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IMMUNE RESPONSES OF THE MICE BEARING TUMOR INDUCED BY DMBA(9,10-Dimethyl-1,2-Benzanthracene)

Chae Woong Rim, Byung Moo Rim and Yun Shin Chung*

College of Veterinary Medicine and *Department of Biology,*
College of Natural Science Chonbuk National University, Jeonju 560-756, Korea

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ABSTRACT: *This study was undertaken to evaluate the immune responses to sheep red blood cell (SRBC) and potential anti-tumor effect of Bacillus Calmette-Guerin (BCG) in the mice bearing tumor induced by DMBA.*

The frequencies of tumor appearances were 62% in DMBA-treated mice, and 14% in DMBA and BCG-treated group, respectively. Cellular immune response such as delayed-type hypersensitivity (DTH) to SRBCs, natural killer (NK) cell activity and antigen-binding cell (ABC) assay were decreased apparently in the tumor bearing mice compared to the normal controls. Humoral immune responses such as hemagglutinin (HA) and hemolysin (HE) were noted to be reduced in the tumor bearing mice, but the spleen index increased in tumor bearing mice.

All the immunological parameters in the DMBA and BCG-group appeared to be higher than those of only DMBA-treated group.

These results indicated that DMBA-induced tumor suppressed host immune responses. Also, they imply the idea that BCG enhanced the immune responses of tumor-bearing host and antitumor effects.

Keywords: *Delayed-type hypersensitivity, Natural killer cell, Antigen-binding cell, Spleen index.*

INTRODUCTION

The substance, DMBA, being commonly found in the environment is an polycyclic aromatic hydrocarbon with potent carcinogenecity. (Elizabeth, 1978). This carcinogenic chemical can cause certain tumors with the two stages through "initiation-promotion model". It can be converted to diol-epoxide by cytochrom P-450 (Sanjoy *et al.*, 1989). This intermediate metabolite activates *c-Ha-ras* oncogene and causes the single point mutation (Allan *et al.*, 1984; Anthony et al., 1984). DMBA is structually related to steroid, bile acid, and sex hormone, which are found normally in higher

organism (Jan Klein, 1982). The fact has led to speculations that abnormal metabolism of these natural compounds may be responsible for some spontaneously developing neoplasms.

Immunodeficient patient who received immunosuppressive therapy can develope cancer (Robert, 1975). However, nude mice and beige (be/be) mice which lack T and NK cell respectively, did not show an increased susceptibility to chemical carcinogens (Stewart, 1987; Ian, 1988).

Successful clinical immunotherapy by BCG appeared in malignant melanoma and superficial bladder carcinoma (Robert, 1974). The development of biological response modifier (BRM) offers the hope for specific treatment of cancer.

The purpose of this work was to evaluate the antitumor effect of BCG and immune responses to SRBC in mice treated with DMBA.

MATERIALS AND METHOD

Animals

Normal *BALB/c* strain mice of both sexes, aged 6 to 7 weeks were used. Experimental groups were the DMBA-treated, both DMBA and BCG-treated, olive oil only treated, BCG only treated. Also normal control group was set up.

Carcinogen

9,10-dimethyl-1,2-benzanthracene (DMBA, Sigma) was used. DMBA- and DMBA and BCG-treated animals were injected into the left knee joint with 0.05 m*l* of 2% DMBA in olive oil. It was injected on day, 0, 32, 46 and 60. 30-G needle was used for injections.

BCG Treatment

BCG (Bacillus Calette Guerin, Japan laboratory) was used. 0.05 m*l* of 0.05% BCG in saline was i.p. inoculated on the DMBA treatment day (5 times in total). 27 G needle was used.

Antigen

Sheep red blood cells (SRBCs) were used. 0.2 m*l* of SRBCs (1×10^8 cells/m*l*) was i.v., injected (immunized) five days before the experiment.

Arthus Reaction and Delayed Type Hypersensitivity (DTH)

0.05 m*l* of 20% SRBCs (1×10^8 cells/mouse) was s.c. injected into the right footpad. Footpad swelling was measured at various times with a caliper (Ha, 1980).

Antibody to SRBCs

The hemagglution (HA) test and hemolysin (HE) titers were measured in order to evaluate the antibody to SRBCs (Naysmith, J.D., *et al.*, 1980).

Spleen Index

The ratio between the spleen and body weight of the normal and experimental mice

were measured at the time of removal of spleen.

Cell Preparation

Mice were sacrificed by cervical dislocation and spleens were excised and immersed in phosphate buffered saline (PBS). The spleen was gently passed through a stainless steel mesh (No. 200) screen and gradient cetrifugation with Ficoll-paque (Phamacia) was conducted. The cells were washed with PBS and adjusted to the cell number as specified.

