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Original Article

In vitro Production of Tumor Necrosis Factor (TNFα/Cachectin) from Human Peripheral Blood Monocytes Stimulated with Bacillus Calmette-Guerin. —A Possible Mechanism of BCG Therapy

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Abstract: Healthy human peripheral mononuclear cells cultured with bacillus Calmette-Guerin (BCG) alone produced cytotoxic factor that lysed L. P3 murine fibroblast cells after 2 hours of culture, and the production reached a peak after 24 to 48 hours. Since L. P3 cells are sensitive both to tumor necrosis factor (TNFα) and lymphotoxin (TNFβ), specific antibodies were used to identify this cytotoxic factor as TNFα, not lymphotoxin. This production of TNFα was shown to be derived from monocytes, because it disappeared after monocytes were depleted from mononuclear cells. Tumor necrosis factor induced with BCG may be a factor in the anti-tumor effect of topical therapy with BCG.

Key words: tumor necrosis factor, Bacillus Calmette-Guerin, human monocytes

INTRODUCTION

Many reports have indicated the beneficial effect of intravesical instillation of bacillus Calmette-Guerin (BCG) on superficial bladder cancer[1-5], though the mechanism has yet to be clarified. Some studies report that certain cytokines induced by BCG exhibit anti-tumor effects[6,7]. Tumor necrosis factor (TFα/Cachectin) is a cytolytic peptides, which was originally found in the sera of mice injected with BCG as a priming agent and endotoxin as an eliciting agent[8]. Recently, it has been demonstrated that human peripheral blood mononuclear cells (PBMC) produce TNFα upon stimulation by other cytokines or mitogens[9-11]. In this study, we demonstrate that human peripheral monocytes stimulated by BCG alone can produce TNFα.

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MATERIALS AND METHODS

Cell lines

Murine L. P3 fibroblast cells, sensitive to both TNF and Lymphotoxin (LT), were a kind gift from Dr. Miyagawa, Department of Microbiology, Yamanashi Medical College, Yamanashi, Japan. Human B lymphoblastoid cell line RPMI 1788, producer of LT, was supplied from the Japanese Cell Resource Bank (JCRB), Tokyo, Japan.

Standard cytotoxins

Human recombinant TNFα, PT-050, with a specific activity of 12.0×10^7 U/mg, was obtained from Dainippon Pharmaceutical Co., Osaka, Japan. Culture supernatant

of the RPMI 1788 cell was used as standard LT[12].

Medium

RPMI-1640 powder (GIBCO Laboratories, Grand Island, NY, USA) was dissolved in ultra pure water in a Milli Q system (Japan Millipore Ltd., Tokyo, Japan) after distilled twice, then sterilized through a filter with a pore size of 0.22 μm (Japan Millipore Ltd.). Complete medium was produced by addition of 100 U/ml penicillin G, 100 mg/ml streptomycin and 2 mM glutamine to the RPMI-1640 medium. Fetal calf serum (FCS) (M. A. Bioproduct. Walkersville, MD, USA) was added as indicated.

Cytotoxic assay

TNFα and LT sensitive L. P3 cells at a concentration of 5×10^4 cells/100 μl were placed in the wells of 96-well flat-bottomed microtiter plates (Falcon 3072, Becton Dickinson, Oxnard, CA, USA) with serially diluted samples in the presence of actinomycin D (1 μg/ml) and cultured at 37°C for 18 hours in a humidified CO_2 incubator. After incubation, the plates were washed, and cell lysis was determined through staining with 0.5% crystal violet in methanol/water (1:4 v/v). Dye uptake was calculated using an automated microplate-reader (Titertek Multiskan, Flow Laboratories Inc. Mclean, VA, USA) at OD_{570}. One unit of TNF activity was defined as the reciprocal of the dilution producing 50% of the total cytotoxicity. Recombinant TNFα and RPMI 1788 culture supernatant were also used as a standard in each assay plate.

Neutralization of TNFα and LT activity

To examine whether cytotoxicity to L. P3 was due to TNFα or LT, neutralization tests were performed with anti-TNFα and anti-LT antibodies. Mouse anti-human TNFα-specific monoclonal antibody was a gift from Dr. Oshima, Teikyo university, Tokyo, Japan. Rabbit anti-human-LT specific antisera was a gift from Dr. Niki, Institute for Applied Biology, Mitsubishi Chemical Industry Co., Yokohama, Japan. Samples were treated with each antibody for 1 hour prior to cytotoxicity assay.

