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Effects of Anti-Inflammatory Agents on Mucosal Inflammation Induced by Infection with Gram-Negative Bacteria

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Gram-negative bacterial infections of the urinary tract elicit a mucosal inflammatory response. Interleukin-6 is secreted into the urine, and polymorphonuclear leukocytes (PMNL) are recruited. In the present study we examined the effect of anti-inflammatory agents on these parameters and on bacterial clearance from the kidneys. Dexamethasone reduced interleukin-6 secretion, the PMNL response, and bacterial clearance. Diclofenac abolished the urinary interleukin-6 response but reduced the PMNL response and bacterial clearance only at the highest concentrations. Indomethacin drastically decreased bacterial clearance without the corresponding effect on interleukin-6 production or the PMNL response. The results demonstrate that the inhibition of inflammation impairs bacterial clearance from the kidneys. This is, however, not a direct function of inhibited interleukin-6 production or PMNL recruitment.

Gram-negative bacterial infections at mucosal surfaces elicit local and systemic inflammation. This inflammation can be quantitated as the recruitment of polymorphonuclear leukocytes (PMNL) (18, 20) or as the level of cytokines such as interleukin-6 (IL-6) in serum and secretions (3). An intact host lipopolysaccharide (LPS) gene, *lps*, found in LPS-responder mice (C3H/HeN [Lps^n Lps^n]), is required for both the PMNL response and the IL-6 response (18, 20). The inflammation is elicited by whole bacteria and the lipid A moiety of endotoxin (18), the presentation of which is facilitated by bacterial attachment to Galα1-4Galβ-containing glycolipid receptors on the epithelial cells (13).

The inflammatory response coincides with the clearance of infection. In LPS-responder mice, the clearance of bacteria from kidneys and bladders occurred within a few days of infection (7, 14, 18). LPS-nonresponder mice (C3H/HeJ and C57BL/10ScCr [Lps^d Lps^d]) remained chronically infected (8). These results suggested that the inflammation was responsible for the elimination of bacteria and infection. Preliminary support for this hypothesis was obtained with indomethacin, which reduced bacterial clearance (14). The aim of the present study was to further evaluate the effects of anti-inflammatory agents on bacterial clearance. Since such effects might be secondary to inhibited production of mucosal mediators or recruitment of leukocytes, the second aim was to analyze the effects of anti-inflammatory agents on IL-6 production and leukocyte recruitment and the relationship to bacterial clearance after mucosal gram-negative bacterial infections.

MATERIALS AND METHODS

Mice. C3H/HeJ mice (original breeding stock; Jackson Laboratory, Bar Harbor, Maine) and C3H/HeN mice (original breeding stock; Charles River Laboratories, Margate, Kent, 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.

Bacteria. *Escherichia coli* Hu734 (O75K5H−) is a Lac⁻ mutant of wild-type *E. coli* GR-12, originating from a patient with acute pyelonephritis (19). Hu734 expressed adhesins specific for two groups of glycoconjugate receptors: the globoseries of glycolipids with the receptor site Galα1-4Galβ determined the ability to attach to mouse uroepithelial and kidney cells (8), and the mannose-specific adhesins conferred a lower degree of attachment to epithelial cells (8).

For infection, bacteria were cultured in Luria broth with 0.1% $CaCl_2$ for 16 h at 37°C, harvested by centrifugation, and diluted in phosphate-buffered saline (PBS; pH 7.2; 300 mosM) to an optical density at 597 nm of 0.96 (Vitatron DCP; Vital Scientific, Dieren, The Netherlands) (approximately 2×10^9 bacteria per ml). The concentration of live bacteria was determined with viable counts.

Receptor specificity of bacterial adhesins. Bacterial suspensions were tested prior to each experiment for the expression of adhesins. Adhesins specific for the globoseries of glycolipid receptors were identified by mannose-resistant agglutination of human P_1 erythrocytes (12) and agglutination of latex beads with synthetic Galα1-4Galβ covalently coupled via a spacer arm (2). Adhesins specific for mannose-containing receptors were identified by agglutination of guinea pig erythrocytes that was reversed in the presence of 0.1 M α-methyl-mannoside (4).

