Butylated hydroxyanisole affects immunomodulation and promotes macrophage phagocytosis in normal BALB/c mice

FANG-MING HUNG[1], YING-YING CHUANG[2,3], CHING-SUNG LEE[4], YUNG-LIANG CHEN[3], JAI-SING YANG[5], JEN-JYH LIN[6,7], KUNG-WEN LU[8], HUI-YING HUANG[9], CHIEN-CHIH YU[10], HSU-FENG LU[2,4*] and JING-GUNG CHUNG[11,12*]

[1]Department of Surgical Intensive Care Unit, Far Eastern Memorial Hospital, New Taipei 220; [2]Department of Clinical Pathology, Cheng Hsin General Hospital, Taipei 112; [3]Department of Medical Laboratory Science and Biotechnology, Yuanpei University, Hsinchu 300; [4]Department of Restaurant, Hotel and Institutional Management, Fu-Jen Catholic University, New Taipei 242; [5]Department of Pharmacology; [6]Graduate Institute of Chinese Medicine; [7]Division of Cardiology; [8]School of Post-Baccalaureate Chinese Medicine; [9]Department of Nutrition; [10]School of Pharmacy; [11]Department of Biological Science and Technology, China Medical University, Taichung 404; [12]Department of Biotechnology, Asia University, Taichung 413, Taiwan, R.O.C.

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Abstract. Butylated hydroxyanisole (BHA), a synthetic antioxidant, has been used in fat and fatty foods to prevent oxidative deterioration. However, the functions of BHA on immune responses in normal mice remain elusive. The aim of the present study was to investigate the effects of oral treatment of BHA on immune responses in normal mice *in vivo*. BALB/c mice received various treatments. Blood samples were collected and analyzed. Flow cytometry was used to determine the levels of the cell markers. Results showed that BHA did not significantly affect the weight of the animal body and spleen in normal mice. BHA promoted macrophage phagocytosis from peripheral blood mononuclear cells, but did not alter this process in the peritoneal cavity. Furthermore, BHA did not influence natural-killer cell cytotoxicity in normal mice. Notably, BHA promoted the levels of CD3 (T cells) and decreased the level of CD19 (B cells), but did not significantly affect the levels of CD11b (monocytes) and macrophages (Mac-3) in normal mice. Based on these observations it can be concluded that BHA promotes immune responses by increasing T cells and activating phagocytosis by macrophages in normal mice. However, the molecular mechanisms require further investigation.

Correspondence to: Dr Jing-Gung Chung, Department of Biological Science and Technology, China Medical University, No. 91 Hsueh-Shih Road, Taichung 40402, Taiwan, R.O.C.
E-mail: jgchung@mail.cmu.edu.tw

Dr Hsu-Feng Lu, Department of Clinical Pathology, Cheng Hsin General Hospital, No. 45, Cheng Hsin St., Taipei 112, Taiwan, R.O.C.
E-mail: ch1835@chgh.org.tw

*Contributed equally

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Introduction

Leukemia is one of the leading causes of death in humans, and numerous efforts towards the development of adjuvant and neoadjuvant therapies have been made to improve survival rate (1). Leukemia is generally poorly responsive to conventional treatment modalities, such as chemotherapy and radiation therapy (2). Chemotherapeutic agents may induce toxicity and inherent resistance, but there is no consensus regarding optimal therapeutic agents in leukemia. Thus, the development of novel approaches to prevent and treat leukemia is essential.

Butylated hydroxyanisole [BHA; 2(3)-tert-butyl-4-hydroxyanisole] is a synthetic phenolic compound, which is widely used as an antioxidant for food products, pharmaceuticals and cosmetics (3,4). BHA has been classified as a 2B compound and a possible carcinogen to humans based on the International Agency for Research on Cancer (5). BHA has been reported to be a forestomach carcinogen in rats, mice and hamsters (6,7). However, findings of other reports have demonstrated that BHA shows no evidence of genotoxic activity (8,9) and it prevents chemically-induced tumorigenesis in certain animal tissues (10,11).

Although many studies described the effects of BHA in cancer cell lines *in vitro*, there is no available information regarding whether BHA affects immune responses of normal mice. Thus, the present study investigated the BHA effect on immune responses in normal mice *in vivo*. We found that BHA promotes the T-cell level and macrophage phagocytosis *in vivo*.

