Arch. Pharm. Res. **10**(4), 223-227 (1987)

In Vivo Immunotoxicities of Butylated Hydroxyanisole (BHA) and Butylated Hydroxytoluene (BHT) in Male Mice

Chang-Kiu Moon, Soo-Hwan Lee, Kwang-Sik Park, Gwi-Seo Hwang, Myung-Soo Mock, Dong-Seok Chung, Dae-Dok Kim and Seok-Ki Min

College of Pharmacy, Seoul National University, Seoul 151, Korea
(Recieved October 29, 1987)

Abstract □ The effects of butylated hydroxyanisole and butylated hydroxytoluene on the immune status in normal male mice were evaluated. They exhibited the significant decrease in the circulating leukocyte counts. Relative spleen and thymus weights were slightly decreased, but not statistically significant. There were, however, significant liver hypertrophies in their exposed mice. Splenic IgM PFCs per one million cells in 1/20 LD50 BHA and BHT exposed mice were significantly reduced. IgM PFCs per spleen were similar to those of control, except in 1/20 LD50 BHA exposed mice, where they were significantly suppressed. The precise nature of the inhibition is not clear. Direct cytotoxicity is not responsible for the depressed antibody response, even following relatively high doses of them, because the changes in spleen cellularity are not significant. Both substances, however, did not show any effects on the arthus reaction and delayed hypersensitivity reaction induced by heat-aggregated bovine serum albumin, and *in vivo* phagocytosis of colloidal carbon. In the light of the present results, *in vivo* antibody response as well as *in vitro,* may be sensitive to BHA and BHT. Further elucidation of the precise nature of antibody suppression in their exposed mice, is warranted.

Keywords □ Butylated hydroxyanisole, Butylated hydroxytoluene, Immunotoxicity, Circulating leukocyte count, IgM plaque forming cell, Arthus reaction, Delayed hypersensitivity reaction, and *In vivo* phagocytosis.

Antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), have been widely used as food additives in various processed foods. Indeed, several tests of acute and chronic toxicities of BHA and BHT have not revealed any adverse effects and from the results, the use of these compounds has generally been believed to be without hazards[1-7]. Although there are several reports of enhancement of tumor promotion in animals[8-10], in many cases, these antioxidants have exhibited cancer protective effects[11-13].

Recently, myriad attention has been paid to the potential *in vitro* immunotoxicities of dairy BHA and BHT[14]. BHA and BHT do not only suppress the humoral immune response but also inhibit T cell mitogenesis and r-interferon production[15-21]. Despite considerable investigation into the immunotoxicities of BHA and BHT in cell culture, the functions of the immune system in BHA and BHT-exposed mice, however, have not been established. In this report, we examined the immunotoxic effects of exposure to BHA and BHT in mice.

EXPERIMENTAL METHODS

Animals

Male CBA/J inbred mice, male ICR mice purchased from Experimental Animal Center of Seoul National University, 6 to 8 weeks of age, weighing 18-22 grams, were used. The animals were housed 5-10 per cage in polyprophylene cages on hardwood chips and acclimatized for at least 5 days prior to use. The animal room was mechanically maintained on a diurnal cycle of 12 hours-interval (lightening; 7:00-19:00). The room temperature was maintained at 20-24 °C and relative humidity at 50-60%. A defined laboratory rodent chow (Sam Yang Ind. Ltd) and municipal tap water were provided *ad libitum.*

Chemicals

BHA and BHT were purchased from Wako pure chemicals (Japan) and dissolved in soybean oil. The doses were 1/200 and 1/20 of lethal dose 50(LD50) and administered orally for ten consecutive days.

Experiments were made at 9:00-10:00 a.m., taking into consideration of the chronobiological aspects of immune responses[22,23].

Circulating leukocytes and relative immunoorgan weights

Blood samples for leukocytes in male ICR mice were collected from retro orbital plexus and the number of leukocytes was counted on hemacytometer. Body, spleen, thymus and liver were weighed and relative spleen, thymus and liver weights were calculated.

IgM plaque forming cells assay

Splenic IgM antibody forming cells to thymic dependent antigen, sheep RBC were quantified by the modified Cunningham's liquid monolayer slide method, 4 days following a single intraperitoneal injection with 0.2m*l* of a 2% suspension of sheep-RBC(8×10^7) in male CBA/J inbred mice[24,25]. Spleen single cell suspension was prepared in BSS (pH. 7.2) using a stainless steel #200 sieve and adjusted to a cell concentration of 2×10^7/m*l*. Reaction mixture was composed of spleen cell suspension (50μl), 12.5% sheep RBC(100μl), 1/5 diluted complement (100μl, Gibco) and BSS (250μl). The capacity of microchamber was slightly more than 50μl. 50μl of reaction mixture was pipetted into each chamber in quadraplicate per sample.

