Urol Res (1988) 16:351–355

Urological Research
© Springer-Verlag 1988

BCG Induced Killer Cell Activity

S. Koga, T. Kiyohara, K. Taniguchi, M. Nishikido, S. Kubota, T. Sakuragi, K. Shindo, and Y. Saitoh

Department of Urology, Nagasaki University School of Medicine, Nagasaki, Japan

Accepted: March 10, 1988

Summary. To investigate the mechanism of Bacillus de Calmette Guérin (BCG) bladder instillation therapy, the killer cell activity induced in peripheral blood mononuclear cells (PBMNCs) after BCG instillation was examined. Significant cytotoxic activity against natural killer (NK) cell resistant target tumor cells was detected after 3 days of instillation. To characterize this BCG induced cytotoxic activity further, human PBMNCs were cultured with BCG in vitro. From 24 h maximum cytotoxicity was obtained and continued for 3 days, then decreased slightly. Neither a DNA synthesis inhibitor Cytosine-arabinoside (Ara-C) nor a cytotoxic T cell (CTL) generation inhibitor Cyclosporine A inhibited this killer cell activation. Monoclonal antibody treatment revealed that both precursor and effector cells are Leu1$^-$, 3a$^-$, 7$^+$, 11b$^+$. The recognition specificity from cold target competition experiments was selective. Taken together NK type precursor was activated with BCG into NK type effector which has wider spectrum of target cells than usual NK cell.

Key words: Intravesical BCG – Peripheral blood mononuclear cells – Killer activity against natural killer cell (NK) resistant tumors, NK type precursor and effector cells

Introduction

Clinical results show that BCG instillation is a satisfactory therapy for superficial bladder cancer since Morales's first report [8]. The mechanism of this therapy is still obscure. Although Ratliff et al. reported that the thymus seemed to be essential for this therapy using murine adoptive transfer system [10], the effectors and how they recognize the target bladder tumors remain unknown. To answer these questions we studied the cytotoxic activity of PBMNC after bladder instillation of BCG to patients with superficial bladder cancer. As the PBMNC from patients who had BCG instillation showed significant lytic activity against various target tumor cells, we constructed an in vitro model. Here we present data showing the presence of an activated NK like population with wider recognition specificity than those cultured with BCG. The possible contribution of these BCG activated killer cells for BCG instillation therapy will be discussed.

Materials and Methods

Effector Cells

PBMNCs from normal volunteers and from bladder tumor patients were isolated by centrifugation over Ficoll-Hypaque gradients. Adherent cells were removed by a 1-h, 37 °C incubation on plastic dishes precoated with autologous plasma.

Target Cells

The cell lines used in this study included NK sensitive human myelogenous leukemia K562 cells, human NK resistant Burkitt's lymphoma line Raji cells, human promyelocytic leukemia cell line HL-60 cells, uterine cancer cell line HeLa cells, bladder cancer cell line T-24 cells, murine mammary cancer cell line MM2 cells and cloned murine lymphoma cells YAC-1.2 cells (YAC-1.2 cells were murine NK sensitive but human NK resistant). All cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum. The cell lines were monitored regularly for contamination and were found to be mycoplasma-free.

Cytotoxicity Assay

Cytotoxicity assays were performed by a standard ^{51}Cr-release assay as described [13].

$$\% \text{ lysis} = \frac{\text{cpm test} - \text{cpm medium}}{\text{cpm max} - \text{cpm medium}} \times 100$$

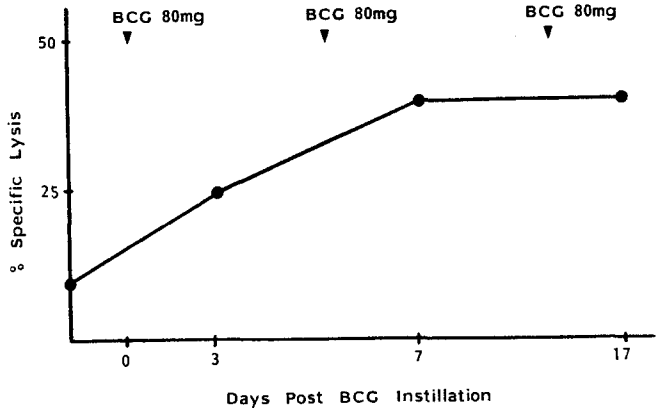

Fig. 1. Appearance of PBMNC killer activity against NK resistant Raji cells after bladder instillation of BCG to a bladder tumor bearing patient: 80 mg of BCG was administered intra vesically to a bladder tumor patient on day 0, 5, 14, and the cytotoxicity of PBMNCs on day 3, 7, 17 were assayed against NK resistant target Raji cells

