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Adhesion-Dependent Activation of Mucosal Interleukin-6 Production

HENRIK LINDER,[1] INGA ENGBERG,[1] HEINTZ HOSCHÜTZKY,[2,3] INGER MATTSBY-BALTZER,[2,3] AND CATHARINA SVANBORG[4]†*

Departments of Clinical Immunology[1] and Bacteriology,[3] University of Göteborg, Göteborg, Sweden; Max Planck Institute für Immunbiologie, D-7800 Freiburg, Germany[2]; and Department of Clinical Immunology, University of Lund, Lund, Sweden[4]

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Mucosal exposure to *Escherichia coli* elicits an inflammatory response in the urinary tract. Interleukin-6 (IL-6) is secreted into the urine, and polymorphonuclear leukocytes (PMNLs) are recruited to the site of infection. This study analyzed the ability of mucosally administered bacterial components to activate IL-6 and PMNL responses. P, S, and type 1 fimbrial preparations with adhesins specific for Galα1-4Galβ, NeuAcα2-3Gal, and mannose, respectively, were inoculated intravesically into lipopolysaccharide (LPS)-responder (C3H/HeN) and LPS-nonresponder (C3H/HeJ) mice. The role of the fimbrial adhesin was examined by comparing P and S fimbriae with (Adh^+) and without (Adh^-) the receptor-binding domain. Isolated lipid A was used in parallel. The urinary IL-6 levels were elevated after challenge with Adh^+ P fimbriae, but not after challenge with the Adh^- P fimbriae, Adh^+ or Adh^- S fimbriae, or type 1 fimbriae. The activation was not a function of contaminating LPS, since it occurred in both LPS-responder and -nonresponder mice and since isolated lipid A was a poor activator of the IL-6 response. In contrast, lipid A was a potent inducer of the PMNL response. The results suggested that the IL-6 and PMNL responses were activated via different pathways; the IL-6 response was activated mainly by an adhesion-dependent interaction with the mucosa, and the PMNLs were activated mainly by lipid A. The results emphasize the active role of the mucosal barrier in the production of mediators in response to diverse bacterial stimulants.

Attaching *Escherichia coli* bacteria induce a mucosal inflammatory response in the urinary tract (36). Interleukin-6 (IL-6) levels in the urine increase within minutes of mucosal challenge with *E. coli* expressing P fimbriae (7), and within hours polymorphonuclear leukocytes (PMNLs) are recruited and excreted into the urine (23, 24, 35). In studies with mice, two bacterial properties have been shown to enhance the inflammatory response: bacterial adhesins binding to the globoseries of glycolipid receptors and the lipid A moiety of lipopolysaccharide (LPS). The inflammatory response to whole *E. coli* bacteria was mimicked by adhesins and lipid A acting in synergy (23, 24). Evidence that bacterial attachment to Galα1-4Galβ-containing receptors increased mucosal inflammation was also obtained from studies with humans. Infections caused by P-fimbriated bacteria gave rise to a higher inflammatory response than infections caused by strains which lacked P fimbriae (6). Taken together, these results suggested that adhesins might promote mucosal inflammation not only by localizing endotoxin to the mucosal surface but also by directly stimulating epithelial cells through ligand-receptor interaction.

Uropathogenic *E. coli* strains coexpress adhesins with several receptor specificities (26, 27, 33). Most strains contain the DNA sequences encoding type 1 fimbriae. The *pap* DNA sequences characterize *E. coli* isolates from the urinary tract (2, 34), and the Galα1-4Galβ binding phenotype is enriched among strains that cause acute pyelonephritis (22, 38). S fimbriae with specificity for NeuAcα2-3Gal occur in certain *E. coli* clones associated with acute pyelonephritis (18). Epidemiologic data also suggest that P fimbriae enhance the induction of mucosal inflammation more than S or type 1 fimbriae (6, 36).

The aim of the present study was to analyze whether mucosal administration of adhesins and/or lipid A can trigger the secretion of IL-6 and the recruitment of PMNLs and to compare the effects of fimbriae differing in receptor specificities.

MATERIALS AND METHODS

Mice. C3H/HeJ mice (original breeding stock; Jackson Laboratory, Bar Harbor, Me.) and C3H/HeN mice (original breeding stock; Charles River Laboratories, Margate, United Kingdom) were bred at the animal facilities in the Department of Clinical Immunology, University of Göteborg, Göteborg, Sweden. Female mice were used at 8 to 10 weeks of age.

