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Enhancing Effect of an Inhibitor of Nitric Oxide Synthesis on Bacillus Calmette-Guérin-induced Macrophage Cytotoxicity against Murine Bladder Cancer Cell Line MBT-2 *in vitro*

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We studied the effect of an inhibitor of nitric oxide (NO) synthesis, N^G-monomethyl-L-arginine (L-NMMA), on the Bacillus Calmette-Guérin (BCG)-induced antitumor activity of murine peritoneal exudate cells (PEC) against murine bladder cancer cell line MBT-2 *in vitro*. L-NMMA enhanced BCG-induced cytotoxic activity of PEC, as well as interferon (IFN)-γ and tumor necrosis factor (TNF)-α production. The L-NMMA-induced enhancement was due to the prolonged survival of BCG in macrophages, because no enhancement of cytotoxicity was observed and neither IFN-γ nor TNF-α production was significantly enhanced by killed BCG. Anti-TNF-α antibody (Ab) and anti-IFN-γAb reduced the L-NMMA-induced enhancement of the cytotoxicity. The depletion of T cells from PEC reduced the production of both IFN-γ and TNF-α, as well as the enhancement of cytotoxicity induced by viable BCG plus L-NMMA. These results suggest that L-NMMA has an enhancing effect on BCG-induced macrophage cytotoxicity and the enhancement is partially mediated by T cells and their soluble products. Accordingly, NO inhibitor should be a valuable adjunct to BCG immunotherapy for bladder cancer.

Key words: Nitric oxide — BCG — Macrophage — Cytotoxicity — Bladder cancer

Intravesical instillation of Bacillus Calmette-Guérin (BCG) is believed to be one of the most effective therapies to prevent recurrence of superficial urinary bladder cancer,[1)] but its mode of action remains unknown. After repeated BCG instillations for superficial urinary bladder cancer, mononuclear cell infiltration is induced in the bladder wall of the patient.[2,3)] Analysis of mucosal bladder mononuclear cells revealed that most of the cells were lymphocytes, associated with some macrophages.[4)] Clinical studies showed a marked increase in the number of macrophages in the urine after repeated BCG instillations.[5)] Various cytokines such as interleukin (IL)-1, IL-2, IL-6, tumor necrosis factor (TNF)-α and interferon (IFN)-γ have also been detected in the urine, and an elevated level of TNF-α seems to play a major role in the cytotoxicity induced by BCG.[6–8)] These studies indicated that macrophages and lymphocytes, which appeared to be the major effector cells, interacted with each other via a complex network of cytokines in the immune response to BCG.

BCG primarily stimulates macrophages, followed by activation of other cell types, such as T cells and NK cells, to induce expression of a large number of Th1-dependent cytokines.[9)] Previous reports suggested that macrophages could inhibit the growth of a wide variety of tumors and *Mycobacterium*, once activated by agents such as IL-2[10)] and IFN-γ.[11)] Cell-mediated immunity against *Mycobacterium* depended upon macrophage activation through T cell cooperation.

Macrophages play a complex role in tumor immunology. First, cytokines induced by some agents prime macrophages, and several products, such as nitric oxide (NO) and TNF-α, can kill tumor cells. It is now well established that NO acts as a potent effector molecule in non-specific immune defense against intracellular pathogens and tumor cells.[12,13)] NO also has other functions, such as inhibition of T cell proliferation.[14)] It was reported that a low concentration of endogenously generated NO stimulates Th1 cell proliferation, but a high concentration prevents the possibly deleterious overexpression of Th1 activities in an infection model.[15)] Conversely, the NO production in macrophages is regulated by T cell and T cell-derived cytokines. In general, Th1-type cytokines (especially IFN-γ and IL-2) up-regulate NO, but Th2-type cytokines (IL-4 and IL-10) inhibit NO.[16)] However, much less is known about the potential role of NO in macrophage- and T cell-dependent regulation of BCG-mediated cytotoxicity.

In this communication, we studied the effect of a specific NO inhibitor, N^G-monomethyl-L-arginine (L-

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NMMA), on BCG-induced macrophage cytotoxicity against murine bladder cancer cells *in vitro*.

