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Inhibition of X-Ray or Chemical Carcinogen-induced Neoplastic Transformation of C3H10T1/2 Fibroblasts by Lipopolysaccharides[1]

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ABSTRACT

Oncogenic transformation of mouse 10T 1/2 fibroblasts induced upon exposure to X-ray or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was suppressed if lipopolysaccharide (LPS) was present in the culture medium. The suppressive effect of LPS was exerted within 24 h after irradiation. Suppression was dependent on the concentration of LPS added and LPS (2 μg/ml) derived from *Salmonella minnesota* R595 reduced the number of transformed type III foci per dish from 0.39 to 0.15. Indomethacin (1 to 30 μM) further enhanced the effect of LPS in a dose-dependent manner.

INTRODUCTION

LPS[3], a major component of the outer membrane of gram-negative bacteria, are known to exert various biological activities in susceptible animals and humans. Various types of cells cultured *in vitro* were also shown to respond to LPS (1); however, no direct effect of LPS has so far been known on oncogenic transformation of mammalian cells, although antitumor activity of LPS *in vivo* was reported by several investigators who suggested that activated macrophages or T-cells might play a key role for the action (2). In this communication, we demonstrate suppressive effects of LPS on neoplastic transformation of 10T1/2 cells induced by X-radiation or MNNG *in vitro*.

MATERIALS AND METHODS

Cell Culture and Transformation Assay. The cell line used in the transformation experiments was C3H/10T1/2 C1.8 (3). A clone (Tf-13) isolated from a type III focus of X-irradiated 10T1/2 cells was also used to examine the effect of LPS on the cell growth. Tf-13 cells are able to grow in soft agar and are tumorigenic in immunosuppressed mice (4).

Irradiation and a subsequent transformation assay were carried out as described previously (5). Briefly, 10T1/2 cells were maintained in Eagle's basal medium supplemented with 10% fetal calf serum by subculturing 5×10^4 cells/60-mm dish every 10 days (3). For the transformation assay, cells were X-irradiated (378 R) 11 days after the inoculation when cell density reached 7×10^5 cells/60-mm dish. Immediately after the irradiation, cells were dispersed with trypsin and inoculated in 100-mm dishes to develop 400 or fewer colonies per dish. The yield of transformation assayed by this method was almost comparable to that of cells irradiated at a sparse density (5). Cells were cultivated for 10 days in the medium containing 10% fetal calf serum, insulin (100 units/ml), and 3,3',5-triiodothyronine (10^{-8} M) (expression medium). At the time of inoculation of irradiated cells, LPS was added to the expression medium to make a final concentration of 1 μg/ml. Unless otherwise stated, cells were exposed to LPS only for the initial 10 days of expression period. For the growth of an additional 7 weeks, fetal calf serum was replaced with newborn calf serum (growth medium) since this replacement did not affect the yield of transformed foci (6).

To determine transformation frequency cells were fixed and stained with May-Grünwald and Giemsa solution and type III foci were counted according to the method of Reznikoff *et al.* (7). We counted only type III foci since most clones (93%) from type III foci were shown to be tumorigenic to immunosuppressed mice whereas only a portion of the clones (20%) from type II foci developed tumors (4).

Chemicals. LPS were obtained from *Salmonella minnesota* R595 (Re type; a chemotype of LPS consisting of 3-deoxy-D-manno-octulosonic acid, lipid A, and some other substituents) and *Yersinia enterocolitica* 161-45a (Re) by extraction with phenol/chloroform/petroleum ether or from *Chromobacterium violaceum* NCTC9694 (S type; a complete LPS, consisting of covalently bound O-side chain polysaccharide, R core oligosaccharide, and lipid A) with phenol/water. The latter was purified by repeated ultracentrifugation. LPS thus obtained were of high purity and free from nucleic acids, proteins, and glycans (1). These LPSs were kindly provided by Dr. E. T. Rietschel, Forschungsinstitut Borstel, Borstel, West Germany. LPS derived from *S. minnesota* R595 was used throughout the experiments except where otherwise noted. MNNG was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI).

RESULTS

The number of type III-transformed foci of 10T1/2 cells irradiated by 378 rads of X-rays was significantly reduced when LPS was present in the assay medium. The suppression of transformation was dependent on doses of LPS and LPS (1 ng/ml or 2 μg/ml) reduced the number of transformed foci per dish to 79 or 38% of that in control cultures, respectively (Table 1). The suppressive effect of LPS was observed to almost equal extent at all radiation dose levels tested with the concentration of LPS (1 μg/ml) (Fig. 1).