Inoculation procedure. Female C3H/HeN (Lps^n/Lps^n) mice and C3H/HeJ (Lps^d/Lps^d) mice were inoculated by urethral catheterization, as previously described (12, 13). Prior to inoculation, the absence of any preexisting inflammation in the urinary tract was confirmed by examination of the urine. Mice with a urinary leukocyte count in uncentrifuged urine of $>20 \times 10^4$ PMNLs per ml were excluded. The intravesical inoculation was performed under ether anesthesia. Each mouse received 100 μl of suspensions containing bacterial components (fimbriae, lipid A, or mixtures of the two) dissolved in phosphate-buffered saline (PBS). The inocula were deposited in the bladder through a soft polyethylene catheter (outer diameter, 0.61 mm; Clay Adams, Parsippany, N.J.) attached to a syringe. Immediately after inoculation, the catheter was withdrawn and no further manipulations were performed. Urine samples were collected prior to inoculation and at 1/2 h, 2 h, and 6 h

* Corresponding author.
† Present address: Department of Medical Microbiology, Division of Clinical Immunology, Sölvegatan 23, S-223 62 Lund, Sweden.

postinoculation by gentle compression of the mouse abdomen. The PMNL counts were determined on the fresh uncentrifuged urine samples with a hemocytometer. IL-6 levels were determined in samples which were kept frozen at −20°C until analyzed. Serum samples were collected by retro-orbital bleeding at 6 h postinoculation under ether anesthesia. The serum samples were allowed to clot and centrifuged at 3,000 × *g* for 10 min, and the serum was collected and stored at −20°C until being assayed for IL-6. The mice were sacrificed by cervical dislocation before awakening from ether anesthesia.

Bacterial strains and fimbrial preparations. (i) *P. fimbriae.* *E. coli* 21624 was a transformant in *E. coli* HB101, used for the isolation of the Adh^+ P fimbriae. It had received pDAL201B, carrying as an insert the chromosomal *pap* DNA region from the wild-type uropathogenic strain *E. coli* C1212 of serotype O4:K12:H^-. The recombinant strain expressed P fimbriae of serotype $F7_1$ (17) and agglutinated Galα1-4Galβ–latex beads.

(ii) *S. fimbriae.* *E. coli* 536(pANN 801-13) (HB101) used for the isolation of the fimbria adhesin complex (FAC) was a recombinant in *E. coli* HB101. It had received the *sfa* DNA sequences encoding S fimbriae and adhesins specific for NeuAcα2-3Gal from wild-type *E. coli* 536 of serotype O6:K15:H31 (11), isolated from a patient with a urinary tract infection (28).

(iii) Type 1 fimbriae. The fimbriae were isolated from *E. coli* B American (Bam) (5, 14) and kindly provided by C. Brinton, University of Pittsburgh, Pittsburgh, Pa.

(iv) Isolation of FAC. The Adh^+ fimbriae were isolated as previously described (17, 28). The fimbriae were released by heating for 30 min at 65°C. Crude FAC was precipitated with ammonium sulfate (20% saturation) and collected by centrifugation. To remove contaminating lipids, the pellet was dissolved in 50% ethanol and precipitated with LiCl (250 mM). After centrifugation, the pellet was suspended in 10 mM Tris chloride (pH 7.8). Deoxycholate (0.5%) was added to remove the residual LPS, and the temperature was elevated to 60°C for 30 min. The Adh^+ fimbriae were precipitated with LiCl (250 mM), collected by centrifugation, dissolved in a small amount of water, and stored at −70°C.

To obtain fimbriae devoid of the adhesin, a fimbrial solution (10 mg/ml) in 10 mM Tris chloride (pH 8) was treated with Zwittergent 3-16 and heated to 80°C for 30 min. This treatment caused a dissociation of the fimbriae and adhesin. The fimbriae devoid of adhesin (Adh^- fimbriae) were collected by precipitation with LiCl (250 mM) and centrifugation. The protein concentrations of the fimbrial preparations were determined spectrophotometrically (Ultrospec II; Biochrom 4050, Cambridge, United Kingdom).

