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Cytokine-mediated antitumor effect of bacillus Calmette-Guérin on tumor cells in vitro

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Abstract. Intravesical instillation therapy of bacillus Calmette-Guérin (BCG) is a useful modality for recurrent superficial transitional-cell carcinoma (TCC) of the urinary bladder. The mechanism of BCG effect has not yet been well characterized. BCG was tested in vitro for cytokine-mediated antiproliferative activity against T24 and KK47 cells (cell lines established from human TCC of the urinary bladder), and ACHN cells (cell line established from human renal cell carcinoma) using a modified human tumor clonogenic assay. Continuous exposure of cells to BCG at concentrations of more than 5 μg/ml in the presence of peripheral blood mononuclear cells (PBMC) consisting of a mixture of 5×10^4 monocytes/dish and 5×10^5 lymphocytes/dish, obtained from healthy donors, significantly inhibited colony formation of T24 and ACHN cells in comparison with growth inhibition in the absence of PBMC ($P < 0.05$). Slightly inhibited colony formation was observed with KK47 cells under the same conditions. At the same time various cytokines were measured in supernatants when BCG and the same conditioned PBMC were co-cultured. Tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β) were detected at markedly high levels at 24 h, and interferon γ (IFNγ) was detected at 120 h. IL-2 and macrophage-colony-stimulating factor were not detected. Neutralizing anti-TNFα monoclonal antibody significantly reduced the anti-proliferative activity of ACHN cells, and anti-IFNγ antibody reduced that of T24 cells. The results obtained suggest that cytokines mediated by BCG play an important role in the antitumor activity of BCG and that the sensitivity of bladder cancer cells to the cytokines induced by BCG may differ considerably.

Key words: BCG – Cytokine – Human tumor clonogenic assay

Introduction

Since the initial report by Morales et al. [13], many reports have described the beneficial effect of intravesical instillation therapy using bacillus Calmette-Guérin (BCG) on refractory superficial transitional-cell carcinoma of the urinary bladder [9, 10, 15]. The mechanism of this therapy, however, has not yet been well characterized. Two main theories have been proposed to explain the mechanism of action of BCG. One is a direct action [17], and the another is an immunological reaction [2, 6]. Immunohistochemical and flow-cytofluorometric studies have shown that T lymphocytes, macrophages and polymorphonuclear leukocytes are the main cells infiltrating the tumor tissue of the urinary bladder after intravesical BCG treatment [4, 11]. Using a murine adoptive-transfer system, Ratliff et al. reported that the T cell seemed to be essential for this therapy [18]. In addition to these hypotheses, the role of cytokines that may be produced in response to BCG has been evaluated [3, 5].

In this study, we investigated whether cytokine-mediated antiproliferative activity can be induced by BCG and what kinds of cytokines are induced in vitro using a modified human tumor clonogenic assay.

Materials and methods

Preparation of target cells. Three cell lines were used in this study as target cells. They were derived from two human transitional cell carcinomas, KK47, T24, and one human renal-cell carcinoma, ACHN. These cell lines were maintained as monolayer cultures in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, and 100 μg/ml streptomycin. The plating density of each cell line was between 5×10^2 and 5×10^4 cells/dish in order to achieve at least 200 tumor colonies/dish.

Source of BCG. The BCG used in this study was Tokyo 172 strain, $(0.5-1.2) \times 10^8$ colony-forming units/mg; Japan BCG Co. Ltd., Tokyo, which was suspended in RPMI-1640 medium supplemented with 10% FCS at concentrations ranging from 20 μg/ml to 2000 μg/ml. The final concentration of BCG ranged from 5 μg to 500 μg/ml.

Preparation of peripheral blood mononuclear cells (PBMC). Samples of 20 ml heparinized human peripheral blood were obtained from healthy donors. Blood samples were layered on 20 ml lymphocyte separation medium (LSM, Litton Bionetics, Kensington, Md., USA)

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and centrifuged at 500 *g* for 30 min at room temperature. The mononuclear cell layer was collected by pipette. After washing with phosphate-buffered saline (PBS) without calcium and magnesium (DPBS, Whittaker, Walerville), the cells were resuspended and decanted into a 75 cm^2 polystyrene tissue-culture flask (Corning 25110, Corning, N.Y.). After 1 h of incubation at 37 °C with 5% CO_2 to allow monocyte attachment to the flask, non-adherent cells were separated and the flask was washed twice with PBS. The adherent cells were removed from the flask by a cell scraper (Falcon 3086, Becton Dickinson), and suspended at a density of 2×10^5 cells/ml in RPMI-1640 medium. More than 90% of this cell population consisted of monocytes as determined by morphological examination using Giemsa-stained preparations. Non-adherent cells (lymphocytes) were also suspended at a density of 2×10^6 cells/ml in RPMI-1640 medium. Mixtures of these monocytes and lymphocytes in RPMI-1640 medium were plated on the upper layer as the liquid layer at a final density of 5×10^4 monocytes/dish and 5×10^5 lymphocytes/dish.

