Jpn. J. Cancer Res. **90**, 829–836, August 1999

Tumorigenic Conversion of a Rat Urothelial Cell Line by Human Polymorphonuclear Leukocytes Activated by Lipopolysaccharide

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Chronic inflammation is a significant risk factor for the development of urinary bladder cancer. We have shown that inflammation induced by killed *Escherichia coli* and also by its lipopolysaccharide (LPS) strikingly enhances N-methyl-N-nitrosourea (MNU)-initiated rat bladder carcinogenesis. Aspirates from the bladder lumen contained a large quantity of hydrogen peroxide (H_2O_2) and several cytokines. In this study, we tested the hypothesis that reactive oxygen intermediates (ROI) released from activated polymorphonuclear leukocytes (PMN) are involved in inflammation-associated bladder carcinogenesis. Using an immortalized nontumorigenic rat urothelial cell line, MYP3, we examined the effect of LPS-activated PMN on malignant transformation. MYP3 cells pretreated with or without MNU were exposed daily to LPS-activated PMN for one week and were then tested for growth in soft agar. In contrast to no colony formation by the parental cells, a varying number of colonies developed from cells treated with LPS-activated PMN. Although combined treatment with MNU and PMN was most effective ($P<0.01$), cells treated with LPS-activated PMN alone also formed a small number of colonies. Addition of catalase, which decomposes H_2O_2, and/or an antioxidant, α-tocopherol, reduced the number of colonies induced by LPS-activated PMN ($P<0.05$). Cells derived from colonies were tumorigenic in athymic nude mice. However, tumorigenicity in mice was greater with cells treated with both MNU and PMN than with cells treated with PMN alone. Our results suggest that ROI released from LPS-activated PMN may be one of the mechanisms involved in the carcinogenesis associated with active urinary tract infection.

Key words: Transformation — PMN — H_2O_2 — LPS — Urothelial tumor

Chronic inflammation is associated with the development of cancer in various organs, including the urinary tract.[1–9] Animal studies[10–12] indicate that urinary tract infection is a significant risk factor for the development of bladder cancer. Although the risk is most significantly associated with chronic infection, it also increases with the number of episodes of acute cystitis and multiplies with tobacco smoking.[13]

Our investigation on the role of chronic inflammation in urinary bladder carcinogenesis was facilitated greatly by the availability of an *in vivo* model, the heterotopically transplanted rat urinary bladder in male Fischer 344 rats, which was developed in our laboratory for investigating tumor promotion by urine components.[14] Using this model, we demonstrated that repeated instillation into the bladder lumen of either killed *Escherichia coli* or its endotoxin, lipopolysaccharide (LPS), strikingly enhanced bladder tumorigenesis initiated with a single dose of N-methyl-N-nitrosourea (MNU).[11, 12, 15] The marked enhancement of tumorigenesis by killed *E. coli* treatment was closely associated with migration of polymorphonuclear leukocytes (PMN) into the urothelium and also into the lumen of the bladder. The inflammatory response was associated with an increase in the hydrogen peroxide (H_2O_2) concentration and several cytokines (IL-1α, IL-6, and tumor necrosis factor-α) in the bladder lumen.[12] These observations *in vivo* suggested the possibility that reactive oxygen intermediates (ROI) and cytokines released during the inflammatory response might contribute to carcinogenesis. When stimulated by LPS, PMN generate a large quantity of H_2O_2 and ROI.[16, 17] H_2O_2 as a source of ROI causes DNA damage and induces formation of 8-hydroxydeoxyguanosine (8-OHdG), an indicator of oxidative DNA damage.[18] 8-OHdG has been shown to increase in the DNA of target tissue, and increased oxidative stress is directly related to increased tumor development.[19, 20]

Furthermore, an immortalized rat bladder epithelial cell line, MYP3 was transformed with H_2O_2 of either an exogenous or an endogenous source.[21, 22] We have also shown

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Abbreviations used: PMN, polymorphonuclear leukocytes; LPS, lipopolysaccharide; MNU, N-methyl-N-nitrosourea; H_2O_2, hydrogen peroxide; 8-OHdG, 8-hydroxydeoxyguanosine; ROI, reactive oxygen intermediates; FBS, fetal bovine serum; dG, deoxyguanosine; PCR-SSCP, polymerase chain reaction-single strand conformation polymorphism.

that TNF-α released in inflammation can induce malignant transformation in MYP3 cells.[23] The present investigation was designed to test the hypothesis that PMN, when stimulated by LPS, can generate ROI and induce malignant transformation in MYP3 cells. Formation of 8-OHdG was measured to assess DNA damage by activated PMN.