Isolation and stimulation of human peripheral blood mononuclear cells (PBMC)

Human PBMC were isolated from the heparinized peripheral blood of healthy donors through Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden) density gradient centrifugation, washed twice with Ca^{++}, Mg^{++} free PBS, and suspended at a concentration of 1×10^6 cells/ml in complete RPMI 1640 medium with 10% FCS. PBMC suspensions were then cultured in the presence of BCG (0–1,000 μg/ml) for the indicated time periods at 37°C in a humidified CO_2 incubator. BCG, Tokyo 172 strain, was purchased from Japan BCG Manufacturing Co., Tokyo, Japan, as dried powder (80 mg/ampule, $5–12\times10^7$ viable bacilli/mg). Cellfree culture supernatants were collected at various time periods and assayed for cytotoxicity. LPS in the medium incubated with BCG (5 μg/ml) was checked by Limulus test (Limulus single test, Wako Chemicals, Tokyo, Japan; Sensitivity > 0.1 ng/ml) and was undetectable.

Inactivation of BCG

BCG, inactivated by heating in a water bath at 65°C for 1 hour, was suspended in RPMI medium. To examine viability, the heat-treated BCG was cultured in Ogawa's culture dish, specific for Mycobacterium tuberculosis.

Preparation of monocyte depleted PBMC

Nonadherent mononuclear cells were obtained from PBMC by depleting monocytes which adhered to plastic flasks for 60 minutes at 37°C in a humidified CO_2 incubator. Nonadherent cells, obtained after three cycles of adherence to plastic flasks were stained with FITC labeled monoclonal

Table 1. Induction of TNFα activity from PBMC with BCG

Donor	Added BCG (μg/ml)	Cytotoxic Activity (U/ml) treated with:		
		Nothing	Anti-TNFα	Anti-LT
A	0	<2	NT[a]	NT
	5	16	<2	16
B	0	<2	NT	NT
	5	21	<2	21
C	0	<2	NT	NT
	5	48	<2	48
Standard rTNF[a]		2000	<2	2000
Standard LT[b]		56	56	<2

[a] Not Tested.
[b] Supernatant of RPMI 1788 cells cultured for 24 hrs.

Table 2. Dose effect of BCG on the induction of L. P3 cytotoxic activity

Donor	Cytotoxic Activity (U/ml)					
	BCG (μg/ml)					
	0	5	50	200	500	1000
A	<1	2	35	66	56	NT[a]
B	<1	39	61	147	105	NT
C	<1	55	185	237	190	151
D	<1	14	16	32	32	30
E	<2	13	21	34	39	25

[a] Not Tested.

son Immunocytometry Systems, Mountain antibody Leu-M3 or Leu4 (Becton Dickin- View, CA, USA) and subpopulations were analyzed using a flow cytometer (EPICS 751, Coulter Electronics, Hialeath, FL, USA). Aliquots of nonadherent cell fractions were then cultured at a concentration of 1×10^6 cells/ml with 200 μg/ml of BCG.

Results

Induction of TNFα activities in PBMC with BCG

The culture supernatant of PBMC, stimulated with BCG (5 μg/ml) for 24 hours, proved cytotoxic to L. P3 cells. This cytotoxicity was completely blocked with anti-TNFα antibody, however, was not blocked with anti-LT antibody (Table 1). The specificity of each antibody was examined at the same time using rTNFα and the culture supernatant of RPMI 1788 as standard TNFα and LT respectively (Table 1). PBMC cultured without BCG produced no such cytotoxic activity.

Dose effect of BCG

The dose effect of BCG added to PBMC for inducing TNF was examined. As shown in Table 2, 200 μg/ml to 500 μg/ml proved optimal for the induction of TNF activity in all five blood donors, however, the induced peak activity of TNF varied widely from 32 to 237 U/ml. A high dose of BCG (1,000 μg/ml) induced less activity than did 200 μg/ml or 500 μg/ml.

Time course of induced TNF activity by BCG

Significant TNF activity first appeared after 2 hours of culture when stimulated with BCG 200 μg/ml. Afterwards, TNF activity gradually increased, the peak level

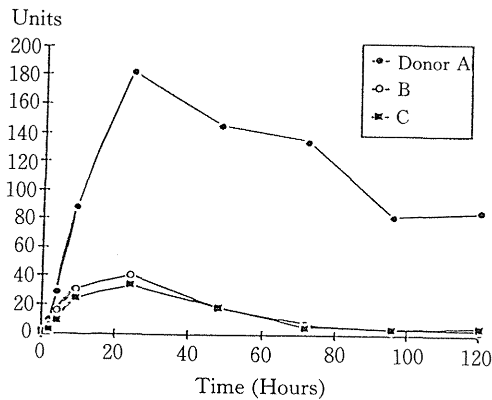

Fig. 1. Time course of TNF induction from PBMC by BCG.

being observed after 24 to 48 hours of culture. The activity then decreased, reaching a plateau after 96 hours (Figure 1). Even when the BCG concentration was decreased to 5 μg/ml, the kinetics were similar (data not shown.).