Anti-(tumor necrosis factor α, TNFα) monoclonal antibody. The neutralizing titer of anti-TNFα monoclonal antibody (Genzyme Corp. Cambridge) was more than 1×10^3 units/μl (5×10^4 pg/μl).

Anti-(interferon γ, IFNγ) monoclonal antibody. The neutralizing titer of anti-IFNγ monoclonal antibody (Genzyme Corp. Cambridge) was more than 1×10^3 units/μl (5×10^4 pg/μl).

Modified human tumor clonogenic assay. The method originally described by Hamburger and Salmon [8] was used in this study with slight modification. Briefly, the lower layer was prepared in 35-mm Petri dishes (Falcon 3801, Becton Dickinson, Sunnyvale, Calif.) using RPMI-1640 culture medium (Flow Laboratories, McLean, Va.) supplemented with 25% heat-inactivated fetal calf serum (FCS, lot no. 0130103, Flow Laboratories), 100 units/ml penicillin, and 100 μg/ml streptomycin (Gibco, Grand Island, N.Y.) in 0.6% agarose. Target cells were suspended in the upper layer with 0.3% agarose prepared with the same medium as the lower layer. Agarose double-layer cultures of tumor cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 2 days. After this short incubation, PBMC obtained from healthy donors and BCG suspended in RPMI-1640 medium were seeded on the upper layer of each dish as a liquid layer, and then incubated under the same conditions for 7–21 days. In order to distinguish non-colony cell aggregates, three plates were fixed with 10% formaldehyde before cultivation as positive controls. Each experiment was conducted in triplicate and six plates were prepared as controls. At the end of incubation, colonies were counted under an inverted phase-contrast microscope (Nikon, Tokyo). Colonies were defined as cell aggregates at least 60 μm in diameter and numbering 30 cells. The true number of colonies was calculated as the number of aggregates on the positive control subtracted from the number of colonies on the experimental plate. Results were expressed as a percentage of the control value.

To examine the influence of BCG and PBMC on cytotoxicity in this assay we compared cytokine activities under various conditions. When BCG and PBMC come into contact in this experimental system, cytokines should be produced from PBMC. First, as a liquid layer, BCG suspended in RPMI-1640 medium supplemented with 10% FCS at a concentration of 500 μg/ml was placed on the upper layer of 0.3% agar containing PBMC under the same conditions described above. Second, as a liquid layer, PBMC suspended in RPMI-1640 medium supplemented with 10% FCS at a concentration of 5×10^5 lymphocytes/ml and 5×10^4 monocytes/ml was placed on the upper layer of 0.3% agar containing BCG. In each culture system, various cytokines in the supernatants were measured after incubation for 24 h.

Measurement of cytokines in the supernatant. In order to measure the various cytokines such as interleukin-1α, β (IL-1α, β), interleukin-2 (IL-2), interleukin-6 (IL-6), TNFα, IFNγ and macrophage-colony-stimulating factor (M-CSF) in the supernatants, 1 ml BCG solution, 1 ml adherent cells, 1 ml non-adherent cells and 1 ml RPMI-1640 medium were mixed and incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 16, 24, 48, 72, 120 h. The final concentration of the supernatants was 5, 50, 500 μg/ml in BCG, 5×10^5 cells/ml in non-adherent cells (lymphocytes) and 5×10^4 cells/ml in adherent cells (monocytes). Supernatants were harvested at each indicated time after incubation and stored at –20 °C until immediately before measurement of cytokine titers.

Measurement of each cytokine was performed by a sandwich enzyme immunoassay using solid-phase reaction methods and coated monoclonal antibodies to each cytokine [22]. The minimum measurable level of each cytokine was 20 pg/ml for IL-1α, β, IL-6, TNFα, and IFNγ, 50 pg/ml for IL-2, and 200 pg/ml for M-CSF. All measurements of cytokines were performed by one of the co-authors (Y. Ohmoto).

