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# Production of Tumor Necrosis Factor by Intravesical Administration of Bacillus Calmette Guérin in Patients with Superficial Bladder Cancer

Chun Il Kim[3], Jeon Soo Shin[1], Hyung Il Kim[4]
Jin Moo Lee[2], and Se Jong Kim[1]

*Although an immune response to bacillus Calmette Guêrin (BCG) has often been associated with antitumor activity, the action mechanism(s) of intravesical BCG therapy for prophylaxis and treatment of superficial bladder cancer is not clearly understood. In an attempt to evaluate the roles of tumor necrosis factor (TNF)-α and lymphotoxin (LT) in the antitumor activity, TNF-α productivities by peripheral blood monocytes, serum levels of TNF-α, and LT productivities by peripheral blood lymphocytes were studied in superficial bladder cancer patients after six intravesical administrations of BCG. TNF-α productivities by peritoneal macrophages of guinea pigs were also studied after six intravesical administrations of BCG. The maximum TNF-α productivities by peripheral blood monocytes of superficial bladder cancer patients were seen after the fourth week of administration of BCG, and the serum TNF-α levels were also slightly increased after intravesical BCG administration in the superficial bladder cancer patients. LT productivities by peripheral blood lymphocytes of superficial bladder cancer patients were significantly enhanced and the maximum LT productivity was also seen after the third or fifth BCG administration. TNF-α productivities by peritoneal macrophages of guinea pigs were significantly enhanced and the maximum TNF-α productivity was seen after the second or third BCG administration. Our data might suggest that six consecutive intravesical BCG administrations could induce the increased productions of TNF-α and LT, which might play an important role in the antitumor activity in superficial bladder cancer.*

**Key Words:** Superficial bladder cancer, TNF-α, Lymphotoxin, BCG

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Department of Microbiology[1], and Urology[2], Yonsei University College of Medicine, Seoul, Korea; and the Department of Urology[3], Kyemyung University College of Medicine, Taegu, Korea; and the Department of Microbiology[4], Ajou University College of Medicine, Suwon, Korea.

Address reprints reguests to Dr. S.J.Kim, Department of Microbiology, Yonsei University College of Medicine, C.P. O. Box 8044, Seoul, Korea, 120-752

This work was supported by development project grant and faculty funds (91-2) and research instructor grant from the Yonsei University College of Medicine. Address correspondence to Se Jong Kim, Department of Microbiology, Yonsei University College of Medicine, 134 Shinchondong Seodaemoonku, Seoul 120-752, Korea.

Intravesical bacillus Calmette Guêrin (BCG) therapy is considered one of the most effective treatments for recurrent superficial bladder cancer and carcinoma *in situ* (Morales *et al.* 1976; Brosman, 1982; Herr *et al.* 1983; Kelley *et al.* 1985; Lamm, 1985). Although an immune response to BCG has often been associated with antitumor activity, the action mechanism of intravesical BCG therapy is not clearly understood. Successful therapy of superficial bladder cancer by intravesical BCG administrations depends on the intact host immune system, especially T lymphocyte functions (Ratliff *et al.* 1987). Clinical studies have supported the associa-

tion between the development of immunological responses to BCG and antitumor activity, such as the correlation between a positive delayed type hypersensitivity (DTH) response to purified protein derivative (PPD) and the prevention of a recurrent tumor by BCG (Lamm *et al.* 1982).

