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# Effect of bacterial lipopolysaccaride on growth of murine bladder cancer, MBT-2

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**Summary.** Intradermal palpable MBT-2 tumor responded with bacterial lipopolysaccharide to hemorrhagic necrosis (LPS) in C3H/HeN (endotoxin sensitive) mice. We have tested LPS fractions isolated from *E. coli, Klebsiella pneumoniae, Salmonella minnesota, Pseudomonas aeruginosa* and *Serratia* culture filtrates. All these LPS preparations showed tumor necrotizing activity accompanied by toxicity (body weight loss) in C3H/HeN mice. However, MBT-2 tumors grown in an endotoxin-resistante strain (C3H/HeJ) of mice did not respond to LPS, even at a very high dose. In vitro, the LPS showed no cytotoxic effect on MBT-2 cells. For comparison, systemic administration of tumor necrosis factor (cachexin) did not affect the i.d. tumor growth. These data indicate that host reactions to LPS (endotoxicity) plays a pivotal role in the expression of tumor necrosis. Accordingly, comparisons of tumor response between endotoxin sensitive and resistant mice avoid potential overestimation of the therapeutic value of certain bacterial products and/or LPS contaminated agents.

**Key words:** Endotoxin – Tumor necrosis factor – MBT-2 tumor – C3H/HeJ mice

## Introduction

It is well known that certain bacterial products cause hemorrhagic necrosis of tumors in experimental animals [3, 8]. However, this effect is not a generalized phenomenon. Tumor necrosis occurs in only a few tumor types [3].

Intradermal growing MBT-2 was found underwent necrosis in response to bacterial culture filtrates of *Serratia* species in C3H/HeN mice. This finding promoted further characterization of the response of MBT-2 tumor to several bacterial products.

## Materials and methods

### *Mice*

Inbred female C3H/HeN (Charles River Laboratory, Pittsburgh, PA), and C3H/HeJ mice (Jackson Laboratory, Bar Harbor, ME), 18 to 20 g, were used for the experiment.

### *Tumor cells and tumor implantation*

The MBT-2 tumor was originally developed by Soloway [11] and was obtained from Drs. Ratliff and Catalona (Washington University, St. Louis, MO). This cell line was maintained by alternate in vivo and in vitro culture (RPMI 1640 – 10% fetal bovine serum – 0.03% glutamine; "RO/10%", GIBCO, Grand Island, NY). Briefly, MBT-2 cells from a 15th to 20th passage of culture flasks were injected i.d. into the flank of C3H/HeN mice. When tumors had grown to 200–600 mg, they were excised, mechanically disaggregated, then plated out for in vitro growth in a T-75 flask (Corning; Corning, NY) to establish a primary culture. Experiments were conducted using cells from the third to 18th in vitro passage. Tumor cell suspensions of $5 \times 10^5$ viable tumor cells were implanted intradermally into both flanks of recipients.

### *Bacterial products and agents*

*E. coli* (026:B6) derived lipopolysaccharide (LPS) was purchased from DIFCO Laboratories (Detroit, MI). LPS (phenol extracted) of *Klebsiella pneumoniae, Salmonella minnesota,* and *Pseudomonas aeruginosa* were purchased from SIGMA (St. Louis, MO). These LPS were dissolved in 0.9% NaCl at 100 μg/ml. *Serratia* culture filtrates was prepared by us. In brief, *Serratia liquifaciens, S. rubidia,* and *S. marcescens* (ATCC, Rockville, MD) were cultivated in a simple salt solution containing glucose (M9) as described by us previously [10]. Culture supernatant was diafiltrated, lyophilized and stored at −20°C. Approximately 13% (Wt/Wt) of the lyophilized culture medium consisted of LPS [9]. A single lot of each lyophilized preparation was used throughout the experiments. Lyophylized culture media were dissolved in 0.9% NaCl at 10 mg/ml and 0.2 μm filtered (Nalgen, Rochester, NY) prior to use. Human recombinant tumor necrosis factor-α(TNF) with a specific activity of $5.02 \times 10^7$ U/mg was kindly supplied by Genentech Inc. (San