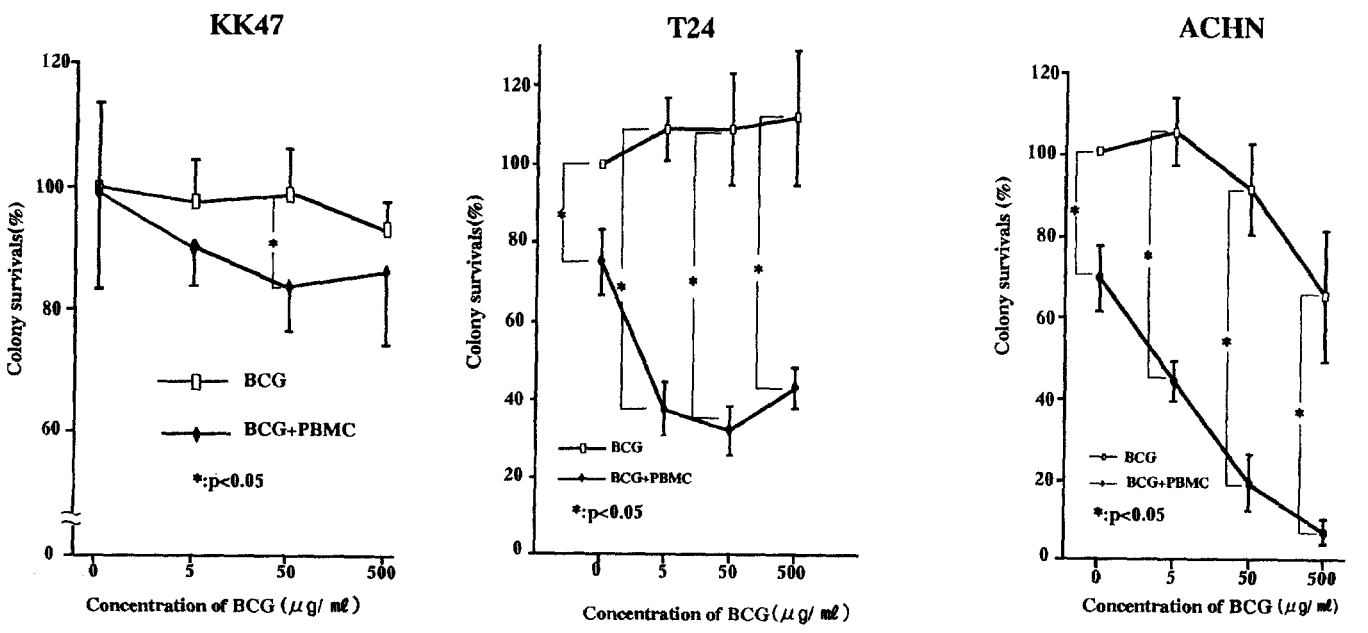

Fig. 1. Effects of bacillus Calmette-Guérin (*BCG*) on survival of KK47, T24, and ACHN cell lines with or without peripheral blood mononuclear cells (*PBMC*). All data represent means ± SD of three separate experiments done in triplicate. * Significant inhibition ($P < 0.05$) of colony growth rate compared to that without PBMC

Statistical analysis. The data obtained were subjected to statistical analysis using the two-sample Wilcoxon test. A P value of less than 0.05 was regarded as statistically significant.

Results

Effects of BCG on survival of tumor cells with or without PBMC

The influence of BCG and/or PBMC on the proliferative activity of tumor cells was investigated. Colony growth of the three cell lines was not significantly inhibited by BCG alone at each indicated dose (Fig. 1). PBMC alone inhibited the proliferative activity of T24 and ACHN cells, but not that of KK47 cells. When a mixture of PBMC and BCG was seeded on the upper layer, colony growth of ACHN and T24 cells was reduced significantly at concentrations ranging from 5 μg/ml to 500 μg/ml BCG compared with those observed with BCG alone ($P < 0.05$). In contrast, although PBMC were seeded with BCG on the upper layer, BCG exposure at concentrations ranging from 5 μg/ml to 500 μg/ml did not significantly reduce the colony growth of KK47 cells, with the exception of inhibition at a concentration of 50 μg/ml (Fig. 1).

Time course of cytokine production by PBMC after contact with BCG

Seven cytokines (IL-1α, IL-1β, IL-2, IL-6, TNFα, IFNγ, M-CSF) in the supernatant were measured with or without BCG 24 h after initiation of cultivation. TNFα, TNFγ, IL-1β, IL-6 were significantly elevated in the supernatants in the presence of PBMC and BCG compared with those in the presence of PBMC alone (Table 1). No remarkable elevations of IL-1α, IL-2 or M-CSF were observed with or without BCG.