The roles of various immunomodulatory proteins or cytokines produced in response to BCG have been evaluated (Droller *et al.* 1986; Haaff *et al.* 1986). Intravesical BCG therapy induced the production of interleukin (IL)-1 (Haaff *et al.* 1986; Böhle *et al.* 1990), IL-2 (Böhle *et al.* 1990), interferon-$\gamma$ (Prescott *et al.* 1990), and tumor necrosis factor-alpha (TNF-$\alpha$) (Nakamura *et al.* 1987; Böhle *et al.* 1990). Bö hle *et al.* (1990) also studied the characteristics of the local cellular infiltrates secondary to BCG instillation and measured TNF-$\alpha$ in the urine of superficial bladder cancer patients treated with intravesical BCG. High titers of TNF-$\alpha$ were measured in the urines of the superficial bladder cancer patients with intravesical BCG therapy. TNF-$\alpha$ very efficiently lyses some tumor cells *in vitro* (Carswell *et al.* 1975; William *et al.* 1983). In addition, lymphotoxin (LT) produced by activated lymphocytes also has cytotoxicity on some tumor cells (Meltzer and Barlett, 1972; Evans and Heinbaugh, 1981; Powell *et al.* 1985). Therefore, it is possible that TNF-$\alpha$ and LT produced by BCG immunotherapy might be associated with antitumor activity. But a better understanding of the changes of TNF-$\alpha$ is not known.

In an attempt to evaluate the changes of TNF-$\alpha$ after intravesical BCG treatment, we performed a prospective study in which the TNF-$\alpha$ productivity by peripheral blood monocytes and LT productivity by peripheral blood lymphocytes, and the serum level of TNF-$\alpha$ were measured in the superficial bladder cancer patients before and after intravesical BCG instillation. And we also studied the TNF-$\alpha$ productivity by peritoneal macrophages in guinea pigs, which seem to be most comparable to humans with regard to their sensitivity for mycobacteria (Meijden *et al.* 1988), after six consecutive intravesical BCG instillations.

## MATERIALS AND METHODS

### Patients and animals

Seven patients diagnosed with superficial bladder cancer were included in this study as well as female guinea pigs weighing from approximately 400 g to 600 g.

### Bacillus Calmette-Guérin (BCG)

Lyophilized BCG (120 mg, Pasteur strain) containing about $1\times10^8$ living bacilli was suspended in saline and administered intravesically to superficial bladder cancer patients once a week for six consecutive weeks. Patients were asked not to urinate for two hours after the administration. In female guinea pigs, BCG was administered intravesically at doses of $2\times10^6$ culturable particles (c.p.) once a week for six consecutive weeks. After disinfecting the vulvas of the guinea pigs, BCG was instilled into the empty bladders of the anesthesized animals using teflon sheaths (Abbocath-T, 24 gauze) as a transurethral catheter, and remained in the bladder for 1 to 2 hours. Guinea pigs were anesthesized by intramuscular injection of 0.8 ml of a mixture of 20 mg/ml KetalarR, 2.5 mg/ml PompunR and 0.05 mg/ml atropine.

### Isolation of peripheral blood lymphocytes, monocytes and serum separation

The isolation of monocytes was done by adhering mononuclear cells on the gelatin-coated flasks as described by Freundilich and Avdalovic (1983). In brief, 5 ml of 2% (wt/vol) gelatin solution was added to a 25 $cm^2$ culture flask (Corning, Corning, NY), and the flask was placed in a 37℃ incubator for 2 hours. After removing the gelatin solution, the flasks were dried overnight. Prior to the purification of monocytes from mononuclear cells, pooled human plasma was added to the gelatin-coated flask, left for 45 minutes, and was washed 3 times with isotonic saline. Peripheral blood was drawn from each patient after four hours of the intravesical BCG administration. Peripheral blood mononuclear cells (PBMC) were separated by Ficoll-

Hypaque (Pharmacia Fine Chemicals, Pistacavay, NJ) density gradient centrifugation, washed three times with ice-cold RPMI 1640 (Hazleton, Lenexa, KS), and then resuspended in the RPMI 1640 supplemented with 2 mM L-glutamine (Hazleton, Lenexa, KS), penicillin (100 IU/ml), streptomycin (100 μg/ml), and 10% horse serum (Gibco laboratories, Grand Island, NY). After allowing the PBMC to adhere to gelatin-coated culture flasks at 37° C with 5% $CO_2$ for 45 minutes, the nonadherent cells were removed with prewarmed RPMI 1640 at 37℃. Adherent cells were detached by $Ca^{++}$, $Mg^{++}$ free PBS contained 4.5 mM EDTA (Flow Lab., North Ryde, Australia). The enriched monocytes preparation contained more than 95% esterase positive cells, and was kept on ice until used. Sera were obtained from each patient after four hours of intravesical BCG administration, and kept at -20℃ until tested.