In vivo phagocytosis

Intravascular phagocytosis by reticuloendothelial system, particularly kupffer cell and splenic macrophage, was evaluated in ICR mice. Colloidal carbon (80mg carbon/m*l*, 2-2.5μm in diameter, Pelikan drawing ink, D-3000 Hannover 1, 17 Black India, Germany) suspended in 1% gelatin-saline was injected intraveously at a dose of 16mg carbon per 100 grams of body weight, as previously described[26,27]. Blood was collected from retro orbital plexus at 5 minutes interval after carbon administration. Aliquot of blood (20μl) was lysed in 2m*l* of 0.1% sodium carbonate and carbon absorbances were determined using a UV spectrometer at 600nm. Phagocytosis was expressed as the phagocytic index and corrected phagocytic index.

Delayed hypersensitivity and arthus reaction

Male ICR mice were sensitized by subcutaneously injecting 100 g of bovine serum albumin (Sigma) emulsified in complete Freund's adjuvants (Sigma) at the base of tail. Seven days later DH and arthus reaction were elicited by challenging mice in the footpad with 30μl of 2% heat aggregated BSA in saline, as previously described[28,29]. At 3 and 24 hours post-challenge, footpad swelling thickness was measured with micrometer (Mitsutoyo, Japan) and the extent of swelling was calculated by substracting the thickness of the negative footpad from that of the antigen injected footpad.

Statistical analysis

The Student's t-test was employed to assess the statistical significance of the treatment effects.

RESULTS

Effects on the circulating leukocytes and relative immunoorgan weights

Table I presents the changes in circulating leukocyte counts and relative immunoorgan weights in normal male ICR mice. There were significant reductions in circulating leukocytes in their exposed mice. Oral exposure to 5mg/kg BHT, 50mg/kg BHT, 10mg/kg BHA and 100mg/kg BHA decreased the leukocyte, 83.6%, 81.8%, 75.0% and 56.1%, respectively. More supressive effects were made in BHA than in BHT-exposed mice. There were no significant differences of relative spleen and thymus weights. Hepatic hyper-

Table I. Effects of BHA and BHT on the circulating leukocytes and immunoorgans in male ICR mice[a]

Group	Leukocytes/mm^3	spleen/body(10^{-3})	thymus/body(10^{-3})	liver/body(10^{-2})
Control	10,763 ± 911	6.28 ± 0.32	1.88 ± 0.16	6.12 ± 0.13
BHT(5mg/kg)	9,000 ± 742	5.38 ± 0.51	2.04 ± 0.16	6.32 ± 0.38
BHT(50mg/kg)	8,800 ± 509[b]	5.45 ± 0.40	1.99 ± 0.30	6.55 ± 0.30[c]
BHA(10mg/kg)	8,070 ± 180[c]	5.07 ± 0.41	1.53 ± 0.30	6.62 ± 0.07[c]
BHA(100mg/kg)	6,038 ± 883[d]	5.59 ± 0.44	1.78 ± 0.33	7.11 ± 0.28[c]

a; Assay was made as described in experimental methods. The data signify the arithmetic mean ± SE in groups of mice 5 in number.

Statistical significance; b *vs* control; $p<0.1$, c *vs* control; $p<0.05$, d *vs* control; $p<0.01$

Table II. Effects of BHT and BHA on the IgM plaque forming cells in normal male CBA/J inbred mice[a]

Group	IgM PFCs/10^6viable spleen cells	IgM PFCs/spleen
Control	383 ± 26	42,801 ± 2,210
BHT(5mg/kg)	308 ± 47	43,365 ± 3,040
BHT(50mg/kg)	234 ± 32[b]	42,035 ± 5,272
BHA(10mg/kg)	304 ± 28	45.967 ± 6,075
BHA(100mg/kg)	165 ± 29[c]	22,407 ± 1,461[c]

a: Mice were immunized with 8×10^7 SRBC intraperitoneally. Four days after immunization, assay was made. The values represent the arithmetic mean ± standard error from 5 mice. Spleen cellularity was not significant.
Statistical significance; b *vs* control; $p<0.05$, c *vs* control; $p<0.01$.

trophies were, however, exhibited in their exposed mice and commensurate with the other reports[2,3].

