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Inhibitory Effect of an Anti-oxidant, Butylated Hydroxyanisole, on the Primary *in Vitro* Immune Response (39656)

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Butylated hydroxyanisole (BHA) is a low molecular weight compound used commercially as an anti-oxidant in foods. BHA is considered generally recognized as safe (GRAS) by the Food and Drug Administration, and foods containing BHA are, therefore, exempt from premarket clearance. In 1974, the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives reported that 0–0.5 mg/kg body weight of BHA, 0–0.5 mg/kg body weight of butylated hydroxytoluene (BHT), or a total of 0.5 mg/kg body weight of both BHA and BHT would be an acceptable daily intake for man (1). The average amount of BHA and BHT consumed daily in the United States was estimated to be 0.2 mg/kg of each food additive for adults (2). The estimated intakes were thought to be higher than actual intakes because a calculation using manufacturing and import data would show an intake of about one-half of the estimated levels. Nonetheless, the intake level is within the range considered acceptable by the FAO/WHO Expert Committee.

This report concerns the effects of BHA on the *in vitro* murine plaque-forming cell (PFC) response to a thymus-dependent antigen, sheep red blood cells (SRBC), and a thymus-independent antigen, *Escherichia coli* 0127:B8 lipopolysaccharide. The results indicate that BHA exerts a significant inhibitory effect early in the *in vitro* primary immune response at anti-oxidant concentrations that may occur in the gut and liver (3–5). This *in vitro* effect and the fact that BHA may be present in the gut and liver at concentrations used in this study warrant further study of this additive as a potential immunosuppressant of some aspect of the immune response. The studies clearly show that more information needs to be obtained on the biological properties of anti-oxidants that are used as food additives.

Materials and methods. Animals. C57Bl/6 female mice, 8- to 12-weeks old, were obtained from Laboratory Supply Company, Indianapolis, Indiana or The Jackson Laboratory, Bar Harbor, Maine. Subsequently, it was shown that male C57Bl/6 mice give equivalent results. Athymic nude mice (NIH Swiss background) were supplied by the Laboratory Supply Company, Indianapolis, Indiana and were used upon delivery.

Antigens. SRBC were obtained from the Colorado Serum Company, Denver, Colorado. All SRBC used were from a single sheep, No. 446. *E. coli* 0127:B8 bacteria were treated exactly as by Johnson *et al.* (6) for the *in vitro* PFC response.

Anti-oxidant. Crystalline BHA (food grade) was obtained from ICN Pharmaceuticals, Cleveland, Ohio. Before use, BHA was solubilized in petroleum ether, washed twice with triple-distilled water, and recrystallized from petroleum ether under negative pressure. The BHA preparation was claimed to have greater than 98.5% purity as supplied, and is considered to be food grade material. Subsequent examination of the BHA preparation by thin layer and column chromatography supported this claim.

Cultures. SRBC *in vitro* PFC response were carried out as described by Mishell and Dutton (7) using 1.5×10^7 spleen cells/ml and 3×10^6 SRBC. Anti-*E. coli* 0127:B8 responses were carried out as previously described (6). The anti-oxidant was added to cultures at the time of SRBC addition in either of two ways. BHA was solubilized in mineral oil and filter sterilized, and appropriate dilutions were made in sterile mineral oil. The volume of added oil was kept constant at 100 μl, and results were compared with controls exposed to mineral oil without added BHA. BHA was also solubilized and

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diluted in absolute ethanol (EtOH). The BHA was then added to sterile 7-mm Whatman No. 1 filter paper disks, from which the EtOH was permitted to evaporate. One disk was added per culture. Results were compared with BHA-free control disks, which were exposed to EtOH alone. Direct PFC assays were performed on microscope slides according to Golub *et al.* (8). A single lot of fetal calf serum (Gibco, Grand Island, New York) No. 640521 was used throughout the study. All PFC responses were determined on Day 5.

