· Original article ·

Comparative immunoregulatory property analysis of genomic DNA and polysaccharide nucleic acid fraction from *Mycobacterium bovis* Bacillus Calmette-Guérin

WANG Jing[1], HU Zhen-Lin[2*], ZHOU Feng-Juan[2], WAN Bin[2], HE Xiao-Wen[2], LING Yi-Ling[1], SUN Shu-Han[2*] (1. Department of Pathophysiology, Hebei Medical University, Shijiazhuang 050017, China; 2. Department of Medical Genetics, College of Basic Medical Sciences, Second Military Medical University, Shanghai 200433)

[**ABSTRACT**] **Objective**: To compare the immunomodulatory property of genomic DNA from *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG-DNA) and polysaccharide nucleic acid fraction from *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG-PSN). **Methods**: Highly purified BCG-DNA was isolated. Murine splenocyte or human peripheral blood mononuclear cell (hPBMC) were incubated with BCG-DNA or BCG-PSN. IFN-γ in cell cultures was tested by ELISA. **Results**: BCG-DNA was extracted; the concentration of DNA was 95.3%. Agarose electrophoresis showed that the size of BCG-DNA is 200-250 bp. ELISA test showed that both BCG-DNA and BCG-PSN could activate murine splenocytes and hPBMC, and significantly increase IFN-γ level ($P<0.01$). Moreover, BCG-DNA induced higher levels of IFN-γ compared to BCG-PSN when the concentration≥10 μg/ml ($P<0.01$), and such effect was dose-dependent. **Conclusion**: Compared with BCG-PSN, BCG-DNA can significantly induce typical Th1 cytokine (IFN-γ) *in vitro*, and it is a potent Th1 immunoregulator.

[**KEY WORDS**] *Mycobacterium bovis* Bacillus Calmette-Guérin; genomic DNA; polysaccharide nucleic acid; immunomodulation; interferon γ

[Acad J Sec Mil Med Univ, 2004, 25(1): 37-40]

Mycobacterium bovis Bacillus Calmette-Guérin (BCG) is a strong non-specific immunostimulator, and local BCG therapy is effective in the treatment of cancer. But severe side effects hampered its further application, so it is a promising strategy to extract effective component without side effects from BCG. Polysaccharide nucleic acid fraction from BCG (BCG-PSN) is considered to be successful clinical application, but there are few researches on immunoregulatory properties of genomic DNA from BCG. Recent studies have shown that a genomic DNA fraction from BCG (BCG-DNA) has strong antitumor activities and little side effects. These prompted us to investigate whether BCG-DNA might be used as a immunostimulator. In this study, we isolated highly-purified BCG-DNA and compared its immunoregulatory property with that of BCG-PSN[1].

1 MATERIALS AND METHODS

1.1 Experimental animal Female BALB/c mice (weighing 20-25 g, 6-8 weeks of age at the time of study initiation) were obtained from BIKAI Animal Corporation.

1.2 Purification of BCG-DNA and product purity analysis Heat-killed BCG were thawed, suspended in STE (0.1 mol/L NaCl, 10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0) and disrupted by sonication with an ultrasonic homogenizer. The disrupted suspension was centrifuged at 15 000 r/min for 15 min, and the collected supernatant was placed in a water bath at 100℃ for 10 min. Then the solution was left at room temperature for 20 min and centrifuged again at 15 000 r/min for 15 min. 10 mg/ml trypsin was added to the supernatant to a final concentration of 1 mg/ml. The mixture was stranded in a 37℃ water bath for 30 min and centrifuged at 15 000 r/min for 15 min. The supernatant was then precipitated with 2 volumes of 99.7% ethanol and kept for 10 min at −20℃. The precipitate was collected, dissolved with PBS, and were placed in a water bath again, the solution after centrifugation was collected. The solution was

[**Foundation**] This work is supported by National Natural Science Foundation of China (30270687).

[**Biography**] WANG Jing (1977-), female (Han nationality), master.