MATERIALS AND METHODS

Cell and cell culture MYP3 is an immortalized urothelial cell line established in our laboratory from a rat bladder treated with MNU. It maintains the characteristics of epithelial cells in culture, is anchorage-dependent for growth, and is nontumorigenic in athymic nude mice.[24] MYP3 cells were grown in Ham's F-12 medium (GIBCO BRL, Gaithersburg, MD) supplemented with 10 *μM* non-essential amino acids (GIBCO), 2.7 mg/ml of dextrose (Sigma Chemical Co., St. Louis, MO), 1 μg/ml of hydrocortisone (Sigma), 5 μg/ml of transferrin (GIBCO), 10 μg/ml of insulin (GIBCO), 10 ng/ml of epidermal growth factor (GIBCO), 100 μg/ml of streptomycin (GIBCO), and 100 U/ml of penicillin (GIBCO). When supplemented with 10% fetal bovine serum (FBS) (GIBCO), the medium was designated as complete medium. Cells were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Preparation of human PMN PMN were isolated from heparinized peripheral blood collected by venipuncture from healthy volunteers. Blood was diluted 1:1 (v:v) in Dulbecco's phosphate-buffered saline (GIBCO) and layered on Ficol-Paque PLUS solution (Amersham Pharmacia Biotech, Piscataway, NJ). After centrifugation at 400*g* for 30 min at room temperature, the cell pellet was collected, and PMN were recovered from the cell pellet by further sedimentation with 3% dextran (Sigma). Erythrocytes were removed from the pellet by hypotonic lysis. PMN were shown to be more than 98% pure by morphologic examination after Giemsa staining.

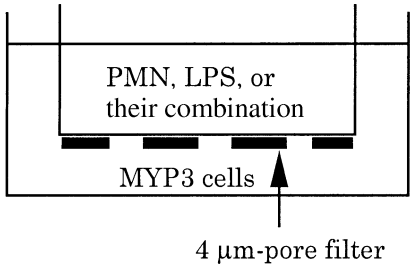

Fig. 1. *In vitro* transformation assay system. MYP3 cells were seeded in the outer well of 6-well plates. After treatment with MNU, MYP3 cells were cocultured with LPS, PMN, or their combination added to the inner well. α-Tocopherol and catalase were added to the inner well.

Measurement of H_2O_2 We modified the colorimetric method described by Pick and Keisari.[25] PMN were seeded on 24-well plates (Beckton Dickinson, Franklin Lakes, NJ) containing phenol red solution (Hanks' balanced salt solution, 100 μg/ml of phenol red (Sigma), 50 μg/ml of horseradish peroxidase (Sigma)) at a density of 1.0×10^6 cells/well with or without 0.1, 1, 10, 50, or 100 μg/ml of *E. coli*-derived LPS (Sigma), 5 or 50 *μM* α-tocopherol (Sigma) in DMSO (Sigma), and 100, 250, or 500 U/ml of catalase (Sigma). After 1 and 6 h, the medium was centrifuged for 5 min at 14,000 rpm, and 1 ml of supernatant was mixed with 10 ml of 1 *N* NaOH. Absorbance was read at 610 nm in a spectrophotometer.

***In vitro* transformation of MYP3 cells** An *in vitro* transformation assay was performed in a double chamber (Falcon) as illustrated in Fig. 1. MYP3 cells were seeded in the outer well of a 6-well plate containing the complete medium at a density of a 5.0×10^4 cells/well. Twenty-four hours after plating, the medium was replaced with serum-free F-12 medium containing MNU (50 μg/ml, Sigma). After 1 h of exposure to MNU or vehicle (DMSO) only, the cells were washed twice with plain medium. After 24 h culture in the complete medium, PMN (4.0×10^6 cells/well) alone, LPS (50 μg/ml) alone, or PMN and LPS in combination suspended in the complete medium were added to the inner well (Fig. 1). The content of the inner well was changed daily. After 7 days of treatment (Fig. 2A), cells derived from each well were seeded separately at 5.0×10^4 cells/35-mm dish in 2 ml of 0.3% Noble agar (Difco, Detroit, MI) in the complete medium. This suspension was layered over 2 ml of 0.6% Noble agar. The cells were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. On days 9, and 18, 0.5 ml of 0.3% agar in the complete medium was added. After 28 days, colonies consisting of more than 20 cells were counted.