Enumeration and viability of cells. The number of cells in each culture was determined by counting in a hemacytometer, and the percentage of viable cells was determined by trypan blue dye exclusion.

[^{3}H]Thymidine incorporation. DNA synthesis of Mishell–Dutton cultures was determined by the addition of 0.5 μCi of [^{3}H]thymidine (New England Nuclear, Boston, Massachusetts) in 10 μl of modified minimal essential medium (MEM) (7) to each culture for the final 18 hr of incubation. Trichloroacetic acid-insoluble material was counted in a Packard liquid scintillation counter (Model 5385). The results are based on the average of triplicate plates.

Mitogens. Concanavalin A (Con A; Nutritional Biochemical Corporation, Cleveland, Ohio), twice recrystallized, was stored at room temperature and diluted in culture medium for addition to cultures. Staphylococcal enterotoxin A (SEA) was produced by the Microbial Biochemistry Branch, Division of Microbiology, Food and Drug Administration, and its purity was estimated to be >99% by extinction coefficient (9).

Results. Effect of BHA on the in vitro PFC response to SRBC. As shown in Fig. 1, BHA inhibits the PFC response to SRBC by 91% at 50 μg/culture when added in an oil phase. BHA, though considered insoluble in water, appears to be capable of interacting with spleen cells in the aqueous phase. As shown, the viable cell recovery was not significantly reduced by levels of BHA in oil giving >90% inhibition of the PFC response. Figure 1 also demonstrates that, when administered as BHA impregnated disks, 25 μg of BHA/culture was sufficient for 90% inhibition of the PFC response.

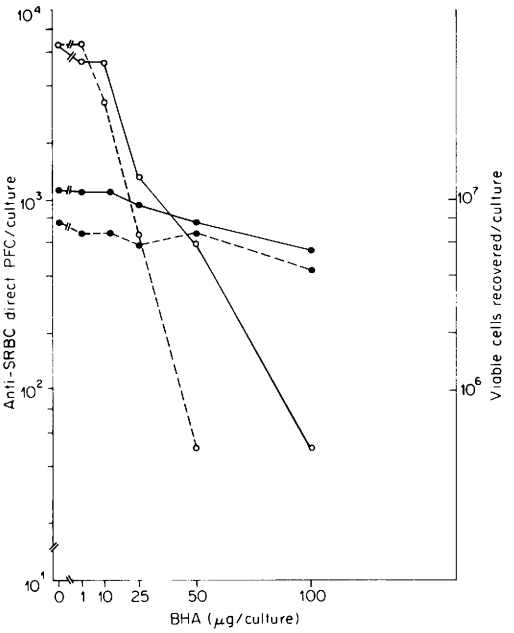

FIG. 1. Effect of butylated hydroxyanisole (BHA) on the *in vitro* primary immune response to SRBC. BHA was added at the time of SRBC addition in oil (BHA-o) and on cellulose pad (BHA-c). The direct anti-SRBC PFC/culture for BHA-o (○——○) and BHA-c (○-----○) as well as the viable cells recovered/culture for BHA-o (●——●) and BHA-c (●-----●) were determined on Day 5.

Again, BHA did not significantly reduce viable cell recovery at concentrations sufficient to give >90% inhibition of the PFC response. BHA also exerted an inhibitory effect on the primary immune response to a thymus-independent antigen, *E. coli* 0127:B8 (data not presented). This inhibitory effect was of the same magnitude as that for the thymus-dependent antigen SRBC. The addition of mineral oil or cellulose disks to antigen-stimulated cultures did not enhance or suppress the PFC response.

