-selectin expression. Cell viability ranged from 70 to 90% in individual samples and did not decrease over a 4-h culture period. More than 95% of cells isolated from the urine were epithelial, as demonstrated by staining with anticytokeratin antibody (Fig. 7A). ICAM-1 expression was observed in 23 to 55% of the epithelial cells (Fig. 7C). The percentage of ICAM-1-expressing cells did not increase after stimulation with *E. coli* or IL-1α. Cells did not stain with anti-E-selectin/P-selectin (data not shown), anti-ICAM-2, or control antibody (Fig. 7B and D).

Involvement of ICAM-1 in transepithelial neutrophil migration. Pretreatment of A-498 epithelial cells with anti-ICAM-1 antibody (BBA 4) reduced neutrophil migration across *E. coli*-, IL-1α-, and medium-stimulated A-498 epithelial layers (Table 1). The effect of anti-ICAM-1 antibody was dose dependent. A concentration of 1 μg of anti-ICAM-1 antibody per ml blocked 70% of the *E. coli*-induced neutrophil migration and 47% of the IL-1α-induced migration. A concentration of 10 μg of anti-ICAM-1 antibody per ml blocked 84% of the *E. coli*-induced neutrophil migration and 74% of the IL-1α-induced migration. The role of ICAM-1 in transepithelial neutrophil migration was confirmed in studies with J82 bladder epithelial layers (Table 1). A 1-μg/ml concentration of anti-ICAM-1 antibody blocked 51% of *E. coli*-induced and 43% of IL-1α-induced neutrophil migration. A 10-μg/ml concentration of anti-ICAM-1 antibody blocked 66 and 61% of *E. coli*- and IL-1α-induced neutrophil migration, respectively. Anti-ICAM-1 MAb LB-2 and LB-2 Fab fragments blocked transepithelial neutrophil migration by 60 to 80%. Anti-HLA-1 antibody (BH-9; Serotec) bound to the epithelial cells to a similar degree as the ICAM-1 antibody LB-2 (data not shown) but had no effect on neutrophil migration.

Involvement of β_2 integrins in transepithelial neutrophil migration. Pretreatment of neutrophils with anti-CD18 or anti-CD11b antibody reduced neutrophil migration across *E. coli*-, IL-1α-, and medium-stimulated A-498 epithelial layers (Table 2). Anti-CD18 antibody (10 μg/ml) blocked 74 and 79% of the neutrophil migration across *E. coli*- and IL-1α-stimulated epithelial layers, respectively. Anti-CD11b antibody (10 μg/ml) blocked 71 and 75% of the neutrophil migration across *E. coli*- and IL-1α-stimulated epithelial layers, respectively. Anti-CD11a (10 μg/ml) antibody had no effect on neutrophil migration across stimulated or unstimulated epithelial cell layers.

LPS does not induce transepithelial migration. A-498 epithelial layers were incubated with 10, 1, or 0.1 μg of LPS (*E. coli* serotype O111:B4) per ml. These concentrations of LPS did not induce transepithelial neutrophil migration or epithelial ICAM-1 expression (Fig. 8). To examine the role of other bacterial products in the induction of ICAM-1, medium preconditioned with *E. coli* for 24 h was added as a stimulant to the A-498 cells. Preconditioned medium stimulated ICAM-1 expression by the A-498 cells compared with medium alone; however, bacteria stimulated higher levels of ICAM-1 expression than preconditioned medium, LPS, or medium alone.

