# Induction of tumour necrosis factor-alpha (TNF-α) mRNA in bladders and spleens of mice after intravesical administration of bacillus Calmette-Guérin

J. S. SHIN, J. H. PARK, J. D. KIM, J. M. LEE* & S. J. KIM *Department of Microbiology and *Urology, Yonsei University College of Medicine, Seoul, Korea*

## SUMMARY

Intravesical bacillus Calmette-Guérin (BCG) therapy is highly effective in the therapy of carcinoma *in situ* of the bladder, but the mechanism of BCG immunotherapy is not clearly understood. We studied the production of TNF-α in spleens and bladders of mice after intravesical BCG or BCG/interferon-gamma (IFN-γ) instillation. Significant change of TNF-α mRNA expression of spleens and bladders of C3H/He mice was observed after intravesical BCG instillation, although intravesical IFN-γ therapy 3 days after BCG instillation to maintain the activated state of monocyte/macrophage lineage cells did not show a significant change of TNF-α mRNA, compared with that of BCG therapy alone. Maximal production of TNF-α mRNA in spleens of mice was seen after the first or second intravesical BCG instillation, and production of TNF-α mRNA in bladders was also increased after intravesical BCG instillation. The increment of TNF-α production by BCG stimulation in HL-60, a promyelocytic leukaemic cell line, and peripheral blood mononuclear cells *in vitro* may support the *in vivo* effect of BCG therapy on the bladder. These data show that local production of TNF-α as well as systemic production by intravesical BCG treatment may correlate with one of the mechanisms of BCG immunotherapy of superficial bladder cancer.

**Keywords** superficial bladder cancer  BCG immunotherapy  tumour necrosis factor-alpha  interferon-gamma

## INTRODUCTION

Intravesical bacillus Calmette-Guérin (BCG) immunotherapy is considered to be one of the effective treatments for superficial bladder cancer and carcinoma *in situ* of the bladder [1–5]. Although the action mechanism of intravesical BCG immunotherapy is not clearly understood, an immune response to BCG has been known to be associated with anti-tumour activity. Congenital T lymphocyte-deficient mice transplanted with bladder tumours fail to respond to intravesical BCG treatment until T lymphocytes are given [6]; this result shows that successful intravesical BCG immunotherapy depends on an intact cellular immune function. Also, many clinical studies of intravesical BCG instillation reveal that the development of immunological responses to BCG is associated with anti-tumour activity [7–11].

Correspondence: Dr Se-Jong Kim, Department of Microbiology, Yonsei University College of Medicine, 134 Shinchon-dong Seodaemoon-ku, Seoul 120-752, Korea.

Intravesical BCG therapy induces the production of lymphokines such as IL-1, IL-2, interferon-gamma (IFN-γ), and TNF-α [12–14] in urine, and BCG-induced lymphokines may be associated with anti-tumour activities [12–13]. The elevated level of TNF-α in urine may play a major role in the cytotoxic activity of BCG [13,15], but the origin of TNF-α in urine has not yet been identified.

IFN-γ is a peptide cytokine produced by antigen-specific T cells during an immune response, and has been shown to have several actions of relevance to tumour immunology. It fulfils a number of immunomodulatory functions, notably activation of macrophages and natural killer (NK) cells, and proliferation and activation of T and B cells [16].

In the present study, we studied production of TNF-α mRNA in spleens and bladders of mice after intravesical BCG instillation to observe the local and systemic immunological effects of intravesical BCG treatment in inducing the production of TNF-α. The changes of TNF-α mRNA level in bladders and spleens of mice were also observed to determine the applicability of IFN-γ in addition to BCG.

## MATERIALS AND METHODS

### Mice and BCG

Female C3H/He mice, 6–8 weeks old, were used, and Pasteur strain BCG, $1 \times 10^5$ colony-forming units (CFU), was suspended in normal saline and instilled into the bladder via a Teflon catheter.