Table 1. Target spectrum of BCG induced killer cell activity

Target	% Specific lysis ± s.e.[a]
K562	48 ± 1
Raji	57 ± 1
Daudi	64 ± 2
HL-60	51 ± 1
Hela	21 ± 1
T-24	35 ± 1
YAC	20 ± 1
MM2	0

[a] calculated from triplicated experiments

Counts per minute (cpm) max was determined by counting an aliquot of resuspended target cells; cpm medium was determined in wells containing targets only with no effectors added. All experiments were done in triplicate in V-shaped 96 well microplates (Nunc, Denmark).

BCG Induced Killer Cell Assay

BCG Tokyo strain (Japan BCG Co Ldt. Tokyo) was added to PBMNCs (5 μg/ml because from 2 μg/ml to 5 μg/ml was optimal to induce Killer activity) and cultured in 10% Fetal Calf Serum supplemented RPMI. 24 h after, non adherent effector cells were harvested and washed three times. Ara-C (Sigma chemicals St. Lois, MO) or Cyclosporine A (Sandimmune, Basel) were added to this 24 h culture system.

Cold Target Competition Assay

To examine recognition specificity (i.e. effector and target cell binding specificity) in a quantitative fashion, we employed a cold target competition assay which was originally developed by M. O. de Landazuri and R. B. Herberman [6]. Briefly, 50 μl of ^{51}Cr-labelled Raji target cells (5×10^3 cells) were incubated with 50 μl of various numbers of BCG induced killer cells in V-shaped microplates (Flow Laboratories, McLean VA). Fifty microliters of varying numbers of nonlabelled competitor cells or medium as control was added and 18 h later, 100 μl of supernatant was harvested and assayed for ^{51}Cr-release.

Results

Appearance of PBMNC Killer Activity After Bladder Instillation

PBMNC from bladder cancer patients were tested in a 18 h ^{51}Cr release assay against Raji cells. As shown in Fig. 1, before BCG instillation % specific lysis of patients was less than 10% as was observed for normal controls. 3 days after BCG instillation cytotoxic activity appeared and increased thereafter. The plastic dish adherent cells showed no lytic activity during therapy (data not shown). These experiments were done with 7 bladder tumor patients who received BCG instillation and with 9 normal controls. Whenever Effector/Target Ratio is not recorded, a 50/1 Effector/Target Ratio, was employed.

Target Spectrum of BCG Induced Killer Activity

First bladder tumor bearing patients' or normal person's PBMNC with BCG were cultured. BCG induced Killer activity from both tumor bearer's PBMNC and normal volunteer's PBMNC in vitro. The following experiments were performed using PBMNC from normal volunteers. This Killer cell induction was dose dependent and the optimal concentration of BCG to induce this Killer activity was from 2 μg/ml to 5 μg/ml (data not shown). Table 1 shows the target spectrum of this BCG induced Killer activity. Both human NK sensitive (K562) and human NK resistant (Raji, Daudi, HL-60, Hela, T-24, YAC-1.2) target tumor cells were sensitive to this BCG induced Killer activity indicating that the target spectrum of this Killer was very wide. But MM2, a murine mammary carcinoma cell line was completely resistant.

Kinetics and Phenotypes of BCG induced Killer Activity

From Fig. 1 we determined the assay time to be 18 h. To activate this Killer cells 24 h were enough, and after 3 days the activity decreased gradually (Fig. 3). Although data are not shown, pre-treatment of PBMNC with monoclonal anti-Leu7 or Leu11b antibody and young rabbit complement diminished Killer activity (−55% for anti-Leu7 and −69% for anti-Leu11b), but pre-treatment with anti-Leu1 or anti-Leu3a did not influence Killer activity (0% for anti-Leu1 and 0% for anti-Leu3a). Treatment of BCG activated Killer cells with these monoclonal antibodies revealed the