In an attempt to neutralize H_2O_2 produced by LPS-treated PMN, MYP3 cells were cultured in the presence of LPS and PMN with or without 50 *μM* α-tocopherol and/or 250 U/ml of catalase added to the inner well (Fig. 1). Cells were then grown in soft agar as described above, and colonies were counted after 28 days.

Measurement of 8-OHdG MYP3 cells were seeded in the outer well of a 6-well plate containing the complete medium at a density of a 2.0×10^5 cells/well. Twenty-four hours after plating, the cells were cultured for 1 h in the presence of LPS (50 μg/ml) alone, or PMN (4.0×10^6 cells/well) and LPS in combination with or without 50 *μM* of α-tocopherol and/or 250 U/ml of catalase, which were added to the inner chamber containing Dulbecco's phosphate-buffered saline (Fig. 1). After 1 h of treatment, DNA was extracted from MYP3 cells with the use of DNA extractor WB kits (Wako Chemical, Richmond, VA). DNA was dissolved in 20 m*M* sodium acetate (pH 5.0) under argon gas, digested to nucleotides by nuclease P1 (Sigma)

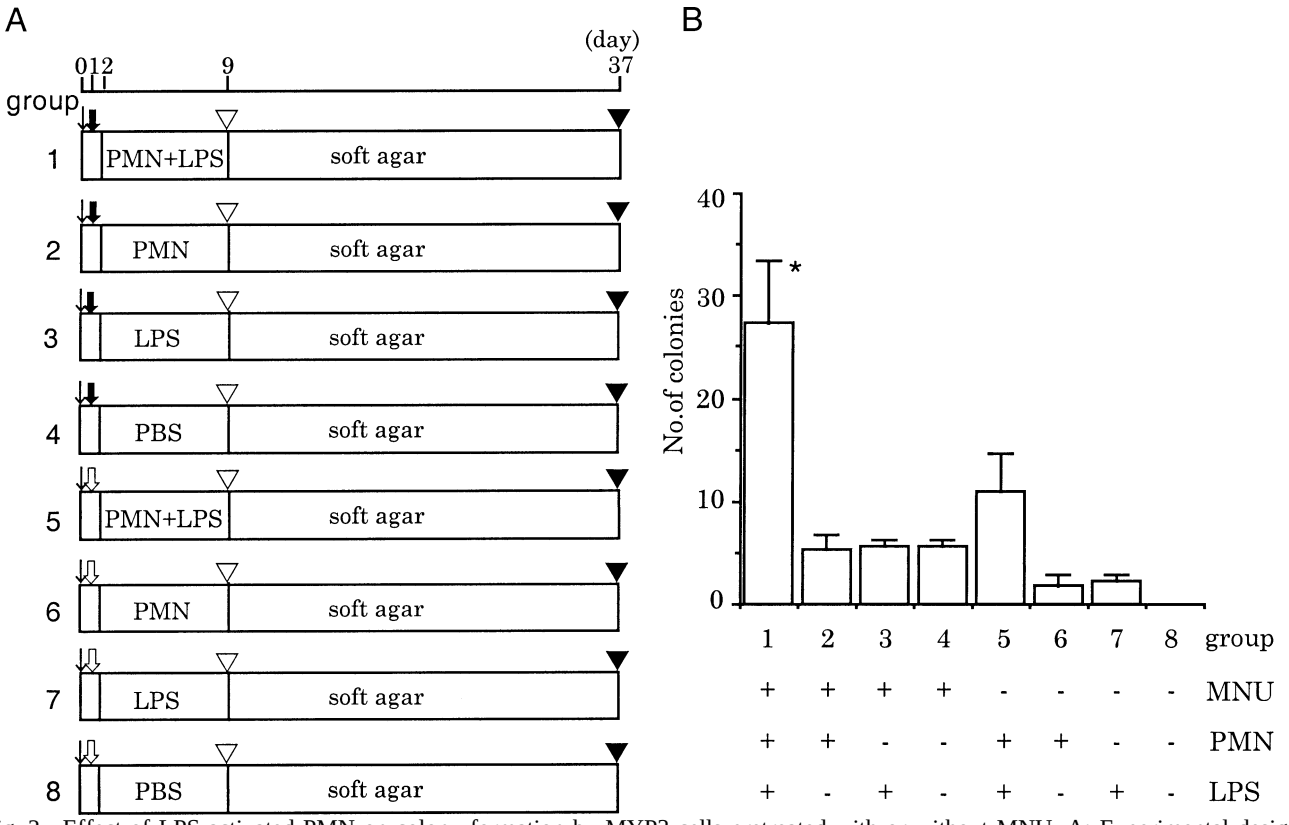

Fig. 2. Effect of LPS-activated PMN on colony formation by MYP3 cells pretreated with or without MNU. A: Experimental design. MYP3 cells (5.0×10^4 cells/well) were seeded on a 6-well plate (↓). Twenty-four hours after plating, cells were exposed to MNU (50 μg /ml) (⬇) or DMSO (⇩) for 1 h. Subsequently, MYP3 cells were cultured for 1 week in medium containing PMN (4.0×10^6 cells/well), LPS (50 μg/ml), or PMN and LPS. The medium was changed daily. MYP3 cells (5.0×10^4 cells/well) were then cultured in soft agar (▽). After 28 days, colonies of more than 20 cells were counted(▼). B: Colony formation by MYP3 cells after treatment with MNU, PMN, and LPS. Bars denote SD of triplicate samples. + or – indicates exposure or no exposure to MNU, PMN, or LPS. * $P<0.01$ as compared with groups 2, 3, 4, 5, 6, and 7.