DISCUSSION

This study demonstrated that *E. coli* or IL-1α can stimulate neutrophil migration across urinary tract epithelial layers. Migration was ICAM-1 dependent: ICAM-1 expression was augmented by *E. coli* or IL-1α, and neutrophil migration across stimulated epithelial layers was blocked with anti-ICAM-1 antibody. CD11b/CD18 (Mac-1) but not CD11a/CD18 (LFA-1) was identified as a possible receptor for ICAM-1 in this process because antibodies to CD11b and CD18 but not CD11a inhibited neutrophil migration. The low level of neutrophil migration across unstimulated epithelial layers constitutively expressing ICAM-1 suggests that additional factors are also involved in the induction of transepithelial neutrophil migration. We have reported previously that *E. coli* stimulates the secretion of IL-8, a neutrophil chemoattractant, by urinary

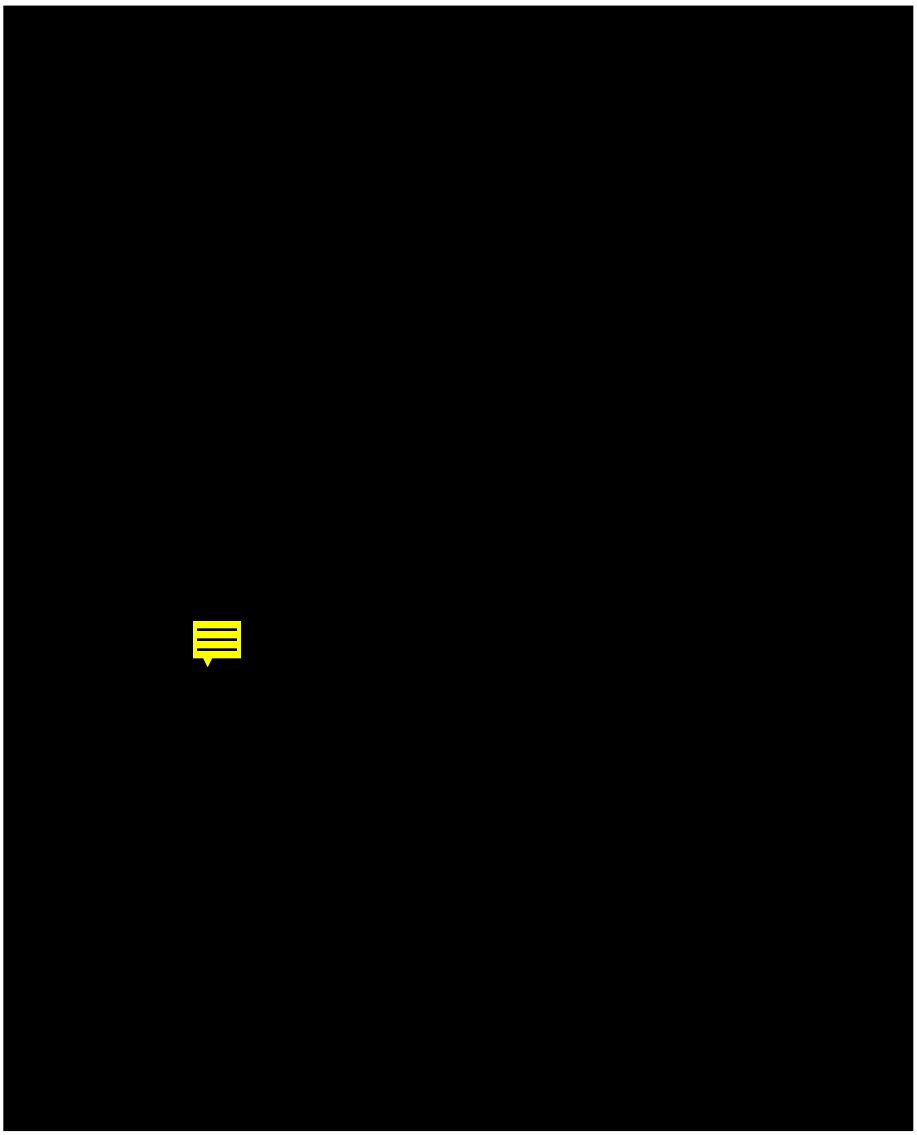

FIG. 7. Expression of adhesion molecules by urinary tract epithelial cells isolated from freshly voided urine. Cells were stained with anti-cytokeratin antibody (A), negative control antibody (B), anti-ICAM-1 antibody (C), or anti-ICAM-2 antibody (D).

tract epithelial cells and that levels of this cytokine correlated strongly with neutrophil influx during UTI (3). Polyclonal antibodies to IL-8 completely block the *E. coli*- and IL-1-induced transepithelial migration (1). Together, these results show that stimulated epithelial cells can initiate two prerequisites for neutrophil migration during mucosal infections, i.e., the release of neutrophil chemoattractants and the induction of adhesion molecules. This evidence suggests that urinary tract epithelial cells play an active role in directing neutrophil influx into the urine during UTI.