Effect of removal of BHA from cultures at 4 and 24 hr. Figure 2 shows that BHA caused a significant reduction of the spleen cell PFC response to SRBC when present for the first 4 or 24 hr of incubation. When spleen cells were removed from contact with the various concentrations of BHA at 4 or 24 hr, no significant loss of viability was noted. A 4-hr exposure to 100 μg of BHA/culture inhibited the PFC response by

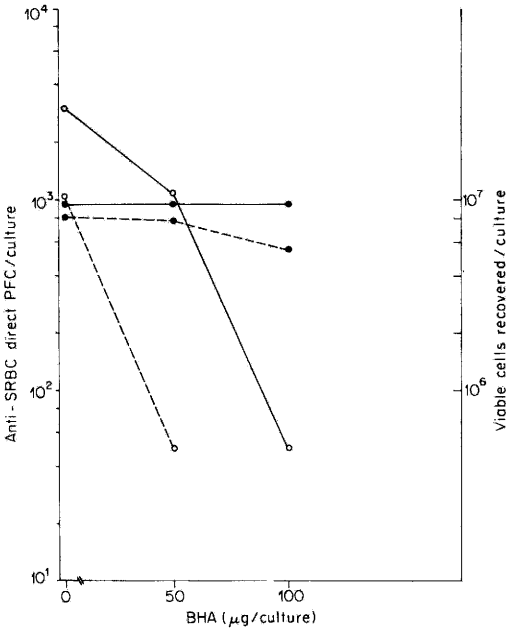

FIG. 2. Effect of removal of BHA at 4 (——) and 24 hr (-----) on the primary *in vitro* immune response to SRBC. The direct anti-SRBC PFC/culture (○) and viable cells recovered/culture (●) were determined on Day 5 for all cultures. BHA was added to cultures on impregnated cellulose disks. Disks were removed at the prescribed times, and the cultures were washed twice in modified MEM and placed in fresh plates and growth medium.

>98%. With a 24-hr exposure of cultures, 50 μg of BHA/culture was sufficient to give >95% inhibition of the PFC response.

Effect of addition of BHA at different times on the PFC response. BHA-impregnated cellulose disks (25 μg of BHA/disk) were added to cultures either at the same time (0 time) or at 24, 48, and 72 hr after SRBC addition. Figure 3 shows that the inhibitory effect of BHA on the PFC response to SRBC was decreased by delayed addition. PFC inhibitions were 99, 69, and 23%, respectively, for 0 time and 24 and 48 hr after addition. Slight stimulation of the PFC response was observed in cultures to which BHA was added at 72 hr. Cell viabilities showed no significant differences in treated versus control cultures.

Effect of BHA on DNA synthesis of C57Bl/6 spleen cells. The effects of BHA, added in oil or by disk, on [^{3}H]thymidine uptake of C57Bl/6 spleen cell cultures containing SRBC are shown in Table I. At 50 μg/culture, BHA in oil gave 43% inhibition of [^{3}H]thymidine uptake, whereas BHA added by disc gave 88% inhibition. Removal of 100 μg of BHA/culture at 4 hr resulted in >90% inhibition of [^{3}H]thymidine uptake. Removal of 50 μg of BHA/culture at 4 hr resulted in stimulation of [^{3}H]thymidine uptake (139%). The addition of mineral oil or cellulose disks did not enhance or suppress DNA synthesis as measured by [^{3}H]thymidine uptake. In general, the data indicate that BHA exerts an inhibitory effect on DNA synthesis at higher concentrations, while having a stimulatory effect at lower concentrations. The data further suggest a complex relationship between the PFC inhibitory concentrations of BHA and its ability to inhibit DNA synthesis. In general, concentrations that inhibited the PFC response also inhibited DNA synthesis, except in the case where 50 μg of BHA was removed from culture. Here, the PFC response was inhibited (Fig. 2), whereas DNA synthesis was slightly enhanced (Table I).