Binding properties and receptor specificity of the fimbrial preparations. Prior to inoculation of the mice, the binding properties of Adh^+ and Adh^- fimbria suspensions were analyzed by the agglutination of erythrocytes and receptor-coated latex beads. Human erythrocytes of blood group AP_1 were obtained from freshly drawn heparinized blood. Guinea pig blood was drawn by cardiac puncture. The erythrocytes were used as 3% suspensions in PBS with and without 0.1 M α-methyl mannoside (α-Man). Hemagglutination reactions inhibited by α-Man were designated as mannose sensitive, and the noninhibitable reactions were designated as mannose resistant. Specificity for the Galα1-4Galβ disaccharide was identified by the agglutination of receptor-coated latex beads (Orion, Helsinki, Finland).

The binding properties of the isolated fimbriae were compared with those of the wild-type strain and the transformants used for fimbrial preparations. Adh^+ P fimbriae induced mannose-resistant agglutination of human and sheep erythrocytes and agglutinated the Galα1-4Galβ latex beads. Adh^- P fimbriae did not. Adh^+ S fimbriae agglutinated human, bovine, and sheep erythrocytes at 4°C. Adh^- S fimbriae did not. Type 1 fimbrial suspensions agglutinated guinea pig erythrocytes in a mannose-sensitive manner.

Quantitation of the inflammatory response. (i) IL-6 assay. The cell line B13.29, which is dependent on IL-6 for growth, has been described previously (16, 21). For IL-6 determinations, the more sensitive subclone B9 was used (1, 16). B9 cells were harvested from the tissue culture flasks, seeded into microdilution plates (Nunc, Roskilde, Denmark) at a concentration of 5,000 cells per well together with urine and serum samples, and cultured in Iscove modified Dulbecco medium supplemented with 5×10^{-5} M mercaptoethanol, 5% fetal calf serum (Sera-Lab, Sussex, United Kingdom), and gentamicin (0.1 mg/ml). [^{3}H]thymidine was added after 68 h of culture, and the cells were harvested 4 h later. The samples were tested in twofold dilutions and compared with an IL-6 standard; 1 U/ml is the concentration required for half-maximal proliferation of the B9 cells. The specificity was tested on occasional samples by inhibition with a monoclonal anti-IL-6 antibody kindly provided by J. van Snick.

(ii) Leukocyte excretion. Prior to each experiment, urine was collected from individual mice, cultured to ensure sterility of the urine, and inspected microscopically for the absence of a preexisting inflammatory response. At various times after the injection of bacteria, urine was collected from individual mice. The number of PMNLs in the uncentrifuged urine was quantitated microscopically with a hemocytometer chamber.

Endotoxin quantitation. The amount of endotoxin in urine and in the fimbrial preparations was analyzed by the quantitative chromogenic *Limulus* amoebocyte lysate assay (9) (Kabi Diagnostics, Mölndal, Sweden). LPS from *E. coli* O111B4 was used as the standard (range, 10 to 100 pg/ml).

Statistics. PMNL counts in the urine and the levels of IL-6 and systemic IL-6 are presented as means. Differences were analyzed for significance with the Spearman rank test. Differences in IL-6 production and PMNL excretion between groups of animals were considered statistically significant for P values of <0.05.

RESULTS

Fimbriae and IL-6 secretion. C3H/HeN mice received 0.1 ml intravesically of suspension containing Adh^+ or Adh^- fimbriae (4 mg/ml; ~400 μg per mouse). A urinary IL-6 response occurred within 30 min of inoculation and remained high for 2 h. It had subsided 6 h after inoculation. The mean IL-6 response to the Adh^+ P fimbriae at 2 h was 230 U/ml compared with 52 U/ml for the Adh^- P fimbriae (Fig. 1). IL-6 activity of >20 U/ml occurred in 14 of 19 mice inoculated with the Adh^+ P fimbriae compared with 5 of 15 mice inoculated with Adh^- P fimbriae ($P < 0.05$). No IL-6 activity was detected in sera at 6 h postinoculation with the Adh^+ or Adh^- P fimbriae.