### Isolation of peritoneal macrophages from guinea pigs

To obtain peritoneal exudate cells, the guinea pigs were sacrificed, and then about 20 ml of cold RPMI 1640 containing heparin (5 U/ml) was injected into the peritoneal cavity. After gentle agitation, the fluid was aspirated. This process was repeated 3 to 5 times and the exudates were pooled. Cells were washed and attached to the gelatin-coated flask, and the adherent peritoneal macrophages were obtained as described above. All cells were kept on ice until used.

### Induction of TNF-α and lymphotoxin

The peripheral monocytes of patients were cultured in 24 well culture plates (Corning) at $1\times10^6$ cells/ml with RPMI 1640 supplemented with 10% (vol/vol) fetal bovine serum (Gibco, Grand Island, NY). Ten μg/ml of lipopolysaccharide (LPS, *E. coli* 055:B5, Sigma Chemicals Co., St Louis, MO), 10 μg/ml of PPD (Statens Serum Institute, Denmark), and 10 μg/ml of BCG were used for stimulants. After 24 hours cultivation, culture supernants were collected, frozen and stored at −20℃ until tested for the quantitation of TNF-α. TNF-α productions by peritoneal macrophages of guinea pigs were also done as described above. For lymphotoxin production, the nonadherent lymphocytes were cultured at $4\times10^6$ cells/ml with complete media with 10% fetal bovine serum. Ten μg/ml of concanavalin A (Con A, Sigma Chemicals Co), 10 μg/ml of BCG, and 10 μg/ml of PPD were used for stimulants. After 3 days cultivation, culture supernants were harvested, frozen and stored at −20℃ until tested.

### Quantitation of TNF-α and lymphotoxin

The TNF-α levels of culture supernatants of peripheral blood monocytes and of sera from patients were measured by using the TNF-α immunoradiometric assay (TNF-IRMA) kit (Medgenix Co., Brussels, Belgium). In brief, culture supernatant or serum and [$^{125}$I] labeled-anti-TNF-α supplied by kit were added to anti-TNF-α antibody coated tubes and incubated at room temperature for 18 hours. After aspirating the contents completely, the tubes were washed twice with 20% (vol/vol) tween 20 solution and then aspirated again. Finally, the tubes were counted in a gamma counter for 60 seconds, and the values were measured using the standard curve.

TNF-α productions by peritoneal macrophages from guinea pigs were measured by L929 cytotoxicity assay (Ruff and Gifford, 1981). In brief, 100 μl of test samples were prepared in a 96-well flat bottomed plate (Corning). One hundred μl of L929 cell suspension ($5\times10^4$ cells per well) treated with actinomycin D (1 μg/ml, Sigma Chemical Co.) were then added to the microtiter plates. The plates were incubated at 37℃ for 18 hours, and incubated for 4 hours further with the addition of methylthiazol tetrazolium bromide (MTT, Sigma Chemical Co.) at a final concentration of 1 mg/ml. The optical density was measured with a spectrophotometer at 570 nm, and the values were measured using the standard curve. One unit of TNF-α was defined as 50% cytotoxicity of L929 cells. The LT productivities of peripheral blood lymphocytes from patients were measured by L929 cytotoxicity assay as described above except for the number of L929 cells ($3\times10^4$ cells/well). For the standard curve, two-fold diluted known amounts of LT (Genzyme, Boston, MA) were used for titration.