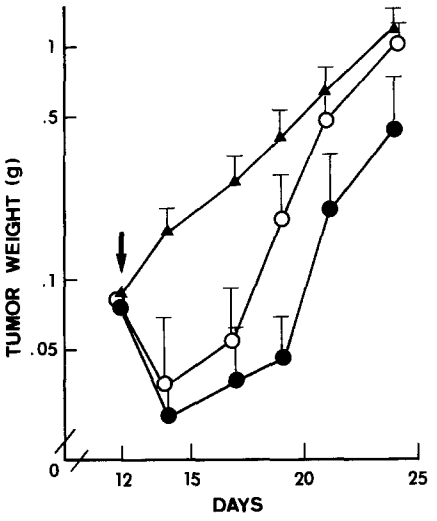


**Fig. 1.** Effect of *Serratia liquifaciens* culture filtrate on MBT-2 tumor growth in C3H/HeN mice. Mice were given 2.5 (○) or 4 mg (●) (maximal tolerated dose) of culture filtrate. Control mice (▲) were given no treatment. Each point indicates mean of 9 to 12 tumors (5 to 6 mice); bar = SD

Francisco, CA; Lot 4407-47). TNF was diluted with 0.9% NaCl containing 1 mg/ml of bovine serum albumin to 5 μg/ml. Aspirin (SIGMA) was dissolved in ammonium acetate (11.5 μg/ml) solution at 10 mg/ml: further dilution of aspirin was made by 0.9% NaCl. Heparin (Elkins-Sinn Inc., Cherry Hill, NJ) was diluted with 0.9% NaCl to 100 units/ml. Cortisone acetate (CA) and cyclophosphamide (CY) were purchased from SIGMA: CA was suspended in 0.9% NaCl with 0.5% ethanol at 25 mg/ml and CY was dissolved in 0.9% NaCl at 10 mg/ml.

### *Evaluation of drug activity*

Prior to treatment, mice were randomized into control and treatment groups consisting of 5 to 6 mice per group. The bacterial products were given once when tumor nodules grew to 100 to 200 mg (on Day 11 or 12). Several bacterial products were given at a maximal tolerated dose (MTD) which had been determined prior to the experiments: the dosage and administration route of drugs are shown in the results section. Tumor nodules were measured 2 to 3 times a week using a sliding caliper by a single person with no knowledge of the treatment. Tumor weight (TW) in mg was estimated as $TW = 0.5 \times W^2 \times L - 0.5 \times uW^2 \times uL$, where W is width and L is perpendicular length of tumor nodule in mm; uW is the width and uL is the length of ulceration. We managed the ulceration of external skin by painting 10% povidone iodine solution to prevent infectious death due to tumor ulceration.

### *In vitro growth study*

MBT-2 cells from subconfluent flasks were suspended in RO/10% at $2 \times 10^4$ viable cells/ml (trypan blue exclusion method) and 0.2 ml plated/well in 96-well flat bottom microculture plates (Falcon). Twenty μl of test materials (in RO/10%) or RO/10% were added to individual wells in quadruplicate. The cells were incubated at 37°C in 5% $CO_2$ for 24 to 72 h. Twenty-four hours before the end of each incubation period the cells were pulsed with 20 μl/well of [$^3$H] thymidine (10 μ Ci/ml, Amersham, Arlington Heights, IL). At the end of the 24 h pulse the cells were harvested with a multiple automated sample harvester and uptake of [$^3$H] thymidine was measured by liquid scintillation counting.

## Results

Figure 1 illustrates the effect of *S. liquifaciens* culture filtrate on MBT-2 tumor growth in C3H/HeN mice (endotoxin sensitive strain). I.p. administration of 2.5 to 4 mg (MTD) of the culture filtrate significantly suppressed tumor growth in a dose dependent manner. Necropsy of representative mice in each dose group showed hemorrhage of tumor nodules which appeared within 6-h of treatment. Ulceration of external skin surfaces developed by 2 to 3 days. The earliest sign of toxicity was diarrhoea, which was noted within 20 min following the injection. Thereafter, mice developed appetite loss, immobility, body weight loss and typical poor looking appearance. These signs of toxicity gradually increased in severity by 2 to 3 days and gradually disappeared from 4 to 7 days (depending on dose). Histological examinations revealed that administration of *S. liquifaciens* filtrates caused tissue hemorrhage not only in the tumor, but also in multi-organ sites including kidney, spleen, (by 8-h) and lung (by 22-h) (data not shown).