at 65°C for 10 min, and treated with calf intestinal alkaline phosphatase (Promega, Madison, WI) at 37°C for 60 min. The content of 8-OHdG in the digested DNA and the total amount of deoxyguanosine (dG) were measured by an electrochemical detector coupled with reverse-phase high-performance liquid chromatography (ESA, Inc., Chelmsford, MA) and a UV detector (ESA). The content of 8-OHdG was expressed as the ratio of 8-OHdG$\times10^5$ to total dG.

Tumorigenicity in athymic nude mice Cells (5.0×10^6) were injected s.c. in each dorsal flank of male athymic BALB/c nude mice (Harlan Sprague-Dawley, Indianapolis, IN) suspended in 0.2 ml of 50% Matrigel (Collaborative Research, Bedford, MA) diluted with serum-free medium. The mice were monitored twice a week for the development of tumors and were killed 36 weeks after inoculation. Tumors were excised and submitted for histologic examination.

Analysis of *H-ras*, *K-ras*, and *p53* gene mutation Because of a recent report of activation of *H-* and *K-ras* genes by PMN,[26] possible mutations of the *H-ras*, *K-ras*, and *p53* genes were examined by the polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP) method.[27] The oligonucleotide primers up- and down-stream, respectively, are shown below (bp of amplified fragments):

H-ras:
exon 1: 5′-ATGACAGAATACAAGCTTGT-3′ and
5′-CTCACCTCTATAGTGGGATC-3′ (116 bp)
exon 2: 5′-AGGACCCTTAAGCTGTGTTC-3′ and
5′-GACTTGGTGTTGTTGATGGC-3′ (184 bp)

K-ras:
exon 1: 5′-CCTGCTGAAAATGACTGAGT-3′ and
5′-GCAGCATTTACCTCTATCG-3′ (125 bp)
exon 2: 5′-CTCCTACAGGAAACAAGTAG-3′ and
5′-ATGGCAAATACACAAAGAAAGCC-3′ (137 bp)

p53:
exon 5: 5′-GCTCTTTGATTCTTTCTC-3′ and
5′-ACCCTGGACAACCAGTTCTAA-3′ (262 bp)
exon 6: 5′-GCCTCTGACTTATTCTTGCTC-3′ and
5′-TGGTATAGTCGGAGCCGAC-3′ (159 bp)
exon 7: 5′-GTGGTACCGTATGAGCCACC-3′ and
5′-CAACCTGGCACACAGCTTCC-3′ (157 bp)
exon 8: 5′-TGTGCCTCCTCTTGTCCCGG-3′ and
5′-GCGCCTCCACCTTCTTTGTCC-3′ (193 bp)

PCR products were generated in a total volume of 20 μl containing DNA (200 ng), 10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.01% gelatin, 20 m*M* dNTP (A, G, C, T), 0.5 unit of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, CT), and 0.25 pmol primer. Thirty-five cycles of reaction at 94, 55–60, and 72°C for 60 s each, were carried out in a DNA Thermal Cycler (Perkin-Elmer Cetus).