The Adh^+ P fimbriae contained 0.7% contaminating endotoxin. In contrast, the Adh^- P fimbriae did not give a positive reaction in the *Limulus* lysate assay at a protein concentration of 4 mg/ml. To differentiate between the effect of the fimbrial protein and endotoxin, parallel experiments were performed in LPS-nonresponder mice (C3H/HeJ). Mucosal administration of the Adh^+ P fimbriae elicited IL-6

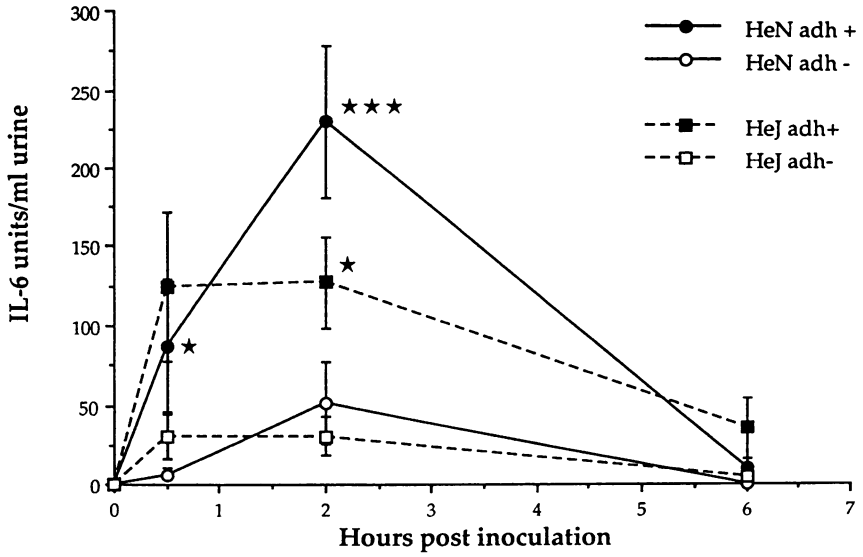

FIG. 1. Kinetics of IL-6 secretion in response to intravesical inoculation of C3H/HeN and C3H/HeJ mice with P fimbriae. The mice were given P fimbriae with (Adh^+) and without (Adh^-) the P-fimbrial adhesin (4 mg/ml; 400 μg per mouse). One unit represents the concentration required for half-maximal proliferation of the B9 cells. Each point represents the mean of 15 to 40 animals. ★, $P < 0.05$; ★★, $P < 0.01$; ★★★, $P < 0.001$.

secretion into the urine of LPS-nonresponder mice as well (15 of 22 mice had a urinary IL-6 level of >20 U/ml). There was no significant difference in the IL-6 response between C3H/HeJ and C3H/HeN mice. At 2 h, the IL-6 response in the C3H/HeJ mice to the Adh^+ P fimbriae was significantly higher (mean, 128 U/ml) than the response to the Adh^- fimbriae (mean, 32 U/ml) (Fig. 1).

Furthermore, the ability of lipid A to elicit an IL-6 response was tested. LPS-responder and -nonresponder mice received intravesically 0.1 ml of suspensions containing 1 mg, 100 μg, 10 μg, 1 μg, and 100 ng of lipid A per ml. In C3H/HeN mice, an IL-6 activity of >20 U/ml occurred in 7 of 15, 1 of 10, 2 of 10, and 0 of 5 mice, respectively. In C3H/HeJ mice, no response occurred in the 10 mice in each group. The mean IL-6 responses were 56 U/ml of urine at 1 mg of lipid A per ml and 7 U/ml of urine at 100 μg of lipid A per ml in the C3H/HeN mice. The contaminating lipid A was, therefore, not likely to have explained the response to the Adh^+ P fimbriae.

The requirements for activation of IL-6 were further analyzed with S fimbriae and type 1 fimbriae (Table 1). A urinary IL-6 level >20 U/ml was found in 1 of 10 C3H/HeN mice inoculated with Adh^+ S fimbriae and in 0 of 10 mice inoculated with Adh^- S fimbriae (4 mg/ml; ~400 μg per mouse). Similarly, the IL-6 response was not efficiently triggered by the type 1 fimbriae. Urinary IL-6 levels of >20 U/ml occurred in 4 of 26 mice at 2 h after inoculation of fimbriae. The level of endotoxin contamination of the fimbrial proteins, as determined by the *Limulus* assay, was not measurable at an S fimbria concentration of 4 mg/ml and was 0.05% for type 1 fimbriae.