Effects on IgM PFCs

The effects on the capacity of B cell, T cell and macrophage to cooperate in the production of antibody to thymic dependent antigen, sheep RBC as humoral immunity, were evaluated in normal male CBA/J inbred mice. Table II indicated that exposures to BHA and BHT significantly tend to depress the IgM PFCs per one million spleen cells. Exposure to 5mg/kg BHT, 50mg/kg BHT, 10mg/kg BHA and 100mg/kg BHA inhibited them, 80.4%, 61.1%, 80.8% and 43.1%, respectively. No reduction of IgM PFCs per spleen was exhibited, except in 100mg/kg BHA exposed mice, where significant suppression of IgM PFCs per spleen was made by 52.3% in 100mg/kg (1/20 LD50) BHA exposed-mice. There were no differences in splenic cellularities (Data not shown).

Effects on the delayed hypersensitivity and arthus reaction

DH and arthus reaction in male ICR mice immunized subcutaneously with 100μg BSA in complete Freund's adjuvant, were determined by challenging their footpad with 30μl of 2% heat aggregated BSA and the results were set out in Table III. BHA and BHT did not show any differences in the DH and arthus reaction.

Effects on the in vivo phagocytosis

The nonspecific phagocytic activities of splenic macrophages and kupffer cells were evaluated in male ICR mice. As set out in Table IV, no significant changes in phagocytic index were found. Significant reductions of corrected phagocytic index in 50mg/kg BHT, 10mg/kg BHA and 100mg / kg BHA-exposed mice, were made by 91.1%, 90.4%, and 85.6%, respectively. Reduction of corrected phagocytic index are thought to be due in part to liver hypertrophies.

Table III. Effects of BHT and BHA on the arthus and delayed hypersensitivity reaction in male ICR mice[a]

Group	Footpad swelling thickness (10^{-1}mm)	
	Arthus (3 hour)	DH (24 hour)
Control	7.18 ± 1.18	4.12 ± 0.46
BHT(5mg/kg)	7.54 ± 0.87	4.33 ± 0.66
BHT(50mg/kg)	7.60 ± 0.46	4.92 ± 0.84
BHA(10mg/kg)	9.46 ± 0.72	4.47 ± 0.83
BHA(100mg/kg)	8.35 ± 0.83	4.43 ± 0.82

a: Groups of ICR mice were sensitized to 100μg BSA in CFA subcutaneously at the base of the tail. Seven days following sensitization, the mice were challenged with saline or 30μl of 2% HA-BSA in the footpad. At 3 and 24 hours after challenge the increase in thickness of the footpad was determined. The values represent the arithmetic mean ± standard error of the swelling response seen in groups of mice 7 in number.

DISCUSSION

As well known, it has become evident that environmental toxicants and food additives have adverse effects on the immune competence or host

Table IV. Effects of BHT and BHA on the *in vivo* phagocytosis of carbon as macrophage function in male ICR mice[a]

Group	Phagocytic index(10^{-3})	Correxted phagocytic index
Control	21.29 ± 1.46	4.17 ± 0.12
BHT(5mg/kg)	19.86 ± 2.47	4.10 ± 0.20
BHT(50mg/kg)	20.16 ± 0.78	3.80 ± 0.14[b]
BHA(10mg/kg)	19.60 ± 2.24	3.77 ± 0.16[b]
BHA(100mg/kg)	20.36 ± 1.02	3.57 ± 0.17[c]

a: *In vivo* phagocytosis of carbon was made as described in experimental methods. The data signify the arithmetic mean ± standard error from 5 mice.
Statistical significance: b *vs* control; $P<0.1$, c *vs* control; $P<0.05$

defence mechanism[30,31]. BHA and BHT have been used as antioxidants in food. Because of continuing public concern about the possible health risks associated with the use of BHA and BHT as well as of the paucity of *in vivo* immunotoxicological informations available, we began to examine the *in vivo* immunotoxicological effects of them. The present studies focus on the immunological status in BHA and BHT-exposed mice.