A-498 kidney and J82 bladder cell lines were chosen for this study because they have been used extensively to model urinary tract epithelial responses to *E. coli* (2, 3, 12, 13, 15, 36). Like normal urinary tract epithelial cells, they express surface glycolipids and mannose-containing carbohydrates which mediate P fimbria- and type 1 fimbria-dependent binding of *E. coli*, respectively (18, 36). A-498 and J82 cell lines secrete the same cytokines as normal urinary tract epithelial cells and as the human urinary tract mucosa after intravesical *E. coli* instillation (3, 11a, 14, 15). In this study, A-498 kidney and J82 bladder epithelial cell lines expressed the same adhesion molecules as those expressed by freshly isolated urinary tract epithelial cells. The urinary tract cell lines formed confluent epithelial layers which restricted PEG movement across polycarbonate filters; however, they showed no evidence of polarization (to our knowledge, a model for polarized human urinary tract epithelial cells has not been published). In addition, neutrophil movement was blocked with antibodies to adhesion molecules expressed on both the epithelial and neutrophil surfaces, suggesting the occurrence of specific epithelial-neutrophil interactions in this model.

Previous studies have examined neutrophil migration across intestinal T_{84} epithelial layers in response to *N*-formylmethionylleucyl phenylalanine gradients (24, 26). Neutrophil migration was observed within 15 min and continued to increase over a 1-h period (24). Recently, *Salmonella typhimurium*, but not *E. coli*, was shown to induce neutrophil migration across T_{84} epithelial layers within 2 h (22). In the present study, *E. coli* failed to induce neutrophil migration within 3 h when added at the same time as neutrophils; however, neutrophils rapidly mi-

TABLE 1. Role of ICAM-1 in transepithelial neutrophil migration

Epithelial cell type	Antibody (concn)	% Neutrophil migration (SE)[a]		
		Medium	*E. coli*	IL-1α
A-498	None	9 (3)	56 (5)	53 (5)
	Anti-ICAM-1 (1 μg/ml)	4 (2)	17 (5)	28 (9)
	Anti-ICAM-1 (10 μg/ml)	2 (1)	9 (3)	14 (5)
	Control (10 μg/ml)	9 (4)	52 (9)	52 (7)
J82	None	34 (6)	81 (6)	59 (8)
	Anti-ICAM-1 (1 μg/ml)	23 (7)	40 (3)	34 (9)
	Anti-ICAM-1 (10 μg/ml)	15 (5)	28 (2)	23 (7)
	Control (10 μg/ml)	36 (5)	75 (2)	50 (7)

[a] Neutrophil migration (3 h) was measured across A-498 kidney and J82 bladder epithelial cell layers prestimulated for 24 h with *E. coli* (10^8 bacteria per ml), IL-1α (1 ng/ml), or medium. Results are the means (± standard errors) of five (A-498 cells) and four (J82 cells) separate experiments.

grated across urinary tract epithelial layers prestimulated with *E. coli* for 24 h. These observations suggested that urinary tract epithelial cell activation was required for the induction of neutrophil migration.