The effect of LPS on the survival rate of irradiated cells was tested to determine any cytotoxic effect of LPS on the cells affected the transformation frequency. As shown in Fig. 2, the presence or absence of LPS did not change the survival rate; furthermore, LPS did not affect the growth rate of nontransformed (10T1/2) or X-ray-transformed (Tf-13) cells (Fig. 3).

Three types of LPS were compared for their inhibitory effects upon transformation. At the concentration of LPS (1 μg/ml) all three types of LPS suppressed the transformation (Table 2). Re type LPSs obtained from either *Y. enterocolitica* or *S. minnesota* were more effective than S type LPS derived from *C. violaceum* under the condition tested.

To determine the effect of the duration of exposure of cells to LPS, irradiated cells were cultivated in the presence of LPS (1 μg/ml) for 1 or 5 days (Table 3). No significant difference in the effect of LPS on the transformation was observed between cells exposed to LPS for 1 (43% of control) or 5 days (44% of control). Considering that a 10-day exposure of irradiated cells to the same concentration of LPS reduced the appearance of transformed foci to approximately 30% of the control (Table 2), LPS was thought to exert its full effect within 24 h postirradiation.

Indomethacin added to the irradiated cell culture at the time of LPS addition enhanced the suppressive effect of LPS. The effect of the enhancement was dose dependent (Table 4). Under

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[3] The abbreviations used are: LPS, lipopolysaccharide; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

Table 1 *Effect of various concentrations of LPS on X-ray transformation*

Cells at plateau phase were X-irradiated with 378 rads, dispersed with trypsin, and inoculated into 100-mm dishes as described in "Materials and Methods." LPS derived from *S. minnesota* R595 was added to the culture medium (final concentration, 1 ng to 2 μg/ml as indicated) at the time of inoculation of irradiated cells. Cells were exposed to LPS for the initial 10 days of the expression period and grown for an additional 7 weeks in the absence of LPS.

Treatment	Plating efficiency (%)	Total no. of surviving cells	No. of dishes with transformed foci/ no. of dishes analyzed	Total no. of transformed foci
Experiment 1				
378 rads	6.8 ± 0.3[a]	18,696	16/46 (0.35)[b]	18
378 rads + LPS (1 ng/ml)	7.4 ± 0.3	20,024	14/45 (0.31)	14
378 rads + LPS (100 ng/ml)	7.5 ± 0.3	19,854	11/44 (0.25)	11
378 rads + LPS (1 μg/ml)	7.0 ± 0.8	18,060	7/43 (0.16)	7
378 rads + LPS (2 μg/ml)	6.9 ± 1.0	21,675	6/48 (0.12)	7
No X-ray	22.4 ± 4.3	36,490	2/115 (0.02)	2
No X-ray + LPS	24.1 ± 2.1	14,317	0/33 (0)	0
Experiment 2				
378 rads	7.0 ± 0.8	17,859	12/39 (0.31)	16
378 rads + LPS (200 ng)	7.1 ± 0.6	22,285	9/48 (0.20)	9
No X-ray	34.5 ± 5.0	20,511	0/46 (0)	0

[a] Mean ± SD.
[b] Numbers in parentheses, percentage.

Table 2 *Comparison of inhibitory effects of LPS derived from various gram-negative bacteria on X-ray transformation*

Cells were X-irradiated (378 R) and cultivated in the presence of each type of LPS (1 μg/ml) or in its absence. Cells were exposed to LPS for 10 days as described in "Materials and Methods."

Lipopolysaccharide from	Type of LPS	Plating efficiency (%)	Total no. of surviving cells	No. of dishes with transformed foci/ no. of dishes analyzed	Total no. of transformed foci
Y. enterocolitica	Re	7.8 ± 0.6[a]	22,515	7/46 (0.15)[b]	8
S. minnesota	Re	7.7 ± 0.0	18,599	6/39 (0.25)	7
C. violaceum	S	7.7 ± 0.5	19,790	13/40 (0.33)	15
No addition		7.2 ± 0.4	15,566	15/34 (0.44)	16

[a] Mean ± SD.
[b] Numbers in parentheses, percentage.