For SSCP, 1 μl of PCR product in 5 μl of loading buffer was prepared. The mixture was heated to 80°C for 5 min and then plunged into ice before it was electrophoresed on the 10–20% polyacrylamide gel containing 10% glycerol under cooling. The gel was stained with a Silver Stain Plus kit (Bio Rad, Hercules, CA).

Statistical analysis Statistical analysis was performed by one-way analysis of variance; $P<0.05$ was considered to indicate significance.

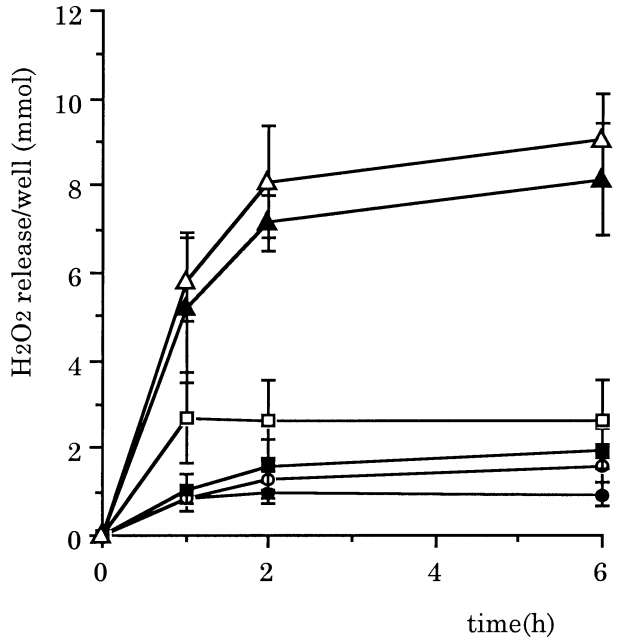

Fig. 3. H_2O_2 generation by LPS-activated PMN (1.0×10^6 cells/well). H_2O_2 release was measured for 1, 2, and 6 h after LPS treatment. Bars denote SD of triplicate samples. LPS 0 (●), 0.1 (○), 1 (■), 10 (□), 50 (▲), 100 (△) (μg/ml).

RESULTS

H_2O_2 production by LPS-activated PMN For determination of the minimal dose of LPS that maximally stimulates H_2O_2 production, PMN (1.0×10^6 cells/well) were treated with LPS at various concentrations for 1, 2, and 6 h, and H_2O_2 released into the medium was measured. When PMN were treated with 50 and 100 μg/ml of LPS, the release of H_2O_2 was maximal after 2 h of incubation (Fig. 3). Because 100 μg/ml of LPS was markedly cytotoxic to MYP3 cells, we used 50 μg/ml of LPS in the subsequent experiment.

Measurement of 8-OHdG by LPS-activated PMN The content of 8-OHdG in cells untreated and cells treated with LPS alone was 0.59 and 0.49 (8-OHdG/10^5 dG), respectively. On the other hand, that in cells treated with LPS-activated PMN was 1.47 (8-OHdG/10^5 dG), which was a 2.5-fold increase over the untreated control.

The content of 8-OHdG enhanced by the treatment with LPS-activated PMN was reduced to 75, 71 and 81% by addition of α-tocopherol, catalase, or their combination, respectively.

Effect of LPS-activated PMN on colony formation by MYP3 cells We examined the effect of LPS-activated PMN on the transformation of MYP3 cells. Combination treatment with MNU, LPS, and PMN was most effective in inducing colonies, as compared with other treatments ($P<0.01$) (Fig. 2B). It is to be noted that colonies were also induced by treatment with PMN and LPS and without carcinogen. After completion of the soft-agar assay, clonal growth was established from 2 to 3 randomly selected colonies from each of groups 1, 4, and 5 shown in Fig. 2B. Cell lines designated as PLM-1 and -2 were established from group 1 (exposed to MNU, PMN, and LPS); M-1 and M-2 from group 4 (exposed only to MNU); and PL-1 and -2 from group 5 (exposed to PMN and LPS). These clones were used in the following experiments.