Infection. Mice were inoculated by urethral catheterization as previously described (6). After ether anesthesia, the suspensions of bacteria were deposited in the bladder through a soft polyethylene cathether (outer diameter, 0.61 mm; Clay Adams, Parsippany, N.J.). Immediately after inoculation the catheter was withdrawn, and no further manipulations were performed. Mice were inoculated with 0.1 ml of live bacteria (2×10^8 per mouse).

Animals were sacrificed 24 h after infection by cervical dislocation. Kidneys and bladders were removed and homogenized in sterile PBS with a Lab Blender Stomacher 80 homogenizer (Seward Medical UAC House, London, United Kingdom). Tenfold serial dilutions of the homogenates (1/1 to 1/1,000) in sterile PBS were plated on lactose-bromthymol blue agar. The dilution of bacteria yielding between 20 and 300 colonies per 0.05 ml was scored. The

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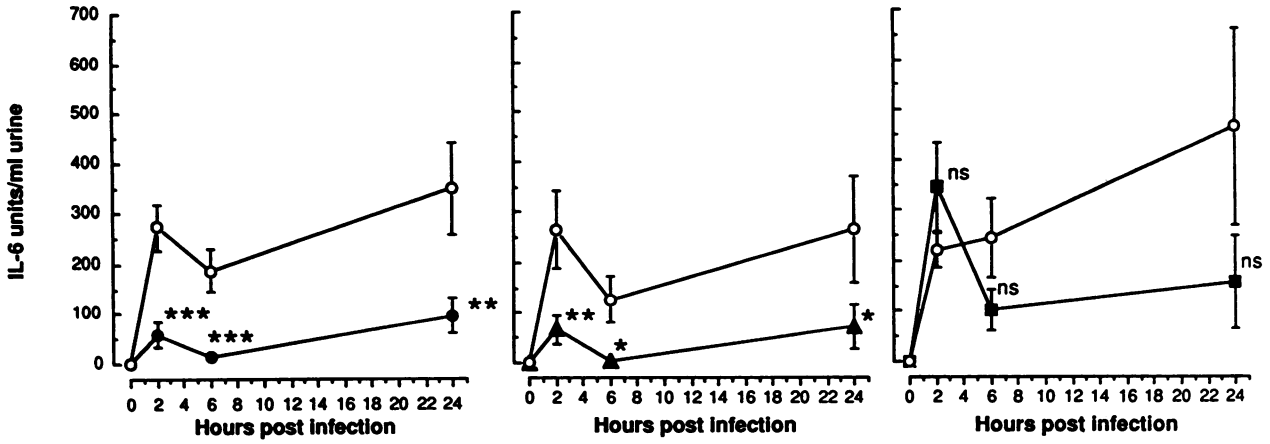

FIG. 1. Kinetics of the IL-6 response to *E. coli* infection in the urinary tracts of mice pretreated with anti-inflammatory drugs. Mice were given intraperitoneal injections (0.1 ml) of dexamethasone (4 mg/ml) (●), diclofenac (10 mg/ml) (▲), indomethacin (5 mg/ml) (■), or PBS (○) at the same time that *E. coli* was inoculated in the urinary tract and at 6 h postinoculation. Each mouse received approximately 10^8 bacteria. One unit represents the concentration required for half-maximal proliferation of the B9 cells. Each point represents a mean of 10 to 40 animals. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

results are expressed as the mean of the logarithm (geometric mean) of bacterial counts for each group of mice.