The effect of heat-inactivated BCG for the induction of TNF

Heat-inactivated BCG (200 μg/ml) induced TNF activity when added to PBMC (Table 3). Moreover, in three out of four cases, this activity proved equal to or stronger than those induced by viable BCG.

TNF producing cells in PBMC

Nonadherent cells were separated from PBMC and cultured with 200 μg/ml of BCG for 24 hours. Table 4 shows the cell populations of each cell fractions analyzed

Table 3. Effect of heat-inactivated BCG on the induction of L.P3 cytotoxic activity

Donor	Cytotoxic Activity (U/ml)	
	viable	heat-inactivated
A	128	36
B	237	277
C	32	59
D	34	86

Added BCG: 200 μg/ml

with Leu-M3 monocyte specific monoclonal antibody and Leu4 pan T specific monoclonal antibody from six donors. Only in the nonadherent cell fraction of donor A, 1.3% of Leu-M3 positive monocytes were contaminated. Next, the culture supernatants of unfractionated PBMC and nonadherent cell fraction with BCG were examined for TNF activity. The culture supernatants of monocyte depleted nonadherent cell fractions showed no TNF production except in the case of donor A, which was probably the result of the slight degree of monocytes contamination in that particular sample. On the other hand, it was shown that the adherent cell fractions stimulated with BCG could produce definite TNF activity (Table 4, donor F). Thus it is concluded that the induced TNF is derived from human peripheral monocytes.

Specificity of TNF activity:

As all the cytotoxic assays mentioned

Table 4. L.P3 cytotoxic activity induced from adherent cells and non-adherent cells

Donor	Cytotoxic Activity (U/ml) induced from			% of non-adherent and adherent (*) cells	
	Unfractionated PBMC	Non-adherent cells	Adherent cells	Leu M3	Leu 4
A	237	35	—	1.3	75.2
B	32	<2	—	0.1	50.3
C	34	<2	—	<0.1	80.8
D	132	<3	—	<0.1	74.7
E	90	<3	—	<0.1	73.6
F	—	—	30	*90.2	*1.3

Table 5. Specificity of TNF induced with BCG

Cell source	Inducer	Incubation time	Cytotoxic Activity (U/ml) after treatment with		
			Nothing	Anti-TNFα	Anti-LT
PBMC	BCG 200 μg/ml	24 hr	32	<1	32
PBMC	BCG 200 μg/ml	72 hr	16	<1	16
PBMC	Heat-inactivated BCG 200 μg/ml	24 hr	32	<1	32
Adherent cells	BCG 200 μg/ml	24 hr	8	<1	8

above were performed using L. P3 cells which are sensitive to both TNFα and LT, neutralization tests were performed using specific antibodies to confirm specificity. As demonstrated in Table 5, all cytotoxic activities induced from PBMC or adherent cells with BCG were confirmed to result from the presence of TNFα and not LT.

Discussion

TNFα was originally found by Carswell et al in the sera of mice primed with BCG and challenged with endotoxin[8]. They also reported that TNFα was induced by neither BCG nor endotoxin alone. On the other hand, many recent studies have shown that TNFα can be induced *in vitro* from human monocytes or lymphocytes by various mitogens or other agents[9–11]. In this study we demonstrated that BCG alone can induce TNFα in vitro from human peripheral monocytes. In Japan, however, most people are primed with Mycobacterium tuberculosis or BCG already, thus BCG stimulation might act as a booster in vitro.

Clinically fever is a usual adverse effect of intravesical administration of BCG[13]. Since TNFα is known to be an endogeneous pyrogen, stimulating synthesis of hypothalamic prostaglandin E_2 synthesis, or inducing IL-1[14], the febrile states observed after intravesical instillation of BCG might be explained by BCG-induced TNFα.

From these things mentioned above we can speculate that intravesical administration of BCG induces TNFα locally or systemically *in vivo* of bladder cancer patients, and the induced TNFα may be one of the factors of its anti-tumor effect, although it is unknown how important TNFα is among those factors.

We have demonstrated that heat-killed BCG exhibits the same TNF-inducing ability as viable BCG. This suggests its use for cancer treatment. Meanwhile, there is a report that the viability of the administered BCG has therapeutic importance[15], and another report said that BCG with reduced viability could not inhibit the growth of murine bladder tumor comparing with rapidly growing BCG[16]. These reports contradict our data. But we assume that TNFα is not only one effective molecule induced by BCG.

The role of cytokines or other immunomodulatory proteins in topical therapy with BCG have been reported[6,7]. TNF produced by human monocytes is possible to be one of those, and the approach to find it *in vivo* of bladder cancer patients treated with topical BCG therapy is needed.

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