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Evidence from *in Vitro* Murine Immunologic Assays That Some Phenolic Food Additives May Function as Antipromoters by Lowering Intracellular Cyclic GMP Levels (41453)

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Abstract. Butylated hydroxyanisole was previously shown to suppress murine *in vitro* immune responses by inhibiting guanylate cyclase function resulting in decreased levels of cyclic GMP. Like butylated hydroxyanisole, butylated hydroxytoluene, methyl paraben, and gallic acid suppress *in vitro* immune responses, the suppression being reversible by adding exogenous dibutyryl cyclic GMP or Ca^{2+}. Since there is a correlation between the strength of tumor promoters and their ability to cause intracellular increases in cyclic GMP, the data suggest that (i) some phenolic food additives may protect from carcinogens by acting as antipromoters in that they lower cyclic GMP levels and (ii) the *in vitro* immune response may be a useful system in which to study promoter–antipromoter interactions.

The phenolic antioxidants butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PrGal), the microbial growth inhibitor methyl paraben (MP), and the natural food constituent gallic acid (GA) are powerful immunosuppressants of murine *in vitro* antibody synthesis and lymphocyte proliferation (1–4). The biochemical mode of action of the phenolic antioxidants in immunosuppression remains undefined, but similarities exist in their immunosuppressive behavior and that of virus-type interferon (IFN) in murine systems. Like IFN, the phenolic food additives exert their suppressive effect at an early stage of the *in vitro* immune response and this, with some of the chemicals, is reversible with sulfhydryl-protective thiols such as 2-mercaptoethanol and dithiothreitol (2, 4, 5). Following a period of interaction with lymphocytes, however, the suppression due to phenolic food additives and IFN is not reversible by thiols; in fact, the lymphocytes become suppressive to fresh lymphocytes in coculture (6, 7). Further evidence for a similar mechanism of action for phenolic antioxidants and IFN was recently demonstrated.Like interferon, GA, MP, and nordihydroguaiaretic acid (NDGA) inhibit clone formation of transformed cells (i.e., anticellular effect), but unlike the species specificity shown by IFNs, the phenolic compounds inhibited clone formation of both human and mouse cells *in vitro* (8). Furthermore, a direct correlation between the compound's ability to exert suppression of the immune response and its ability to inhibit clone formation was shown, and both phenomena were manifest at similar doses of chemical (8).

There have been reports concerning an *in vivo* antitumor effect of phenolic antioxidants (9). Wattenberg (reviewed in (10)), in an extensive series of reports, has shown that BHA and BHT protect against a variety of tumor types induced *in vivo* by a variety of carcinogens in both the mouse and rat (reviewed in (10)). Wattenberg has demonstrated that BHA selectively activates portions of the microsomal monooxygenase system which prevent reactive metabolite formation of some carcinogens (10), and additionally, that BHA also activates the conjugating enzymes glutathione-*S*-transferase and UDP-glucuronyl transferase, resulting in accelerated detoxification and excretion of carcinogens (10).

Coffey and Hadden (11), utilizing human lymphocytes, recently showed that the relative strength of tumor promoters is dependent on their ability to activate guanylate cyclase and raise cellular cyclic GMP levels. We recently reported that BHA's immunosuppressive activity in mouse lymphocytes could be reversed by exogenous

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cyclic GMP or extracellular calcium; the data suggested that BHA interfered with guanylate cyclase (12). We herein report that, like BHA, the immunosuppressive effects of BHT, MP, and GA are also reversed by exogenous cyclic GMP and calcium. The data suggest that these phenolic compounds may protect from carcinogens by an additional mechanism to those proposed by Wattenberg, that is, they may function as antipromoters.

Materials and Methods. BHA, BHT, GA, MP, NDGA, and PrGal were obtained from ICN Pharmaceuticals, Cleveland, Ohio. $N^2,O^{2'}$-dibutyryl guanosine 3′:5′-cyclic monophosphoric acid (dbc-GMP) was purchased from Sigma, St. Louis, Missouri. $CaCl_2$ was obtained from Mallinckrodt, St. Louis, Missouri.