### Human and mouse TNF-α cDNA

Human TNF-α cDNA clone was obtained from the American Type Culture Collection (ATCC, Rockville, MD), and mouse TNF-α cDNA clone was kindly given by Dr P. Vassalli (Departement de Pathologie, Centre Medical Universitaire, Geneva, Switzerland), which contains the 696 Taq-EcoRI fragment of the mouse TNF gene [17]. The human γ-actin probe was a generous gift from Dr S. Y. Yang (Memorial Sloan Kettering Cancer Centre, New York, NY).

### TNF-α mRNA expression in vitro

HL-60 (ATCC CCL 240), a promyelocytic leukaemic cell line, and human peripheral blood mononuclear cells (PBMC) were used to observe the effect of BCG and IFN-γ on TNF-α mRNA induction in vitro. The HL-60 cells were cultured in RPMI 1640 medium (supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine) with 10% fetal bovine serum (FBS; GIBCO BRL, Grand Island, NY). The TNF-α mRNA was induced by recombinant human (rh) IFN-γ (Genzyme Co., Cambridge, MA) 50 or 500 U/ml, BCG $1 \times 10^5$ CFU/ml with rhIFN-γ 50 or 500 U/ml, and 20 μg/ml lipopolysaccharide (LPS; Escherichia coli 055:B5; Sigma, St Louis, MO), and cells were cultured for 4 h in humidified 5% $CO_2$, 37°C incubator. Cytoplasmic total RNA was isolated by a caesium chloride gradient centrifugation [18] after lysing the cells by guanidine isothiocyanate solution [19].

PBMC were obtained from the heparinized (20 U/ml) peripheral blood of two normal persons by Ficoll–Hypaque (Pharmacia, Piscataway, NJ) density gradient centrifugation at 4°C. PBMC were washed with ice-cold RPMI 1640, and cultured in RPMI 1640 with 10% human AB serum. TNF-α mRNA was induced by BCG 20 μg/ml with rhIFN-γ 50 or 500 U/ml, and LPS 20 μg/ml for 4 h [20]. Cytoplasmic total RNA was isolated by the guanidine isothiocyanate–phenol chloroform method [21]. TNF-α mRNA were detected by the Northern blot hybridization method [22].

### TNF-α mRNA in spleens and bladders of mice

The mouse bladder tumour cell line, MBT-2, from which signal of TNF-α mRNA could not be detected in our experiment (data not shown), was maintained in RPMI 1640 (Hazleton, Lenexa, KS) with 10% FBS. MBT-2 cells were washed with RPMI 1640 and implanted into the bladders as described previously [23]. Briefly, mice were placed under light general anaesthesia with i.p. sodium pentobarbital (0·05 mg/g animal weight), and bladders were catheterized with a cautery electrode which was guided by a 24 G teflon catheter under 10-fold magnification power. The electrode was activated with a Bovie unit for 4 s at the lowest coagulation setting, and $5 \times 10^5$ viable MBT-2 cells were injected into the bladder. Intravesical BCG treatments were started 24 h after tumour implantation, and BCG $1 \times 10^5$ CFU was instilled weekly for 5 weeks. Recombinant mouse IFN-γ (Genzyme) 3000 U per injection was instilled into the bladder 3 days after intravesical BCG treatment. Spleens and bladders of mice were removed to observe the expression of TNF-α mRNA after 1 week of final BCG treatment. Total cellular RNA of spleens were isolated by a caesium chloride gradient centrifugation, and those of bladders by the guanidine isothiocyanate–phenol–chloroform method [21] after homogenizing the tissue. Each test group consisted of three or four mice, and the expressions of TNF-α mRNA of spleens and bladders were detected by the slot blot hybridization method [24,25]. Control mice were also catheterized with a cautery electrode, and then MBT-2 cells were injected into the bladders as in the experimental mice, and spleens and bladders were removed after 1 week.