MATERIALS AND METHODS

Reagents The culture medium was RPMI-1640 (Nissui Pharmaceutical Co., Tokyo) containing 10% heat-inactivated fetal calf serum (FCS), L-glutamine (2 m*M*) and penicillin-G (100 U/ml) (Grand Island Biological Co., Grand Island, NY). OK432 was a generous gift from Chugai Pharmaceutical Co., Tokyo. L-NMMA was purchased from Sigma Chemical Co., St Louis, MO. Rat anti-murine Pan-NK cell monoclonal antibody (mAb) and rat anti-murine IFN-γ mAb were purchased from Pharmingen Co., San Diego, CA. Anti-murine Thy1.2 mAb was purchased from Serotec Ltd., Oxford, UK. Low-toxicity rabbit complement was purchased from Cosmobio Co., Tokyo. Rat anti-murine TNF-α mAb was purchased from Dainihon Pharmaceutical Co., Tokyo.

Animals Female C3H/HeN mice were purchased from Seac Co., Ohita and were maintained for at least 1 week in our laboratory, then used for experiments at 6 to 8 weeks of age.

BCG and culture medium BCG (Tokyo 172 strain, 7.5×10^7 viable units/mg) was kindly supplied by Japan BCG Production Co., Tokyo. BCG was grown to mid-log phase in Middlebrook 7H9 broth (Difco Laboratories Inc., Detroit, MI) supplemented with 10% albumin-dextrose-catalase (ADC, Difco Laboratories Inc.), 0.2% glycerol, and 0.5% Tween 80. The grown bacteria were washed and suspended in phosphate-buffered saline (PBS) pH 7.4. The concentration of the bacterial suspension was adjusted based on the absorbance at 590 nm.

The number of BCG was counted by culturing on Middlebrook 7H10 agar (Difco Laboratories Inc.) supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC, Difco Laboratories Inc.), 0.5% glycerol, 400 U/ml penicillin, and 100 mg/ml cycloheximide.

Killed BCG was prepared by heating the viable bacteria at 121°C for 30 min. After extensive washing of killed bacteria with PBS, the concentration was adjusted to equal that of viable BCG. Viability was tested by culturing on 7H10 agar, and Ziel-Nealsen staining confirmed that the structure of the bacteria was intact.

Target cells The target cell line used in this study was N-[4-(5-nitro-2-furyl)-2-thiazolyl]-formamide (FANFT)-induced transitional cell carcinoma MBT-2 from C3H/HeN mice.[17] The cells were maintained continuously by *in vitro* culture.

***In vitro* culture of peritoneal exudate cells (PEC)** PEC were harvested from C3H/HeN mice that had been injected intraperitoneally with 2 ml of thioglycollate (Difco Laboratories Inc.) medium, or 1 ml of OK432 (1KE) 3 days previously. Cells were collected by washing out the peritoneal cavity with PBS. The cells were washed several times with PBS, then suspended in RPMI-1640-10% FCS medium. One hundred microliters of PEC (1×10^5 cells/well) suspension was cultured in 96-well flat-bottomed microtiter culture plates (Falcon #3072, Becton Dickinson Co., Franklin Lake, NJ). PEC were composed of more than 90% macrophages and less than 10% lymphocytes as evaluated by flow cytometric analysis. After 2 h incubation at 37°C in 5% CO_2 and 95% air, BCG (1×10^5 bacilli/well) and L-NMMA or mAb were added and the final volumes in wells were adjusted to 200 μl.

The culture supernatants were harvested after 36 h and the amount of IFN-γ was assayed by an enzyme-linked immunosorbent assay (ELISA) using anti-IFN-γ capture mAb and biotinylated detection mAb, streptavidin-conjugated alkaline phosphatase and *p*-nitrophenyl phosphate as a substrate (Zymed Laboratory Inc., San Francisco, CA). TNF was measured by L929 bioassay.[18]

NO_2^- concentration was determined by using the Griess reagent.[19] Assays were carried out at 36 h post-stimulation.

Depletion of T cells and NK cells from PEC The PEC (1×10^5 cells/well) were seeded in 96-well culture plates (Falcon #3072) for 1 h at 37°C in 5% CO_2 and 95% air. After that, anti-Thy-1 mAb and/or anti-NK mAb with complement were added to each well. Culture was continued for 2 h, then the medium was slowly removed and replaced with fresh RPMI-10% FCS medium.