Effect of BHA on the PFC response and

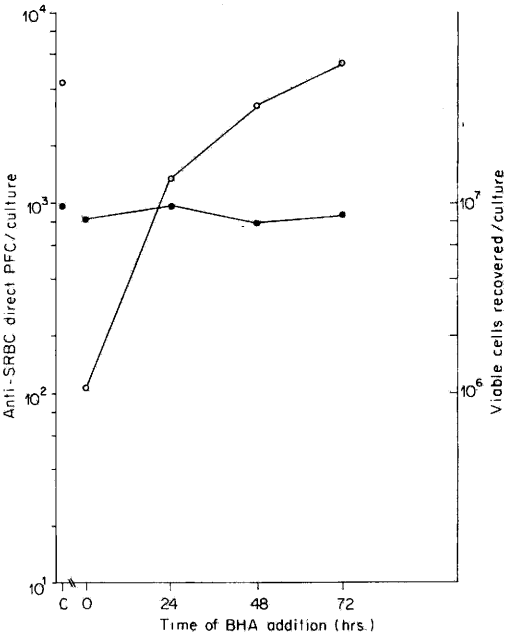

FIG. 3. Effect of addition of 25 μg of BHA (on cellulose) to mouse spleen cell cultures at the same times (0 time), and at 24, 48, and 72 hr after SRBC addition. The anti-SRBC PFC response (○——○) and viable cells recovered/culture (●——●) were determined on Day 5.

TABLE I. EFFECT OF BHA ON DNA SYNTHESIS OF C57Bl/6 SPLEEN CELLS

BHA (μg/culture)	[³H]Thymidine uptake (cpm × 10²)		
	BHA in oil[a]	BHA on discs[b]	BHA removed at 4 hr[c]
200	4.5[d]	not determined	1.2
100	29.6	1.4	28.2
50	260	43.9	667
25	363	274	727
10	635	456	366
1	299	not determined	not determined
control[e]	455	360	480

[a] Appropriate concentrations of BHA added to cultures in 100 μl of oil at the time of SRBC addition. Cultures harvested on Day 5.

[b] Appropriate concentrations of BHA added to cultures on disks at the time of SRBC addition. Cultures harvested on Day 4.

[c] Cultures washed free of BHA with two washes of modified MEM at 4 hr and placed in fresh plates and medium. Cultures harvested on Day 4.

[d] Data correspond to a BHA concentration of 250 μg.

[e] Contains 100 μl of BHA-free oil or EtOH-exposed disks, as indicated in Materials and methods.

DNA synthesis of athymic nude mouse spleen cells. Table II shows the effect of BHA on the PFC response of female athymic nude mouse spleen cells to the thymus-independent antigen, *E. coli* 0127:B8. BHA, 25 μg/culture, inhibited the PFC response by approximately 53%, and higher concentrations inhibited the response by >90%. In these experiments, BHA was added to cultures on disks and remained in contact with the cells for the full 5 days of culture. No significant loss of viability occurred in any of the cultures exposed to BHA. BHA, at 25 μg/culture or more, inhibited the uptake of [³H]thymidine by >99%. Interestingly, a corresponding decrease in the PFC response did not occur. Staphylococcal enterotoxin A, a T-cell mitogen (10), had no stimulatory effect on [³H]thymidine uptake by athymic nude mouse spleen cells. The same concentration of enterotoxin A increased [³H]thymidine uptake of C57Bl/6 cultures by 245% (data not shown). This is evidence of a lack of functional T cells in the nude mouse spleen cell cultures. High control counts in this experiment (Table II) were probably due to the large number of cells used per culture (1.5×10^7) and the presence of *E. coli O* antigen.

TABLE II. THE EFFECT OF BHA ON THE PFC RESPONSE TO *E. COLI* 0127:B8 AND DNA SYNTHESIS OF ATHYMIC NUDE MOUSE SPLEEN CELLS.

BHA (μg/culture)	Anti-*E. coli* 0127:B8 (direct PFC/culture)[a]	[³H]Thymidine uptake (cpm)[b]
100	<10	55
50	<10	58
25	340	52
Control[c]	725	13,380[d]
Enterotoxin A[e]	–	8,006

[a] PFC determination and [³H]thymidine uptake were separate experiments.

[b] Cultures consisted of 1.5×10^7 cells and were stimulated with 1×10^5 *E. coli* 0127:B8.

[c] BHA omitted.

[d] Unstimulated control (absence of *E. coli* 0127:B8) gave 10,200 cpm.