### Northern blot and slot blot analysis

Northern blot analysis was performed to observe TNF-α mRNA induction in HL-60 cells and PBMC. Briefly, isolated total cellular RNA was denatured with 50% formamide/17·5% formaldehyde/20 mM 3-[N-morpholino] propanesulphonic acid (MOPS) at 65°C for 15 min, and electrophoresed on a formaldehyde denaturing gel (1% agarose, 5·4% formaldehyde), and then transferred to a nylon membrane (Bios Co., New Haven, CT). The transferred RNA was fixed on the nylon membrane by an ultraviolet crosslinker (Hoeffer Scientific Instruments, San Francisco, CA) and prehybridized at 42°C overnight. The prehybridization solution contained 50% formamide/5 × SSC/ 5 × Denhardt's solution (0·1% bovine serum albumin (BSA)/ 0·1% polyvinylpyrrolidone/0·1% Ficoll)/1% SDS/250 μg/ml denatured herring sperm DNA/50 mM sodium phosphate buffer, pH 7·0. Hybridization was performed at 42°C overnight in the hybridization solution (50% formamide/5 × SSC/ 1 × Denhardt's solution/0·1% SDS/10% dextran sulphate/ 250 μg/ml denatured herring sperm DNA/50 mM sodium phosphate buffer, pH 7·0).

Slot blot analysis was done to observe TNF-α mRNA expression in spleens and bladders of mice. Briefly, a wet nylon membrane with 20 × SSC was fixed on the slot blot kit (Hoeffer) and prewashed with 10 × SSC. Heat-denatured RNA in 50% formamide/7% formaldehyde/1 × SSC was blotted to a nylon membrane through the slots, each slot was washed with 10 × SSC, and the RNA was fixed on the membrane by an ultraviolet crosslinker. The nylon membrane was prehybridized, and then hybridized overnight. α-$^{32}$P-labelled TNF-α cDNA probes by a random primer labelling kit (Amersham International, Aylesbury, UK) were used to detect signals [26,27]. The specific activity of labelled DNA, which was calculated by the formula of ((total ct/min incorporated)/(μg DNA synthesized + μg of input DNA)), was $1 \sim 2 \times 10^9$ ct/min per μg. The hybridized nylon membrane was washed with solution A (6 × SSC/0·2% SDS) at room temperature for 20 min, and with solution B (1 × SSC/1% SDS) at 42°C for 20 min, and with solution C (0·1 × SSC/1% SDS) at 50°C for 60 min. The intensity of each band was expressed by the value of optical density and compared with a laser scanning densitometer (Pharmacia LKB, Uppsala, Sweden) at 633 nm of absorbance after autoradiography.

## RESULTS

### TNF-α mRNA induction in HL-60 and PBMC

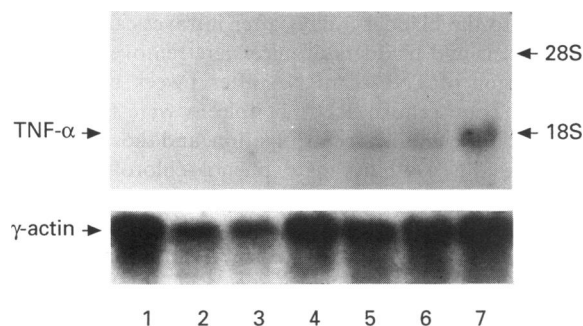The human promyelocytic cell line, HL-60, produces barely

**Fig. 1.** Northern blot analysis of TNF-$\alpha$ mRNA of HL-60 cells. Each lane was loaded with 20 $\mu$g/well of total cellular RNA. Lane 1, non-treated cells; lane 2, bacillus Calmette–Guérin (BCG) $1 \times 10^5$ colony-forming units (CFU)/ml; lanes 3 and 4, rhIFN-$\gamma$ 50 and 500 U/ml, respectively; lane 5, BCG $1 \times 10^5$ CFU/ml and rhIFN-$\gamma$ 50 U/ml; lane 6, BCG $1 \times 10^5$ CFU/ml and rhIFN-$\gamma$ 500 U/ml; lane 7, lipopolysaccharide (LPS) 20 $\mu$g/ml.

detectable levels of TNF-$\alpha$ mRNA without stimulation. TNF-$\alpha$ mRNA was not clearly detected after 4 h of stimulation with BCG or rhIFN-$\gamma$ 50 U/ml alone. The maximum signal came out when the cells were stimulated by BCG combined with IFN-$\gamma$ 50 U or 500 U (Fig. 1). PBMC also showed barely detectable levels of TNF-$\alpha$ mRNA without stimulation, but the expression of TNF-$\alpha$ mRNA greatly increased with the stimulation of BCG. TNF-$\alpha$ mRNA production of PBMC was more enhanced by the addition of IFN-$\gamma$ to BCG stimulation; however, there was no significant difference between the addition of IFN-$\gamma$ 50 U/ml and of 500 U/ml to BCG (Fig. 2). The *in vivo* effect of BCG on the production of TNF-$\alpha$ in bladders and spleens shown below was then observed.