Materials and methods

Materials and reagents. BHA, dimethyl sulfoxide (DMSO), propidium iodide (PI) and trypan blue were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). Pharm Lyse™ lysing buffer was purchased from BD Biosciences (San Jose,

CA, USA). FITC anti-mouse CD3, PE anti-mouse CD19, PE anti-mouse Mac-3 and FITC anti-mouse CD11b antibodies were purchased from BD Pharmingen Inc. (San Diego, CA, USA). The PHAGOTEST kit was obtained from Glycotope Biotechnology GmbH (Heidelberg, Germany). RPMI-1640, fetal bovine serum (FBS), penicillin-streptomycin and L-glutamine were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA).

Male BALB/c mice. Forty male BALB/c mice, 8 weeks of age and 22-28 g in weight were obtained from the National Laboratory Animal Center (Taipei, Taiwan, R.O.C.)

BHA treatment. BALB/c mice were randomly divided into four groups to receive different treatments. Group I served as a control (n=10). Group II mice were treated with olive oil as vehicle (n=10). Group III mice were treated with BHA (100 mg/kg) in olive oil (n=10). Group IV animals were treated with BHA (200 mg/kg) in olive oil (n=10). BHA was administered by oral gavage to the treatment groups daily for up to 3 weeks before being weighed (12).

Body and spleen tissue weights. At the end of treatment, the animals were weighed and blood was withdrawn. Spleen samples were isolated and weighed individually (12).

Immunofluorescence staining for surface markers from normal mice after exposure to BHA. At the end of the treatment, blood samples from the animals were collected and analyzed and individually exposed to 1X Pharm Lyse™ lysing buffer (BD Pharmingen Inc.) for lysing of the red blood cells. The samples were then centrifuged for 15 min at 1,500 rpm at 4˚C. The isolated white blood cells from each animal were stained using FITC anti-mouse CD3, PE anti-mouse CD19, PE anti-mouse Mac-3 and FITC anti-mouse CD11b antibodies before being analyzed to determine the levels of the cell markers using flow cytometry (FACS Calibur™ ; Becton-Dickinson, NJ, USA) as previously described (13,14).

Quantification of phagocytic activity of macrophages. Macrophage phagocytosis used the PHAGOTEST kit, as previously described (15). At the end of the treatment, a total of $1x10^5$ leukocytes from peripheral blood mononuclear cells (PBMCs) or peritoneal cavity in 100 μl whole blood from individual animals of each group were incubated for 1 h at 37˚C with fluoresce in isothiocyanate-labelled *E. coli* (20 μl). The quenching solution (100 μl) was added to the reaction according to the manufacturer's instructions. After the completion of phagocytosis by monocytes/macrophages, DNA was stained according to the manufacturer's protocol. Cells from each animal were analyzed by flow cytometry, as previously described (16). Fluorescence data were collected on 10,000 cells and analyzed using the BD CellQuest software (15,16).

Quantification of natural-killer (NK) cell cytotoxicity. A total of $1x10^5$ leukocytes from the spleens of BHA-treated animals in 1 ml of RPMI-1640 were individually cultured in each well of 24-well culture plates for 24 h. Then, $2.5x10^7$ of YAC-1 cells were cultured in 15-ml tubes with serum-free RPMI-1640 medium. PKH-67/Dilunt C buffer (Sigma-Aldrich Corp.) was added to the cells, mixed thoroughly for 2 min at 25˚C, then 2 ml PBS was added for 1 min. Finally, 4 ml of RPMI-1640 was added for a 10-min incubation, followed by centrifugation at 1,200 rpm at 25˚C. At the same time, the YAC-1 cells in 100 μl were placed on 96-well plates before the addition of the leukocytes from each treatment to the wells for 6 h and determination of NK cell activity by flow cytometry, as previously described (13,17).

Statistical analysis. The results were shown as the means ± SD and the difference between the BHA and control groups was analyzed using the Student's t-test. $P<0.05$ was considered to be significant.

Results

BHA affected the body and spleen weight in normal BALB/c mice. To investigate whether BHA affects the body and spleen weights of normal mice, body weights of each group were weighed, then spleen tissues were isolated and individually weighed at the end of the BHA treatment. Results shown in Fig. 1 indicate that BHA did not affect the body (Fig. 1A and B) and spleen (Fig. 1C and D) weights of each treatment group, compared to the untreated control group.

BHA affected the surface markers of whole blood cells from normal BALB/c mice. To investigate whether BHA affects the levels of the cell surface markers, leukocytes were isolated from BHA-treated or untreated groups, and then the levels of CD3, CD19, Mac-3 and CD11b were measured. Data are shown in Fig. 2A-D, and indicate that BHA significantly increased the levels of CD3 (100 and 200 mg/kg/day; 43.57 and 44.89%; Fig. 2A) and decreased the levels of CD19 (100 and 200 mg/kg/day; 16.53 and 22.74%; Fig. 2B), but did not significantly affect the levels of Mac-3 (Fig. 2C) and CD11b (Fig. 2D) when compared to the untreated groups.