Spleens from 6- to 8-week old female BDF_1 ($C57B1/6 \times DBA/2)F_1$ mice (Harlan Laboratories, Indianapolis, Ind., or Laboratory Supply Co., Indianapolis, Ind.) were prepared for culture and lymphocytes were cultured as previously described (4). Phenolic compounds were added to cultures by applying the desired dose as a 10-μl spot in absolute ethanol on plastic 35-mm culture dishes and permitting the ethanol to evaporate prior to addition of splenic lymphocytes (see tables for dose levels). RPMI 1640 medium (Microbiological Associates, Walkersville, Md.) containing 10% fetal calf serum and 10 μg/ml gentamycin was used for culturing cells. Otherwise, culture conditions were identical to those described by Mishell and Dutton (13). Cultures were immunized with 3×10^6 sheep erythrocytes (SRBC) and direct plaque-forming cell (PFC) assays were performed on Day 5 of culture by the method of Cunningham and Szenberg (14). dbc-GMP or Ca^{2+} (as $CaCl_2$) were added to cultures directly after immunization in culture medium and distilled water, respectively. Results are presented as mean direct PFC/culture ± SEM normalized to 1000 PFC/culture in controls to permit pooling of several experiments. Culture viabilities on Day 5 were determined by trypan blue dye exclusion; at the doses of inhibitors used, no effect on culture viability relative to control cultures was noted.

Results. The data presented in Table I demonstrate that BHT, MP, and GA effectively suppress the *in vitro* PFC response to the thymus-dependent antigen SRBC. Two other phenolic compounds, NDGA and PrGal, both antioxidants, were likewise potent suppressants. The data also show that 1 m*M* dbc-GMP added to MP-, BHT-, or GA-suppressed cultures effected at least partial reversal of suppression. In a previous report, it was demonstrated that BHA could completely offset the 3.7-fold increase in cGMP induced by lipopolysaccharide (LPS) when lymphocytes were incubated with BHA for 4 hr prior to LPS activation (12). Unlike BHA, BHT, MP, and GA, the suppressions induced by NDGA or PrGal were not reversed by dbc-GMP; this suggests that other mechanisms unrelated to cyclic nucleotides may be involved in NDGA- or PrGal-induced

TABLE I. EFFECT OF EXOGENOUS dbcGMP ON PHENOLIC CHEMICAL-INDUCED INHIBITION OF THE *in Vitro* ANTI-SRBC PFC RESPONSE

Culture additions	Inhibitors:	Direct PFC/culture normalized to 1000 PFC/culture[a]				
		MP, 25 μg/ml	BHT, 50 μg/ml	GA, 10 μg/ml	PrGal, 10 μg/ml	NDGA, 10 μg/ml
None[b]		1000 ± 165	1000 ± 142	1000 ± 165	1000 ± 165	1000 ± 162
Inhibitor		<80	264 ± 25	80 ± 80	<80	<80
Inhibitor + 1 m*M* dbcGMP		863 ± 61	1387 ± 77	1325 ± 115	<80	<80

[a] Data presented as the mean ± SEM of triplicate determinations normalized to 1000 PFC/culture, based on immunized controls, to enable pooling of multiple experiments. Plaque counts are background corrected prior to normalization (background PFC/culture varied from <80 to 300). Direct PFC/culture varied from 10,200 to 12,567 in immunized cultures.

[b] Control cultures immunized with SRBC; control cultures to which dbcGMP were added generally showed from 10 to 25% depression of PFC.

PFC suppression. In our previous report (12), it was also shown that Ca^{2+} could reverse BHA-induced PFC suppression in a dose-dependent manner. The data presented in Table II indicate that, as with BHA, the PFC suppression induced by BHT, MP, and GA are also reversed by addition of exogenous CA^{2+} (3–10 m*M*). The NDGA- and PrGal-induced suppressions of PFC were unaffected by Ca^{2+}, further suggesting a similarity in the modes of action of BHA, BHT, MP, and GA, and a different inhibitory mechanism manifest by NDGA and PrGal.

At the concentrations used in these experiments, none of the inhibitors, Ca^{2+} or cGMP reversible or irreversible, were cytotoxic (data not shown); this supports previous observations (1–3, 8, 12).