*TNF-$\alpha$ mRNA induction in spleens and bladders of mice*

The bladders of mice were electrocoagulated, and intravesically implanted with MBT-2 cells, and were weekly instilled with BCG for 5 weeks. The mice were killed 7 days after each BCG instillation, and spleens and bladders were dissected out to observe TNF-$\alpha$ mRNA expression. The maximum signal of TNF-$\alpha$ mRNA of spleens was observed at the first or second intravesical BCG instillation, and a higher level of TNF-$\alpha$ mRNA was sustained during BCG treatment, but the signal slightly decreased with time (Figs 3 and 4). Maximum production of TNF-$\alpha$ mRNA in bladder tissues was observed after the first and fifth intravesical BCG treatments. The signals of TNF-$\alpha$ mRNA of bladders were highly sustained during BCG treatment. The L(S) cell, a TNF-$\alpha$-sensitive L cell line which produces little TNF-$\alpha$ mRNA, and the L(R) cell, a TNF-$\alpha$-resistant L cell line which produces high amounts of TNF-$\alpha$ mRNA, were used as controls [28] (data not shown).

The productions of TNF-$\alpha$ mRNA in the spleens and bladder tissues were studied to observe the effect of IFN-$\gamma$ in addition to BCG treatment. rmIFN-$\gamma$ (3000 U/mouse) was instilled into mice bladders 3 days after BCG instillation, in the expectation that IFN-$\gamma$ maintains the activated state of monocyte/macrophage cell lineage of bladder tissue by BCG treatment. However, there was no significant change of TNF-$\alpha$ mRNA production in spleens and bladders compared with the BCG-alone treatment group (Figs 4 and 5).
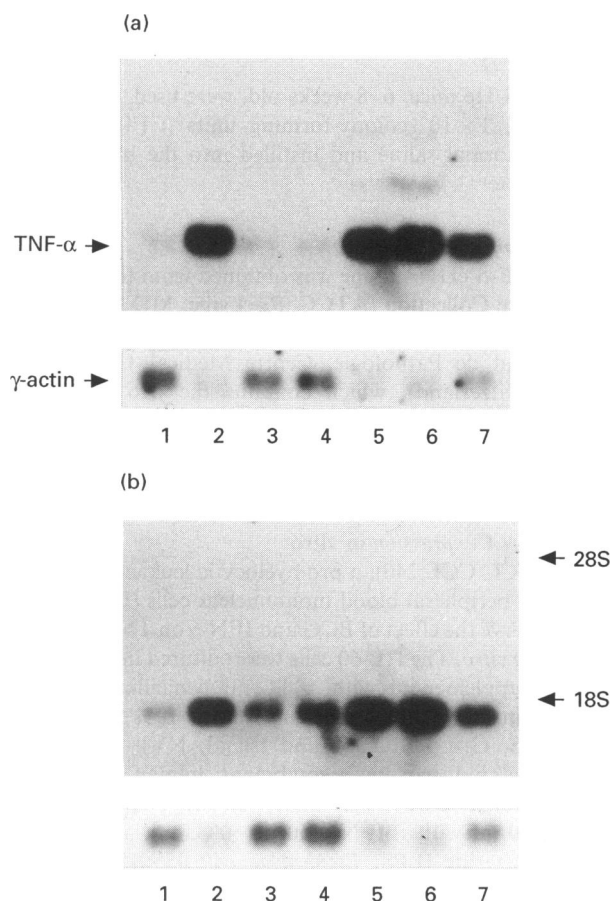