Fimbriae and leukocyte excretion. C3H/HeN mice were inoculated intravesically with either Adh^+ or Adh^- P fimbriae (4 mg/ml; ~400 μg per mouse). A urinary PMNL

TABLE 1. Urinary IL-6 and PMNL responses to isolated *E. coli* P, S, and type 1 fimbriae in C3H/HeN and C3H/HeJ mice

Fimbriae and treatment	Concn (mg/ml)	Median (range) response			
		IL-6, U/ml[a]		PMNL, cells/ml, 10^{4b}	
		C3H/HeN	C3H/HeJ	C3H/HeN	C3H/HeJ
P					
Adh^+	1	187 (0–899)(n = 35)[c]	61 (0–400)(n = 30)	200 (0–500)(n = 28)	21 (0–150)(n = 10)
	0.1	0 (0–73)(n = 10)	0 (0–105)(n = 9)	121 (0–500)(n = 10)	0 (0–55)(n = 8)
Adh^-	1	0 (0–350)(n = 15)	0 (0–200)(n = 15)	2 (0–100)(n = 15)	0 (0–155)(n = 14)
S					
Adh^+	1	0 (0–62)(n = 10)	0 (0)(n = 10)	0 (0)(n = 7)	0 (0)(n = 9)
Adh^-	1	0 (0)(n = 10)	0 (0)(n = 10)	15 (0–500)(n = 10)	0 (0–20)(n = 10)
Type 1	9	0 (0–100)(n = 10)	0 (0–20)(n = 17)	0 (0–500)(n = 25)	0 (0–10)(n = 10)

[a] Results were taken 2 h postinfection.
[b] Results were taken 6 h postinfection. 0, $<10^4$ cells per ml.
[c] n, number of mice.

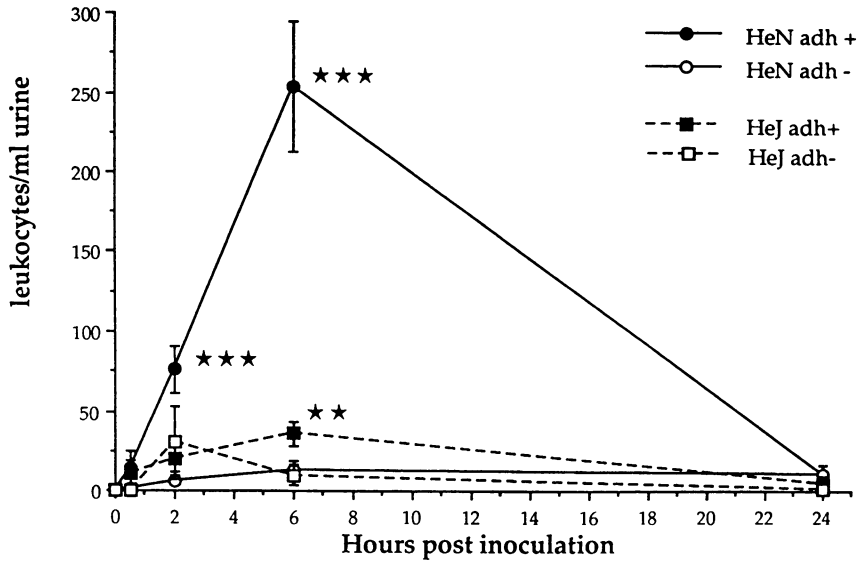

FIG. 2. Kinetics of the PMNL response to intravesical inoculation of C3H/HeN and C3H/HeJ mice with P fimbriae. Mice were inoculated intravesically with P fimbriae with (Adh^+) and without (Adh^-) the P-fimbrial adhesin (4 mg/ml; 400 μg per mouse). Leukocyte data are presented as 10^4 PMNLs per ml of urine. Each point represents the mean of 15 to 40 animals. **, $P < 0.01$; ***, $P < 0.001$.

response occurred within 2 h postinoculation of Adh^+ P fimbriae, increased between 2 and 6 h, and subsided at 24 h (Fig. 2). The Adh^+ P fimbriae gave a significantly greater leukocyte response at 2 h (76 × 10^4 PMNLs per ml) and 6 h (mean, 254 × 10^4 PMNLs per ml) than did Adh^- P fimbriae (6 × 10^4 PMNLs per ml at 2 h and 13 × 10^4 PMNLs per ml at 6 h, respectively).