Cytotoxicity assay

Experiment 1: One hundred microliters of PEC (1×10^5 cells/well) suspension was seeded in 96-well flat-bottomed microtiter culture plates. After 2 h culture, BCG (1×10^5 bacilli/well) and L-NMMA or mAb were added and the final volumes in the wells were adjusted to 200 μl. The plates were incubated at 37°C in 5% CO_2 and 95% air for 24 h. MBT-2 cells were radiolabeled with 100 μCi of [^{51}Cr]sodium chromate (NEN Life Science Co., Tokyo) for 12 h at 37°C in 5% CO_2 and 95% air. After incubation, the cells were washed 3 times with warm PBS, detached with 0.25% trypsin (Difco Laboratories Inc.) and suspended at a concentration of 1×10^5/ml in RPMI-10% FCS medium. Fifty microliters of target cells (5×10^3 cells/well) was added to 96-well flat-bottomed microtiter culture plates containing 200 μl of effector cells which had been incubated with several agents for 24 h.[20–22]

Experiment 2: Five hundred microliters of PEC (5×10^5 cells/well) was seeded in 24-well flat-bottomed microtiter culture plates (Falcon #3047, Becton Dickinson Co.). After 2 h incubation, BCG (5×10^5 bacilli/well) and L-NMMA or mAb were added and the final volumes in wells were adjusted to 1 ml. The plates were incubated at 37°C in 5% CO_2 and 95% air for 24 h with or without a cell culture insert (Falcon #3095, Becton Dickinson Co.). BCG was

added at the same number as PEC. Two hundred and fifty microliters of ^{51}Cr-labeled target cells (2.5×10^4 cells/well) was added to the wells or to the cell culture insert.

After a further 20 h incubation, the supernatants were harvested and released ^{51}Cr was counted with a γ counter. All assays were performed in triplicate. Spontaneous ^{51}Cr release was determined by incubating radiolabeled target cells in the absence of PEC. Maximal ^{51}Cr release was determined by incubating the same amount of target cells in 1% Triton X-100 (Sigma Chemical Co.). The percentage of specific cytotoxicity was calculated as follows:

$$\%\text{Cytotoxicity} = [(\text{Experimental release} - \text{Spontaneous release}) / (\text{Maximal release} - \text{Spontaneous release})] \times 100$$

Detection of BCG survival in macrophages Five hundred microliters of thioglycollate-elicited PEC (5×10^5 cells/well) was cultured in 24-well flat-bottomed culture plates. After 2 h incubation at 37°C, viable BCG (5×10^5 bacilli/well) and L-NMMA were added. Final volumes in wells were adjusted to 1 ml with RPMI-10% FCS medium and the plates were incubated at 37°C in 5% CO_2 and 95% air for 36 h. After incubation, the wells were washed several times with warm PBS to remove all reagents and extracellular bacteria. Adherent cells were solublized with 0.2% sodium dodecyl sulfate (SDS) (Sigma Chemical Co.) and then suspended in RPMI-10% FCS medium. The lysate was plated on Middlebrook 7H10 agar, and incubated at 37°C for 3 weeks, then colony-forming units (CFU) were counted.

Statistical analysis All determinations were made in triplicate and each result was expressed as a mean±standard deviation (SD). Statistical significance was determined by using the paired Student's *t* test. A *P*-value of 0.05 or less was considered significant.

RESULTS

NO production and cytotoxicity of PEC stimulated with viable or killed BCG Thioglycollate or OK432-elicited PEC were stimulated with viable or killed BCG *in vitro*. The culture supernatants were collected at 36 h and the amount of NO was determined. The preliminary experiment showed that NO was increased until 48 h after stimulation, then gradually decreased. PEC-mediated cytotoxicity was detected between 24 and 48 h after stimulation. The 36 h incubation time was the optimum time to investigate the relation between NO and cytotoxicity against target cells in our experimental system. As shown in Fig. 1, BCG enhanced both cytotoxic activity and NO production by OK432- and thioglycollate-elicited PEC. BCG-induced enhancement of cytotoxic activity was observed at 40:1 and 20:1 effector-to-target cell ratio. Since the 20:1 ratio gave the clearest results, only the results obtained at 20:1 ratio are presented here. OK432-elicited PEC showed a lower level of cytotoxicity and a higher level of NO production, while thioglycollate-elicited PEC showed a higher level of cytotoxicity and a lower level of NO production. There is no significant difference between the enhancements of cytotoxic activity by killed BCG and viable BCG. Killed or viable BCG alone without PEC had no direct cytotoxic effect on MBT-2 (less than 3%).

