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Anticellular and Immunosuppressive Activities of Foodborne Phenolic Compounds (41185)

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Abstract. Several phenolic compounds were examined for their ability to inhibit *in vitro* antibody production and human and mouse cell clone formation. Those compounds (gallic acid, nordihydroguaiaretic acid, methylparaben, and propyl gallate) which inhibited antibody production also suppressed clone formation. These effects occurred well below toxic concentrations. Other closely related compounds (*p*-hydroxybenzoic acid, shikimic acid, and vanillic acid) were without either effect. The data suggest that certain phenolic compounds, present either as normal constituents of food or as additives, may alter various cellular functions at or below concentrations found in the gut.

Several foodborne phenolic compounds have been shown to inhibit the *in vitro* antibody response of mouse spleen cells (1). It has been proposed that the immunosuppressive action of a number of these agents may be similar if not identical to interferon's immunosuppressive action (2). For example, the phenolic food additives and interferon have been shown to suppress the *in vitro* antibody response with similar kinetics (3, 4). The suppression is reversed by reducing agents such as 2-mercaptoethanol (2-ME) under the same conditions, such as in the early period of the immune response (5, 6). Both phenolic food additives and interferon induce suppressor lymphocytes and/or a soluble factor(s) that mediates immunosuppression (7, 8). The biological activity of the suppressor cell is not inhibited by 2-ME. Furthermore, it is thought that interferon's immunomodulatory effects are related to other biological actions of interferon, such as anticellular activity, but are independent of its antiviral properties (6, 9). It was the intent of this study to determine if, like interferon, immunosuppressive phenolic compounds also have anticellular activity.

Materials and Methods. *Chemicals.* Gallic acid, propyl gallate, methylparaben, (ICN Pharmaceuticals, Cleveland, Ohio), vanillic acid, *p*-hydroxybenzoic acid, nordihydroguaiaretic acid (Aldrich, Milwaukee, Wisc.), and shikimic acid (Sigma, St. Louis, Mo.) were diluted in the appropriate culture media and tested at various concentrations.

In vitro antibody response. The primary *in vitro* antibody method was performed exactly as described (10) for the anti-sheep erythrocyte (SRBC) plaque-forming-cell (PFC) response. All PFC responses were determined on Day 5. C57BL/6 female mice, 6–8 weeks old (Jackson Laboratories, Bar Harbor, Maine) were used throughout. Enumeration and viability of cells were determined by counting in a hematocytometer and staining with trypan blue dye. All compounds were added at the time of antigen (SRBC) addition. The quantity of compound inhibiting the PFC response by $\geq$90% was termed the 90% PFC suppressive dose ($PFCSD_{90}$). Control anti-SRBC PFC responses ranged from 10,000 to 25,000 PFC/culture and were background-corrected before the mean was calculated. Cultures were done in triplicate.

Clone inhibition assay. Anticellular activity was assayed by a slight modification of our previously described clone inhibition assay (11). Human WISH or mouse L cells can be readily grown at low cell densities and form clones from single cells. The number of cells plated is directly proportional to the number of clones which develop and the plating efficiency in fortified MEM (Eagles' minimum essential medium supplemented with two times the usual

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amount of essential and nonessential amino acids and vitamins) with 2% fetal calf serum is about 50%. One-half milliliter of a L- or WISH-cell suspension (200 cells/ml) was added to each well of a Multiwell tissue culture plate (Falcon Plastics, Oxnard, Calif.) followed by 0.5 ml of dilutions of the various compounds. The cells were then incubated undisturbed for 8 to 10 days at 37° in 4% CO_2 to allow clone formation. The clones are then stained with crystal violet and counted. The quantity of compound that inhibits 50% of clones from developing as compared with media controls was termed the clone inhibitory dose 50% (CID_{50}).

For toxicity studies, dilutions of the compounds were placed on WISH- or L-cell cultures (7.5×10^5 cells/well, Multiwell tissue culture plates). Cultures were incubated for 24 hr at 37° in 4% CO_2. Cells were then removed from the wells with trypsin and counted in trypan blue dye. The amount of compound that reduced culture viability by 50% was termed the viability reducing dose 50% (VRD_{50}).

Results. Table I shows a comparison of the immunosuppressive and clone inhibitory (anticellular) activity of seven phenolic compounds on mouse spleen cells and mouse L cells or human WISH cells, respectively. Gallic acid, nordihydroguaiaretic acid, methylparaben, and propyl gallate were all potent inhibitors of the *in vitro* antibody response and also inhibited L- and WISH-cell clone formation. The doses required for inhibition of antibody and clone formation were similar and were well below toxicity levels for these compounds. *p*-Hydroxybenzoic acid, vanillic acid, and shikimic acid (a cyclohexene with a structure similar to gallic acid) lacked immunosuppressive activity and also did not inhibit L- or WISH-cell clone formation. These results clearly show that phenolic compounds that are immunosuppressive also have anticellular activity. The immunosuppressive and anticellular activities are not due to toxicity since the inhibitory concentrations are far lower than

TABLE I. COMPARISON OF THE IMMUNOSUPPRESSIVE AND ANTICELLULAR ACTIVITIES OF VARIOUS PHENOLIC COMPOUNDS[a]

Compound	CID_{50}[b] (μg/ml)		$PFCSD_{90}$[b] (μg/ml)	VRD_{50}[b] (μg/ml)	
	WISH cells	L cells	Mouse spleen cells	WISH cells	L cells	Mouse spleen cells
Gallic acid	<1	<1	1–2	100	100	>200
Nordihydroguaiaretic acid	2	1	5–10	100	100	>200
Methyl paraben	20	20	25	>100	>100	>200
Propyl gallate	20	20	5	100	100	50
p-Hydroxybenzoic acid	>100	>100	>200	>100	>100	>200
Shikimic acid	>100	>100	>200	>100	>100	>200
Vanillic acid	>100	>100	>200	>100	>100	>200

[a] Each compound was tested at six concentrations from 100 to 1 μg/ml and 200 to 0.01 μg/ml for the CID and PFCSD, respectively. These same doses were used for the appropriate cell type in the VRD. Endpoints were obtained by extrapolation from the resulting dose responses. There were about 100 WISH or L-cell clones/well and 10,000 to 25,000 PFC/culture in the nontreated controls.

[b] Endpoints for clone inhibition (CID_{50}), viability (VRD_{50}), and suppression of PFC ($PFCSD_{90}$) are different from no inhibition at a $P < 0.05$, $P < 0.05$, and $P < 0.01$, respectively.

those which are toxic. These effects are also not a general property of phenolic compounds since *p*-hydroxybenzoic acid, vanillic acid, and shikimic acid were neither immunosuppressive nor anticellular.

Discussion. These studies provide further evidence that there is a close relationship between the action of certain phenolic compounds and interferon (2). Thus, in addition to the similarities in the immunosuppressive activities of interferon and phenolic food additives (see introduction), they are now shown to possess anticellular activity. While the mechanisms are presently unknown, the results suggest that a understanding of the action of these compounds will be interesting in and of themselves as well as may be enlightening relative to the actions of interferon. From a practical point of view, many phenolic compounds are commonly ingested as food additives and natural constituents of food. Many of these compounds are currently being examined for possible mutagenic or carcinogenic potential. However, some may prove to be beneficial. For instance, butylated hydroxyanisole (BHA) has been reported to inhibit chemical carcinogenesis (reviewed in (12)) as well as exert direct antitumor effects (13). Our results showing inhibition of tumor cell clone formation indicate that other foodborne compounds may also have antitumor activity and should be evaluated.

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