Quantitation of the inflammatory response by the IL-6 assay. Cell line B13.29, which is dependent on IL-6 for growth, has been described previously (11). For IL-6 determinations, the more sensitive subclone B9 was used (1, 9). The B9 assay can detect IL-6 concentrations in picograms. B9 cells were harvested from tissue culture flasks, seeded into microdilution plates (Nunc, Roskilde, Denmark) at a concentration of 5,000 cells per well, and cultured in Iscove modified Dulbecco medium supplemented with 5×10^{-5} M β-mercaptoethanol, 5% fetal calf serum (Sera-Lab, Sussex, United Kingdom), and gentamicin (100 mg/ml). [^{3}H]thymidine was added after 68 h of culturing, and the cells were harvested 4 h later. The samples were tested in twofold dilutions and compared with an IL-6 standard. One unit per milliliter is the concentration required for half-maximal proliferation of the B9 cells, and 1 U is approximately equivalent to 1 pg.

The specificity of the assay was tested on random samples. B9 cells react with interleukin-4 (IL-4) but with an activity 1,000-fold lower than that for IL-6 (9). Monoclonal anti-IL-6 antibodies reduced the activity of the samples, but monoclonal anti-IL-4 antibodies did not. The monoclonal anti IL-6 antibodies were provided by J. van Snick, Ludwig Institute for Cancer Research, Brussels, Belgium, and the rat hybridoma 11B11, developed by W. E. Paulwas, was the source of the monoclonal anti-IL-4 antibodies (16).

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 leukocytes in the uncentrifuged urine was quantitated microscopically with a hemacytometer chamber.

Anti-inflammatory agents. Dexamethasone (Decadron) was purchased from Merck Sharp & Dohme (Rahway, N.J.). Indomethacin (Confortid) was purchased from A/S Dumex (Dumex Ltd., DK 2300, Copenhagen, Denmark). Diclofenac was kindly provided by R. Björkman, Ciba-Geigy Läkemedel AB (Fröfästegatan 5, S-421 26 Västra Frölunda, Sweden). The chemicals were dissolved in PBS (pH 7.2; 300 mosmol) by end-over-end rotation with a Heto RK 20 apparatus (Heto Lab Equipment, Klintehøjevenge 3, 3460 Birkerød, Denmark) at 37°C for 30 min. Animals were injected intraperitoneally with drugs as specified for each experiment in the Results section.

Statistics. PMNL counts in urine and the local IL-6 response are presented as means, the systemic IL-6 response is presented as the geometric mean, and bacterial persistence is presented as the geometric mean for the group of animals treated with each drug or with PBS.

Differences were analyzed for significance by Student's *t* test. Differences in leukocyte excretion and IL-6 production between groups of animals were considered statistically significant at *P* values of <0.05.

RESULTS

Effect of anti-inflammatory agents on the IL-6 response. IL-6 levels in urine increased from undetectable levels prior to colonization with *E. coli* Hu734 to a mean response of about 270 U/ml 2 to 5 h after infection (Fig. 1). No IL-6 response occurred in LPS-nonresponder mice (data not shown).

To test the effect of pharmacologic inhibition on the IL-6 response in urine and serum, we injected C3H/HeN mice intraperitoneally with PBS or suspensions of dexamethasone, indomethacin, and diclofenac (0.1 ml per mouse) at various concentrations. Injections were given concomitantly with intravesical infection with *E. coli* Hu734 and 6 h later. Urine and serum samples were obtained at 2, 6, and 24 h after infection.

Dexamethasone (4 mg/ml) significantly reduced IL-6 levels in urine as compared with the PBS control at 2, 6, and 24 h (Fig. 1) ($P < 0.001$ at 2 h, $P < 0.001$ at 6 h, and $P < 0.01$ at 24 h). Diclofenac significantly reduced IL-6 activity in urine at 2, 6, and 24 h as compared with the PBS control ($P < 0.05$ at 2, 6, and 24 h) (Fig. 1). Indomethacin (5 mg/ml) treatment did not significantly reduce IL-6 levels in urine at any time tested (Fig. 1). The concentrations of dexamethasone and diclofenac required to significantly reduce IL-6 levels in urine at 2 h were 4 and 5 mg/ml, respectively.