L-NMMA decreased NO production but increased cytotoxity of PEC It is well known that NO is an important effector molecule for the destruction of pathogens and cancer cells. To study whether NO alone is responsible for the BCG-mediated tumoricidal activity, the effect of L-NMMA on cytotoxic activity of PEC was examined. As shown in Fig. 2A and 2B, L-NMMA enhanced the cytotoxicity of PEC stimulated with viable BCG in a dose-

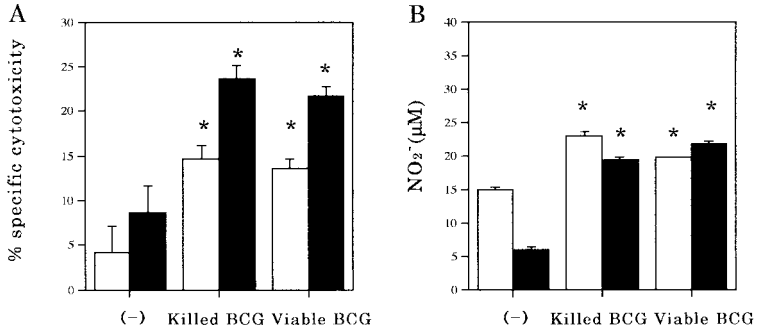

Fig. 1. Cytotoxicity and NO production of PEC stimulated with BCG. PEC elicited with thioglycollate medium (■) or OK432 (□) were cultured with viable or killed BCG for 24 h. Then, ^{51}Cr-labeled MBT-2 cells were added. After a further 20 h incubation, the released ^{51}Cr in the supernatant was counted and specific lysis of MBT-2 cells was calculated (A). The culture supernatants from PEC were harvested after 36 h and assayed for NO (B). * Significantly enhanced.

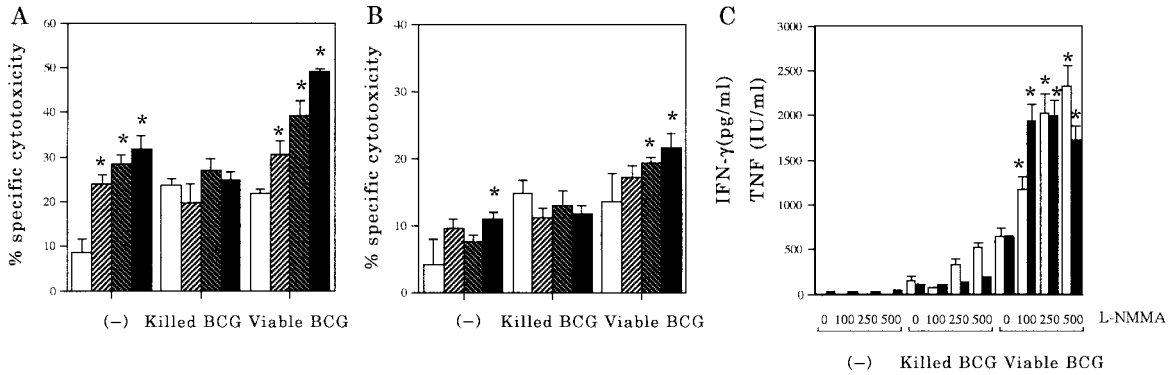

Fig. 2. Effect of L-NMMA on cytotoxicity and cytokine production of PEC stimulated with BCG. Thioglycollate- (A) and OK432- (B) elicited PEC were stimulated with BCG in the presence of various concentrations of L-NMMA for 24 h and their cytotoxicity against MBT-2 cells was determined. □ L-NMMA (0 *μM*), ▨ L-NMMA (100 *μM*), ▩ L-NMMA (250 *μM*), ■ L-NMMA (500 *μM*). The culture supernatants from thioglycollate-elicited PEC were harvested after 36 h and assayed for IFN-γ (□) and TNF (■) (C). ∗ Significantly enhanced.