Effect of α-tocopherol and catalase on H_2O_2 release by LPS-activated PMN and on transformation of MYP3 cells We examined the effect of α-tocopherol and catalase on H_2O_2 release into the medium by LPS-activated PMN. The level was reduced by the addition of α-tocopherol, catalase, and their combination in a dose-dependent manner after both 1 and 6 h of treatment (groups 3–9, $P<0.005$; group 10, $P<0.05$; respectively, as compared with group 2, Fig. 4). The inhibitory effects were, however, maximal after 1 h, and further incubation had little additional effect (only the 1 h data are shown) (Fig. 4). Because catalase was cytotoxic when tested at the highest concentration, we chose 50 μM α-tocopherol and 250 U/ml of catalase in the subsequent experiment.

The number of colonies induced by LPS-activated PMN (group 2) was reduced by addition of α-tocopherol, catalase, or their combination (groups 3, 4, and 5) ($P<0.05$,

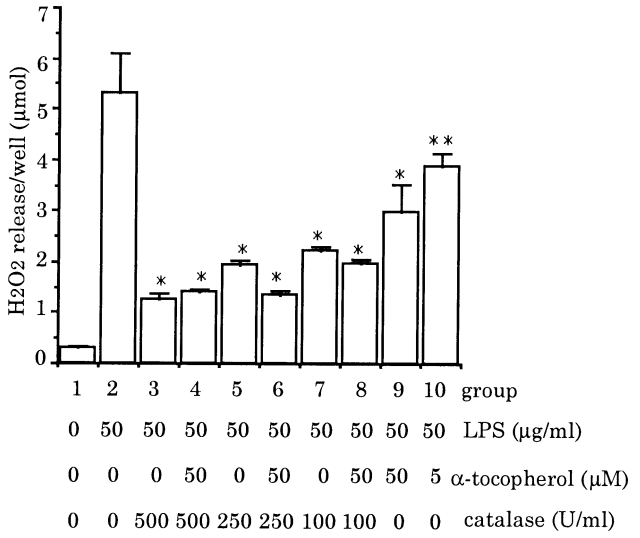

Fig. 4. Effect of α-tocopherol and catalase on H_2O_2 production by LPS-activated PMN. α-Tocopherol (5 and 50 *μM*) and/or catalase (100, 250, and 500 U/ml) were added to the dish containing PMN treated with LPS. H_2O_2 levels were measured 1 h later. Bars denote SD of triplicate samples. Any combination of α-tocopherol and catalase treatment reduced H_2O_2 generation significantly. * $P<0.001$ and ** $P<0.05$ as compared with group 2.

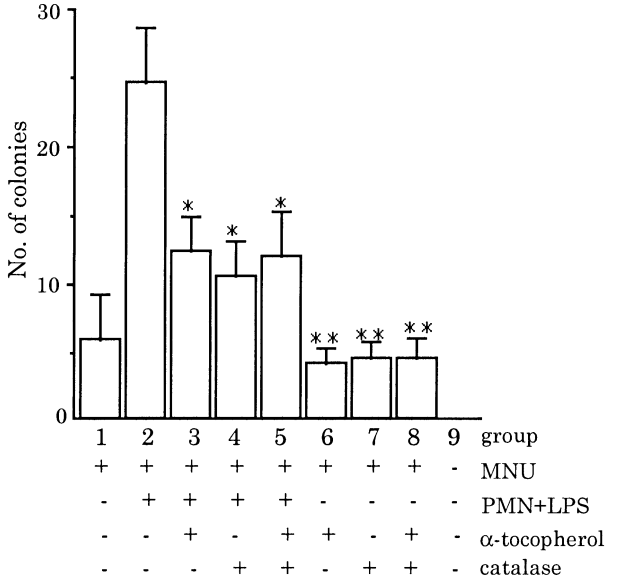

Fig. 5. Effect of α-tocopherol and catalase on transformation of MYP3 cells induced by LPS-activated PMN. The experimental protocol used was identical to that in Fig. 2 except for addition of 50 *μM* α-tocopherol and/or 250 U/ml of catalase. Bars denote SD of triplicate samples. + or − indicates exposure or no exposure to MNU, α-tocopherol, or catalase. * $P<0.05$ and ** $P<0.001$ as compared with group 2.

Fig. 5). The colony formation initiated by MNU alone (group 1) was not affected by addition of α-tocopherol, catalase, or their combination (groups 6, 7, and 8).