## RESULTS

**TNF-α productions by monocytes of superficial bladder cancer patients after intravesical administration of BCG.**

The TNF-α levels of monocytes culture supernatants were measured by using the TNF-IRMA kit to observe the changes of TNF-α productions after intravesical administration of BCG (Table 1). TNF-α productions were gradually increased according to intravesical BCG administration from 10.72, 8.82 ng/ml (before BCG administration) to 26.05, 28.53 ng/ml (after the fourth BCG administration) in two patients (H03, H04) among the 7 patients in this group. TNF-α levels of monocytes culture supernatant in one patient (H01) changed from 16.29 ng/ml (after first BCG administration) to 36.94 ng/ml (after second BCG administration). And TNF-α productions were gradually increased in 3 patients (H02, H05, H06), but there was no marked change in one patient (H07).

**Changes of the serum TNF-α levels in superficial bladder cancer patients after intravesical administration of BCG**

Serum TNF-α levels of five patients were observed 4 hours after intravesical BCG trial. The TNF-α level peaked in the second or sixth BCG administration in 2 patients (H02, H06). TNF-α levels of two other patients (H03, H07) were gradually increased from 8.72, 5.51 pg/ml to 12.24, 9.59 pg/ml, respectively (Fig. 1).

**LT productions by peripheral blood lymphocytes of superficial bladder cancer patients after intravesical administration of BCG**

LT productions by peripheral blood lym-

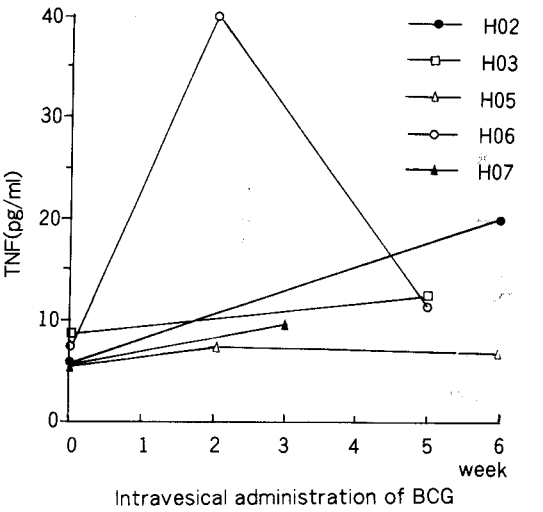


***Fig. 1.*** *Levels of serum TNF-α in superficial bladder cancer patients after intravesical administration of BCG. Sera were obtained from five patients after four hours of intravesical BCG administration, and quantitation of TNF-α were done by using the TNF-IRMA kit.*

**Table 1. Productions of TNF-α (ng/ml) by peripheral blood monocytes stimulated with LPS in superficial bladder cancer patients after intravesical administrations of BCG**

| Patients | Intravesical administration of BCG(weeks) | | | | |
|---|---|---|---|---|---|
| | 0 | 1st | 3rd | 4th | 6th |
| H01 | NT* | 16.29 | 36.94 | NT | 28.18 |
| H02 | 21.98 | 25.62 | 27.54 | NT | NT |
| H03 | 10.72 | 11.20 | 12.76 | 26.05 | 10.18 |
| H04 | 8.82 | 11.74 | 17.66 | 28.53 | 11.81 |
| H05 | 21.66 | NT | NT | 27.54 | NT |
| H06 | 14.99 | 11.75 | NT | NT | 19.55 |
| H07 | 12.31 | 13.52 | NT | 10.30 | 15.08 |

The peripheral monocytes ($1 \times 10^6$ cells/ml) of seven patients with intravesical administration of BCG in the presence of LPS (10 μg/ml), and culture supernatants were tested for the quantitation of TNF-α by using the TNF-IRMA kit. *NT: Not tested

phocytes stimulated with Con A were measured by L929 cytotoxicity assay in 6 patients after intravesical BCG administration. LT productions of 3 patients (H03, H04, H05) peaked to 680.8, 451.8, 560.6 U/ml at the fourth intravesical BCG administration compared to 10.5, 10.2, 309.1 U/ml before intravesical administration of BCG, respectively. And there were two patients (H06, H07) who produced maximum LT at the sixth intravesical administration of BCG (Table 2).