The tumor necrotizing and host toxicity (as measured by body weight loss) of bacterial products are summarized in Table 1. Culture filtrates from other strain of *Serratia (S. rubidia* and *S. marcenscens)* caused tumor necrosis and host toxicity similar to that described above.

I.P. administration of LPS fractions from *E. coli* (15 μg) (MTD), *K. pneumoniae* (50 μg), *S. minnesota* (50 μg), and *P. aeruginosa* (50 μg) mediated tumor regression accompanied by necrosis and body weight loss (Table 1).

For comparison, tumor bearing C3H/HeN mice were injected with a MTD of TNF (2.5 μg, i.v.). TNF did not affect tumor growth (Table 1). The progress of TNF induced toxic signs was different from that due to LPS preparations. The mice did not show any signs of toxicitity by 8 to 12-h following TNF i.v. thereafter they developed immobility. Even at higher doses of TNF administration (5 to 10 μg), which caused toxic death by Day 1, mice remained healthy looking by 8 to 12-h (data not shown).

The influence of anti-coagulation and immunosuppressive treatments on the tumor necrotizing and toxic effect of LPS was studied. Tumor bearing mice were treated with aspirin (100 mg/kg, i.p.; −2 h and 0 h),

**Table 1.** Influence of bacterial products on tumor growth and body weight change

| Host | Bacterial products[a] | Dose (per mouse) | Tumor necrosis | Body weight loss[b] | |
|---|---|---|---|---|---|
| | | | | Day 2 | Day 4 |
| C3H/HeN | Culture filtrates | | | | |
| | *Serratia liquifaciens* | 4 mg (MTD)[c] | + | ++ | + |
| | *Serratia rubidia* | 1.5 mg | + | + | + |
| | *Serratia marcescens* | 1.5 mg | + | + | + |
| | LPS | | | | |
| | *E. coli* | 15 μg (MTD) | + | ++ | + |
| | *Klebsiella pneumoniae* | 50 μg | + | + | − |
| | *Salmonella minesota* | 50 μg | + | + | − |
| | *Pseudomonas aeruginosa* | 50 μg | + | + | − |
| | TNF | 2.5 μg (MTD) | − | + | − |
| C3H/HeJ | Culture filtrate | | | | |
| | *Serratia liquifasciens* | 4 mg | − | − | − |
| | *Serratia liquifasciens* | 20 mg | − | + | − |

[a] Bacterial products were given once when tumor grew to 100 to 200 mg
[b] The degree of body weight loss was graded on scale of minus (less than 5% loss), + (5 to 15% loss), and ++ (15 to 20%) compared to their initial body weights
[c] Maximal tolerated dose

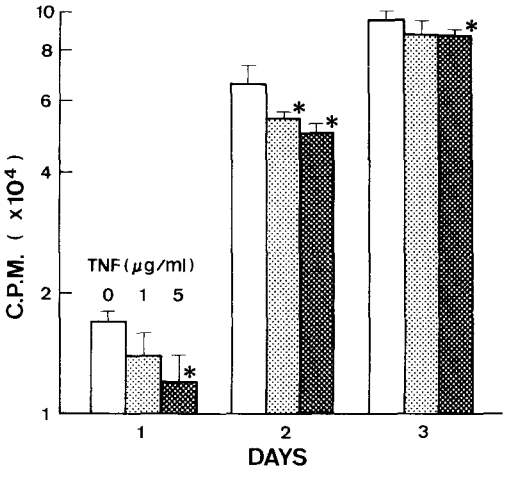


Fig. 2. Influence of TNF on [$^3$H] thymidine uptake of MBT-2 cells. MBT-2 cells ($4 \times 10^3$ cells/well) were cultured in the presence of 1 μg or, 5 μg of TNF, or in culture medium alone. Each point indicates mean of 4 wells; bars = SD, * $P < 0.05$; control vs TNF