Neutrophil extravasation is controlled by cell adhesion molecules expressed on endothelial cells lining the blood vessel wall. ICAM-1 expression peaks at 18 to 24 h, ICAM-2 levels remain constant, and E-selectin expression is induced maximally at 2 to 6 h after endothelial stimulation with inflammatory cytokines such as IL-1α (25, 28, 29). Urinary tract epithelial cells also express ICAM-1. Proliferating ureter epithelial cells expressed ICAM-1, and LPS stimulation of these cells induced ICAM-1 clustering at the cell surface (10). Kidney tubular epithelial cells expressed ICAM-1 constitutively, and expression was augmented by IL-1α (16). In the present study, kidney and bladder epithelial cell lines and freshly isolated urinary tract epithelial cells constitutively expressed ICAM-1 but not ICAM-2, E-selectin, or P-selectin. *E. coli* and IL-1α augmented ICAM-1 expression; however, expression of ICAM-2, E-selectin, or P-selectin was not induced. Antibodies to ICAM-1 blocked from 61 to 85% of the neutrophil migration across *E. coli*- or IL-1α-stimulated urinary tract epithelial layers. These results showed *E. coli* or IL-1α to be potent stimulators of urinary tract epithelial ICAM-1 expression and suggested a role for ICAM-1 in neutrophil migration across urinary tract epithelial layers.

Previous reports have shown the importance of the β_2 integrin CD11b/CD18 in neutrophil migration across intestinal epithelial cells (26). Neutrophils from leukocyte adhesion deficiency patients, which lack CD11/CD18 surface expression, failed to migrate across T_{84} epithelial layers in response to an *N*-formylmethionylleucyl phenylalanine gradient. Moreover, antibodies to CD11b, but not CD11a, blocked transepithelial

TABLE 2. Role of β_2 integrins in neutrophil migration across A-498 cell layers

Antibody (10 μg/ml)	% Neutrophil migration (SE)[a]		
	Medium	*E. coli*	IL-1α
None	27 (5)	79 (5)	63 (4)
Anti-CD11a	27 (4)	73 (9)	53 (4)
Anti-CD11b	9 (2)	23 (4)	16 (5)
Anti-CD18	3 (3)	21 (3)	13 (3)

[a] Neutrophil migration (3 h) was measured across A-498 kidney epithelial cell layers prestimulated for 24 h with *E. coli* (10^8 bacteria per ml), IL-1α (1 ng/ml), or medium. Results are the means (± standard errors) of four separate experiments.

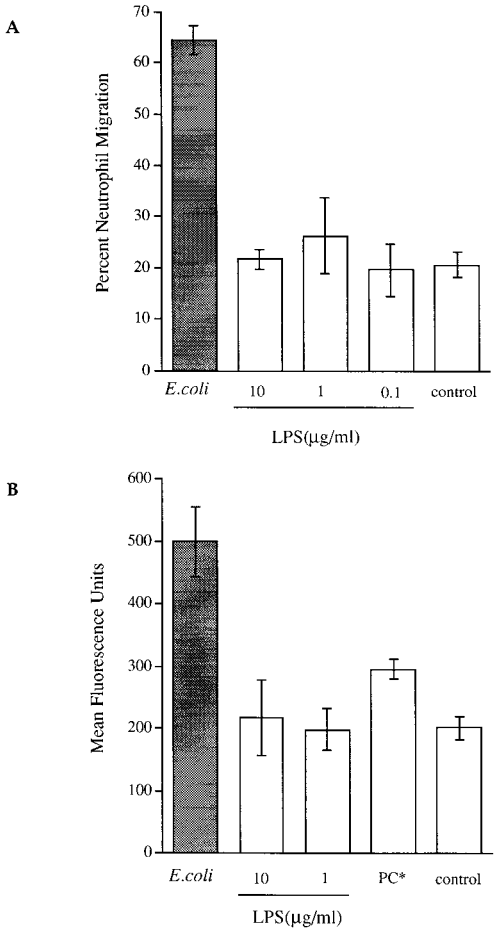

FIG. 8. LPS and preconditioned medium are less effective than *E. coli* as inducers of ICAM-1 expression and neutrophil transmigration. (A) Neutrophil migration (3 h) across A-498 kidney epithelial cell layers stimulated for 24 h with LPS; (B) ICAM-1 expression by A-498 epithelial cell lines stimulated for 24 h with LPS or preconditioned medium (PC*). Preconditioned medium was prepared by suspending *E. coli* in culture medium containing gentamicin at a concentration of 10^8 bacteria per ml. The medium was incubated overnight at 37°C in a 5% CO_2 atmosphere. Bacteria were removed by filtration through a 0.2-μm-pore-size filter (Schleicher & Schuell, Dassel, Germany), and the supernatants were used as medium for the cells. Results are the means (± standard errors) of two separate experiments.