**Fig. 2.** Northern blot analysis of TNF-$\alpha$ mRNA of peripheral blood mononuclear cells (PBMC) of two normal persons. Each lane was loaded with 2 $\mu$g/well of total cellular RNA. Lane 1, non-treated cells; lane 2, bacillus Calmette–Guérin (BCG) 20 $\mu$g/ml; lanes 3 and 4, rhIFN-$\gamma$ 50 and 500 U/ml, respectively; lane 5, BCG 20 $\mu$g/ml and rhIFN-$\gamma$ 50 U/ml; lane 6, BCG 20 $\mu$g/ml and rhIFN-$\gamma$ 500 U/ml; lane 7, lipopolysaccharide (LPS) 20 $\mu$g/ml.

## DISCUSSION

This study shows that C3H/He mice instilled with intravesical BCG produce TNF-$\alpha$ mRNA in the spleen. TNF-$\alpha$ mRNA was highly induced by the first or second intravesical BCG instillation in the spleen. This result suggests that the systemic immune response may be influenced by local treatment of BCG. In our previous study, peritoneal macrophages of guinea pigs treated weekly with intravesical BCG for 6 weeks produced high amounts of TNF-$\alpha$, especially at the second or third week [15]. The serum TNF-$\alpha$ level was also slightly increased after BCG immunotherapy in superficial bladder cancer patients [15]. According to these data, it is considered that intravesical BCG treatment systemically influences the immune system. The positive conversion of purified protein derivative (PPD) skin tests in patients treated with intravesical BCG correlates with the tumour-free status [29–31]; therefore, the TNF-$\alpha$ produced in the spleen may play an important role in the mechanisms of BCG immunotherapy in superficial bladder cancer.

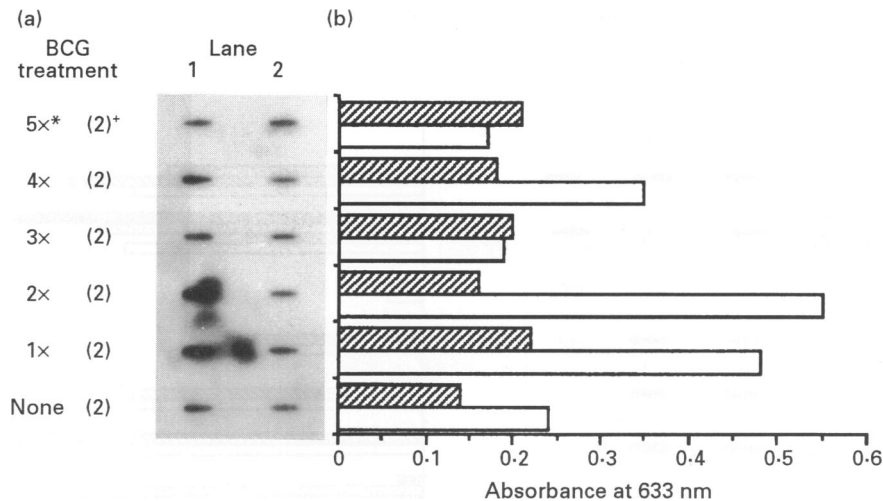The therapeutic role of interferon has been evaluated in

**Fig. 3.** Slot blot analysis of TNF-α mRNA of spleens of mice treated with bacillus Calmette–Guérin (BCG) only. MBT-2 cells ($5 \times 10^5$) were implanted into the bladders of mice after electrocauterization, and BCG ($1 \times 10^5$ colony-forming units (CFU)) was intravesically injected weekly. Spleens were removed to observe the expression of TNF-α mRNA after 1 week of final BCG instillation. Each slot was loaded with 20 μg of total cellular RNA. (a) Slot blot analysis of TNF-α mRNA of spleens of mice[*][+]. Numbers indicate the number of BCG treatments and the number of mice, respectively. (b) Band intensity of each slot is expressed on absorbance by laser scanning densitometry.