BHA promoted phagocytosis by macrophages from PBMC and peritoneal cavity in normal BALB/c mice. To examine whether BHA affects phagocytosis, the leukocytes from PBMC and the peritoneal cavity of the BHA-treated or oliver oil-treated groups were isolated and phagocytic activity was determined. Results are shown in Fig. 3A and B, indicating that BHA promoted the activity of phagocytosis from PBMC (100 mg/kg/day, 12.86%; 200 mg/kg/day, 13.72%; Fig. 3A), but did not significantly affect the activity of phagocytosis from the peritoneal cavity (Fig. 3B).

BHA affected NK cell cytotoxicity of splenocytes from normal BALB/c mice. The results from NK cell cytotoxicity are shown in Fig. 4, indicating that both target YAC-1 cell ratios of 50:1 and 25:1 were not killed by NK cells from the BHA-treated group when compared to the untreated groups. Moreover, BHA at 100 and 200 mg/kg/day did not cause any significant difference when compared to the untreated groups (Fig. 4).

Discussion

In rodents, BHA and its demethylated metabolite tert-butylhydroquinone (tBHQ) act as a potent chemopreventive agent against tumor formation of a variety of carcinogens (14-16,18-21).

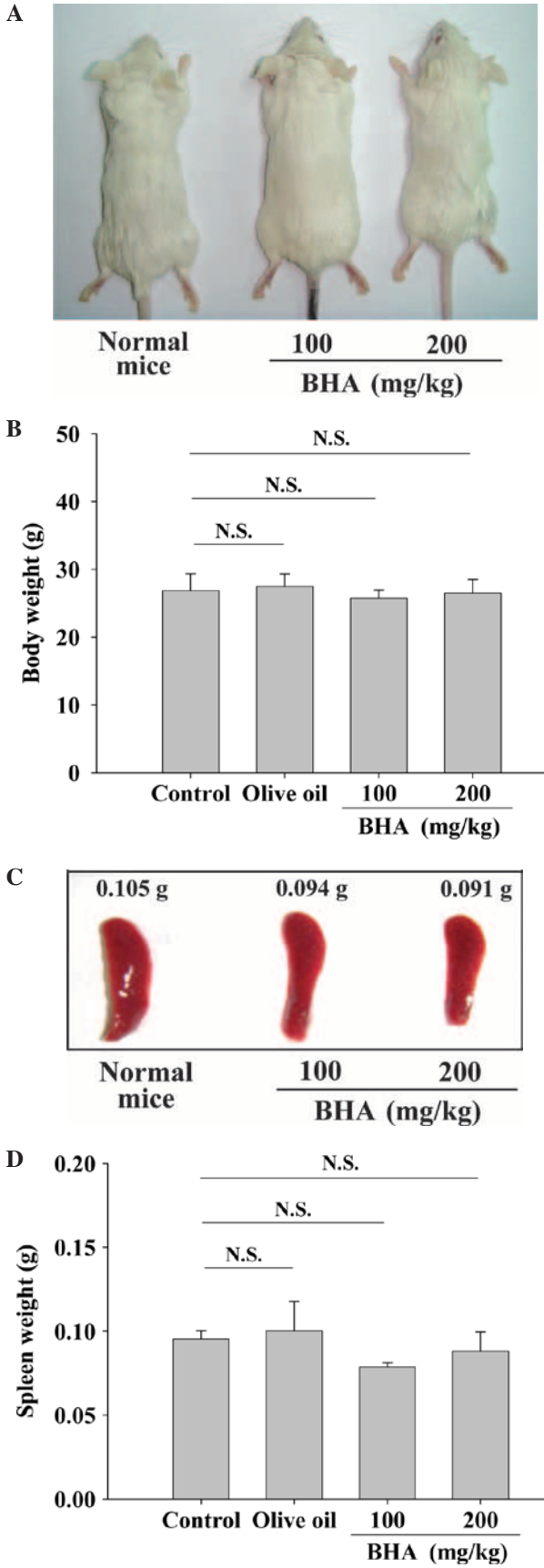

Figure 1. BHA affected the body and spleen weights of normal BALB/c mice. The mice were kept in the animal center for another 3 weeks and then orally treated with or without BHA (100 and 200 mg/kg/mouse) for 3 weeks. The (A) representative animal, (B) body, (C) representative spleen and (D) spleen weights were weighed as described in Materials and methods. Each point is the mean ± SD (n=10). *P<0.05 was considered significant when compared to the untreated normal mice.