To differentiate between the effects of endotoxin and the adhesin-receptor interaction on PMNL excretion, the experiments were performed in parallel in LPS-nonresponder mice (C3H/HeJ). Mucosal administration of the Adh^+ P fimbriae in C3H/HeJ mice did not elicit a PMNL response of >50 × 10^4 PMNLs per ml of urine (Fig. 2).

To further investigate the role of lipid A as a stimulus for the PMNL response, C3H/HeN and C3H/HeJ mice were inoculated with decreasing concentrations of lipid A (1 mg/ml, 100 μg/ml, 20 μg/ml, and 100 ng/ml). A significant PMNL response occurred in C3H/HeN mice at 20 ng/ml (Fig. 3), but not in C3H/HeJ mice (results not shown).

The role of receptor specificity for the PMNL response to the adhesins was further analyzed with S fimbriae and type 1 fimbriae. C3H/HeN mice were inoculated intravesically with Adh^+ or Adh^- S fimbriae (4 mg/ml). A urinary leukocyte response of >50 × 10^4 PMNLs per ml of urine was found in 4 of 10 mice inoculated with Adh^+ S fimbriae and in 0 of 10 mice inoculated with Adh^- S fimbriae (Table 1). The leuko-

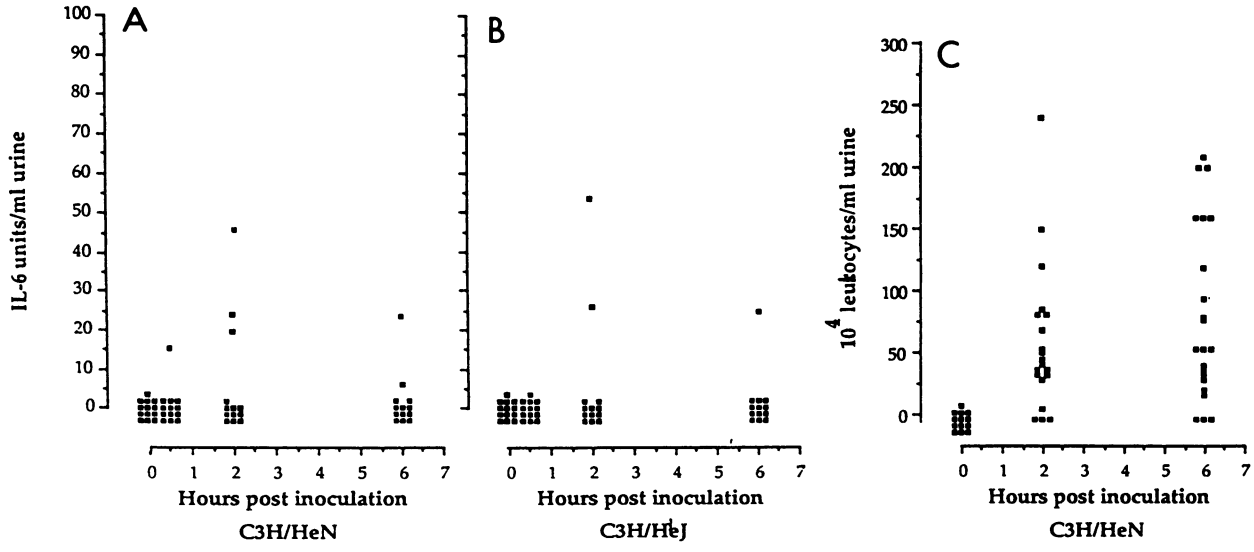

FIG. 3. Individual IL-6 (A and B) and PMNL (C) responses of C3H/HeN and C3H/HeJ mice inoculated with lipid A (20 ng/ml). One unit of IL-6 represents the concentration required for half-maximal proliferation of the B9 cells. Leukocyte data are presented as 10^4 PMNLs per ml of urine. Each datum point represents one animal.

cyte response was not efficiently triggered by type 1 fimbriae. A response of $>50 \times 10^4$ PMNLs per ml of urine occurred in 0 of 23 mice (Table 1).

DISCUSSION

Attaching *E. coli* activate mucosal inflammation. Our previous studies suggested that the attachment enhances the toxicity of other bacterial surface components such as the LPS (23, 24). The present study provided evidence for a more direct role of P fimbriae as inducers of mucosal IL-6 secretion. Exposure of the urinary tract mucosa to adhesive P fimbriae elicited an IL-6 response; fimbriae lacking the receptor-binding domain did not. The stimulation of IL-6 production was not solely a result of synergy with lipid A, since the Adh^+ P fimbriae also elicited an IL-6 response in C3H/HeJ (Lps^d/Lps^d) mice and since purified lipid A did not induce IL-6 secretion at a level comparable to that induced by the fimbriae.