When the peripheral blood lymphocytes were stimulated with BCG, LT productions of five patients (H03, H04, H05, H06, H07) were increased from 0.7, 22.1, 51.0, 115.8, 30.5 U/ml to 626.7, 295.4, 339.1, 800.6, 140.2 U/ml respecifvely. The peak time of LT production was the third or fourth intravesical BCG administration, after which production gradually decreased (Table 2). LT productions of peripheral blood lymphocytes stimulated with PPD in 3 patients (H03, H04, H06) showed similar patterns to those of BCG stimulation, but no significant change was observed in one patient (H07)(Table 2).

**Correlation between TNF-$\alpha$ production by monocytes and LT production by lymphocytes from patients**

The TNF-$\alpha$ and LT productions peaked at

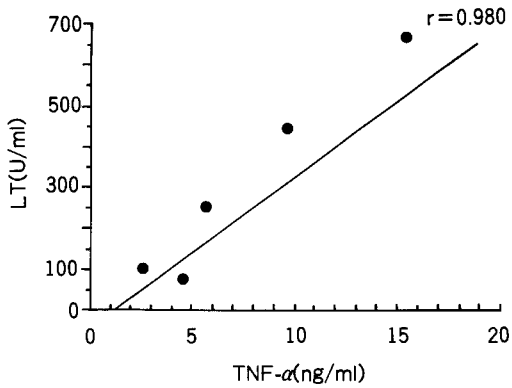


***Fig. 2.*** *Correlation between the increments of TNF-$\alpha$ productions by peripheral blood monocytes and LT productions by peripheral blood lymphocytes in superficial bladder cancer patients after the 4th or 6th intravesical BCG administration.*

**Table 2. Productions of LT (units/ml) by peripheral blood lymphocytes stimulated with Con A, BCG or PPD in superficial bladder cancer patients after intravesical administration of BCG**

| Patient | Stimulant | Intravesical administration of BCG (weeks) | | | |
|---|---|---|---|---|---|
| | | 0 | 1st | 4th | 6th |
| H01 | ConA | NT* | 618.4 | 306.2 | NT |
| | BCG | NT | 2.5 | 399.4 | NT |
| H03 | ConA | 10.5 | NT | 680.8 | 424.3 |
| | BCG | 0.7 | NT | 626.7 | 248.9 |
| | PPD | 1.0 | NT | 170.8 | NT |
| H04 | ConA | 10.2 | 0 | 451.8 | 32.0 |
| | BCG | 22.1 | 9.8 | 295.4 | 27.0 |
| | PPD | 1.6 | 2.1 | 415.4 | 6.5 |
| H05 | ConA | 309.1 | NT | 560.6 | NT |
| | BCG | 51.0 | NT | 339.1 | NT |
| H06 | ConA | 360.5 | 257.2 | NT | 439.7 |
| | BCG | 115.8 | 117.5 | NT | 800.6 |
| | PPD | 117.9 | 41.7 | NT | 489.9 |
| H07 | ConA | 111.5 | 121.2 | 34.5 | 168.8 |
| | BCG | 30.5 | 6.5 | 140.2 | 113.7 |
| | PPD | 28.8 | 2.8 | 0 | 4.6 |

The peripheral lymphocytes ($4\times10^6$ cells/ml) of six patients with intravesical administration of BCG in the presence of Con A (10 $\mu$g/ml), BCG (10 $\mu$g/ml), and PPD (10 $\mu$g/ml), culture supernatants were tested for the quantitation of LT by L929 cytotoxicity assay. *NT: Not tested

**Table 3. Productions of TNF-α (units/ml) by peritoneal macrophages stimulated with LPS or BCG in guinea pigs after intravesical administration of BCG**

| Stimulant | Intravesical administration of BCG (weeks) | | | | | |
|---|---|---|---|---|---|---|
| | 0 (n=2)* | 1st (n=3) | 2nd (n=2) | 3rd (n=3) | 4th (n=3) | 6th (n=4) |
| LPS | 393.7**±75.8 | 428.8±221.3 | 1445.1±654.4 | 1258.9±720.7 | 421.1±329.5 | 498.4±380.5 |
| BCG | 304.9±108.6 | 872.0±498.4 | 1283.1±433.6 | 1715.1±152.0 | 717.9±119.1 | 695.0±445.0 |

Guinea pigs were intravesically instilled with BCG ($2\times10^6$ c.p.), and the peritoneal macrophages ($1\times10^6$ cells/ml) one week after final administration of BCG were cultured with LPS or BCG for 24 hours. The TNF-α levels were measured by L929 cytotoxicity assay. * Number of guinea pigs. **Mean±standard error.