Tumorigenicity of transformants in athymic nude mice The tumor yield was highest in the group receiving the PLM clones (Table I). Both clones induced tumors in 4 of 6 mice, and the tumors ranged in size from 3×2 to 10×5 mm. Microscopically, they were squamous cell carcinomas with desmoplastic stroma (Fig. 6A). Although the tumors were in general poorly differentiated, keratin cyst formation was observed in 2 of 4 tumors derived from each clone. The mitotic frequency was 1 to 2 per high-power field. Both PL-1 and PL-2 clones yielded a small tumor in one of 6 recipients. The tumor cells were confined within Matrigel, arranged in small strands or nests, and were bounded by dense desmoplastic stroma (Fig. 6B). Mitotic figures were absent, and apoptotic bodies were present. One of the tumors showed pseudoglandular architecture focally. One of the M clones (M-1) gave rise to a small tumor in 2 of 6 mice. These tumors resembled those in the PL tumors in that their growth was confined within Matrigel. Small, uniform-sized tumor cells were arranged in single file (Fig. 6C). Pseudoglandular or microcystic architecture and mitotic figures were absent. The tumors of the M-1, PL-1, and PL-2 clones appeared far less aggressive in their growth potential than did tumors of PML clones.

Table I. Tumorigenicity in Athymic Nude Mice of MYP3 and Its Transformants

Group	Cells	Treatment	Mice with tumor/ total mice
1	MYP3	Parental	0/6
2	PLM-1	MNU and PMN	4/6
	PLM-2	MNU and PMN	4/6
3	PL-1	PMN	1[a)]/6
	PL-2	PMN	1[a)]/6
4	M-1	MNU	2[a)]/6
	M-2	MNU	0/6

a) Tumor confined to Matrigel.
Cells (5×10^6) were injected subcutaneously into the dorsal flank of athymic nude mice. All mice except one in group 2 were killed 253 days after inoculation. The mouse in group 2 was killed after 182 days because it became moribund. Autopsy examination did not demonstrate tumor in that mouse.

Analysis of *H-ras*, *K-ras*, and *p53* gene mutation Mutation involving exons 1 and 2 of the *H-ras* and *K-ras* genes, and exons 5–8 of the *p53* gene, was not detected in

