Enhancement of Rat Urinary Bladder Tumorigenesis by Lipopolysaccharide-induced Inflammation

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ABSTRACT

Chronic inflammation of the urinary tract is a significant risk factor for the development of urinary bladder cancer in humans. We previously demonstrated that weekly treatment with killed Escherichia coli enhanced rat urinary bladder tumorigenesis initiated by the carcinogen N-methyl-N-nitrosourea. We conducted the present study to determine whether lipopolysaccharide (LPS), a major cell wall component of E. coli, had a tumor-enhancing effect. LPS was instilled twice a week at three doses (100, 1.0, and 0.01 µg/ml) into heterotopically transplanted rat urinary bladders which were treated with a single low dose (0.25 mg) of N-methyl-N-nitrosourea or vehicle. Rats treated with 100 µg/ml of LPS showed a significant increase in the incidence and number of tumors in the bladders pretreated with N-methyl-N-nitrosourea. Treatment with LPS alone did not induce tumors. The enhancing effects were associated with a marked increase in the numbers of polymorphonuclear leukocytes and an increase in the H2O2 concentration in the bladder lumen. Oxidative stress by reactive oxygen intermediates and a proliferative response of the carcinogen-exposed urothelium to the inflammatory stimulation appeared to play a significant role in tumor enhancement by LPS.

INTRODUCTION

Chronic inflammation has been implicated in the pathogenesis of several forms of cancer, including gastric carcinoma after atrophic gastritis (1), colon carcinoma after ulcerative colitis (2), and squamous carcinoma in a draining sinus of chronic osteomyelitis (3). Epidemiological studies (4, 5) have suggested that urinary tract infection is a significant risk factor for the development of bladder cancer. Deeply invasive squamous cell carcinomas in patients with spinal cord injury (6) and in patients with urinary tract schistosomiasis (7) are examples of bladder cancers related to chronic inflammation. Recent studies on rats seem to support the causal relationship between bacterial infection and development of neoplasia (8–10).

In a previous study, we used the HTB3 system which we developed as an animal model to examine the role of urine components in neoplasia. Administration of KEC to HTBs that had been pretreated with a low dose of the carcinogen MNU caused a significant increase in the incidence of tumors and a 6–40-fold increase in the number of tumors/bladder (11). Because LPS is resistant to boiling, the action of KEC may be due to LPS.

Our primary purpose in the present investigation was to determine whether LPS, an endotoxin derived from the cell wall of Escherichia coli, has a tumor enhancing effect. In a preliminary experiment, we demonstrated that injection of LPS caused migration of PMNs into the HTB lumen and that the number of inflammatory cells was roughly proportional to the dose of LPS. In the present experiment, we administered LPS at three different doses to test the relationship between the severity of the inflammatory response and the tumor-enhancing effect. The results indicate that LPS plays a significant role in the enhancement of tumorigenesis only in bladders which have been pretreated with the carcinogen and that the incidence of tumor development is correlated with the severity of the inflammatory response in the bladder.

MATERIALS AND METHODS

Animals. A total of 174 young male Fischer 344 rats weighing 160–190 g (Harlan Sprague-Dawley Inc., Indianapolis, Ind.) with an HTB (see below) were housed in plastic boxes, four or five/cage, in an air-conditioned room at 22°C with 50% humidity under a 12-h light-dark cycle. They had free access to pellet diet (Purina 5012;Ralston Purina Co.,St. Louis, MO) and tap water.

HTB System. We used the HTB system which we developed in our laboratory for investigating the role of urine in bladder carcinogenesis. This system consisted of a rat urinary bladder transplanted aseptically into the gluteal muscle of a recipient rat (12). The system has advantages over others in which the natural bladder is used in that it is free from bacterial contamination and stone formation (8).

LPS. Butanol-extracted LPS was prepared in our laboratory from E. coli strain 3921–1 (a gift from Dr. John R. Warren, Northwestern University Medical School, obtained from a patient with urosepsis) by the method described by Morrison and Leive (13). The LPS was dissolved in pyrogen-free PBS at concentrations of 100, 1.0, and 0.01 µg/ml and stored at −20°C in portions sufficient for single use. Once a sample was thawed, the remainder was discarded.

Determination of H2O2 Production in HTBs. We modified the colorimetric method described by Pick and Keisari (14) to adapt it for measuring the H2O2 production in HTBs. Seventy-two h after instillation of LPS or PBS, the fluid in HTBs was aspirated completely for measurement of the volume. The aspirate was mixed with one quarter volume of H2O2. The mixture was instilled back into the HTB. The content of the HTB was aspirated 3 h later. The aspirate was centrifuged for 10 min at 14,000 rpm at 4°C and 1 ml of supernatant was mixed with 100 µl of 1 N NaOH. Its absorbance was read at 610 nm in a spectrophotometer against a blank of 1 ml at one-fifth the concentration of the phenol red solution to which 100 µl of 1 N NaOH was added. A standard curve was drawn based on 1–100 µM H2O2.