The kinetics of cytokine values in the supernatants was measured with the exposure period of PBMC to BCG ranging from 16 h to 120 h (Fig. 2). Markedly high levels of TNFα and IFNγ were observed at all experimental periods. Although a high level of IL-1β was observed, it was lower than the levels of TNFα and IFNγ. The maximum values were found at 24 h for IL-1β and TNFα, and 120 h for IFNγ.

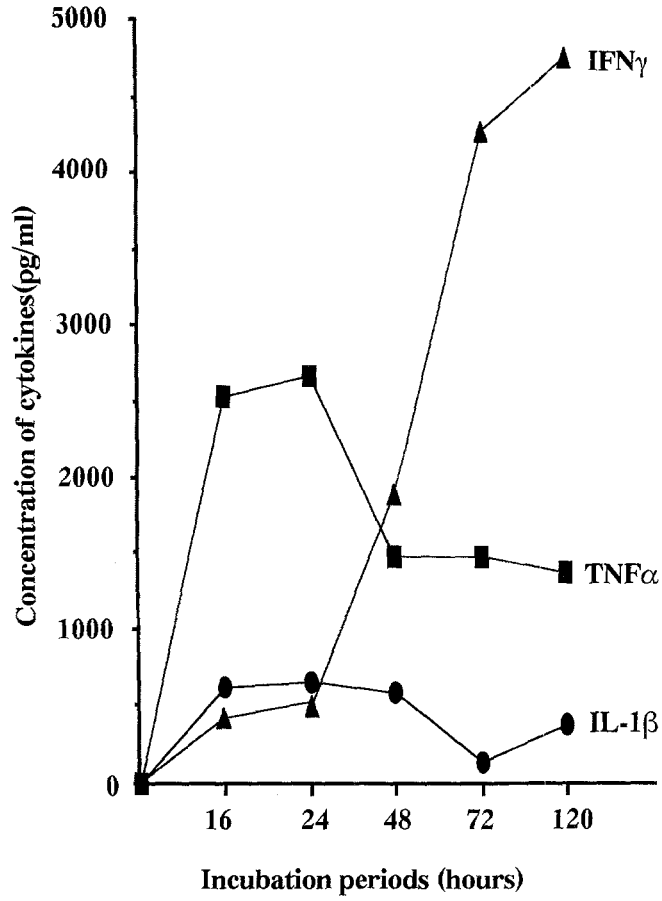

Fig. 2. Time course of cytokine production by PBMC after contact with BCG. BCG was applied at a concentration of 500 μg/ml, and PBMC were applied at a density of 5×10^4 monocytes/dish and 5×10^5 lymphocytes/dish

Movement of BCG and PBMC into the upper layer of 0.3% soft agar

The production of three cytokines (TNFα, IFNγ, IL-1β) by PBMC consisting of monocytes and lymphocytes in the supernatant were measured with or without BCG under various cultivation conditions (Table 2). When BCG and PBMC were seeded as a liquid layer on the upper layer consisting of 0.3% agar alone, the three cytokines were detected at high levels. In contrast, when BCG alone was

Table 1. Effect of bacillus Calmette-Guérin (BCG) on the production of various cytokines

Cytokines observed	PBMC[a]	PBMC+BCG (5 μg/ml)	PBMC+BCG (50 μg/ml)	PBMC+BCG (500 μg/ml)
TNFα	50±29	751±212*	1106±520*	2030±1289**
IFNγ	<20	58±25*	128±52*	422±318**
IL-1β	56±34	961±307*	1205±304*	1191±488**
IL-6	59±25	1849±83*	9017±169*	9661±355**

BCG and peripheral blood mononuclear cells (PBMC) were co-cultured for 24 h. All data represent means ± SD ($n = 5$). TNFα, tumor necrosis factor α; IFNγ, interferon γ; IL, interleukin

* Significant difference ($P < 0.05$) compared to PBMC alone

** Significant difference ($P < 0.01$) compared to PBMC alone

[a] PBMC were applied at a density of 5×10^4 monocytes/dish and 5×10^5 lymphocytes/dish