The P fimbriae showed the highest capacity to activate mucosal IL-6 production. The S and type 1 fimbrial preparations used in this study were not as efficient inducers of IL-6. These results are consistent with observations from epidemiological studies. Patients infected with P-fimbriated bacteria had higher levels of fever and C-reactive proteins than patients infected with type 1-fimbriated strains or with S-fimbriated strains (6, 36). These differences may be due both to the fimbriae and to their receptors. First, P fimbriae differed from type 1 and S fimbriae in that the P adhesin strongly binds LPS, as shown with immunogold-labeled anticore antibodies in immunoelectron microscopy (17a). The adhesin-receptor interaction might, therefore, lead to the direct approximation of toxin to the epithelial surface and form a basis for the synergistic activation of mucosal inflammation by P fimbriae and lipid A, which has been demonstrated previously (23). In contrast, S and type 1 fimbriae did not contain LPS as determined by the *Limulus* assay.

Second, there are differences among the receptors. Receptors for the P, S, and type 1 fimbriae have been reported to occur on urinary epithelial cells and in the renal tissue of mice (4, 12, 13, 19, 20, 30). The globoseries oligosaccharide sequences occur mainly in glycolipid form bound in the lipid bilayer of the epithelial cells. In contrast, the oligosaccharide sequences recognized by the S and type 1 fimbriae occur on glycoproteins, which may be secreted like Tamm Horsfall glycoprotein (31, 32). The injected S and type 1 fimbriae may therefore bind to the soluble receptor and be prevented from reaching mucosal receptor sites. Their capacity to enhance the activation of IL-6 production would then be reduced. Third, there may be functional differences between the globoseries and the sialic acid containing glycolipids in their capacity to participate in transmembrane signalling. This remains to be demonstrated.

P fimbriae and lipid A were found to activate different pathways of mucosal inflammation. The Adh^+ P fimbria-lipid A complex activated both the secretion of IL-6 and the accumulation of PMNLs, whereas lipid A alone mainly triggered the PMNL response. Even at high concentrations of lipid A, the level of IL-6 in urine and the number of responding animals were low. Thus, even though LPS is known to activate IL-6 production in other cell types (3, 29), the mucosa appeared to prefer activation via bacterial adhesins. In contrast, lipid A was a sufficient stimulus for the PMNL response. Mucosal exposure to lipid A activated the PMNL influx in LPS-responder mice but not in LPS-nonresponder mice. Lipid A contamination probably explained the fimbrial activation of the PMNL response, since the Adh^+ P fimbrial preparation which activated the PMNL response had the highest concentration of contaminating lipid A. The existence of different pathways for the activation of IL-6 and of chemoattractants recruiting PMNLs to the local site was also consistent with our previous studies using pharmacologic inhibitors of inflammation. Cyclosporin A reduced IL-6 but not the PMNL response (15). Dexamethasone and diclofenac lowered both the IL-6 level and the PMNL response, but indomethacin did not reduce either parameter (15).

The two pathways of mucosal inflammation have different biological consequences. IL-6 is an endogenous pyrogen which activates the febrile response in rabbits at least as rapidly as IL-1. It stimulates hepatocytes to produce C-reactive protein and fibrinogen (8, 10, 37) and mucosal B cells to produce immunoglobulin A (3, 8). It may, therefore, be responsible for the induction of symptoms and immunity which characterize patients with the systemic urinary tract infection. On the other hand, IL-6 does not appear to determine the defense against urinary tract infection in the mouse. Bacteria are cleared efficiently in mice in which the IL-6 response has been suppressed by cyclosporin A (15). The PMNLs, on the other hand, probably are directly involved in the clearance of bacteria from the urinary tract. In vivo susceptibility to infection is increased in mice with a defective PMNL response, and anti-inflammatory agents which suppress phagocytosis enhance susceptibility to experimental infection (25, 32). The identification of these two pathways and the bacterial components responsible for their activation means that it may be possible to selectively influence the symptoms without reducing host resistance to infection.

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