Experimental Design. Five weeks after the establishment of the HTB system (Fig. 1), rats were divided randomly into eight groups: rats of groups 1, 3, 5, and 7 each received a single dose of 0.25 mg of MNU freshly dissolved in 0.5 ml of 0.9% NaCl; and rats of groups 2, 4, 6, and 8 received a single dose of the vehicle. One week later, injection of LPS twice a week at 0.01 µg/ml (groups 5 and 6), 1.0 µg/ml (groups 3 and 4), or 100 µg/ml (groups 1 and 2) 0.5 ml of PBS was begun and continued until the end of the experiment. To evaluate the initial effect of LPS on MNU-exposed urothelium, we killed seven rats each of group 1 (LPS, 100 µg/ml) and group 7 (PBS) at 2 and 4 weeks after the start of LPS treatment. The remaining rats were killed at 31 weeks. Every 2 weeks, bladder aspirates of three rats randomly selected from each group were subjected to PMN counting with hemocytometer. In addition, at 10–15 weeks from the start of LPS treatment, PMNs in aspirates from all rats in groups 1, 3, 5, and 7 were counted once a week for 3 weeks (weeks 12, 13, and 14 for group 1; weeks 10, 12, and 13 for group 3; weeks 12, 14, and 15 for groups 5 and 7). We routinely cultured aspirates from all animals once every 6 weeks to ensure their sterility. Rats were killed 4 days after the last regularly scheduled instillation of test samples except for four rats randomly selected...
Long-Term Effects of LPS treatment on MNU-treated Urothe-
lum. There was no difference in body weight among groups. Thir-
ten rats were removed from the study: two female rats delivered by
oversight and assigned to group 1; four rats with positive microbial
cultures of aspirates; and seven rats with the closure of the reservoir-
bladder communication. Thus, our analysis was based on 147 rats.

The incidence and number of tumors are shown in Table 1. Bladder
tumors were detected only in MNU-treated groups. LPS treatment
enhanced the incidence and the number of tumors. The effect was
dependent on the LPS dose in that total number of tumors was en-
hanced at LPS concentrations of 100 and 1 µg/ml, respectively. As
compared to group 7 (MNU + PBS), both the tumor incidence and the
number of tumors per HTB were significantly higher in group 1 (P <
0.0001). In addition, a significant linear trend in tumor incidence was
noted in MNU-treated groups as the LPS dose increased [group 7
(6%), group 5 (9%), group 3 (20%), group 1 (81%); P < 0.001]. All
tumors were noninvasive transitional cell carcinomas of grade 1 and
rarely of grade 2. They were characterized by intraepithelial clus-
terings of PMNs and by chronic inflammation and capillary proliferation
in the tumor stroma. The findings were similar to those of tumors
induced by KEC treatment (11).

PMN Numbers in Aspirates from HTBs. Exudation of PMNs
into the HTB lumen was a prominent feature. The initial high numbers
of PMNs, however, decreased gradually to 60–70% of the original
level at the late phase of the experiment in group 1 (MNU+LPS, 100
µg/ml) and group 3 (MNU+LPS, 1.0 µg/ml) (Fig. 2A). Fig. 2B shows
PMN numbers during weeks 10–15 of the experiment in the aspirates
of MNU-treated groups. There was a significant difference in numbers
of PMNs in the aspirates across the groups (P < 0.0001), and each
group was significantly different from each other group (P < 0.005).
However, there was no difference between the MNU-treated and
untreated groups in the numbers of PMNs at each LPS dose level
data not shown). Fig. 3 shows the numbers of PMNs of all 25 rats in
group 3 (MNU+LPS, 1.0 µg/ml) during weeks 10–13 of the experi-
ment. The five rats which developed tumors showed significantly
higher numbers of PMNs than did the remaining 20 rats which did not
develop tumors [4.75 ± 0.91 (SEM) versus 1.55 ± 0.27 × 10⁶
cells/ml; P = 0.003].

H₂O₂ Concentration in the Aspirates. A significantly higher con-
centration of H₂O₂ was detected in the aspirates in group 1 (63.1 ±
4.4 µM) than in all other MNU-treated groups (P < 0.001) (Fig. 4).