Table 2. Cytokine production under various cultivation conditions

Cultivation conditions		Cytokines in supernatant (pg/ml)[a]		
Liquid layer	Upper layer	TNFα	IFNγ	IL-1β
BCG[b] + PBMC[c]	–	2329±168	1470±145	1103±12
BCG + PBMC	0.3% agar alone	1731±400	442±11	462±88
PBMC alone	0.3% agar alone	68±400	<20	69±5
BCG alone	PBMC in 0.3% agar	137±35	39±4	75±15
PBMC alone	BCG in 0.3% agar	523±65	41±2	688±68

[a] Each cytokine was measured in supernatants after 24 h incubation

[b] BCG was applied at a concentration of 500 μg/ml

[c] PBMC were applied at a density of 5×10^4 monocytes/dish and 5×10^5 lymphocytes/dish

seeded in this way on the upper layer consisting of 0.3% agar and PBMC, the three cytokines were detected at low levels. When PBMC alone was seeded as a liquid layer on the upper layer consisting of 0.3% agar and BCG, these cytokines except for IFNγ were detected at moderate levels.

Effect of monoclonal antibodies of cytokines on antiproliferative activity of BCG with PBMC

To examine which cytokine reduced the colony growth of ACHN and T24 cells, anti-TNFα monoclonal antibody or anti-IFNγ monoclonal antibody was added to the liquid layer (Fig. 3). In ACHN cells, although approximately 70% of the antiproliferative activity of BCG with PBMC was neutralized by adding anti-TNFα monoclonal antibody, about 25% of that of BCG with PBMC was neutralized by adding anti-IFNγ monoclonal antibody. In contrast, in T24 cells, approximately 95% of the antiproliferative activity of BCG with PBMC was neutralized by adding anti-IFNγ monoclonal antibody. Adding anti-TNFα monoclonal antibody to the liquid layr did not neutralize the antiproliferative activity of BCG with PBMC in T24 cells.

Discussion

Although the mechanism of antitumor activity of BCG for transitional-cell carcinoma of the urinary bladder has not yet been elucidated, it is obvious that an immunological response is intimately involved [2, 6]. Recent reports state that various cytokines such as IL-1, IL-2, IL-6, TNFα and IFNγ have been detected in the urine after intravesical BCG immunotherapy [3, 5, 16]. These results suggested that cytokines were produced by monocytes and lymphocytes which were induced by BCG in the bladder wall. TNFα was detected in supernatant when BCG and monocytes were co-cultured in vitro, but there have been few reports concerning the roles of cytokines in instillation therapy of BCG for transitional-cell carcinoma of the urinary bladder. We investigated what kinds of cytokines are induced in vitro when BCG and PBMC are co-cultured, and evaluated cytokine-mediated antitumor effects on urological tumor cell lines using a modified human clonogenic assay (HTCA). HTCA is easily modified to evaluate cytokine-mediated antitumor effects because the target cells in the upper layer and effecter cells, such as monocytes or lymphocytes, in the feeder layer are separated by semi-solid agarose. We have recently reported the cytokine-mediated antitumor effect of interferon α using a modification of HTCA [12]. First, in the present study, we evaluated the cytokine-mediated antitumor effect of BCG using a method reported elsewhere [12]: BCG and PBMC were seeded in 0.6% agar as the lower layer and target cells in 0.3% agar as the upper layer. Also, we replaced the upper layer to the culture medium only and measured various cytokine activities in the medium. In these systems, however, colony survival of target cells was only slightly inhibited, and no cytokines were detected in the medium (unpublished data). From the results obtained, we considered that BCG could not move in 0.6% agar, and could not come into contact with PBMC. Cytokines, therefore, were not produced from PBMC, and the colony formation of target cells was only slightly inhibited. On the other hand, when BCG and PBMC were co-cultured in the culture medium, high concentrations of TNFα, IFNγ, IL1-β and IL-6 were detected after 16–120 h culture (Table 1 and Fig. 2).

Second, we established a new modified HTCA consisting of three layers. We seeded 0.6% agar as the lower layer, target cells in 0.3% agar as the upper layer, and seeded BCG and PBMC in the culture medium on the upper layer as a liquid layer after the agar became gel. With this assay system, the colony growth of ACHN and T24 cells was reduced significantly compared with that observed in BCG alone in the liquid layer, whereas the colony growth of KK47 cells was only slightly reduced. Inhibition of the growth of tumor cells by production of cytokines from PBMC was observed even when BCG and the tumor cells were not in contact. To confirm that BCG really did not come into contact with tumor cells we examined the movement of BCG into the soft agar of the upper layer by observation of cytokine activities in the supernatants (Table 2). These results showed that BCG moved only slightly in 0.3% agar, suggesting that a direct cytotoxic effect of BCG on tumor cells was negligible in this assay. It is, however, impossible to distinguish the cytotoxic effects of cytokines induced from PBMC by stimulation of BCG from the cytotoxicity of another mechanism, because PBMC were considered to be in contact with tumor cells in our system (Table 2). We must consider the cytotoxic ef-