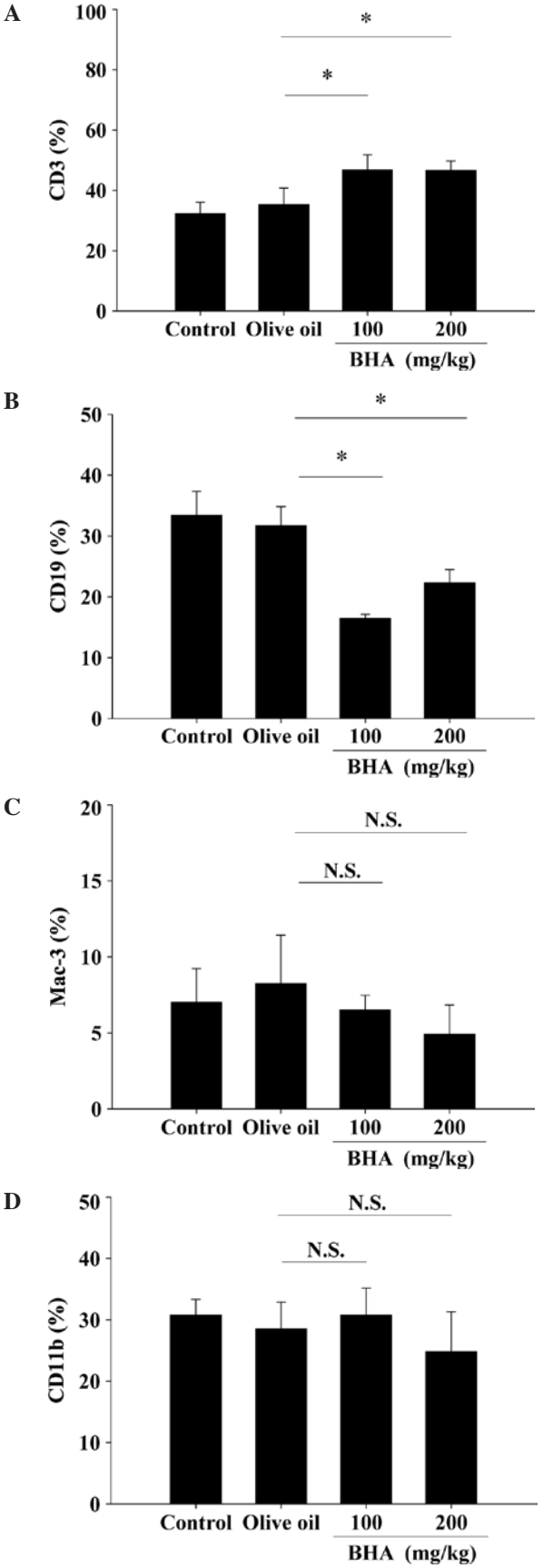

Figure 2. BHA affected the levels of cell markers in white blood cells from normal BALB/c mice at the end of the oral treatment with or without BHA for 3 weeks. Blood was collected from each group and was analyzed for cell markers using flow cytometry as described in Materials and methods: (A) CD3; (B) CD19; (C) Mac-3; (D) CD11b. Data are expressed as the means ± SD of three experiments. Each point is the mean ± SD (n=10). *P<0.05 was considered significant when compared to the untreated normal mice.