migration of normal neutrophils, indicating participation of CD11b/CD18 at the neutrophil surface. The results of the present study confirmed a role for CD11b/CD18 in transepithelial neutrophil migration. Antibodies to CD18 and CD11b, but not CD11a, blocked from 70 to 79% of neutrophil migration. In contrast to our results, neutrophil migration across T_{84} epithelial layers was ICAM-1 independent, suggesting the presence of other ligands for CD11b/CD18 on the intestinal epithelial surface.

The bacterial properties involved in *E. coli*-induced transepithelial neutrophil migration and epithelial ICAM-1 expression remain undefined. *E. coli* protein synthesis was not required since gentamicin, an inhibitor of protein synthesis, was present in the medium throughout the experiments. Furthermore, epithelial cell activation was not a result of LPS stimulation alone. LPS was unable to stimulate transepithelial neutrophil migration, and LPS and medium preconditioned with bacteria were poor inducers of ICAM-1 expression. These results agree with previous studies showing LPS to be a poor activator of

uroepithelial cytokine production compared with whole bacteria (15). *E. coli* strains that attached to the epithelial cell surface induced a higher epithelial IL-6 and IL-8 response than isogenic nonattaching *E. coli* strains (3, 15). *E. coli* Hu734, used in this study, attached to J82 bladder and A-498 kidney epithelial cells and stimulated higher levels of cytokine secretion than nonattaching strains (15). It is likely that the induction of ICAM-1 and transepithelial neutrophil migration was potentiated by the specific binding of *E. coli* Hu734 to the urinary tract epithelial layers. The role of specific adhesins for the activation of these responses is under further study.

Neutrophil migration to the urinary tract mucosa is a characteristic of UTI. Once at the urinary tract mucosa, neutrophils are thought to play an important role in the clearance of infection. Intravesical inoculation of *E. coli* into the bladders of C3H/HeN mice induced a rapid neutrophil influx into the urine followed by a clearance of the infection. In contrast, C3H/HeJ (LPS-low-responder) mice, which showed a reduced neutrophil response to intravesical inoculation of *E. coli*, were unable to clear the infection (32). The anti-inflammatory agent dexamethasone reduced the neutrophil response of C3H/HeN mice to UTI and reduced their ability to clear the bacteria (19). In addition, in a rat UTI model, administration of antineutrophil serum led to a 1,000-fold increase in bacterial numbers in the acute phase of the infection (23). A more selective blocking of neutrophil influx may help provide direct evidence for the role of neutrophils in the clearance of UTI. The importance of ICAM-1 in transuroepithelial neutrophil migration suggests that interference of ICAM-1 expression, either in ICAM-1 knockout mice or by using blocking antibodies to murine ICAM-1 (30), may reduce neutrophil influx into the urine and provide a useful tool in studying the role of neutrophils in the clearance of UTI.