DISCUSSION

The present investigation demonstrates that LPS treatment en-
hanced tumorigenesis, but the effect was limited to the bladders that
were pretreated with MNU. The enhancement of tumorigenesis by
LPS was reflected in an increase in the incidence of tumors, in total

RESULTS

Short-Term Effects of LPS Treatment on MNU-treated Urothe-
lum. Microscopic examination showed that 100 µg/ml of LPS in-
duced focal and diffuse hyperplasia (4–7 layers) in the MNU-treated
urothelium when examined at 2 and 4 weeks of the experiment.
Intraepithelial infiltration of PMNs was a common finding within
hyperplastic urothelium. Neither hyperplasia nor PMN infiltration was
observed in the urothelia of the control groups, which were not
exposed to LPS.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rats</th>
<th>Treatment</th>
<th>MNU</th>
<th>LPS (µg/ml)</th>
<th>Rats with tumors (%)</th>
<th>Total no. of tumors/group</th>
<th>No. of tumors/bladder (mean ± SEM)</th>
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<tr>
<td>1</td>
<td>21 (25)ab</td>
<td>+</td>
<td>100</td>
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<td>17 (81)c</td>
<td>94c</td>
<td>4.48 ± 0.86c</td>
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<tr>
<td>2</td>
<td>15 (15)</td>
<td></td>
<td>100</td>
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<td>0 (0)</td>
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<td>3</td>
<td>25 (25)</td>
<td></td>
<td>1.0</td>
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<td>5 (20)</td>
<td>8d</td>
<td>0.32 ± 0.14</td>
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<tr>
<td>4</td>
<td>14 (15)</td>
<td></td>
<td>1.0</td>
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<td>0 (0)</td>
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<td>5</td>
<td>22 (25)</td>
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<td>2 (9)</td>
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<td>6</td>
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<td>7</td>
<td>17 (20)</td>
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<td>0</td>
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<td>1 (6)</td>
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<td>0.06 ± 0.06</td>
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<tr>
<td>8</td>
<td>19 (20)</td>
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<td>0 (0)</td>
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</table>

ab No. of rats assigned to the group.
bc The seven rats used for the short-term experiment are not included.
c P < 0.0001 compared to group 2 or group 7.
d P = 0.03 compared to group 4.
ec P < 0.0001 compared to group 3, 5, or 7.
INFLAMMATION ENHANCEMENT OF RAT BLADDER TUMORIGENESIS

Fig. 2. Number of PMNs in exudates into HTB lumen. A, time course of PMN exudation in groups 1, 3, 5, and 7. Points, mean from six to ten counts based on three randomly chosen rats. B, PMN exudation at 10–15 weeks of experiment. PMNs in aspirates from all rats were counted once a week for 3 weeks. The results are expressed as mean ± SD (bars).

Fig. 3. Number of PMNs in exudates in HTB lumen in rats in group 3. Points, mean ± SD from three counts at 10–13 weeks of the experiment. ○, rats that did not develop tumors; ●, rats that developed tumors. The rat of circle a showed an extraordinarily large SD.

We suggest three mechanisms to account for the tumor-enhancing effect of inflammation in the present experiment. First, repeated LPS treatment may induce a prolonged oxidative stress which results in DNA damage (18) and mutation (19). We demonstrated that LPS at the highest dose (100 µg/ml, group 1) was associated with a significant increase in the concentration of H2O2, one form of active oxygen species, in aspirates (Fig. 4). However, it is significant that tumors did not develop in the groups treated with LPS alone. This suggests that oxidative stress by itself is insufficient to induce tumors but may be sufficient to augment neoplastic changes induced by MNU. A promotional effect of active oxygen is reported in an in vitro model consisting of irradiated (20) and carcinogen-treated (21) cells; however, the molecular mechanism of the effect remains unclear.

Second, it is possible that nitrosamine(s) formed in the HTB may be involved; it is well documented that the synthesis of nitric acid and nitrosamine(s) can be stimulated greatly by the cytosol of macrophages activated by LPS and γ-interferon (22, 23).

Third, repeated LPS treatment accelerated urothelial proliferation. This may have augmented the mutagenic effect of the carcinogen (24, 25). The mechanism whereby inflammation induced by LPS stimulates urothelial cell proliferation is not clear at present. It does not appear to be a direct effect of LPS because LPS is cytotoxic to cultured normal urothelial cells (26). It is possible that epithelial proliferation is caused by cytokines. In psoriatic skin, which is characterized by accelerated proliferation of keratinocytes, several kinds of cytokines, including IL-1, TNF, and IL-6, are suggested to be involved in the pathogenesis (27, 28). IL-6 and IL-8 are known to stimulate keratinocyte proliferation in culture (29, 30). We have demonstrated a significant increase in IL-1 and TNF concentration in aspirates from KEC-treated HTBs. These observations suggest that the cytokine network induced by LPS may play a significant role in inflammation-induced urothelial hyperplasia. Furthermore, there is a possibility that cytokines such as TNF (31) and IL-8 (32), which have chemotactic and angiogenic activity, may be involved in the development of the marked vascular proliferation in the stroma and of PMN aggregation within the epithelium, which are characteristic of the tumors observed in the present and previous studies (11).

REFERENCES


4 Unpublished data.
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