The primary finding of the present investigation is that humoral immunity was significantly reduced in normal male CBA/J inbred mice exposed orally to BHA and BHT, as well as *in vitro* (Table II). IgM PFCs per one million spleen cells were significantly reduced. The precise nature of the inhibition is not clear. No changes in DH and *in vivo* phagocytosis suggest that Helper T cell and macrophage are not particularly sensitive to their exposure. Direct cytotoxicity is not responsible for the depressed antibody response, even following relatively high doses of them, because the changes in spleen cellularity are not statistically significant. Those suppressive effects, therefore, might be based in part on the increased stress by the indirect toxicities in the host[1-7,32]. Care should be taken of no reduction of IgM PFCs per spleen (Table II). It is, however, noteworthy that significant suppression of IgM PFCs per spleen was made in 100mg/kg (LD50) BHA-exposed mice.

Circulating leukocyte count of control group in ICR mice was $10,763 \pm 911$ per μl of blood (Table I) and commensurate with the other report[33]. There were, however, significant leukocytopenia in BHA and BHT exposed mice. The precise nature of this leukocytopenia is not clear. It should be, especially, important to note that high percentage of lymphocytes (above 70%) in leukocyte counts is characteristic of rodents, which is different from the other animals[33]. It is, therefore, possible that those leukocytopenia may result from lymphocytopenia.

DH as representitive of cell mediated immunity, and carbon clearance as *in vivo* phagocytosis of splenic macrophage and kupffer cell were not changed. Because reductions in corrected phagocytic index are supposed to be derived from liver hypertrophies, nonspecific phagocytosis is considered not to be affected in BHA and BHT-exposed mice.

In the light of the present results, *in vivo* antibody response as well as *in vitro*, may be sensitive to BHA and BHT. Further elucidation of the precise nature of antibody suppression in their exposed mice, is warranted.

LITERATURE CITED

1. Deichmann, W.B., Gables, C., Clemmer, F.J.J., Rakoczy, R., and Bianchne, J.: Toxicity of ditertiary butylmethyl phenol. *Arch. Ind. Health.* **11,** 93 (1955).
2. Hodge, H.C., Fassett, D.W., Mayard, E.A., Downs, W.L., and Coye, R.D.: Chronic feeding studies of butylated hydroxyanisole in dogs. *Toxicol. Appl. Pharmacol.* **6,** 512 (1964).
3. Kerr, R., Lefeure, A., Lane, B.P., and Lieber, C.S.: Liver growth induced by butylated hydroxytoluene (BHT). *Clin. Res.* **14,** 229 (1967).
4. Marino, A.A., and Mitchell, J.T.: Lung damage to mice following intraperitoneal injection of butylated hydroxytoluene. *Proc. Soc. Exp. Biol. Med.* **140,** 122 (1972).
5. Witschi, H., and Saheb, W.: Simultation of DNA synthesis in mouse lung following intraperitoneal injection of butylated hydroxytoluene. *Proc. Soc. Exp. Biol. Med.* **147,** 690 (1974).
6. Branen, A.L.: Toxicology and biochemistry of butylated hydroxyanisole and butylated hydroxytoluene. *J. Am. Oil Chem. Soc.* **52,** 59 (1975).
7. Witschi, H., and Lock, A.: Toxicity of butylated hydroxytoluene in mouse following oral administration. *Toxicology* **9,** 137 (1978).
8. Ito, N., Fukushima, S. and Tsuda, H.: Carcinogenecity and modification of the carcinogenic response by BHA, BHT and other antioxidants. *CRC. Crit. Rev. Toxicology* **15,** 109 (1985).
9. Fukushima, S., Mirasaki, G., Hirose, M., Nakanish, K., Hasegawa, R. and Ito, N.: Histopathological analysis of preneoplastic changes during Nibutyl-N-(4-hydroxybutyl)-nitrosamine induced urinary bladder carcinogenesis in rats. *Acta. Pathol. Jpn.* **32,** 243 (1982).
10. Ito, N., Fukushima, S., Tamano, S., Hirose, M. and Hagiwara, A.: Dose response in BHA induction of forestomach carcinogenesis in F344 rats. *J. Natl. Cancer. Inst.* **77,** 1261 (1986).
11. Williams, G.M., Maeura, Y. and Weiberger. J.H.: Simultaneous inhibition of liver carcinogenecity and enhancement of bladder carcinogenecity of N-2-fluorenylacetamide by BHT. *Cancer. Lett.* **19,** 55 (1983).
12. Prochaska, H.J., deLong, M.J. and Talalay,