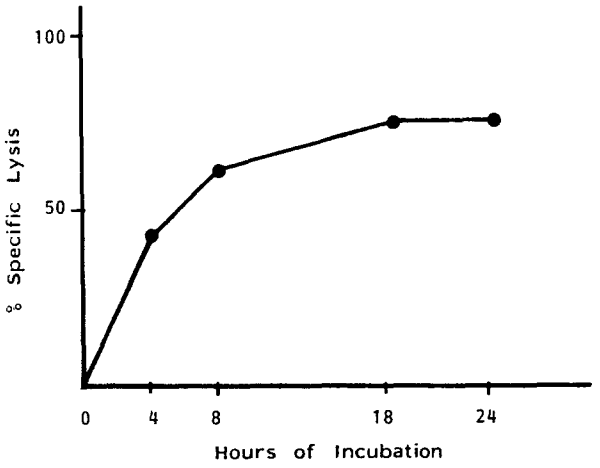

Fig. 2. Assay time kinetics of BCG induced killer cell activity: 5 μg/ml of BCG was added to a normal volunteer's PBMNCs. After 24 h, PBMNCs were washed three times and ^{51}Cr-labelled Raji target cells were added. ^{51}Cr-release assay was done at 4, 8, 18, 24 h incubation

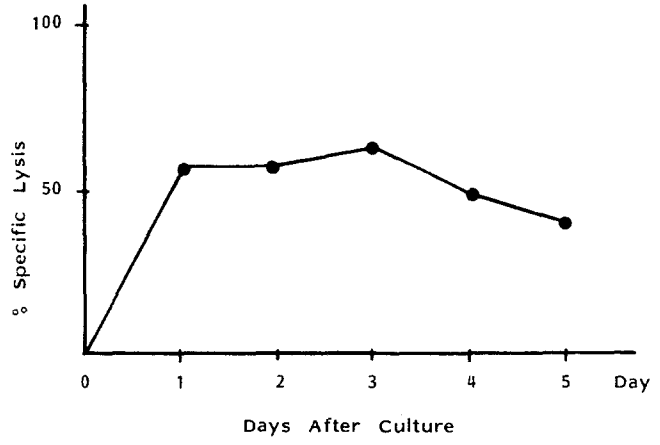

Fig. 3. Kinetics of BCG induced killer cell activation: 5 μg/ml of BCG was added to PBMNCs from a normal volunteer in vitro and activated killer activity was assayed against ^{51}Cr-labelled Raji cells on each day. 18 h ^{51}Cr-release assays were done

same results (–50% for anti-Leu7, –75% for anti-Leu11b, 0% for anti-Leu1 and 0% for anti-Leu3a). From the results of these experiments, both precursors and effectors were thought to have NK like phenotypes.

Effect of Ara-C or Cyclosporine on the Activation

In order to investigate this effect, which may have been due to an activation of LAK precursor cells, we used Ara-C as an inhibitor of DNA synthesis or Cyclosporine A as a blocker of IL-2 dependent activation, and assayed against Raji target tumor cells. As shown in Table 2, 4 μg/ml of Ara-C (which was not cytotoxic and no DNA synthesis was seen by ^{3}H-Thymidine uptake assay) did not influence BCG induced Killer cell activation. Also 1 μg/ml of Cyclosporine A a did not influence BCG induced Killer cell activation, indicating that this activation seemed to be different from LAK activation.

Table 2. Effect of Ara-C or Cyclosporine on the activation of BCG induced killer cells

Experiment # 1	% Specific lysis ± s.e.[a]
BCG-K	51 ± 1
BCG-K + CsA (1 μg/ml)	49 ± 1
Experiment # 2	**% Specific lysis ± s.e.[a]**
BCG-K	31 ± 1
BCG-K + Ara-C (4 μg/ml)	36 ± 2

[a] calculated from triplicated experiments

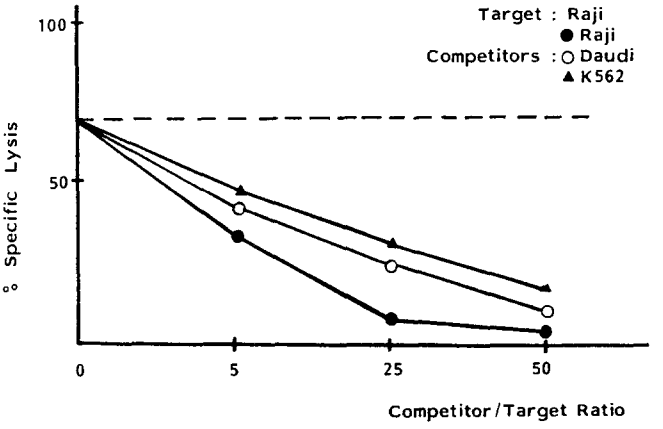

Fig. 4. Cold target competition test of BCG induced killer cells with labelled Raji cells and unlabelled competitors: % specific lysis of BCG induced killer cells against Raji cells without competitor is shown as dotted line (70% at Effector/Target Ratio = 50/1)