**Table 4. Productions of TNF-α (units/ml) by peritoneal macrophages stimulated with LPS or BCG in guinea pigs according to days after intravesical administration of BCG**

| Stimulant | Intravesical BCG administration | | | | | |
|---|---|---|---|---|---|---|
| | Days after 2nd administration | | | Days after 5th administration | | |
| | 1 day (n=2)* | 4 day (n=3) | 7 day (n=1) | 1 day (n=2) | 4 day (n=2) | 7 day (n=2) |
| LPS | 131.7**±110.0 | 1529.6±385.6 | 1777.4 | 1409.0±325.2 | 1212.6±484.7 | 1476.4±424.8 |
| BCG | 292.7±351.3 | 1379.4±448.6 | 1025.4 | 1608.9 | 1511.1 | 1492.9±7.1 |

Guinea pigs were intravesically instilled with BCG ($2\times10^6$ c.p.), and sacrificed on the first, fourth and seventh day after the second or fifth administration of BCG. Peritoneal macrophages ($1\times10^6$ cells/ml) of guinea pigs were cultured with LPS or BCG for 24 hours. The TNF-α levels were measured by L929 cytotoxicity assay. *Number of guinea pigs, **Mean±standard error.

the time of the fourth (H03, H04, H05) or sixth (H06, H07) intravesical BCG administration. A significant correlation (r=0.980) was observed between TNF-α and LT productions of the patients (Fig. 2).

### TNF-α productions by peritoneal macrophages of guinea pigs

Guinea pigs were intravesically treated with $2\times10^6$ c.p. of BCG once a week for six weeks. One week after BCG administration, peritoneal macrophages were isolated and then stimulated with LPS or BCG *in vitro* to observe TNF-α productions. TNF-α levels were measured by L929 cytotoxicity assay. When peritoneal macrophages were stimulated with LPS or BCG, TNF-α productions peaked at the time of the second or third intravesical BCG administration and then gradually decreased. These changes were similar to those of TNF-α or LT of peripheral blood monocytes or lymphocytes from superficial bladder cancer patients after intravesical BCG administrations (Table 3).

TNF-α productions by peritoneal macrophages were observed on the first day, fourth day and seventh day after the second or fifth intravesical BCG administration to evaluate how long the TNF-α productivity was sustained. TNF-α productions on the first day, fourth day and seventh day after the second intravesical BCG administration were respectively 131.7, 1529.6, 1777.4 U/ml with LPS stimulation, and 292.7, 1379.4, 1025.4 U/ml with BCG stimulation. After the fifth intravesical BCG administration, TNF-α productions were respectively 1409.0, 1212.6, 1476.4 U/ml with LPS stimulation, and 1608.9, 1511.1, 1492.9 U/ml with BCG stimulation (Table 4).