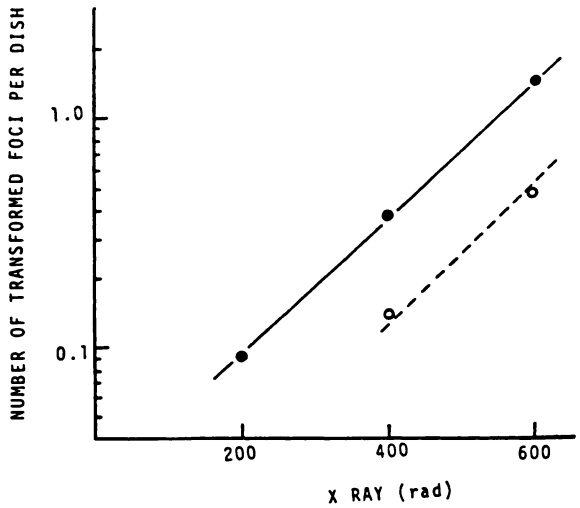

Fig. 1. Effect of LPS on transformation induced by various doses of X-rays. Cells were X-irradiated with various doses and exposed to LPS (1 μg/ml) for the initial 10 days as described in "Material and Methods." ●, X-rays only; ○, X-rays plus LPS. No foci were detected in cells irradiated with 200 R and treated with LPS. Number of dishes analyzed was 24, 38, and 22 for 200, 400, and 600 R, respectively.

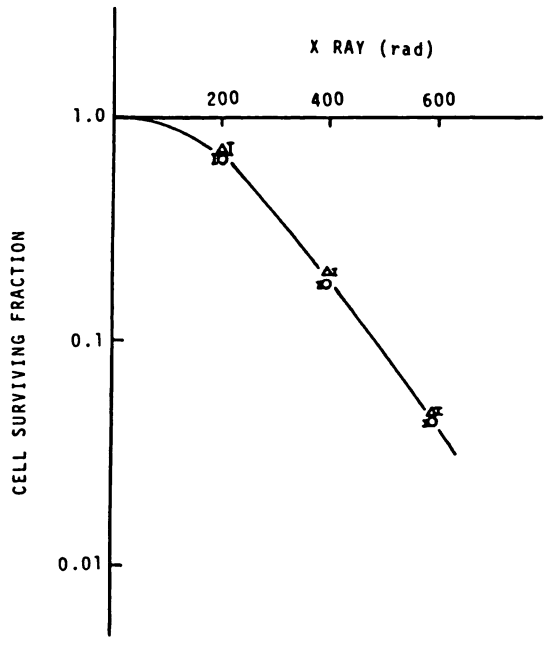

Fig. 2. Effect of LPS on survival rate of 10T1/2 cells. △, cells cultured in the presence of LPS (1 μg/ml); ○, control culture without LPS. Values are average of determinations of two dishes. *Bars*, variations.

the condition where LPS lowered the transformation to 40% of the control, 30 μM indomethacin reduced the rate further to 17% of the control. Exposure of irradiated cells to 30 μM indomethacin alone caused reduction of the transformation to 78% of control culture without indomethacin.

LPS suppressed the transformation induced not only by X-irradiation but also by exposure to a chemical carcinogen. Logarithmically growing cells were treated with MNNG (1.3 μg/ml) for 3 h and inoculated into dishes under identical conditions to assay X-ray-induced transformation. As shown in Table 5, the presence of LPS for the initial 10 days of the expression period inhibited the appearance of transformed foci significantly.

DISCUSSION

In this paper we showed that LPS added to the culture medium suppressed the neoplastic transformation of 10T1/2

cells induced either by X-irradiation or by exposure to MNNG. To our knowledge, such a direct effect of LPS on transformation has not been reported.

The mechanism by which LPS inhibits the transformation of cells is not clear at the moment. Because LPS affected neither the survival of irradiated 10T1/2 cells nor the growth rate of 10T1/2 and Tf-13 cells, the effect of LPS did not result from a simple selective killing or growth inhibition of LPS on potentially transforming cells. Since LPS is known to induce production of interferon which might affect the transformation, we assayed the level of interferon in the culture medium of 10T1/2 cells irradiated and subsequently exposed to LPS (1 μg/ml) by the plaque reduction method using L-cells and vesicular stomatitis virus. No detectable amount of interferon was induced by LPS.

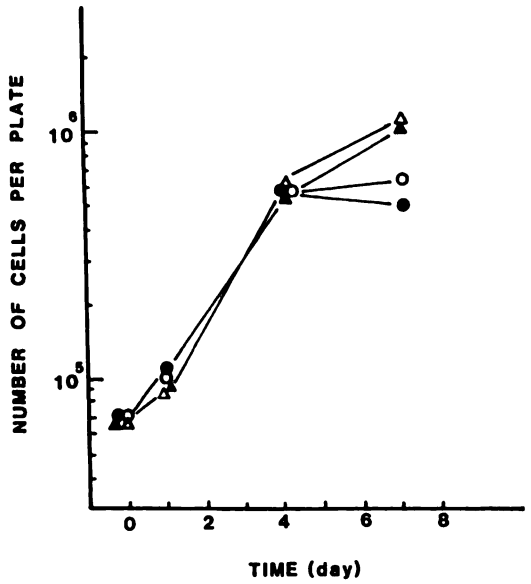

Fig. 3. Effect of LPS on growth of 10T1/2 and Tf-13 cells. Cells (7×10^4 cells/plate) were inoculated into 30-mm dishes with or without LPS (1 μg/ml), yielding cell numbers grown in culture in the presence (▲, ●) or absence (△, ○) of LPS. ●, ○, 10T1/2 cells; ▲, △, Tf-13 cells.