several urological malignancies [32], but the studies of intravesical IFN-γ therapy for superficial transitional cell carcinoma are primitive, and most studies have been performed with IFN-α [33–34]. In this study, the applicability of IFN-γ combined with BCG immunotherapy was evaluated by observing the change of TNF-α mRNA level. *In vitro*, IFN-γ augments TNF-α mRNA production by BCG in HL-60 and PBMC, compared with that of IFN-γ or BCG stimulation alone. This synergy may be due to the activation of macrophages and the increased number of TNF receptors by IFN-γ

[35]. Since many toxicities are manifested by *in vivo* treatment of TNF-α [36], although combined stimulation of TNF-α and IFN-γ has demonstrated synergistic cytotoxic activity [37–40], we treated the mice with BCG followed by IFN-γ after 3 days to maintain continuously the activated state of macrophages and other immune cells. Unexpectedly, however, there was no significant increase of TNF-α mRNA production in mice spleens and bladders. This result may be due to poor absorption of IFN-γ into the bladder tissue, or loss of the priming effect of IFN-γ at the time of sacrifice [38].
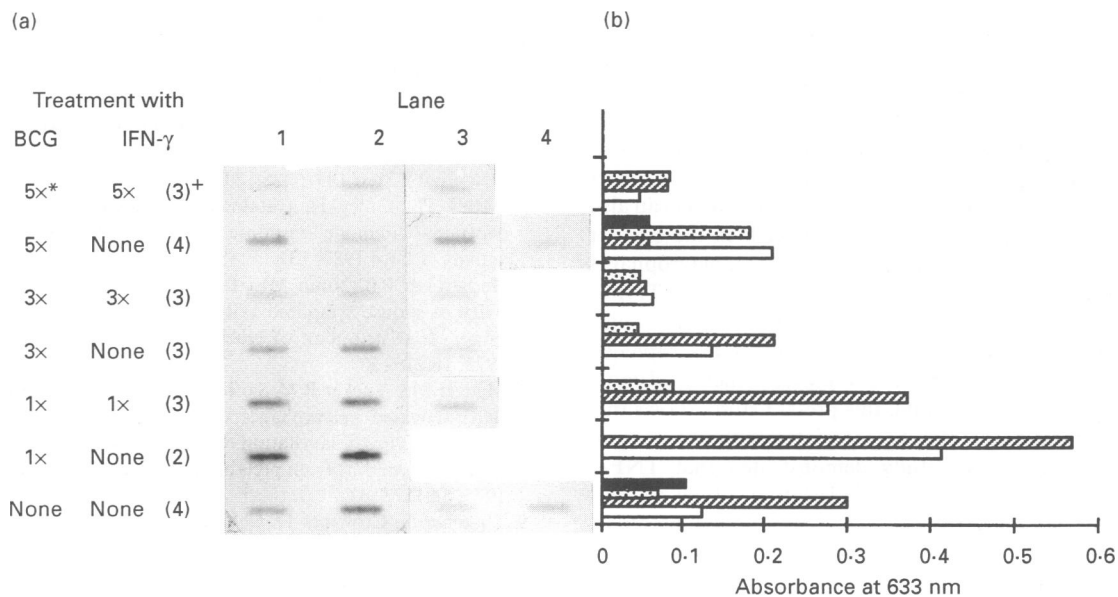