## DISCISSION

The mechanism by which intravesical BCG instillations for superficial bladder cancer inhibits tumor growth is not known. Considerable evidence suggests that immunological mechanisms could play an important role; however, definitive data associating the immune response to antitumor activity have not been reported. There were histopathological changes of cellular infiltrate in bladder tissues instilled with BCG (Guinan *et al.* 1986). An intense inflammatory reaction secondary to the BCG, associated with local proliferation of T and B cells and macrophages was noted (Guinan *et al.* 1986). Ratliff *et al.* (1987) proposed that BCG-related antitumor activity is a T cell dependent mechanism, because athymic nude mice were incapable of BCG-related tumor rejection but its function was restored after the transfer of syngeneic T lymphocytes. BCG is a potent activator of macrophages (Carswell *et al.* 1975). The monocyte-macrophage lineage is believed to play an important role in antitumor activity. Böhle *et al.* (1990) observed the elevated levels of IL-1, IL-2, TNF-$\alpha$ in the urine of patients treated with intravesical BCG, and the stimulation of TNF-$\alpha$ production may play a major role in the cytotoxic activity of BCG. TNF-$\alpha$ is produced predominantly by activated macrophages, and LT is produced by activated lymphocytes (Ruddle and Wakesman, 1968[a,b]), and both have a direct cytotoxic action on some tumor cells and on the vasculature of tumors (Ruddle and Schmid, 1987; Beutler and Cerami, 1989).

In this study, BCG was intravesically administered once a week for six consecutive weeks to superficial bladder cancer patients and to guinea pigs to observe the changes of productions of TNF-$\alpha$ and LT. The TNF-$\alpha$ productivities by peripheral blood monocytes were significantly enhanced in the superficial bladder cancer patients after intravesical administrations of BCG. The maximum TNF-$\alpha$ productivity was generally seen after the third or fourth week of intravesical BCG administration; and the TNF-$\alpha$ productivities by peritoneal macrophages of guinea pigs were also significantly enhanced after the second or third intravesical BCG administration; and the productivities of LT by peripheral blood lymphocytes in superficial bladder cancer patients were elevated at the fourth BCG administration in accordance with intravesical BCG administrations. These results suggest that the systemic immune system is sensitized by BCG despite its local application into the bladder. Nissenkorn *et al.* (1987) showed an activation of peripheral monocytes after intravesical BCG instillation. The conversion of the PPD skin test from negative to positive in patients treated with intravesical BCG (Lamm, 1985; Kelly *et al.* 1986; Netto and Lemos, 1983) suggests that a systemic immune response is related with intravesical BCG therapy, even though the PPD skin test conversion has not been uniformly linked with an antitumor response in humans. Although further investigations may be necessary to elucidate whether the enhanced TNF-$\alpha$ productivity contributes to the antitumor activity of BCG, it might be suggested that the increased production of TNF-$\alpha$ of peripheral blood monocytes and of LT of peripheral lymphocytes are believed to play an important role in the antitumor activity of superficial bladder cancer.

From the fourth day after the second BCG instillation, we found a high level of TNF-$\alpha$ productivity of peritoneal macrophages in the guinea pigs. And the TNF-$\alpha$ productivity of peritoneal macrophages after BCG administration was maintained up to seven days till the next BCG was given. These results suggest that repeated BCG instillations once a week were an appropriate schedule for the induction of TNF-$\alpha$ production. Protocols for intravesical BCG treatment for superficial bladder cancer is variable, but six week instillations are usually given initially (Herr *et al.* 1983; Lamm 1985). In this study, six consecutive BCG treatments are thought to be necessary for intravesical BCG immunotherapy in the production of TNF-$\alpha$ and LT. These experiments showed that intravesical BCG administration stimulated lymphocytes and monocytes to produce LT and TNF-$\alpha$, respectively. TNF-$\alpha$ production induced by intravesical administration of BCG may contribute to one of the mechanisms of BCG immunotherapy on patients with superficial

bladder cancer. The data showing sustained TNF-$\alpha$ productivity up to the seventh day after BCG administration in guinea pigs support the rationale of the one week interval for BCG administration.

Increased levels of TNF-$\alpha$ were measured not only in sera but also in urine (data not shown) of patients with superficial bladder cancer after BCG administrations. These data suggested that intravesical BCG administrations stimulate the production of TNF-$\alpha$ by both a systemic and local immune response. An attempt must be made to determine whether intravesical BCG administrations for the treatment of superficial bladder cancers can stimulate not only a systemic immune response, such as TNF-$\alpha$ and LT production, but also a local immune response to produce TNF-$\alpha$ from the bladder wall.

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