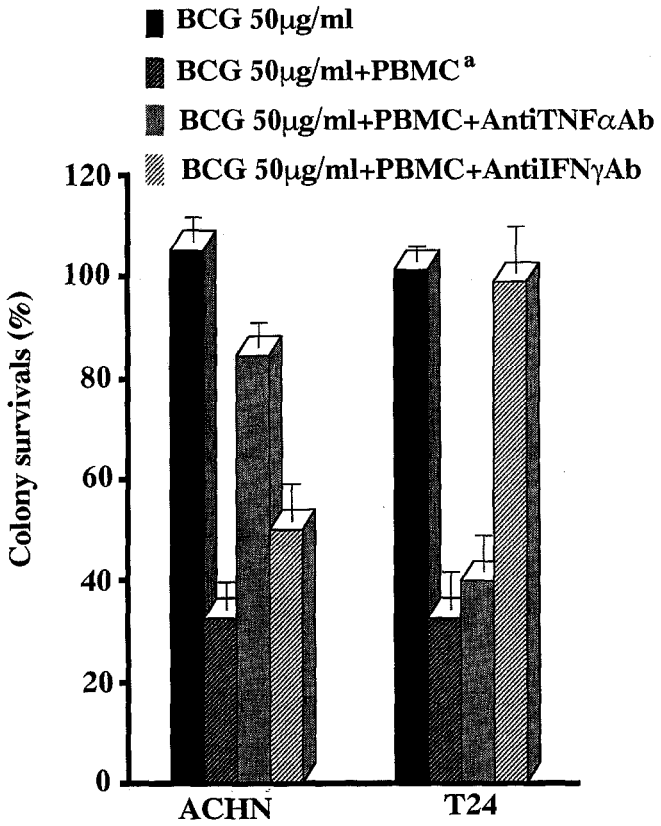

Fig. 3. Effect of anti-(tumor necrosis factor α) (*AntiTNFαAb*) monoclonal antibody and anti-(interferon γ) (*AntiIFNγAb*) monoclonal antibody on antiproliferative activity of BCG. All data represent means ± SD in triplicate. [a] PBMC were seeded at a density of 5×10^4 monocytes/dish and 5×10^5 lymphocytes/dish

fects mediated by cell-to-cell contact, such as phagocytosis by activated macrophages, specific cytotoxity by cytotoxic T lymphocytes or cytotoxity by natural killer (NK) cells. But approximately 70% of the antiproliferative activity of BCG with PBMC was neutralized by neutralizing anti-TNFα monoclonal antibody in ACHN cells, and 95% was neutralized by neutralizing anti-IFNγ monoclonal antibody in T24 cells (Fig. 3). We therefore considered that a large part of the antiproliferative activity observed in this study is due to a direct action of TNFα and IFNγ mediated by BCG. The three cell lines studied had different sensitivities to cytokines induced from PBMC by stimulation of BCG: T24 cells were sensitive to IFNγ and ACHN cells were sensitive to TNFα, while KK47 cells were resistant to both cytokines. These results are compatible with the reported sensitivities of these cell lines in vivo or in vitro [1, 12, 21].

One of the reasons why so many cases have been resistant to intravesical immunotherapy with BCG might be that individual cancers have different sensitivities to cytokines such as TNFα or IFNγ.

Concerning IL-1 and IL-6, a direct effect of both cytokines on the cell lines studied is not obvious because a blocking assay for those cytokines was not done in this study. But IL-1 and IL-6 have been reported to be immunomodulators rather than direct cytotoxic cytokines for tumor cells. IL-1 exhibits several mechanisms by which it may exert an inhibitory effect on tumor growth, for example by induction of IL-2 [20], and by activation of macrophages [14], and cytotoxic T cells [7]. It was reported that urinary IL-6 was a useful marker for monitoring the inflammatory response after BCG instillation [19].

As far as the direct action of cytokines induced is concerned, the clinical efficacy of intravesical immunotherapy with BCG will depend on two factors. One is the activity of various cytokines mediated by BCG, and the another is the sensitivity of individual tumors to the cytokines induced.

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