**Fig. 4.** Slot blot analysis of TNF-α mRNA of spleens of mice treated with bacillus Calmette–Guérin (BCG) and/or IFN-γ. Mice were treated with the same method as in Fig. 3, while IFN-γ (3000 U) was intravesically instilled on day 3 after each BCG instillation. Each slot was loaded with 20 μg of the total cellular RNA. (a) Slot blot analysis of TNF-α mRNA of spleens of mice treated with BCG and/ or IFN-γ[*][+]. Numbers indicate the number of BCG and/or IFN-γ treatments and the number of mice, respectively. (b) Band intensity of each slot is expressed on absorbance by laser scanning densitometry. Hybridization was performed on the same filter.
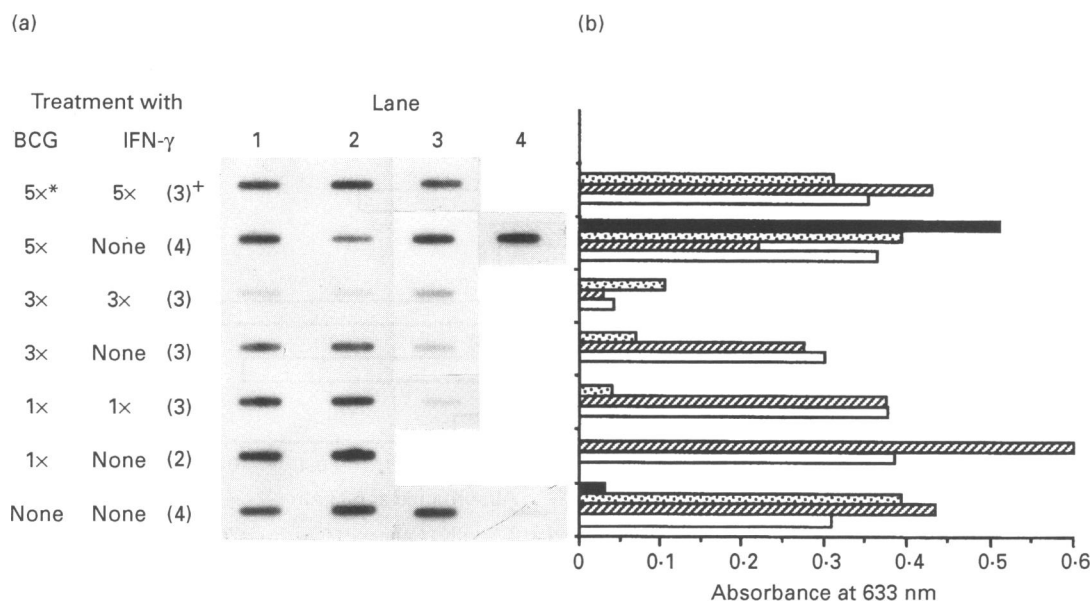
(a)

(b)



**Fig. 5.** Slot blot analysis of TNF-α mRNA of bladders of mice treated with bacillus Calmette–Guérin (BCG) and/or IFN-γ. Refer to the legend of Fig. 4 for the treatment of mice. Each slot was loaded with 5 μg of the total cellular RNA. (a) Slot blot analysis of TNF-α mRNA of bladders of mice treated with BCG and/or IFN-γ* +. Numbers indicate the numbers of BCG and/or IFN-γ treatments and the number of mice, respectively. (b) Band intensity of each slot is expressed on absorbance by laser scanning densitometry. Hybridization was performed on the same filter.

The high expression of TNF-α mRNA of bladder tissue by intravesical BCG treatment shows that TNF-α produced in bladder tissue may play an important role in direct killing of nearby tumour cells. A preliminary attempt at intravesical therapy of superficial bladder cancer with TNF-α was made, and the therapeutic effect was observed [41,42]. The concentration of TNF-α in the urine was increased from 4 to 8 h after intravesical BCG treatment [13]. Our results show that TNF-α in the urine may be the direct effect of BCG on the bladder wall as well as on the spleen. Histologically, small suburothelial granulomas consist of mononuclear cells, and many mononuclear cells can be found in the suburothelium after intravesical BCG treatment [43,44], and the number of leucocytes, monocytes/macrophages and T lymphocytes are significantly increased in the urine of patients with superficial bladder cancer after intravesical immunotherapy with BCG [45]. BCG can also be found in the phagosome of phagocytic cells in the bladder wall after intravesical BCG treatment; therefore, macrophages and immigrated monocytes in bladder tissue produce TNF-α by BCG treatment. Along with our previous report, which showed that production of TNF-α by alveolar macrophages from patients with pulmonary tuberculosis is increased more than that of normal persons [46], this present study shows that TNF-α from local tissue may be associated with anti-tumour activity. As a result, this study demonstrated that TNF-α production after intravesical administration of BCG may possibly come not only from systemic production by BCG-primed splenocytes, but also from local production by mononuclear cells infiltrated on the bladder wall.

## ACKNOWLEDGMENTS

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