Intravesical injection of *E. coli* Hu734 also resulted in detectable IL-6 levels in serum within 2 h of infection (Table

TABLE 1. IL-6 levels in serum at 2, 6, and 24 h after treatment with dexamethasone, diclofenac, and indomethacin prior to infection

Treatment	Geometric mean (SEM) IL-6 level at a sample time of:		
	2 h	6 h	24 h
Dexamethasone (4 mg/ml)	2 (1)[a]	16 (2)	9 (3)
Diclofenac (10 mg/ml)	52 (1)	ND[b]	45 (1)[c]
Indomethacin (5 mg/ml)	26 (2)	ND	74 (6)[c]
PBS	49 (5)	34 (8)	8 (2)

[a] $P < 0.001$ versus PBS control (Student's t test).
[b] ND, Not done.
[c] $P < 0.05$ versus PBS control (Student's t test).

1). Dexamethasone treatment significantly reduced IL-6 levels in serum at 2 h. Diclofenac and indomethacin had no significant effect on IL-6 levels in serum at 2 h, but the levels were significantly increased 24 h after treatment (Table 1).

Effect of anti-inflammatory agents on the urinary leukocyte response. After intravesical injection of *E. coli* Hu734 in to C3H/HeN mice, leukocytes were excreted into the urine. Dexamethasone (4 mg/ml at infection and at 6 h postinfection) inhibited the leukocyte response at 6 h but not 24 h after infection (Fig. 2). The decrease was linear and dose dependent. The concentration required for a significant reduction at 6 h was 4 mg/ml. Pretreatment and prolonged treatment with dexamethasone (4 mg/ml at 2 h before infection, at infection, and at 6 and 12 h postinfection) also reduced the 24-h leukocyte response. High concentrations of diclofenac (20 mg/ml) reduced the leukocyte response at 6 and 24 h (Fig. 2), but lower concentrations had no significant effect. Indomethacin treatment (10 mg/ml) as compared with the PBS control did not reduce the influx of PMNL.

Effect of anti-inflammatory agents on bacterial clearance. Dexamethasone treatment reduced bacterial clearance from the kidneys of C3H/HeN mice. The effect was most pronounced after a total dose of 16 mg per mouse (four 4-mg doses) given at 2 h before infection, at infection, and at 6 and 12 h after infection (Fig. 3). Diclofenac treatment reduced bacterial clearance from the kidneys of C3H/HeN mice (Fig. 3) at concentrations of 10 to 20 mg/ml but not at lower concentrations. Indomethacin treatment markedly decreased bacterial clearance (Fig. 3). C3H/HeN mice given 0.5 to 10 mg of indomethacin per ml as compared with the PBS control showed a 150-fold decrease in bacterial clearance from the kidneys.

DISCUSSION

The mucosal inflammatory response has been proposed to mediate the clearance of gram-negative bacteria from mucosal sites. Thus, C3H/HeJ mice, which lack the ability to mount such a response, remain chronically infected. In the present study, we have used pharmacologic agents with known anti-inflammatory activity to test further the hypothesis of a causal relationship between inflammation and bacterial clearance. The results demonstrated that dexamethasone, diclofenac, and indomethacin reduced the clearance of *E. coli* from the kidneys and bladders of C3H/HeN mice. The reduced clearance was, however, not simply related to the inhibition of mediators such as IL-6 or effectors of inflammation such as PMNL.

Dexamethasone and diclofenac inhibited local and systemic IL-6 responses. This result is consistent with those of in vitro studies showing that dexamethasone inhibited IL-6 mRNA production after interleukin-1 (IL-1) induction (15). Indomethacin did not inhibit IL-6 levels in urine. Since indomethacin and diclofenac, which share the ability to block cyclooxygenase, had different effects on IL-6 production, cyclooxygenase is not likely to be of major importance for the IL-6 response. In contrast, both diclofenac and indomethacin increased IL-6 levels in serum at 24 h. This effect may be explained by the high bacterial load of the animals at that time.