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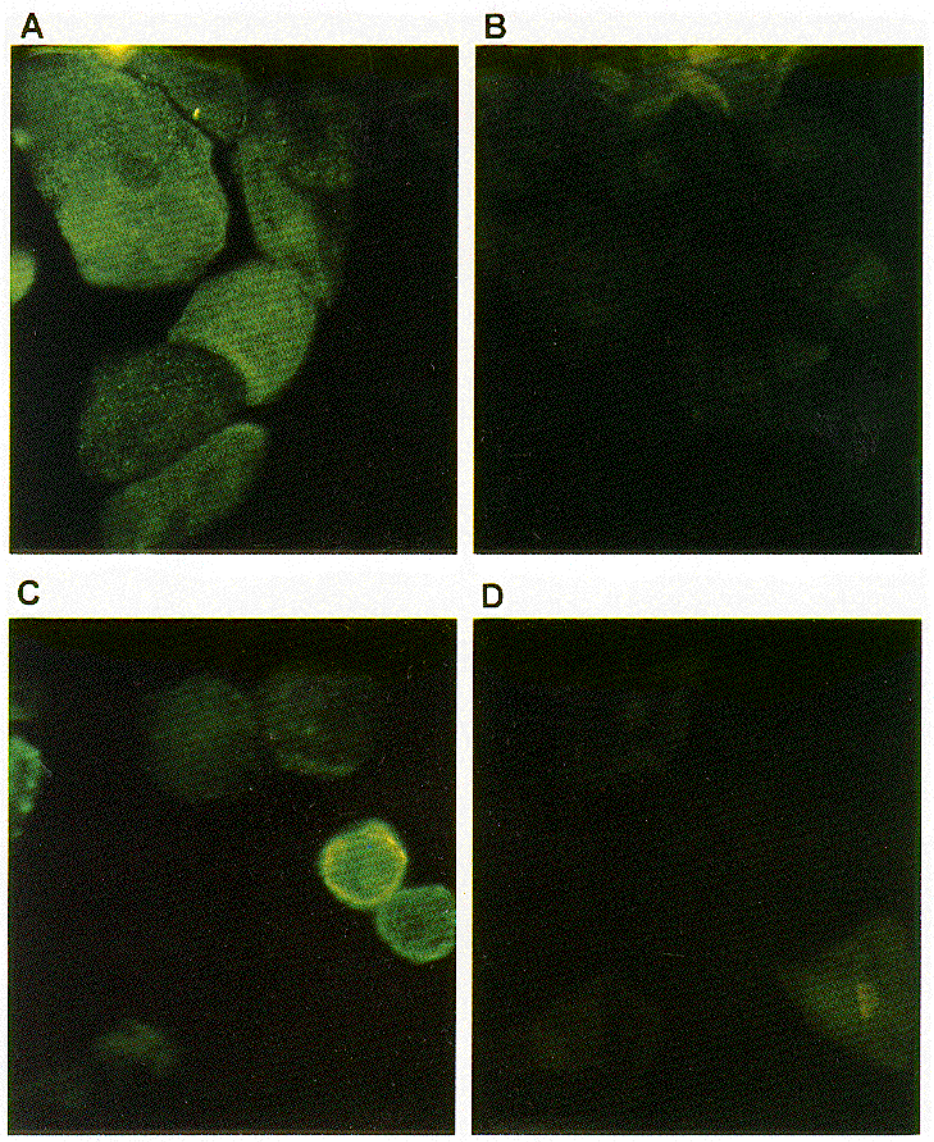
REFERENCES

1. **Agace, W., et al.** Unpublished data.
2. **Agace, W., S. Hedges, U. Andersson, J. Andersson, M. Ceska, and C. Svanborg.** 1993. Selective cytokine production in epithelial cells following exposure to *Escherichia coli*. Infect. Immun. **61:**602–609.
3. **Agace, W., S. Hedges, M. Ceska, and C. Svanborg.** 1993. Interleukin-8 and the neutrophil response to mucosal gram negative infection. J. Clin. Invest. **92:**780–785.
4. **Andersson, P., I. Engberg, G. Lidin-Janson, K. Lincoln, R. Hull, S. Hull, and C. Svanborg.** 1991. Persistence of *Escherichia coli* bacteriuria is not determined by bacterial adherence. Infect. Immun. **59:**2915–2921.
5. **Bevilacqua, M., and R. Nelson.** 1993. Selectins. J. Clin. Invest. **91:**379–387.
6. **Bevilacqua, M., S. Stengelin, M. Gimbrone, Jr., and B. Seed.** 1989. Endothelial leucocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. Science **243:**1160–1165.
7. **Colgan, S., C. Parkos, C. Delp, M. Amin Arnout, and J. Madara.** 1993. Neutrophil migration across cultured intestinal epithelial layers is modulated by epithelial exposure to IFN-γ in a highly polarized fashion. J. Cell Biol. **122:**785–798.
8. **Diamond, M., D. Staunton, A. Fougeerolles, S. Stacker, J. Garcia-Aguilar, M. Hibbs, and T. Springer.** 1990. ICAM-1 (CD54): a counter receptor for Mac-1 (CD11b/CD18). J. Cell Biol. **111:**3129–3139.
9. **Dohlsten, M., G. Hedlund, P. Lando, J. Trowsdale, D. Altmann, M. Pattaroyo, H. Fischer, and T. Kalland.** 1991. Role of adhesion molecule ICAM-1 (CD54) in staphylococcal enterotoxin mediated cytotoxicity. Eur. J. Immunol. **21:**131–135.
10. **Elgavish, A.** 1993. Effects of *Escherichia coli* and *E. coli* lipopolysaccharides on the function of human ureteral epithelial cells cultured in serum-free medium. Infect. Immun. **61:**3304–3312.
11. **Fougerolles, A., S. Stacker, R. Schwarting, and T. Springer.** 1991. Characterization of ICAM-2 and evidence for a third counter receptor for LFA-1. J. Exp. Med. **174:**253–267.