P.: On the mechanism of induction of cancer protective enzymes, a unifying proposal. *Proc. Natl. Acad. Sci.* **82**, 8232 (1985).
13. Slaga, T.J.: Can tumor promotion be effectively inhibited?, in "Models, Mechanisms and etiology of tumor promotion". Borzsozyi, M and Lapid, L(ed). *IARC. Sci. Publish.* No. 56, pp. 497-506 (1984).
14. Ball, S.S., Weindruch, R. and Walford, R.C.: Antioxidants and the immune responses. *Mod. Aging. Res.* **8**, 427 (1986).
15. Archer, D.L., Wess, J.A. and Smith, B.G.: Inhibitory effect of an antioxidant, BHA, on the primary *in vitro* immune response. *Proc. Soc. Exp. Biol. Med.* **154**, 289 (1977).
16. Archer, D.L. and Wess, J.A.: Chemical dissection of the primary and secondary *in vitro* antibody responses with BHA and gallic acid. *Drug. Chem. Toxicol.* **2**, 155 (1979).
17. Hoffeld, J.T.: Agents which block membrane lipidperoxidation enhance mouse spleen cell immune activities. *In vitro* relationship to the enhancing activity of 2-Mercaptoethanol. *Eur. J. Immunol.* **11**, 371 (1981).
18. Johnson, H.M. and Torres, B.A.: Leukotrienes; positive signals for regulation of r-interferon production. *J. Immunol.* **132**, 413 (1984).
19. Sciorra, L.J., Kaufmann, B.N. and Maier, R.: The effect of BHT on the cell cycle and chromosome morphology of PHA stimulated leukocyte cultures. *Food. Cosmet. Toxicol.* **12**, 33 (1974).
20. Wess, J.A. and Archer, D.L.: Restoration by cyclic monophosphate and extracellular calcium of BHA-suppressed primary murine thymus dependent antibody response. *Immunopharmacol.* **3**, 361 (1981).
21. Wess, J.A. and Archer, D.L.: Evidence from *in vitro* murine immunologic assays that some phenolic food additives may function as antipromotors by lowering cyclic GMP levels. *Proc. Soc. Exp. Biol. Med.* **170**, 427 (1982).
22. Fernandes, G., Yunis, E.J. and Halberg, F.: Circadian aspects of immune responses in the mice, in "Chronobiology of allergy and immunity". Macgovern, T(ed). Thomas, (1976).
23. Fernandes, G., Halberg, F., Yunis, E.J. and Good, R.A.: Circadian rhythmic plaque forming cell response of spleen from mice immunized with SRBC. *J. Immunol.* **117**, 962 (1977).
24. Cunningham, A.J. and Szenberg, A.: Further improvements in the plaque techniques for detecting single antibody forming cells. *Immunol.* **14**, 599 (1968).
25. Kissinger, R. and David Myl, A.: Improvements to the plaque assay for antibody secreting cells. *J. Immunol. Method* **66**, 377 (1984).
26. Biozzi, G., Benacerraf, B. and Halpern, B.N.: Quantitative study of the granulopectic activity of the reticuloenthelial system. II: A study of the kinetics of the granulopectic activity of the RES in relation to the dose of carbon injected. Relationship between the weight of the organs and their activity. *Brit. J. Exp. Pathol.* **34**, 441 (1954).
27. Stuart, A.E., Habesham, J.A. and Davison, A.E.: The carbon clearance test for *in vivo* phagocytosis, in "Handbook of experimental immunology (vol. 2)". Weir, D.M.(ed). Blackwell Sci. Publish. pp. 28.1-28.25 (1978).
28. Titus, R.G. and Chiller, J.M.: A simple and effective methods to assess murine Delayed type hypersensitivity to proteins. *J. Immunol. Method* **45**, 65 (1981).
29. Henningsen, G.M., Koller, L.D., Exon, J.H., Talcott, P.A. and Osborne, C.A.: A sensitive delayed type hypersensitivity model in the rats for assessing *in vivo* cell mediated immunity. *J. Immunol. Method* **70**, 153 (1984).
30. Vos, J.G.: Immune suppression as related to toxicology. *CRC. Crit. Rev. Toxicol.* **5**, 67 (1977).
31. Sharma, R.P.(ed): Immunological considerations in toxicology. vol. 1-2. CRC press (1981).
32. Riley, V.: Psychoneuroendocrine influences on immunocompetence and neoplasia. *Sci.* **212**, 1100 (1981).
33. Mitruka, B.M., and Rawnsley, H.M.: Hematological reference values, in "Clinical biochemical and hematological reference values in normal experimental animals and normal humans(2ed). Masson. Pub. Co. p. 60 (1981).