The PMNL response was independent of the IL-6 response. Whereas dexamethasone inhibited both, diclofenac

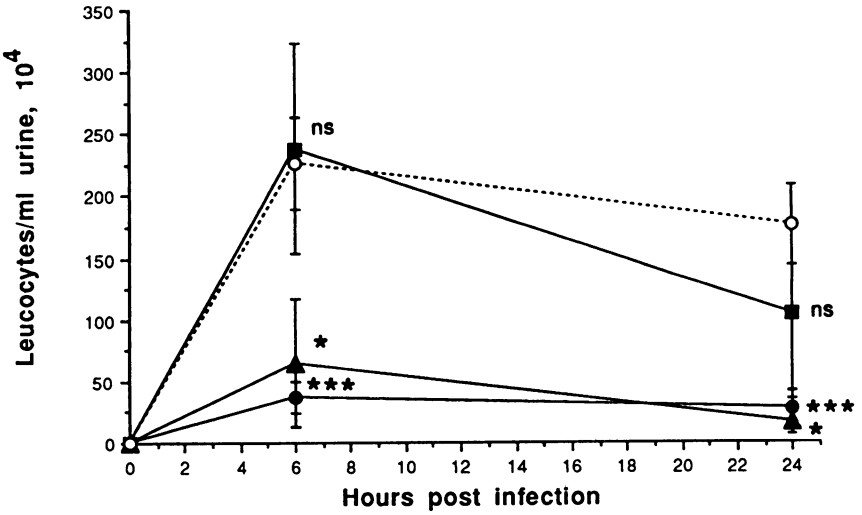

FIG. 2. Kinetics of the PMNL response to *E. coli* infection in the urinary tracts of mice pretreated with anti-inflammatory drugs. Mice were given intraperitoneal injections (0.1 ml) of dexamethasone (four 4-mg/ml doses) (●), diclofenac (20 mg/ml) (▲), indomethacin (10 mg/ml) (■), or PBS (○) at the same time that *E. coli* was inoculated in the urinary tract and at 6 h postinoculation. Each mouse received approximately 10^8 bacteria. Results are presented as numbers of PMNL per milliliter of urine. Each point represents a mean of 10 to 40 animals. *, $P < 0.05$; ***, $P < 0.001$; ns, not significant.