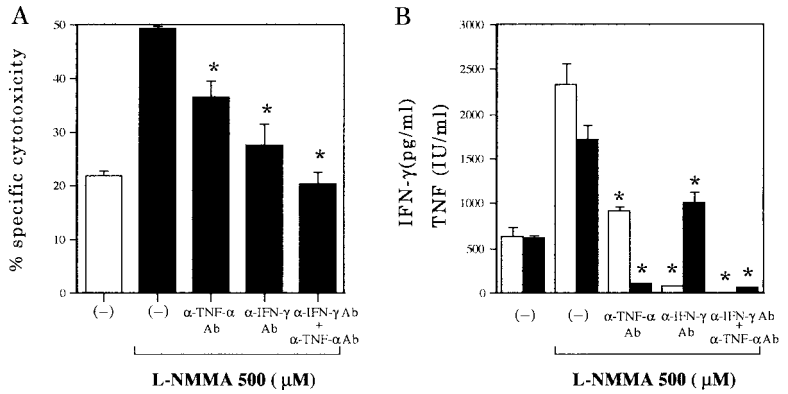

Fig. 3. Effect of anti-IFN-γ and/or anti-TNF-α Ab on cytotoxicity and cytokine production of PEC stimulated with BCG and L-NMMA. Thioglycollate-elicited PEC were stimulated with viable BCG plus L-NMMA (500 *μM*) in the presence or absence of anti-IFN-γ (5 *μ*g/ml) and/or anti-TNF-α (5 *μ*g/ml) Ab for 24 h and cytotoxicity against MBT-2 cells (A) and IFN-γ (□) and TNF (■) (B) in the culture supernatant were assayed. ∗ Significantly suppressed.

dependent manner. However, L-NMMA did not enhance killed BCG-induced cytotoxicity. In the case of thioglycollate-induced PEC, the cytotoxicity was increased about 30% and total cytotoxicity was up to 50% at the maximum concentration of L-NMMA. We also confirmed that L-NMMA alone without PEC had no direct cytotoxic effect on MBT-2 (less than 1%). As shown in Fig. 2C, L-NMMA markedly enhanced IFN-γ and TNF-α production by thioglycollate-elicited PEC stimulated with viable BCG in a dose-dependent manner, while L-NMMA only slightly enhanced IFN-γ and TNF-α production by killed BCG-stimulated PEC. IL-2 was not detected and neither IL-10 nor IL-12 was enhanced by L-NMMA (data not shown). L-NMMA decreased killed and viable BCG-induced NO production in a dose-dependent manner and the maximum concentration of L-NMMA (500 *μM*) reduced NO production by 90% (Fig. 6). L-NMMA alone without BCG also enhanced the cytotoxicity of PEC in a dose-dependent manner, but did not affect IFN-γ and TNF-α production. The stereoisomer of L-NMMA, monomethyl-D-arginine (D-NMMA) had no effect or cytotoxic activity or cytokine production by PEC (data not shown).

Anti-IFN-γ and/or TNF-α antibody reduced cytotoxicity of PEC stimulated with BCG plus L-NMMA To determine more directly the participation of IFN-γ and TNF-α in the L-NMMA-induced enhancement of cytotoxic activity, we added anti-IFN-γ and/or anti-TNF-α Ab to the culture with BCG plus L-NMMA. As shown in Fig. 3A and 3B, anti-IFN-γ or anti-TNF-α Ab significantly inhibited the cytotoxicity of PEC against MBT-2. The

combination of both antibodies completely inhibited the cytotoxicity to the control level. Both IFN-γ and TNF-α in the culture supernatant were also diminished by these two antibodies. These results indicate that the enhanced cytotoxicity of PEC induced with L-NMMA is mediated by IFN-γ and TNF-α.

Enhanced cytotoxicity of PEC stimulated with BCG plus L-NMMA was mediated by both soluble factors and cell-to-cell contact To differentiate the role of soluble factors and cell-to-cell contact in the L-NMMA-induced cytotoxicity, we used a cell culture insert to separate target cells from effector cells and assayed the cytotoxicity. As shown in Fig. 4A, the cytotoxicity was again enhanced by BCG and L-NMMA. In the presence of a cell culture insert, the cytotoxic activity was diminished by 50%. However, the cytotoxic activity induced with viable BCG was still significantly higher than the control and L-NMMA enhanced the cytotoxicity to almost the same level as that in the absence of a cell culture insert. As shown in Fig. 4B, BCG plus L-NMMA-induced cytotoxicity in the presence of a cell culture insert was completely inhibited by the presence of both anti-IFN-γ and anti-TNF-α Ab. These results suggest that BCG plus L-NMMA-induced cytotoxicity is mediated by both direct cell-to-cell contact and soluble factors such as IFN-γ and TNF-α.