* Corresponding author. E-mail: shsun888@hotmail.com

applied to a DEAE Sepharose Fast Flow column (200 mm×25 mm, Pharmacia Biotech, Sweden). A stepwise gradient of 100% solvent A (10 mmol/L Tris-HCl, 0.2 mol/L NaCl, 1 mmol/L EDTA, pH 5.0), 80% solvent A+20% solvent B (10 mmol/L Tris-HCl, 2 mol/L NaCl, 1 mmol/L EDTA, pH 5.0) and 62% solvent A + 38% solvent B at 6 ml/min were used. The BioCAD workstation was used to carry out the experiments. The detector wavelength was set at 254/280 nm. The solution achieved after ion-exchange chromatography was applied to a Sephadex G-25 column (200 mm× 25 mm, Pharmacia Biotech, Sweden). The column was equilibrated and eluted with solvent A at a flow rate of 5 ml/min. The same BioCAD workstation was applied with the detector wavelength set at 254/280 nm. The solution was lyophilized with a lyophilizer (type MAXI Dry Lyo, HETO-HOLTEN A/S. Denmark)

DNA and RNA were quantified by the diphenylamine reaction with calf thymus DNA and by the orcinol reaction with yeast RNA, respectively[1]. The size of DNA fragment was examined with 0.8% agarose electrophoresis. Protein and polysaccharide were determined by a commercial kit (Micro BCA Protein Assay Reagent Kit, PIERCE) according to the manufacturer's instruction and by the anthrone-sulfuric acid reaction with glucose[1].

1.3 Effects of BCG-DNA and BCG-PSN on IFN-γ production by murine splenocyte Spleens were obtained from 6- to 8-week-old BALB/c mice under sterile conditions and single-cell suspensions were prepared in RPMI 1640 supplemented with 10% FBS[2]. The splenocytes were plated at 5×10^6/ml for the cytokine assays (1 ml/well) in triplicate in 24-well round-bottom polystyrene plates. BCG-DNA and BCG-PSN (polysaccharide 350 μg/ml, SIQI Pharmaceutical Co. Ltd., Hunan) were suspended in RPMI 1640 and plated to final concentrations of 0, 10, 50, and 100 μg/ml for IFN-γ evaluation. The splenocytes were incubated with 5% CO_2 at 37°C for 72 h. The level of IFN-γ was determined by ELISA kits (Jingmei Biotech Co. Ltd., Shenzhen).

1.4 Effects of BCG-DNA and BCG-PSN on IFN-γ production by hPBMC 20 ml peripheral blood from healthy volunteers was heparinized by heparin sodium (250 U/ml, No. 1 Pharmaceutical Co. Ltd., Shanghai) and then one volume of PBS was added for dilution. The hPBMC were obtained by Ficoll-Hypaque density gradient centrifugation and were suspended in RPMI 1640 supplemented with 10% FBS[3]. The hPBMC (2×10^6/ml) were plated in triplicate in 24-well round-bottom polystyrene plates for the cytokine assays (1 ml/well). BCG-DNA and BCG-PSN were suspended in RPMI 1640 and plated to final concentrations of 0, 10, 50, and 100 μg/ml for IFN-γ evaluation. The hPBMC were incubated with 5% CO_2 for 72 h at 37°C. The level of IFN-γ was determined by human-specific ELISA kits (Jingmei Biotech Co. Ltd., Shenzhen).

1.5 Effects of BCG-DNA digested by DNase on IFN-γ production by murine splenocyte or hPBMC

BCG-DNA (500 μg) and DNase I (2 400 U, HUASHUN Biotech Co, Ltd., Shanghai) were dissolved in the solution containing 5 mmol/L $MgCl_2$ and 0.1 mol/L Tris-HCl (pH 8.0) and the concentration of BCG-DNA was adjusted to 1 mg/ml and incubated at 37°C for 5 h. The enzymatic reaction was stopped by shaking with an equal volume of chloroform-isoamyl alcohol (24 : 1, *V/V*), and the mixture was centrifuged at 10 000 r/min for 10 min. The same procedures were repeated until the protein interphase disappeared.

Murine splenocyte and hPBMC were prepared according to the method described above. BCG-DNA and BCG-DNA digested by DNase were suspended in RPMI 1640 and plated to final concentrations of 0, 10, 50, and 100 μg/ml for IFN-γ evaluation. The cells were incubated for 72 h at 37°C and 5% CO_2. The level of IFN-γ was determined with ELISA kits (Jingmei Biotech Co. Ltd., Shenzhen).

1.6 Statistical analysis Levels of IFN-γ were expressed as $\bar{x}\pm s$ and analyzed with statistical software. The significance of differences between values was determined by *t* test.