Recognition Specificity

Finally, recognition specificity of BCG induced Killer cells were tested with 1) cold target competition test and 2) α-Mannosidase treatment. Raji cells were labelled with ^{51}Cr and various non-labelled target cells were added (Fig. 4). Non-labelled competitor targets such as Raji cells themselves, Daudi cells and K562 cells competed to Raji cell recognition of BCG induced Killer cells. As shown in (Fig. 5) Raji cells and Daudi cells also competed for K562 cell recognition. Criss cross competition (data not shown) showed that BCG induced Killer cells had specific binding receptors and that the sensitive targets had common target structures which mediate binding. α-Mannosidase is known to inactivate NK cell recognition by cleaving off the carbohydrate moiety of the NK cell surface receptor [4]. To discover whether this BCG induced Killer operated via a

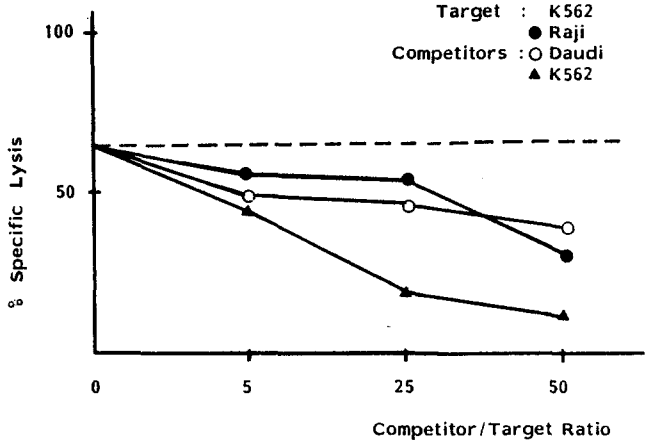

Fig. 5. Cold target competition test of BCG induced killer cells with labelled K562 cells and unlabelled competitors: % specific lysis of BCG induced killer cells against K562 cells without competitor is shown as dotted line (64% at Effector/Target Ratio = 50/1)

Table 3. Effect of α-Mannosidase treatment to induced BCG killer cell lytic activity

Target cell	not treated	α-Mannosidase treated
K562	72 ± 3%[a]	11 ± 1%
Raji	60 ± 2%	13 ± 1%
T-24	43 ± 1%	4 ± 1%

[a] calculated from triplicated experiments

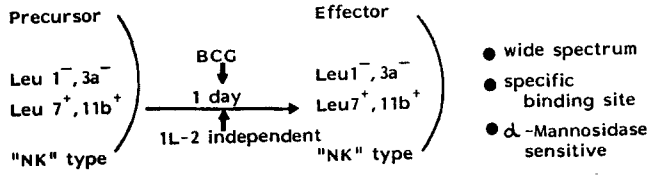

Fig. 6. Schematic representation of characters of BCG induced killer cells

carbohydrate signalling system [5], we pre-treated activated effector cells with 1 Unit/ml of α-Mannosidase at room temperature for 1 h and tested their cytotoxicity. As shown in (Table 3) BCG induced Killer cell activity was impaired with α-Mannosidase treatment.

Discussion

The results presented here suggested that 1) after bladder instillation of BCG, some Killer cells which can lyse NK resistant tumor cells were activated in PBMNC, 2) within 24 h in the presence of BCG, NK type precursors were activated in vitro into effectors which were of NK type but had a wider spectrum.

A. S. D. Pang and A. Morales have reported that peritoneal exudate cells from mice after intraperitoneal infection of BCG demonstrated cytolytic activity in vitro against the syngeneic bladder tumor cell line MBT-2 [9]. They concluded that the effector cells in their experiments were NK like cells. In our experiments, not only in vivo BCG administration but also in vitro BCG, induced wider spectrum of Killing activity than was usual with NK cells. Sensitive target cells were not uniform as shown in Table 1, because murine lymphoma YAC cells as well as human solid tumor cells HeLa cells, T-24 and human hematogenous promyelocytic leukemia cells HL-60 were all sensitive. Although both precursors and effectors seemed to be NK type, the sensitive target spectrum was wider. This seems to be a crucial difference. Data are not shown here but, murine spleen cells have be activated in vitro with BCG as in the human system. There was enough BCG induced Killer cell activity from bg/bg mutant spleen cells which lacked NK Cytolytic activity completely [11, 12].