Depletion of T cells and/or NK cells from PEC slightly decreased BCG-induced cytotoxicity in the presence or absence of L-NMMA It is believed that the cytotoxic

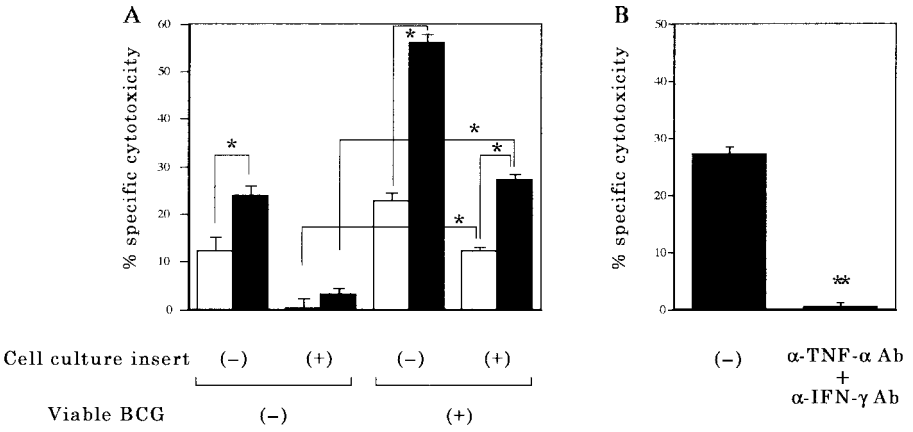

Fig. 4. Cytotoxicity of PEC stimulated with BCG plus L-NMMA in the presence of cell culture insert. Thioglycollate-elicited PEC were cultured with viable BCG in the presence (■) or absence (□) of L-NMMA (500 *μM*) for 24 h in 24-well plates. ^{51}Cr-labeled MBT-2 cells were seeded on the effector cells directly or indirectly by using a cell culture insert (A). PEC were cultured with BCG plus L-NMMA in a cell culture insert in the absence or presence of anti-IFN-γ (5 μg/ml) and anti-TNF-α (5 μg/ml) Ab for 24 h (B). After a further 20 h incubation, released ^{51}Cr in the supernatant was counted. * Significantly enhanced. ** Significantly suppressed.

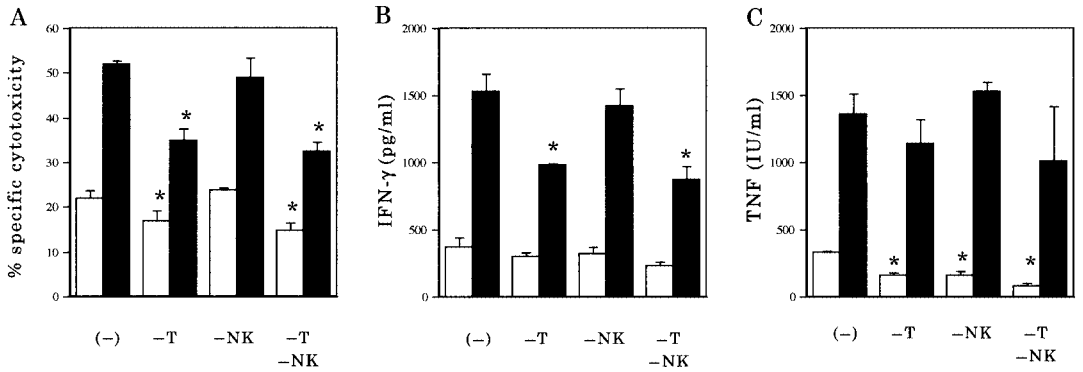

Fig. 5. Effect of T cell and NK cell depletion on cytotoxicity and cytokine production of PEC stimulated with BCG and L-NMMA. Thioglycollate-elicited PEC were treated with anti-Thy-1 Ab or anti-NK Ab with complement for 2 h, then stimulated with viable BCG in the presence (■) or absence (□) of L-NMMA (500 *μM*) for 24 h and their cytotoxic activity against ^{51}Cr-MBT-2 cells was assayed after 20 h. The amounts of IFN-γ (B) and TNF-α (C) in the culture supernatant were also assayed. * Significantly decreased.