2 RESULTS

2.1 Product purity analysis The ratio of absorbencies at 260 nm and 280 nm was 1.80. 0.8%

agarose electrophoresis showed that the size of BCG-DNA is 200-250 bp and there existed no nuclear acid when BCG-DNA was digested by DNase (Fig 1). BCG-DNA was highly purified (DNA 95.3%, RNA 0.5%, protein 0.1%, polysaccharide 3.5%) and differed from BCG-PSN which mainly consisted of polysaccharide (>85%).

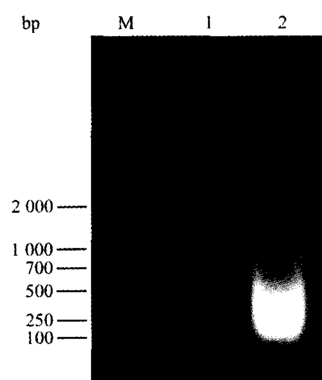

Fig 1　Agarose gel electrophoresis analysis of BCG-DNA/BCG-DNA digested with DNase

M: DL2000 marker;

1: BCG-DNA digested with DNase; 2: BCG-DNA

2.2　BCG-DNA activate murine splenocyte to produce IFN-γ　Both BCG-DNA and BCG-PSN activated IFN-γ producing in murine splenocyte compared to non-BCG-DNA or non-BCG-PSN ($P<0.01$). Moreover, the level of IFN-γ in BCG-DNA group was higher than that in BCG-PSN group when the concentration was above 10 μg/ml ($P<0.01$), and such effect was dose-de pendent (Tab 1).

Tab 1　Effects of BCG-DNA/BCG-PSN on IFN-γ level in mice splenocyte

($n=3, \bar{x}\pm s, \rho_B$/pg • ml^{-1})

Group	IFN-γ	
	BCG-DNA	BCG-PSN
0	52±2.5	52±2.0
10 μg • ml^{-1}	118±2.5**△△	80±3.5**
50 μg • ml^{-1}	142±2.5**△△	95±3.0**
100 μg • ml^{-1}	179±3.6**△△	112±2.5**

** $P<0.01$ *vs* 0 μg/ml group; △△ $P<0.01$ *vs* the same concentration group of BCG-PSN

2.3　BCG-DNA activating hPBMC to produce IFN-γ　The result was the same with murine splenocyte experiment (Tab 2).

Tab 2　Effects of BCG-DNA/BCG-PSN on IFN-γ level in human PBMC

($n=3, \bar{x}\pm s, \rho_B$/pg • ml^{-1})

Group	IFN-γ	
	BCG-DNA	BCG-PSN
0	189±3.6	186±4.0
10 μg • ml^{-1}	243±3.0**△△	198±3.0**
50 μg • ml^{-1}	296±3.6**△△	224±4.0**
100 μg • ml^{-1}	375±3.5**△△	300±2.0**

** $P<0.01$ *vs* 0 μg/ml group; △△ $P<0.01$ *vs* the same concentration group of BCG-PSN

2.4　BCG-DNA digested by DNase have no effect on IFN-γ levels　There was no significant elevation in the IFN-γ levels both in murine splenocyte group and hPBMC group after BCG-DNA was digested by DNase (Tab 3).

Tab 3　Effects of BCG-DNA/BCG-DNA digested with DNase on IFN-γ level in mice and human splenocyte

($n=3, \bar{x}\pm s, \rho_B$/pg • ml^{-1})

Group	BCG-DNA		BCG digested with DNase	
	Mice	Human	Mice	Human
0	52±2.5	189±3.5	52±2.5	187±4.0
10 μg • ml^{-1}	118±2.5**	243±3.0**	50±2.0	186±4.0
50 μg • ml^{-1}	142±2.5**	296±2.5**	48±2.5	186±4.0
100 μg • ml^{-1}	179±3.6**	375±3.5**	48±2.1	186±4.1

** $P<0.01$ *vs* 0 μg/ml group

3　DISCUSSION

Mycobacteria, especially *Mycobacterium bovis* strain BCG, have been investigated widely. But severe side effects hampered its further application in human[4]. Efforts have also been made to isolate bacterial components with antitumor activity from *Mycobacterium bovis* strain BCG or from other mycobacteria, such as the active components wax D, cell-wall skeleton, and polysaccharide nucleic acid

fraction[1]. But DNA from BCG has been scarcely studied. In 1984, Tokunaga *et al* found that a DNA fraction extracted from BCG had strong antitumor activity without direct cytotoxicity[1,5]. These results suggested further investigation for BCG-DNA as a new immunoregulator.