11a. **Hedges, S.** Personal communication.

12. **Hedges, S., W. Agace, M. Ceska, and C. Svanborg.** 1994. Uroepithelial cells are a part of a mucosal cytokine network. Infect. Immun. **62:**2315–2321.
13. **Hedges, S., W. Agace, M. Svensson, and C. Svanborg.** 1993. Cyclosporin A does not inhibit IL-1α induced epithelial cell IL-6 secretion. Scand. J. Immunol. **37:**581–586.
14. **Hedges, S., P. Anderson, G. Lidin-Janson, P. de Man, and C. Svanborg.** 1991. Interleukin-6 response to deliberate colonization of the human urinary tract with gram-negative bacteria. Infect. Immun. **59:**421–427.
15. **Hedges, S., M. Svensson, and C. Svanborg.** 1992. Interleukin-6 response of epithelial cell lines to bacterial stimulation *in vitro*. Infect. Immun. **60:**1295–1301.
16. **Ishikura, H., C. Takahashi, K. Kanagawa, H. Hirata, K. Imai, and T. Yoshiki.** 1991. Cytokine regulation of ICAM-1 expression on human renal tubular epithelial cells *in vitro*. Transplantation **51:**1272–1275.
17. **Lawrence, M., and T. Springer.** 1993. Neutrophils roll on E-selectin. J. Immunol. **151:**6338–6346.
18. **Leffler, H., and C. Svanborg-Eden.** 1980. Chemical identification of a glycosphingolipid receptor for *Escherichia coli* attachment to human uroepithelial cells and agglutinating human erythrocytes. FEMS Microbiol. Lett. **8:**127–134.
19. **Linder, H., I. Engberg, C. van Cooten, P. de Man, and C. Svanborg-Edén.** 1990. Effects of anti-inflammatory agents on mucosal inflammation induced by infection with gram-negative bacteria. Infect. Immun. **58:**2056–2060.
20. **Luscinkas, F., M. Cybulsky, J. Kiely, C. Peckins, V. Davis, and M. Gimbrone, Jr.** 1991. Cytokine activated human endothelial layers support enhanced neutrophil transmigration via a mechanism involving both endothelial-leucocyte adhesion molecule-1 and intercellular adhesion molecule-1. J. Immunol. **146:**1617–1625.
21. **Marlin, S., and T. Springer.** 1987. Purified intercellular adhesion molecule-1 is a ligand for lymphocyte function-associated antigen-1 (LFA-1). Cell **51:**813–819.
22. **McCormack, B., S. Colgan, C. Delp-Archer, S. Miller, and J. Madara.** 1993. *Salmonella typhimurium* attachment to human intestinal epithelial layers: transcellular signalling to subepithelial neutrophils. J. Cell Biol. **123:**895–907.
23. **Miller, T., G. Findon, and S. Cawley.** 1987. Cellular basis in host defence in pyelonephritis. III. Deletion of individual components. Br. J. Exp. Pathol. **68:**377–388.
24. **Nash, S., J. Stafford, and J. Madara.** 1987. Effects of polymorphonuclear leukocyte transmigration on the barrier function of cultured intestinal epithelial cell layers. J. Clin. Invest. **80:**1104–1113.
25. **Nortama, P., L. Rui, R. Renkonen, T. Timonen, J. Prieto, M. Patarroya, and C. Gahmberg.** 1991. The expression of intercellular adhesion molecule-2 is refractory to inflammatory cytokines. Eur. J. Immunol. **21:**2629–2632.