LPS is known to enhance arachidonic acid metabolism in mouse and rabbit macrophages and to produce prostaglandins with the aid of an enzyme system in the cyclooxygenase pathway (8). Such metabolic changes of arachidonates were also reported to take place with 10T1/2 cells upon exposure to a peptide toxin produced by *Staphylococcus* (δ-toxin) (9). A very similar peptide toxin, mellitin (10) was reported to suppress X-ray- or UV-induced transformation (11). It is therefore possible that LPS affects 10T1/2 cells and induces release of arachidonic acid, which is subsequently metabolized in the cyclooxygenase and/or lipoxygenase pathway and that the products of such a pathway affect in turn the transformation frequency.

Indomethacin lowered further the X-ray transformation when added to the culture along with LPS. Indomethacin slightly lowered the yield of transformed cells induced by X-rays when present alone for the initial 10 days of the expression period in this investigation. This is in contrast to the published report showing that indomethacin by itself induced a fairly high yield of transformation if the agent was present in culture medium for the entire 7 weeks of the assay period (11).

Terasima *et al.* (5) have reported that significant variations were observed in transformation efficiencies in various serum lots used for the transformation assay. Serum contains various kinds of components including LPS which potentially affect the transformation frequency. These components possibly exert their effects either alone or in combinations.

Table 3 *Effect of duration of exposure to LPS on X-ray transformation*

Cells were X-irradiated (378 R) and cultured with or without LPS as described in "Materials and Methods." At 1 or 5 days after inoculation the expression medium was switched to growth medium without LPS.

Exposure time (day)	Plating efficiency (%)	Total no. of surviving cells	No. of dishes with transformed foci/no. of dishes analyzed	Total no. of transformed foci
0	2.7 ± 0.4[a]	9,975	11/30 (0.37)[b]	13
1	2.8 ± 0.4	11,280	5/32 (0.16)	6
5	3.7 ± 0.2	14,896	7/37 (0.19)	7

[a] Mean ± SD.
[b] Numbers in parentheses, percentage.

Table 5 *Effect of LPS on transformation induced by MNNG*

Cells at logarithmic growing stage were treated with MNNG (1.3 μg/ml) for 3 h. The cells were washed, trypsinized, and inoculated into 100-mm dishes with or without LPS as described in "Material and Methods."

Treatment	Plating efficiency (%)	Total no. of surviving cells	No. of dishes with transformed foci/no. of dishes analyzed	Total no. of transformed foci
MNNG	7.8 ± 0.7[a]	17,390	6/37 (0.16)[b]	6
MNNG + LPS	9.0 ± 0.0	16,524	1/36 (0.03)	1

[a] Mean ± SD.
[b] Numbers in parentheses, percentage.

Table 4 *Effect of indomethacin on the suppressive effect of LPS on X-ray-induced transformation of 10T1/2 cells*

In experiment 1, cells were irradiated with X-ray (378 R) and inoculated into dishes with LPS (1 μg/ml) together with various doses of indomethacin dissolved in dimethyl sulfoxide. Ten days after the inoculation the expression medium was changed to regular growth medium containing 10% newborn calf serum. Addition of indomethacin up to 30 μM did not affect the plating efficiency of the cells. In experiment 2, the effects of indomethacin alone on transformation were examined as described in experiment 1.

Addition	Plating efficiency (%)	Total no. of surviving cells	No. of dishes with transformed foci/ no. of dishes analyzed	Total no. of transformed foci
Experiment 1				
None	3.3 ± 0.3[a]	14,406	19/37 (0.51)[b]	24
LPS	3.4 ± 0.4	13,806	9/34 (0.26)	10
LPS + dimethyl sulfoxide	3.3 ± 0.1	15,519	10/38 (0.26)	10
LPS + indomethacin (1 μM)	3.9 ± 0.0	16,491	9/36 (0.25)	9
LPS + indomethacin (10 μM)	3.8 ± 0.3	17,042	7/38 (0.18)	7
LPS + indomethacin (30 μM)	3.8 ± 0.1	17,883	4/38 (0.11)	4
Experiment 2				
Dimethyl sulfoxide	3.8 ± 0.3	11,560	23/34 (0.68)	31
Indomethacin (30 μM)	5.4 ± 0.3	12,021	19/34 (0.56)	24

[a] Mean ± SD.
[b] Numbers in parentheses, percentage.

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