Discussion. The data demonstrate that, like BHA (4), BHT, MP, and GA exert an immunosuppression *in vitro* to the thymus-dependent antigen SRBC. The suppression induced by these phenolic compounds is reversed by the addition to cultures of either dbc-GMP (1 to 2 m*M*) or Ca^{2+} (3–10 m*M*). We previously established that BHA causes a reduction in the cGMP content of murine splenic lymphocytes, and can totally abrogate LPS-induced increase in cGMP (12). The same pattern of dbc-GMP and Ca^{2+} reversal was observed in BHT-, and MP-, and GA-suppressed cultures as is observed for BHA. This is suggestive of a similar mechanism for the action of these four phenolic compounds, all involving cGMP metabolism. Recently, Coffey and Hadden (15) have proposed a model for guanylate cyclase activation in which the membrane-bound, Ca^{2+}-dependent enzyme lipoxygenase converts arachidonic acid to hydroxy- and hydroperoxy- derivatives (HETE and HPETE). HETE and HPETE then activate guanylate cyclase probably by oxidizing sulfhydryl groups on the enzyme. The lipidsoluble phenolic antioxidants used in these experiments may function by lipoxygenase inhibition. The fact that NDGA- and PrGal-induced inhibitions were not reversed by exogenous cGMP or Ca^{2+} may merely indicate that NDGA and PrGal adversely affect other enzyme systems, while BHA, BHT, MP and GA demonstrate specificity for guanylate cyclase or lipoxygenase. NDGA has recently been shown to be capable of binding to DNA when activated by molecular oxygen in aqueous media; mercaptoethanol could prevent the activation (18).

Preliminary evidence obtained in this laboratory also indicates that phorbol myristate acetate (PMA) added to GA-suppressed cultures at 1.6 μM can also effect reversal of PFC suppression (unpublished). This same concentration of PMA was shown to optimally activate soluble guanylate cyclase of human peripheral lymphocytes (11), resulting in a dramatic increase in intracellular cGMP.

Evidence has been presented by Coffey and Hadden (11) that a direct relationship exists between the strength of a tumor promoter and its ability to activate guanylate cyclase, resulting in intracellular in-

TABLE II. EFFECT OF EXOGENOUS Ca^{2+} ON PHENOLIC CHEMICAL-INDUCED INHIBITION OF THE *in Vitro* ANTI-SRBC PFC RESPONSE

Inhibitor added (μg/ml)	Direct PFC/culture normalized to 1000 PFC/culture[a]	
	Without added Ca^{2+}	With added Ca^{2+} (3 m*M*)
None (control)[b]	1000 ± 20	699 ± 76
BHT, 50	197 ± 120	899 ± 87
MP, 25	380 ± 86	838 ± 70
GA, 10	419 ± 37	961 ± 40
PrGal, 10	<80	<80
NDGA, 10	<80	<80

[a] Data presented as the mean ± SEM of triplicate determinations normalized to 1000 PFC/culture, based on immunized controls, to enable pooling of multiple experiments. Plaque counts are background corrected prior to normalization (background PFC/culture varied from <80 to 300). Direct PFC/culture were 14,400 in immunized control cultures.

[b] SRBC-immunized controls.

creases in cGMP. Strong promoters such as PMA induce high levels of cGMP while weaker diesters such as phorbol diacetate induced very little change in intracellular cGMP.

Taken together, the data concerning BHA, BHT, MP, and GA suggest a mechanism whereby these compounds act as antipromoters through their interference with guanylate cyclase- or lipoxygenase-mediated cGMP production. The data would further suggest that *in vitro* immune response systems, both human and murine, which are very well-characterized systems, may be well suited to the study of promoter–carcinogen and promoter–antipromoter interactions.

The evidence presented by Wattenberg (reviewed in (10)) concerning the protective effects (anti-initiation) of the antioxidants BHA and BHT is well documented. Wattenberg's proposed mode of action for BHA involves (i) induction of microsomal enzymes which prevent activation of precarcinogens and (ii) induction of enzymes which catalyze conjugation of carcinogens, resulting in their rapid excretion. The data presented herein suggest a third mechanism: some foodborne phenolic chemicals may function as antipromoters by a mechanism involving inhibition of cGMP formation.

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