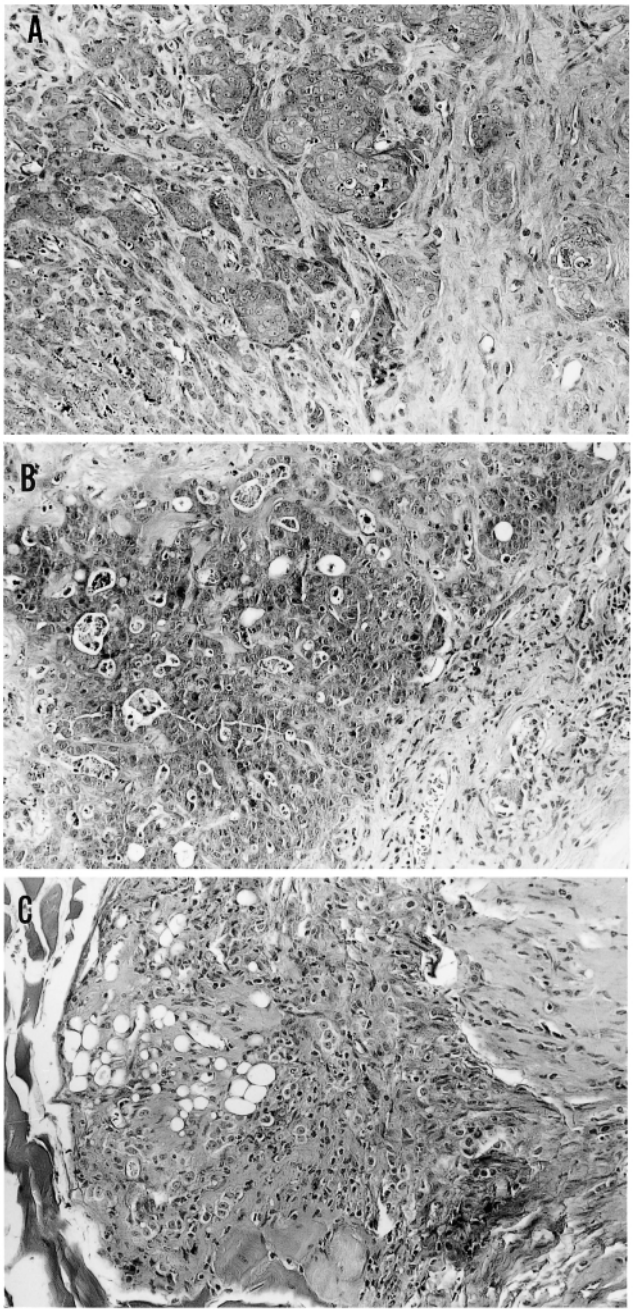

Fig. 6. Histologic appearance of tumors in athymic nude mice. A: Tumor induced by PLM-1 clone (treated with MNU and PMN) is a poorly differentiated squamous cell carcinoma with desmoplastic stroma. B: Tumor induced by PL-1 clone (treated with PMN only). It is confined within Matrigel and forms a pseudoglandular to microcystic architecture. Apoptosis is frequent. Mitotic figures are not seen. C: Tumor induced by M-1 clone (treated with MNU only) consists of uniform small cells arranged in single file, accompanied by eosinophils. The growth is confined within Matrigel. Mitotic figures are absent. HE stain (×88).

any of the cell clones used for the *in vivo* tumorigenicity assay.

DISCUSSION

We previously showed that continuous inflammatory stimuli induced by KEC (killed *E. coli*) or LPS treatment strikingly accelerated MNU-initiated rat urinary bladder carcinogenesis.[11, 12, 15] Because PMN were found in the bladder mucosa and because aspirates from the bladder lumen contained H_2O_2 and several cytokines at high concentrations,[12] we hypothesized that ROI including H_2O_2 and several cytokines were involved in tumor formation. Two inflammation-associated cytokines and H_2O_2 were tested: IL-6 was found to be a potent growth factor in stimulating the growth *in vitro* of MYP3-derived tumor cell lines, but not of the parental cell line MYP3.[21] Tumor necrosis factor-α, on the other hand, was found to be a potent mutagen by generating H_2O_2 *in vitro*.[23] H_2O_2, when given exogenously as well as when generated intracellularly by transfection of cells with an H_2O_2-generating expression vector *in vitro*,[22, 28] was found to be a potent inducer of malignant phenotype, yielding poorly differentiated transitional to squamous carcinoma in athymic nude mice.[28]

The present investigation was an extension of our previous work and was designed to test the hypothesis that PMN are indeed a source of ROI involved in tumor formation. To assess the generation of ROI and DNA damage, we measured H_2O_2 released by activated PMN and the content of 8-OHdG in MYP3 cells. We observed that a) H_2O_2 release was stimulated strikingly by LPS, as was reported by other investigators[16]; b) LPS-activated PMN enhanced the level of 8-OHdG and accelerated colony formation by MNU; c) PMN alone were likewise mutagenic; and d) catalase, which decomposes H_2O_2, and α-tocopherol, an antioxidant, reduced H_2O_2 release, the level of 8-OHdG, and colony formation by LPS-activated PMN.

The present investigation clearly supports the contention that acute inflammation in the bladder can generate ROI in the presence of *E. coli* (or its LPS) and can induce DNA damage and malignant transformation in urothelial cells. ROI have been reported to induce single-strand breaks in cellular DNA, oxidation of DNA bases, chromosomal aberration, and DNA-protein cross-links.[4, 29] Because of the report that stimulated human leukocytes cause activating mutations in the *K-ras* proto-oncogene,[26] we examined our transformed cells for the presence of mutation in the *H-ras*, *K-ras*, and *p53* genes. None was found. It remains to be determined which gene(s) is the target of transformation by ROI generated by PMN.

It is to be noted that, in the present investigation, LPS-activated PMN alone were only weakly tumorigenic in MYP3 cells and that the tumorigenic potential of the trans-

formed cells was far less than that of transformants induced by the combination of MNU and LPS-activated PMN. The finding indicates that the tumorigenicity associated with acute and chronic urinary tract infection is the sum of various causative factors involved in the inflammation process.

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ACKNOWLEDGMENTS

This study was supported by NIH grants CA14649 and 33511 and the Joseph L. Mayberry Sr. Endowment Research Fund.

(Received March 4, 1999/Revised June 7, 1999/Accepted June 10, 1999)

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