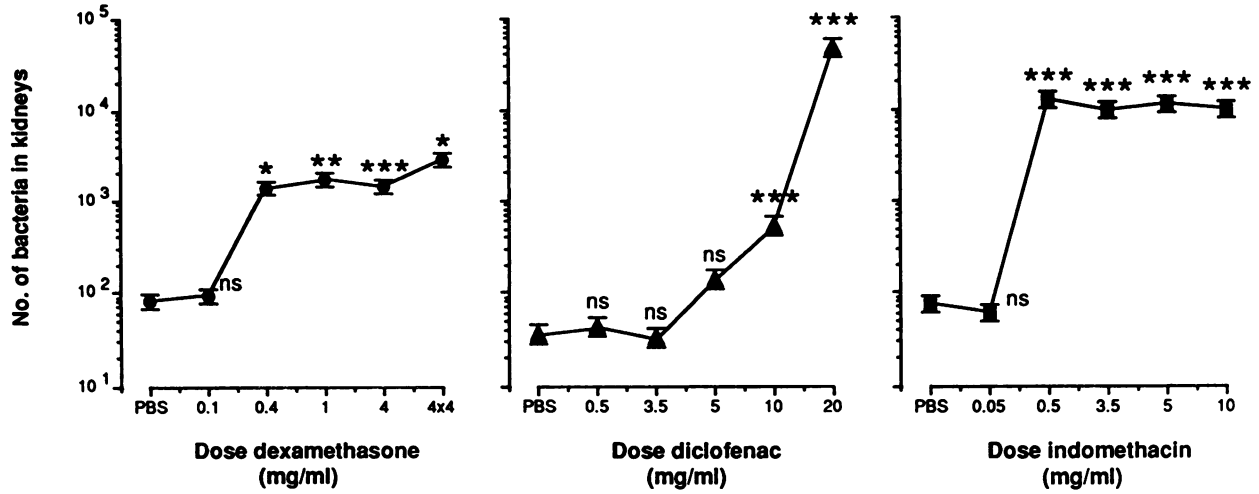

FIG. 3. Dose-dependent clearance of gram-negative bacteria from the urinary tracts of mice pretreated with anti-inflammatory drugs. Mice were given intraperitoneal injections (0.1 ml) of different concentrations of dexamethasone (●), diclofenac (▲), and indomethacin (■) or of PBS at the same time that *E. coli* was inoculated in the urinary tract and at 6 h postinoculation. Each mouse received approximately 10^8 bacteria. Results are presented as the geometric means of the numbers of persisting viable bacteria. Each point represents a mean of 10 to 40 animals. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

blocked the IL-6 response while leaving the PMNL response intact. These results are analogous to those obtained with cyclosporin A, which blocked IL-6 production at 2 h but did not inhibit the PMNL response or bacterial clearance (8a). Recently, different bacterial components were shown to trigger IL-6 secretion and PMNL recruitment. The former required the presence of bacterial adhesins binding to the Galα1-4Galβ disaccharide receptor in synergy with lipid A, while the latter required only lipid A (H. Linder, I. Engberg, I. Mattsby-Baltzer, K. Jann, and C. Svanborg-Edén, *in* T. MacDonald, ed., *Proceedings of the International Congress of Mucosal Immunology*, in press). Taken together with the results of pharmacologic inhibition, these results suggest that IL-6 production and PMNL recruitment are activated by different mechanisms and are independent products of the mucosal inflammatory response. Cytokines other than IL-6 have been shown to act as chemoattractants (17). Interleukin-8 (IL-8) is a chemoattractant for PMNL, is inhibited by dexamethasone, and is activated by LPS (17). The PMNL response may thus be a function of IL-8.

Although bacterial clearance was impaired by the three anti-inflammatory drugs tested here, the inhibition of clearance did not correlate with either the inhibition of IL-6 levels or PMNL recruitment. The suppression of IL-6 by dexamethasone and diclofenac was more marked and occurred at lower concentrations than those required to reduce clearance. Furthermore, indomethacin, which resulted in the greatest impairment of bacterial clearance, had no effect on IL-6 production. The lack of association between clearance and the PMNL response in indomethacin-treated animals was surprising, since previous studies have demonstrated a parallelism between susceptibility to phagocytosis in vitro and killing of *E. coli* in the mouse urinary tract. It is, however, well known that anti-inflammatory agents reduce uptake and killing by phagocytes without decreasing the number of PMNL. Therefore, the presence of PMNL in the urinary tracts of the indomethacin-treated mice should not be equated with intact phagocytic capacity.

The anti-inflammatory agents had little effect on bacterial clearance from the bladders of the same mice that showed increased susceptibility in the kidneys. This result suggests that mucosal inflammation is less critical for the elimination of bacteria from the bladder. Consistent with this result, LPS-nonresponder C3H/HeJ mice, which mount neither an IL-6 response nor a PMNL response to gram-negative bacterial infections, do not have significantly increased bacterial numbers in their bladders as compared with LPS-responder mice.

IL-6 is an endogenous pyrogen (10) and an activator of the acute-phase response (5). After systemic inoculation with LPS, IL-6 is activated in concert with tumor necrosis factor and IL-1. This may also be the case at mucosal surfaces. The segregation between IL-6 production and bacterial clearance, demonstrated here, raises the possibility of defining pharmacologic agents which inhibit the symptoms of infection, such as fever and systemic inflammation, without blocking the beneficial effects of inflammation on the clearance of infection.

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