Antigen Binding Cell (ABC) Assay

Equal volume (0.5 ml) of splenocytes (1×10^6 cells/ml) and SRBCs (1×10^8 cells/ml) suspensions were mixed. The mixture was maintained at 4°C for 18 hours. ABCs were determined as splenocytes attached 3 or more SRBCs.

Natural Killer (NK) Cell Activity

Cell culture medium was RPMI 1640 (Sigma) containing 10% fetal calf serum (Sigma), 2 mM glutamin, 2 mM pyruvate, 6 mM HEPES buffer, 10 units/ml penicillin and 100 ug/ml streptomycin. YAC-1 cell line was used as a target cell. NK cell activity was measured by 4-hour ^{51}Cr release assay (Uchida, A., *et al.*).

RESULTS

Tumor Incidence

Tumor developed around the knee joint. The developement of tumor (62%) was noted in DMBA-treated mice (8/13) and 14% in DMBA and BCG-treated mice (2/14). Seven mice of the former group and 6 of the latter were killed before the tumor was palpated, and were excluded from the incidence analysis (Table 1).

Spleen Index

Ratio of spleen weight(mg) to body weight(g) was 82% in DMBA treated group and 68% in DMBA and BCG-treated mice. These are much higher than that of normal (48%). Those of BCG-treated and olive oil-treated group were 46% and 50%, respectively (Table 2).

Arthus Reaction and DTH

Four days after immunization, mice were challenged in the right footpad with SRBCs and the footpad thickness was measured with caliper (Table 3). DTH levels of

Table 1. Frequency of tumor appearance in mice treated with DMBA and/or BCG

Group	% of tumor production
DMBA	62(8/13)*
DMBA and BCG	14(2/14)

*(numbers of tumor developed/total numbers of mice)

Table 2. Comparison of spleen indices of DMBA and/or BCG treated mice

Group	Spleen Index*
Nomral	48.5± 8
DMBA	82.4±12
DMBA and BCG	68.4± 9
BCG	46.1±11
Olive oil	50.8±12

*Spleen index = [spleen wt. (mg)/body wt.(g)] × 100
Each value is the Mean ± S.D.

Table 3. Effect of DMBA and/or BCG treatment on footpad swelling reaction to SRBC in mice

	% increase of thickness*		
Group	4 hr-Arthus	24 hr-DTH	48 hr-DTH
Normal	25.5±2.1	29.9±2.5	21.2±1.9
DMBA	15.2±2.5	17.5±2.5	13.5±2.2
DMBA and BCG	20.0±1.5	21.3±1.8	17.7±1.6
BCG	31.8±2.9	34.2±1.6	24.1±1.6
Olive oil	26.0±2.5	28.3±2.1	22.9±1.8

*Footpad thickness was measured just before the challenge(T_0) and 4 hr(T_4). 24 hr(T_{24}) and 48 hr(T_{48}) after the challenge and calculated by following formula; % increase = ((T_4, T_{24}, or T_{48})/T_0) × 100
Each value is Mean ± S.D.

DMBA- and DMBA and BCG-treated group were lower than those of normal control group. Mice received BCG developed much higher level of DTH in response to SRBC. A sligt increase also occurred in olive oil treated mice.

HA and HE

Antibody titer tests were carried out in microtiter plates by serial two-fold dilutions. Results were expressed as the highest dilution of an antiserum which gave a definitely positive reaction (Fig. 1). Antibody titers were suppressed in both DMBA- and DMBA and BCG-treated group. But the titers of DMBA and BCG-treated group were higher than those of only DMBA-treated mice. On the contrary, the titers of BCG-treated group appeared to increase.

Antigen Binding Cell (ABC)

The percentages of ABC in DMBA- and DMBA and BCG-treated mice were lower than those of normal contral group. The percentage of BCG treated mice was higher than that of normal contral group. This result is opposite to that of spleen index but similar to that of antibody titer (Fig. 2).

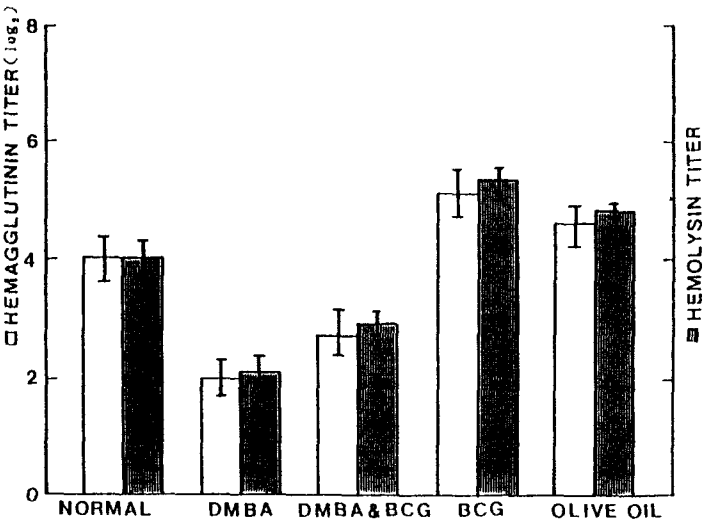

Fig. 1. Antibody titers to SRBC in the DMBA and / or BCG treated mice. Mice were sensitized i.v. with 0.2 ml (1×10^8 cells / ml SRBC 6 days before sacrifice. Each bar represents Mean ± SD.