We developed practical strategies for purifying genomic DNA fraction from BCG by ion-exchange chromatography. The size of BCG-DNA is 200-250 bp because of the procedure of cell disruption by sonication. The purity of the product is more than 95% with only a small amount of protein and polysaccharide.

On the basis of these researches, we compared immunoregulatory activity of BCG-DNA and BCG-PSN. Bioactivity assays showed that BCG-DNA acted as a Th1 immune response immunostimulator, significantly increasing Th1-type cytokine (IFN-γ) level, which was significantly higher than that of BCG-PSN. To determine whether the DNA in BCG-DNA is essential for the immunoregulatory activity, BCG-DNA was digested with DNase and then tested for immunoregulatory activity. We found that DNA in BCG-DNA was essentially responsible for its strong immunoregulatory activity.

In addition to its antitumor activities, BCG-DNA also shows a promising application in atopic disorders. This study laid a foundation for further researches in treatment of atopic disorders by BCG-DNA.

[REFERENCES]

[1] Tokunaga T, Yamamoto H, Shimada S, *et al*. Antitumor activity of deoxyribonucleic acid fraction from *Mycobacterium bovis* BCG. Ⅰ. Isolation, physicochemical characterization, and antitumor activity[J]. *J Natl Cancer Inst*, 1984, 72(4): 955-962.

[2] Horner AA, Datta SK, Takabayashi K, *et al*. Immunostimulatory DNA-based vaccines elicit multifaceted immune responses against HIV at systemic and mucosal sites[J]. *J Immunol*, 2001, 167(3): 1584-1591.

[3] Roman M, Matin-Orozco E, Goodman JS, *et al*. Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants[J]. *Nat Med*, 1997, 3(8): 849-854.

[4] 雪 华，舒雪辉. 卡介苗的不良反应[J]. 医药导报，1999，18(6)：455-456.

[5] Shimada S, Yano O, Inoue H, *et al*. Antitumor activity of the DNA fraction from *Mycobacterium bovis* BCG. Ⅱ. Effects on various syngeneic mouse tumors[J]. *J Natl Cancer Inst*, 1985, 74(3): 681-688.

[Received] 2003-07-15 **[Accepted]** 2003-11-25
[Editor] YIN Cha

卡介菌基因组DNA和卡介菌多糖核酸免疫调控活性的比较

王 静[1]，胡振林[2*]，周凤娟[2]，万 斌[2]，何晓文[2]，凌亦凌[1]，孙树汉[2*]
（1. 河北医科大学病理生理学教研室，石家庄 050017；2. 第二军医大学基础医学部医学遗传学教研室，上海 200433）

[摘要] **目的**：比较研究卡介菌(*Mycobacterium bovis* Bacillus Calmette-Guérin，BCG)基因组DNA(BCG-DNA)和卡介菌多糖核酸(BCG-PSN)的免疫调控活性。**方法**：制备高纯度的BCG-DNA，小鼠脾细胞（splenocyte)和人外周血单个核细胞(peripheral blood mononuclear cell，PBMC)分别与BCG-DNA和BCG-PSN共同培养72 h，采用ELISA方法检测细胞培养上清中IFN-γ的水平。**结果**：所制备的BCG-DNA纯度较高，DNA含量达95.3%。琼脂糖电泳显示：BCG-DNA片断大小集中于200～250 bp。ELISA结果表明BCG-DNA与BCG-PSN均可显著激活小鼠脾细胞和人外周血单个核细胞，有效诱导IFN-γ的产生($P<0.01$)。当浓度达10 μg/ml以上时，BCG-DNA免疫调控作用优于BCG-PSN($P<0.01$)。**结论**：BCG-DNA可显著诱导Th1型细胞因子IFN-γ的产生，具有较强的免疫调控活性。

[关键词] 卡介苗；基因组；DNA；多糖核酸；免疫调控；干扰素-γ
[中图分类号] R 392.11 **[文献标识码]** A **[文章编号]** 0258-879X(2004)01-0037-04

欢迎订阅
《第二军医大学学报》 ISSN 0258-879X CN31-1001/R
上海市翔殷路800号(邮编：200433) 邮发代号：4-373
JOURNAL OF MEDICAL COLLEGES OF PLA ISSN 1000-1948X CN31-1002/R
上海市翔殷路800号(邮编：200433) 邮发代号：4-725