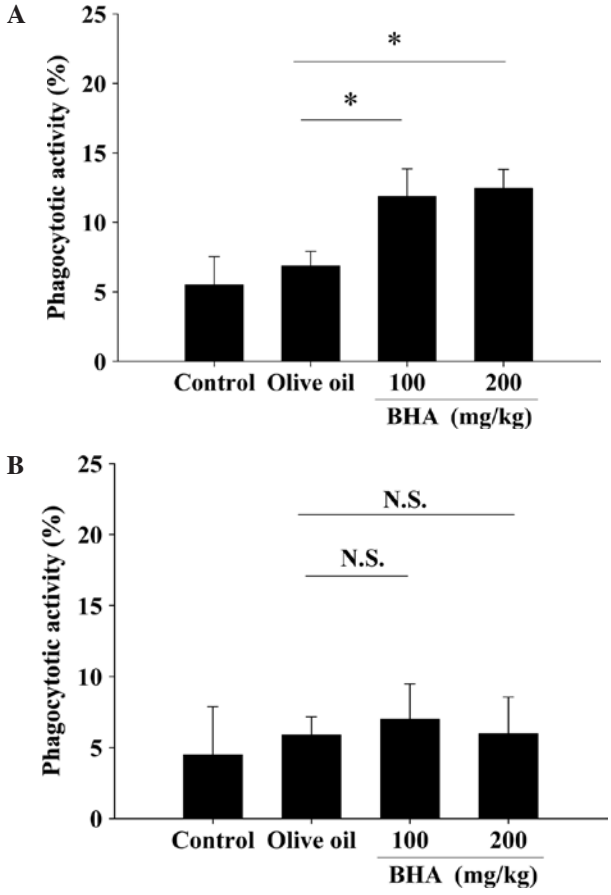

Figure 3. BHA affected the macrophage phagocytosis from normal BALB/c mice. The mice were orally treated with or without BHA for 3 weeks. Macrophages were isolated from (A) PBMC and (B) peritoneal cavity, and the percentages of phagocytosis with phagocyte green fluorescent particles (FITC-*E. coli*) were determined using flow cytometry, as described in Materials and methods. Each point is the mean ± SD. *P<0.05 was considered significant when compared to the untreated normal mice (n=10).

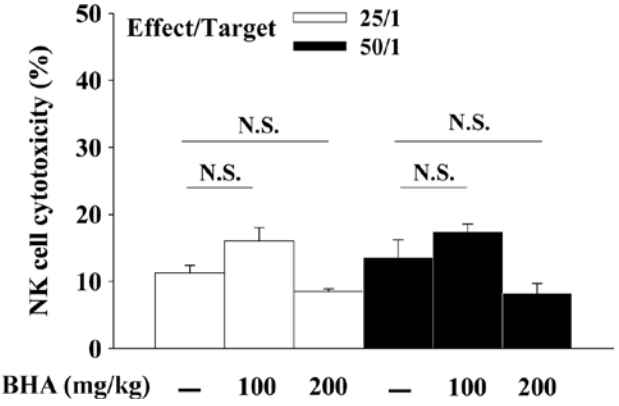

Figure 4. BHA affected the NK cell cytotoxicity from normal BALB/c mice. The mice were orally treated with or without BHA for 3 weeks. The YAC-1 target cells were killed by NK cells of splenocytes from the mice after treatment with or without BHA by oral administration at 100 and 200 mg/kg/day in target cell ratios of 25:1 and 50:1, as described in Materials and methods. Each point is the mean ± SD. *P<0.05 was considered significant when compared to the WEHI-3 leukemia mice (n=10).

BHA is also a potent inducer of phase II detoxifying enzymes, such as glutathione S-transferase (GST) and quinine reductase (QR) (22-24). Although various studies have demonstrated the protective effects of antioxidant BHA on carcinogen-induced carcinogenesis in animal model, these findings are also controversial. In particular, the protective effect was established to determine the influence of certain carcinogens acting upon tissues of given strains of animals. however, the carcinogens that were examined did not protect other tissues of different strains of animals against other types of carcinogens (4). There is currently no available information showing that BHA affects immune responses in normal mice *in vivo*. Thus, in the present study we investigated the oral administration of BHA, and then examined the immune responses of normal mice *in vivo*.

Results from flow cytometric assay for cell markers from normal mice after oral treatment of BHA indicated that the percentages of CD3 (T cells) were significantly increased and the levels of CD19 (B cells) were significantly decreased, but there was no significant difference in the levels of CD11b (monocytes) and Mac-3 (macrophages) (Fig. 2A-D). Furthermore, there was no effect on the body, liver (data not shown) or spleen weight of normal mice after oral treatment with BHA (Fig. 1A-C). We also found that BHA (100 and 200 mg/kg) promoted the phagocytosis of macrophages from PBMC (Fig. 3A) rather than that from peritoneal cavity (Fig. 3B). Both macrophage phagocytosis and NK cell cytotoxicity are involved in immune responses after animals are exposed to antigen (14-16). However, BHA (100 and 200 mg/kg) treatment did not alter the cytotoxicity of NK cells (Fig. 4). It has been reported that the estimated no-effect-level of BHA in food is approximately 250 mg/kg body weight per day (4).

BHA may fully exert its chemopreventive action by rendering the toxic free radicals, enhancing the process of excretion of foreign compounds, inhibiting the metabolic conversion of carcinogens to harmful derivatives, inhibiting the binding of these carcinogenic metabolites to the nuclear DNA and modifying the immune defense mechanisms of the organism (4).

Based on these observations, a conclusive result regarding BHA promoted immune responses could not be reached. Although we observed macrophage phagocytosis in mice after BHA exposure, there was a decrease of NK cell cytotoxicity. Further studies are required to determine whether or not BHA acts as a potent immunological adjuvant in animal models *in vivo*.

Acknowledgements

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