[e] Staphylococcal enterotoxin A added at 2 μg/culture.

Effect of BHA on [³H]thymidine uptake by Con A- and SEA-stimulated cultures. [³H]Thymidine uptake by C57Bl/6 spleen cells, stimulated with mitogenic doses of SEA (2 μg/ml) or Con A (5 μg/ml), was inhibited >99% by 100 μg of BHA/culture (data not shown). BHA at this concentration demonstrated no cytotoxic effect as determined by trypan blue dye exclusion. This suggests that BHA inhibits T-lymphocyte function.

Discussion. The results demonstrate that, in the Mishell–Dutton system, BHA has immunosuppressant activity at levels not cytotoxic. It appears that BHA affects both B- and T-cell function. (a) BHA inhibits the PFC response of C57Bl/6 spleen cells to both SRBC (Fig. 1) and to *E. coli* 0127:B8.

(b) BHA inhibits DNA synthesis by both nude mouse spleen cells (Table II) and Con A- and SEA-stimulated C57Bl/6 spleen cells. (c) BHA can inhibit the PFC response of athymic nude mouse spleen cells to *E. coli* 0127:B8 antigen (Table II).

Treatment of spleen cell cultures with BHA resulted in inhibition of both the PFC response to SRBC (Fig. 1). and [^{3}H]thymidine uptake by the same cultures (Table I) when BHA was present for the entire incubation period. BHA had to be added to cultures at the same time as SRBC, or no later than 1 day after SRBC, for inhibition of the PFC to occur (Fig. 3). When BHA was present for only the first 4 hr of culture, a concomitant inhibition of the PFC response and stimulatory effect on [^{3}H]thymidine uptake was observed (Table I). The mechanism of the PFC inhibitory effect of BHA appears, then, to be complex. Studies are currently in progress to determine if the immunosuppressant activity of BHA is due to loss of macrophage function. PFC suppression could possibly, in some cases, involve the induction of suppressor-cell activity; the measurement of possible mediators of suppressor-cell activity (12, 13) is in progress.

BHA is a commonly used food additive which is readily absorbed in the intestine and conjugated with glucuronic acid in the liver to form the o-glucuronide which is excreted in the urine (3, 5). Studies in humans using ^{14}C-labeled BHA have shown that, even 2 days after ingestion, the compound is not entirely cleared from the body (3), and residual BHA is present for prolonged periods after a single oral dose (4). The delay in excretion may be due to enterohepatic circulation (3–5), but, of all body tissues, the liver and small intestine are probably exposed to the highest levels of BHA and its metabolic products (5). The effect of ingested BHA on the immune response of the gut has not been determined; however, the gastrointestinal mucosa is rich in immunoglobulin-producing cells (14), and the consequences of BHA suppressive activity within the microenvironment of the gut are unknown.

The widespread use of BHA and its pattern of metabolism warrants further exploration into the mechanism of the biological effects of BHA on the immune response.

Summary. Butylated hydroxyanisole (BHA), an anti-oxidant food additive, inhibited the primary *in vitro* antibody (PFC) response of C57Bl/6 spleen cells to both a thymus-dependent antigen (SRBC) and a thymus-independent antigen (*E. coli* 0127:B8); the PFC response of athymic nude spleen cells to *E. coli* 0127:B8 was inhibited to the same degree. BHA inhibited both B- and T-lymphocyte function. BHA needed to be present only for the first 4 hr of culture to exert its inhibitory effect on the PFC response. DNA synthesis in C57Bl/6, athymic nude, and T-cell mitogen-induced C57Bl/6 spleen cell cultures was inhibited by BHA; however, short-term exposure of cultures to BHA at certain PFC inhibitory concentrations resulted in stimulation of DNA synthesis. The mechanism of the inhibitory effect of BHA on the *in vitro* PFC response is unknown, but may possibly involve activation of regulatory cell activity. Certainly, the data warrant further study of the effect of this ubiquitous food additive on the immune response.

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