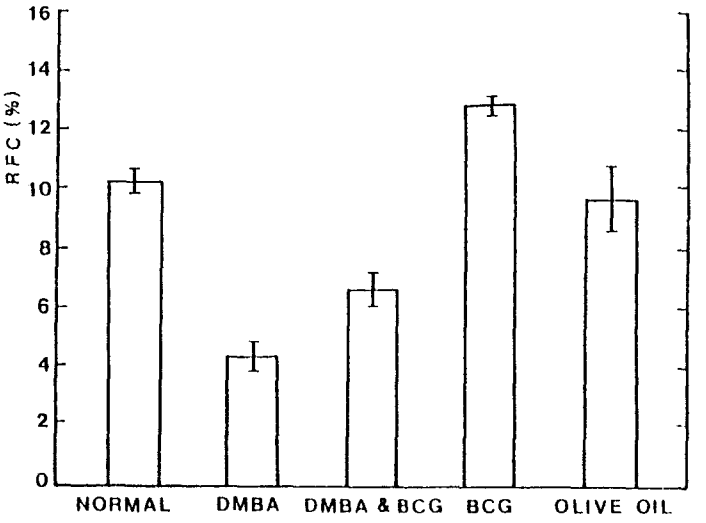

Fig. 2. Effect of DMBA and / or BCG on the antigen binding avidity of splenocyte. Lymphocytes binding three or more SRBC were regarded as rosette forming cell (RFC) and calculated as following formula: RFC(%) = (No. of RFC / No. of total cell counted) × 100. Each bar represents Mean ± SD.

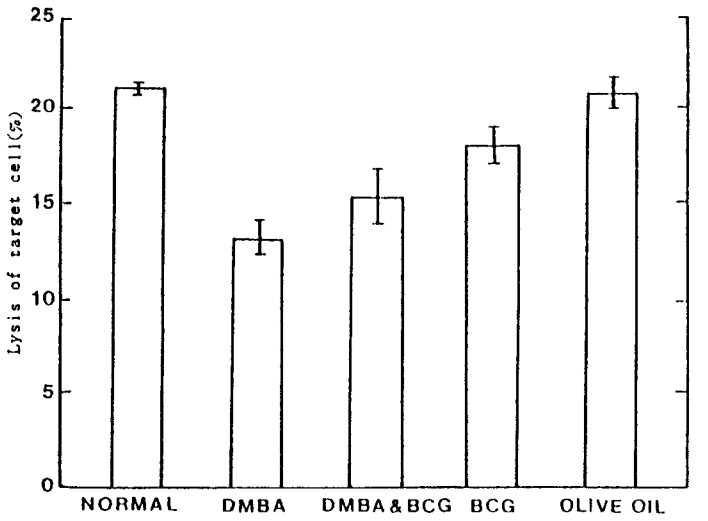

Fig. 3. Cytotoxicity of natural killer cell in mice treated by DMBA and/or BCG. Splenic NK cell assay measured using the 4 hr ^{51}Cr release method. Effector/target ratio = 100 : 1. Each bar represents Mean ± SD.

NK Cell Activity

Effect of DMBA or BCG on NK cell activity was examined. DMBA-treated group was mostly inhibited in comparison with any other groups (Fig. 3). Splenic NK cell activity was shown to be 13.4% in DMBA-, 15.3% in DMBA and BCG-, 18.6% in BCG-, and 20.7% in olive oil-treated mice. These results suggeted that BCG did not increase the activity of splenic NK cells.

DISCUSSION

NK cells comprise apparently heterogeneous population of the cells having a capability of lysing transformed cells that are not exposure previously to specific antigen (Barry, 1982). In present study, we noted that splenic NK cell activity in DMBA-treated mice showed cytotoxicity (13%) to YAC-1. The mice inoculated with BCG exhibited a low splenic NK cell activity (17.8%). The BCG treatment did not apparently increase the NK cells activity.

Biological therapy is considered as an new approach for the treatment of cancer. BCG has been known as a nonspecific immunostimulator. Lymphocytes immunized with BCG secreted macrophage activating factor (MAF), enhancing phagocytosis of RES (reticuloendothelial system) (John *et al.*, 1979). The course of cytotoxic cell appearance after BCG administration was rapid, peaking at 4 to 5 days. In contrast, BCG-activated macrophage was known to last for several weeks (Daniel *et al.*, 1977).