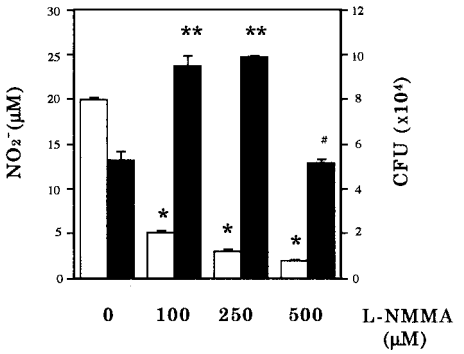

Fig. 6. Effect of L-NMMA on NO production and BCG survival in PEC. Thioglycollate-elicited PEC were cultured with viable BCG and various concentrations of L-NMMA. The supernatants were harvested after 36 h and assayed for NO (□). The wells were washed, adherent cells were solublized and the lysate was plated on Middlebrook 7H10 agar. It was cultured for 3 weeks and CFU (■) was counted. * Significantly decreased. ** Significantly increased. # Significantly decreased compared to L-NMMA (250 *μM*).

activity of macrophages is regulated by T cells and NK cells and their soluble products, such as IFN-γ. To study the participation of T cells and NK cells in BCG and L-NMMA-induced cytotoxicity of PEC, we depleted these cells by Ab plus complement treatment. As shown in Fig. 5A, the depletion of T cells decreased the cytotoxicity about 23% (without L-NMMA) and 33% (with L-NMMA), but the depletion of NK cells had no effect on the cytotoxicity in the absence or presence of L-NMMA. The amount of IFN-γ (Fig. 5B) and TNF-α (Fig. 5C) was also decreased by T cell depletion, but not by NK cell depletion. However, the reduction of TNF-α by T cell depletion was not statistically significant.

Effect of L-NMMA on BCG survival in macrophages Finally, we studied the survival of BCG in macrophages treated with various concentrations of L-NMMA. As shown in Fig. 6, NO production was decreased by L-NMMA in a dose-dependent manner and 500 *μM* L-NMMA reduced it by almost 90%. In contrast, CFU of BCG was increased up to 250 *μM* L-NMMA. However, at 500 *μM* L-NMMA, CFU remained at the level seen without L-NMMA. L-NMMA (500 *μM*) is not toxic to PEC.

DISCUSSION

NO is predominantly produced by macrophages and acts as a potent effector molecule in nonspecific immune defense against infectious agents and cancer cells.[12, 13] It also functions as a regulator for many biological activities including Th1/Th2 balance.[14–16] In this study, we investigated the effect of an NO production inhibitor, L-NMMA, on the cytotoxic activity and cytokine production of PEC stimulated with viable or killed BCG *in vitro*.

Several investigators have examined the biological difference between killed BCG and viable BCG. One reported that there was no difference between the cytotoxicity induced with killed and viable BCG *in vitro*.[23] In contrast, viable BCG was found to be superior to killed BCG *in vivo* and in a clinical study.[24] However, the precise mechanism involved is unclear. One reason why viable BCG is superior to killed BCG is that viable BCG continuously stimulates macrophages by secreting various kinds of immunopotentiating substances such as 32 kDa protein, so-called α antigen,[25] which strongly modifies the host immune response.[26] In short-term culture, as in *in vitro* study, BCG can not grow rapidly and can not secrete large amounts of various proteins. To investigate the difference between viable and killed BCG in an *in vitro* system, we first used mid-log-phase growing BCG. Secondly, we added L-NMMA, an inhibitor of NO and a strong scavenger of intracellular pathogens such as BCG.

L-NMMA significantly enhanced cytotoxic activity and both IFN-γ and TNF-α production by PEC stimulated with viable BCG, but not with killed BCG (Fig. 2). One of the mechanisms of enhancement of IFN-γ and TNF-α production by L-NMMA is a depletion of inhibitory factors such as prostaglandins, which are induced by NO. Another mechanism is the prolongation of survival of BCG in macrophages, causing continuous stimulation of macrophages and T cells. Both anti-TNF-α and anti-IFN-γ Ab reduced BCG plus L-NMMA-induced cytotoxicity. This suggests that both IFN-γ and TNF-α are essential to kill tumor cells. Furthermore, both IFN-γ and TNF-α seem to have regulatory functions on IFN-γ and TNF-α production, because each antibody suppresses not only the corresponding cytokine production, but also the other cytokine production.