heparin (20 U/mouse i.p., −15 min), CA (250 mg/kg, s.c −2 h), or CY (125 mg/kg, i.p., −2 h) prior to *S. liquifaciens* culture filtrate administration (4.0 mg, i.p.). Pretreatment of mice with CA prolonged the advent of toxic signs by 6 to 12 h, though all of them subsequently developed toxic signs. Aspirin, heparin or CY did not influence the development of toxicities. These prophylactic treatments did not influence the tumor necrotizing activity of LPS (data not shown).

The MBT-2 tumor grown in endotoxin-resistant hosts (C3H/HeJ mice) was treated with high doses of *S. liquifaciens* culture filtrate (MTD to lethal dose in C3H/HeN mice). There was no difference in the tumor growth rate between C3H/HeN and C3H/HeJ mice (data not shown). None of the tumors responded to the therapy. Mice remained healthy looking during the observation, although higher dose of culture filtrates (20 mg) caused body weight loss (Table 1).

MBT-2 cells were cultivated in the presence of *S. liquifaciens* culture filtrate (1 to 10 μg/ml), *E. coli* LPS (1 to 10 μg/ml), or TNF (1 to 5 μg/ml). In vitro administration of the culture filter and *E. coli* LPS did not influence the tumor growth as measured by [$^3$H] thymidine incorporation (data not shown), whereas TNF significantly suppressed it (Fig. 2).

## Discussion

The mechanism of endotoxin induced tumor necrosis has been explained by well-known actions of endotoxins on the circulatory system (anaphylactic shock) [1]. Systemic administration of LPS causes hypotension accompanied by arterial dilatation, sluggish blood flow and hemoconcentration. Prolonged hypotension (more than several hours) and sluggish microcirculation result in tissue anoxia, which mediate hemorrhagic necrosis of tumor [1, 13]. Vascular occlusion by platelet aggregation may enhance tissue damage, although this

is not mandatory [1]. The reason why only a few tumor types respond with necrosis to endotoxin is unknown as yet, but one likely explanation involves differences in the vascular structure of tumors. Vascular structure varies among tumor types, tumor stages, and anatomic locations [5]. Tumors which are rich in certain vascular type(s) may be sensitive to anaphylactic shock. It is also possible, but unlikely, that endogenous production of cytokines including TNF [2] is responsible for tumor necrosis. TNF causes hemorrhagic necrosis of certain tumor types and in vitro it has cytostactic activity on MBT-2 cells (Fig. 2). However, systemic administration of TNF did not affect i.d. MBT-2 growth (Table 1).

Since the late 1800 s, bacterial endotoxins have been used as antitumor agents for the treatment of cancer patients with some successful results [7]. However, endotoxins fell into disfavor because of a lack of consistency and fatal side effects. We have combined several potential or established antianaphylactic shock treatments with LPS (*Serratia* culture filtrate). None of these agents affected the tumor necrotizing activity. More importantly, among the treatments we tested, glucocorticoid prolonged the advent of toxic signs, however none of these treatments protected mice from toxic effects. Thus, these anti-shock treatments, when used as prophylaxis, were not able to control lethal side effects of LPS.

Accordingly, a translation of our results into a bladder cancer model is extremely dangerous. Rather, a significane of our findings is that i.d. MBT-2 tumor in endotoxin sensitive strain of mice responds with necrosis to LPS from a variety of bacteria strains. Thereby, the use of this model may be limited for evaluating therapeutic effect of certain bacterial products and/or LPS contaminated agents, since this potentially overestimates the therapeutic benefits. However, this tumor model provides a useful tool for understanding the mechanism of LPS induced tumor necrosis and possibly endotoxic shock. Because MBT-2 tumor is syngenic to C3H mice, the tumor responses between endotoxin sensitive and in resistant mice can be compared directly. Moreover, the role of host components (such as bone marrow and spleen) [4, 6, 12] for the expression of antitumor and toxic effects, could be investigated using this model.

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