These data suggest that not only competent NK cells but also defective NK cells could be activated into BCG induced Killer cells. Then, what characters do the BCG induced Killer cells have? LAK cells [1, 7] and allo-reactive anomalous Killer cells [3] are known to be cytotoxic to various target tumor cells in vitro. Hence the difference between LAK cells and this BCG induced Killer cells were examined. LAK cells were originally defined as IL-2 dependent and $Leu1^+$, $OKT3^+$, $T8^+$, $T4^+$, $F2^+$, $OKM1^-$, T cell population [7]. 1 day culture supernatant of PBMNCs with BCG contained less than 2 Unit/ml of IL-2 using Radioimmuno-assay and less than 2 Unit/ml of Interferron (Unpublished data). Hence, as shown in the schema in Fig. 6 not IL-2 dependent LAK cells but IL-2 independent NK cell activation may be characteristic of this BCG activated Killer cell phenomenon.

E. A. Grimm et al. [2] reported that Cyclosporine A inhibited CTL (autologus tumor specific cytotoxic T Lymphocytes) activation but did not inhibit IL-2 dependent LAK cell activation. In our experiments BCG induced Killer activity was not inhibited with cyclosporine A, showing that this phenomenon is not polyclonal CTL activation. Indeed, this was re-examined using Ara-C which inhibited CTL generation. Aca-C had no influence on BCG Killer induction. NK cells but not T cells seems to have crucial role in BCG induced Killer cell cytotoxicity. Why should the thymus be necessary for the BCG therapeutic effect as shown by Ratliff et al.?

We believe that this is an important and essential question to be solved in future.

References

1. Grimm EA, Mazumdar A, Zhang HZ, Rosenberg SA (1982) Lymphokine activated killer cell phenomenon, lysis of natural killer-resistant fresh solid tumor cells by Interleukin2-activated autologous human peripheral blood lymphocytes. J Exp Med 155:1823–1841

2. Grimm EA, Muul LM, Wilson DJ (1985) The differential inhibitory effects exerted by cyclosporine and hydrocortisone on the activation of human cytotoxic lymphocytes by recombinant Interleukin-2 versus allospecific CTL. Transplantation 39:537–540
3. Kärre K, Seeley JK, Eriksson E, Burton RC, Kiessling R (1983) "Anomalous" Thy-1$^+$ killer cells in allogeneic and F_1-antiparental mixed leukocyte culture. Relation to natural killer cells and allospecific T lymphocytes. J Exp Med 156: 385–397
4. Kiyohara T, Dennis JW, Boegman RJ, Roder JC (1985) An exoglycosidase-sensitive triggering site on NK cells which is coupled to transmethylation of membrane phospholipids. J Immunol 135:659–664
5. Kiyohara T, Dennis JW, Roder JC (1987) Double restriction in NK cells recognition is linked to transmethylation and can be triggered by asparagine-linked oligosaccharides on tumor cells. Cell Immunol 106:223–233
6. De Landauzuri MO, Herberman RB (1972) Specificity of cellular immune reactivity to virus-induced tumors. Nature New Biol 238:18–19
7. Lotze MT, Grimm EA, Mazumder A, Strausser JL, Rosenberg SA (1981) Lysis of fresh and cultured autologous tumor by human lymphocytes cultured in T cell growth factor. Cancer Res 41:4420–4425
8. Morales A, Eidinger D, Bruce AW (1976) Intracavitary BCG in the treatment of superficial bladder tumors. J Urol 116: 180–183
9. Pang ASD, Morales A (1982) BCG induced murine peritoneal exudate cells; cytotoxic activity against a syngeneic bladder tumor cell line. J Urol 127:1225–1229
10. Ratliff TL, Gillen D, Catalona WJ (1987) Requirement of a thymus dependent immune response for BCG-mediated antitumor activity. J Urol 137:155–158
11. Roder JC, Duwe A (1979) The beige mutation in the mouse selectively impairs natural killer cell function. Nature 278: 451–453
12. Roder JC (1979) The beige mutation in the mouse. I. A. stem cells predetermined impairment in natural killer cell function. J Immunol 123:2168–2173
13. Werkmeister J, Roder JC, Curry C, Pross HF (1983) The effect of unphosphorylated and phosphorylated sugar moieties on human and mouse natural killer cell activity: is there selective inhibition at the level of target recognition and lytic acceptor site? Cell Immunol 80:172–186

S. Koga, MD
Department of Urology
Nagasaki University School of Medicine
7-1 Sakamoto-machi
Nagasaki
852 Japan