IFN-γ is an important molecule to activate macrophages and kill intracellular bacteria. It was reported that *Mycobacterium* antigen-specific $CD4^+$ T cells produce large amounts of IFN-γ and they are thought to activate macrophages to control intracellular microbes in human and mouse.[27] The depletion of $CD4^+$ T cells resulted in disseminated infection of *Mycobacterium* complex in mouse, while competent mouse had restricted infection.[28] On the other hand, $CD8^+$ T cells were also activated in a BCG infection model *in vitro*[29] and they are essential components for protective immunity against *Mycobacterium tuberculosis* in human.[30] Another report claimed that NK cell-derived IFN-γ was essential to stimulate macrophages to induce NO production in spleen cells stimulated with BCG.[31]

In this report we also studied the participation of T cells and NK cells in BCG plus L-NMMA-induced cytotoxicity

and cytokine production and found that T cells, but not NK cells, regulate the cytotoxicity and cytokine production (Fig. 5). However, T cell-mediated regulation accounts for only 30% of total PEC-mediated cytotoxicity and cytokine production. These results suggest that non T cells and non NK cells, mainly macrophages, are the major source of cytotoxicity and cytokine production reported by some investigators,[32, 33] and the macrophage activity seems to be regulated by BCG and L-NMMA. We did not separate $CD4^+CD8^-$ and $CD4^-CD8^+$ cell populations, because T cells consisted of only 5% PEC.

The survival of BCG in macrophages increased with increasing concentration of L-NMMA (Fig. 6). However, the survival of BCG was not increased at the maximum concentration of L-NMMA (500 μM). This may reflect excessive activation of macrophages by BCG; IFN-γ and TNF-α, in the absence of NO, cause not only an increase of cytotoxicity against cancer cells, but also augmentation of macrophage death, which results in the scavenging of intracellular BCG.[34–36] In fact, the inhibition of NO is reported to induce cell apoptosis.[37, 38]

It is well known that BCG-induced macrophage cytotoxicity is mediated by at least two mechanisms: cell-to-cell contact killing and secretion of effector lytic substances.[39] The study using cell culture inserts suggests that L-NMMA enhances both mechanisms. Another interesting finding in this study is that L-NMMA alone without BCG also enhances cytotoxic activity (Fig. 2), and this activity is reduced by a cell culture insert (Fig. 4), suggesting that this enhanced cytotoxicity is mediated by direct cell-to-cell contact, although the presence of high concentrations of soluble factors at the site of cell-to-cell contact can not be excluded. The mechanism of enhancement of cytotoxicity by L-NMMA without BCG is under investigation.

Various effector mechanisms are considered to be involved in cancer cell killing by macrophages. NO is thought to play a major role.[40] In this study we did not examine the role of NO in the cytotoxic activity against cancer cells. In our preliminary study, anti-TNF-α and anti-IFN-γ Ab reduced viable BCG-induced cytotoxicity about 49% and also decreased NO production about 50%. The decrease of NO may be due to the decrease of IFN-γ, which is a strong inducer of NO from macrophages.

In this report we did not study the *in vivo* activity of BCG and L-NMMA. In a preliminary experiment, we found that BCG activated macrophages upon *in vivo* administration (data not shown). Recently, Medot-Pirenne *et al*.[41] and Koblish *et al*.[42] reported that NO inhibitor suppressed suppressor macrophages and augmented antitumor activity upon *in vivo* administration. Thus, we consider that BCG and L-NMMA should also work *in vivo*.

In conclusion, inhibition of NO results in long-lasting survival of BCG in macrophages and this BCG can continuously activate T cells and macrophages to produce IFN-γ and TNF-α. The increased amounts of IFN-γ and TNF-α showed enhanced cytotoxic activity against cancer cells. It is established that BCG used for vaccine induces not only specific immunity, but also nonspecific inflammation in the host, which augments inflammatory cell functions to scavenge target cells as a bystander effect. The combination of small amounts of BCG and NO inhibitor seems promising as a therapy for superficial bladder cancer. Furthermore, this study has shown for the first time that viable BCG can induce cytotoxicity superior to that of killed BCG *in vitro* by activating macrophages.

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