26. **Parkos, C., C. Delp, M. Armin Arnout, and J. Madara.** 1991. Neutrophil migration across cultured intestinal epithelium. J. Clin. Invest. **88:**1605–1612.
27. **Patarroyo, M., E. Clark, J. Prieto, C. Kantor, and C. Gahmberg.** 1987. Identification of a novel adhesion molecule in human leukocytes by monoclonal antibody LB-2. FEBS Lett. **210:**127–131.
28. **Pober, J., M. Bevilacqua, D. Mendick, L. Lapierre, W. Fiers, and M. Gimbrone, Jr.** 1986. Two distinct monokines, interleukin-1 and tumor necrosis factor, each independently induce biosynthesis and transient expression of the same antigen on the surface of cultured human vascular endothelial cells. J. Immunol. **136:**1680–1687.
29. **Pober, J., M. Gimbrone, Jr., L. Lapierre, D. Mendick, W. Fiers, R. Rothlein, and T. Springer.** 1986. Overlapping patterns of activation of human endothelial cells by interleukin-1, tumor necrosis factor and immune interferon. J. Immunol. **137:**1893–1896.
30. **Prieto, J., F. Takei, R. Gendelman, B. Christenson, P. Biberfeld, and M. Patarroyo.** 1989. MALA-2, a mouse homologue of human adhesion molecule ICAM-1 (CD54). Eur. J. Immunol. **19:**1551–1557.
31. **Salcedo, R., and M. Patarroyo.** 1995. Constitutive $\alpha_V\beta_3$ integrin-mediated adhesion of human lymphoid B cells to vitronectin substrate. Cell. Immunol. **160:**165–172.
32. **Shahin, R., I. Engberg, L. Hagberg, and C. Svanborg-Edén.** 1987. Neutrophil recruitment and bacterial clearance correlated with LPS responsiveness in local Gram-negative infection. J. Immunol. **138:**3475–3480.
33. **Somersalo, K., J. Tarkkanen, M. Patarroyo, and E. Saksela.** 1992. Involvement of the B_2 integrins in the migration of human NK cells. J. Immunol. **149:**590–598.
34. **Staunton, D., M. Dustin, and T. Springer.** 1989. Functional cloning of ICAM-2, a cell adhesion ligand for LFA-1 homologous to ICAM-1. Nature (London) **339:**361–364.

35. **Svanborg-Edén, C., R. Freter, L. Hagberg, R. Hull, S. Hull, H. Leffler, and G. Schoolnik.** 1982. Inhibition of experimental ascending urinary tract infection by receptor analogue. Nature (London) **298:**560–562.
36. **Svensson, M., R. Lindstedt, N. S. Radin, and C. Svanborg.** 1994. Epithelial glucosphingolipid expression as a determinant of bacterial adherence and cytokine production. Infect. Immun. **62:**4404–4410.
37. **Weiland, J., W. Davis, J. Holter, J. Mohammed, P. Dorinsky, and J. Gadek.** 1986. Lung neutrophils in the adult respiratory distress syndrome. Clinical and pathophysiologic significance. Am. Rev. Respir. Dis. **133:**218–225.
38. **Yardley, J., and M. Donowitz.** 1977. Colo-rectal biopsies in inflammatory bowel disease, p. 50–94. *In* J. H. Yardley and B. Morson (ed.), The gastrointestinal tract. The Williams & Wilkins Co., Baltimore.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