NK cell activity in DMBA treated-group was lower than any other group, and BCG did not increase the activity of splenic NK cells.

Rosette forming technique has been used method for the enumeration of antibody-forming cells in mice (Chung and Ha, 1986). ABC of DMBA treated mice was 4.3%, whereas 10.4% in normal. In contrast, ABC in BCG treated mice was 13%. These results showed that BCG could initiate a potential of antibody production of B cells. Also, the results of HA and HE titers corresponded to those of ABCs. These data suggest that the level of antibody titer is related to the level of ABC.

Arthus reaction and DTH, which are mediated by activation of complement with antibody and population of specifically sensitized T cell, have been used to measure the immune reaction. It was reported that correlation between DTH and tumor immunity is possible (Halliday and Mary, 1969). In this connection, we observed that the levels of DMBA treated group decreased, whereas those of BCG treated group increased, suggesting a possibility of the increase of humoral and cellular immunity as proposed previously (Eugenia, 1980).

Splenic hyperplasia was concomitant with tumor growth and decreased mitogen responsiveness. Numbers of macrophage and Ts cell increased in the spleen of tumor bearing mice (Klaus, William, 1978). Spleen index of DMBA treated-mice was to be 82% in current experiment, which was responsible for immune suppression.

While tumor induced by DMBA suppresses host immune response, BCG reverses the immune response and may have antitumor effects. Some publication have described the use of BCG to faciliated tumor growth (Robert *et al.*, 1974). Therefore, on the basis 8 the present experiment, it is desirable to establish an optimal dose for future study.

REFERENCES

Allan, B., Martin, R., Geoige, T. and John, S. (1984): Activation of the mouse cellular Heavy-ras gene in chemically induced benign skin papillomas, *Nature*, **307,** 658-660.

Anthony, D., Margaret, P., Robert, C. and Nina, C. (1983): Evidence That Binding of 7,12-Dimethylbenz(a)anthracene to DNA in mouse embryo cell cultures results in extensive substitution of both adenine and guanine residues, *Cancer Res.*, **43,** 4132-4135.

Barry, R.B. (1982): Natural killers to rescue immune surveillance?, *Nature*, **300,** 214-215.

Daniel, E.T., Seth, A.W., Jeannine, M.D. and Christopher, S.H. (1977): BCG-induced murine effector cells, *J. Immun.*, **119,** 1145-1158.

Elizabeth, C.M. (1978): Some current perspectives on chemical carcinogenesis in humans and experimental animals, *Cancer Res.*, **48,** 1479-1496.

Eugenia, H. (1980): Induction of delayed-type hypersensitivity and antitumor immunity by systemic BCG, *Cell. Imm.*, **50,** 136-152.

Ha, T.Y., Park, Y.M., Kim, C.K. (1987): Effect of thermal injury on cellular and humoral immune responses, *Korean J. Immun.*, **9,** 155-167.

Halliday, W.J. and Marry, W. (1969): Delayed hypersensitivity to chemically induced tumors in mice and correlation with an *in vitro* test, *J. Nat. Cancer Inst.*, 434, 141-149.

Ian, R.T. (1988): *Immunology: an introduction.* (Saundersw college, Philadelphia), 422-430.

Jan, K. (1982): *Immunology, the science of self-nonself discrimination* (Wily-Interscience New York), 623-648.

John, B.H., Read, R.T., Harold, A.C. and Weinberg, J.B. (1977): Macrophage tumor killing: influence of the local environment, *Science*, **197,** 279-282.

Chung, H.T. and Ha, T.Y. (1986): Cellular interactions in the immune responses of the snakehead (Channa asiatica), *Korea J. Immun.*, **2,** 127-136.

Klaus, D.E. and William, L.F. (1978): Suppressor cell activity in tumor-bearing mice, *J. Immunol.*, **120,** 1345-1353.

Naysmith, J.D., Elson, C.J., Margaret, D., Elain, F. and Maria, G.O. (1980): Anti-erythrocyte autoantibody production in mice associated with the injection of rat erythrocytes, *Immun.*, **39,** 469-479.

Robert, C.B., Berton, Z., Tibor, B. and Herbert, J.R. (1974): BCG and cancer, *New England J. Med.*, **290,** 1413-1420.

Sanjoy, K.D., Christopher, R.D., Abhik M.B., Michelle, M., William, M.B. and Mihir, R.B. (1989): Fate of 7,12-Dimethylbenz(a)anthracene in the mouse mammary gland during initiation and promotion stages of carcinogenesis *in vitro*, *Cancer Res.*, **49,** 920-924.

Stewart, S. (1987): *Immunology, immunopathology and immunity.* (Elsevier, New York), 725-771.