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Accurate Assessment of the Bioactivities of Redox-Active Polyphenolics in Cell Culture

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Phenolic compounds are widely known for their roles as antioxidants and anti-inflammatory agents, as well as for their epidemiological association with reduced risks for certain types of diseases. In the present study, we used rabbit peripheral blood mononuclear cells (PBMCs) to evaluate possible artifacts that result from the reactivity of polyphenolics. We evaluated several common methods for cytotoxicity tests using nine polyphenolics, representing several major classes of tannins and their subunits. For three of those phenolics, we investigated whether or not the bioactivities of the phenolics were altered by spontaneous oxidation. Our study showed that many of the nine tested tannins interfered with the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay, which is commonly used to measure cell viability. A better method for determining cell viability is the luciferin/luciferase ATP assay, and using that method, we found that several tannins are toxic to PBMCs. We measured TNF-α production to assess possible anti-inflammatory activity, and found that only apigenin inhibited TNF-α production in LPS-stimulated cells (EC_{50} 1.0 μg/mL). The other polyphenolic compounds we tested either had no effect on TNF-α or increased its production. However, our data indicated that spontaneous oxidation altered the activity of phenolics, eliminating their toxicity. This study shows that the chemical reactivity of phenolics can significantly affect attempts to evaluate bioactivity in cultured cells and that particular attention should be paid to both methods for determining toxicity and to spontaneous oxidation of tannins during cell testing.

KEYWORDS: Tannins; proanthocyanidins; hydrolyzable tannins; polyphenolics; monocytes; PBMC; cell viability; MTT assay; phenolic oxidation

INTRODUCTION

Polyphenolics are found in various fruits, vegetables, and beverages such as green and black tea, and red wine. The term refers to any compound that contains one or more phenolic groups, and can be further divided into categories, such as flavonoids, stilbenes (e.g., resveratrol), and tannins. The tannins are differentiated from other phenolics by their relatively high molecular weight and their high affinity for protein (*1*). There are several different classes of tannins, including the gallotannins and ellagitannins, which are galloyl esters, and the proanthocyanidins, which are flavan-3-ol polymers (*2*). Procyanidins and proanthocyanidins are found in grapes and berries, while ellagitannins and gallotannins come from fruits such as pomegranate, herbal teas, and traditional medicines. In The Netherlands, approximately 50% of the total daily phenolic intake is obtained by drinking green or black tea (*3*). Some of the major phenolics found in tea include the tannin (−)-epigallocatechin-3-*O*-gallate (EGCG) and the related flavan-3-ols (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), (−)-epicatechin (EC), and (+)-catechin (*4*).

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Many of the health benefits attributed to polyphenolics or polyphenolic-rich diets are thought to result from their ability to scavenge free radicals, function as antioxidants, and chelate metals. It has also been suggested that tannins are anti-inflammatory, antibacterial, and antiallergic (*4−6*). However, there is much debate as to the effects of dietary tannins in humans compared to the effects seen in model systems (*7*). For example, tea polyphenolics are active in cultured cells at relatively high concentrations (5 μM to 100 μM), which surpass plasma concentrations found *in vivo* by 10- to 100-fold (*8*). We propose that the potent redox activity of tannins and other polyphenolics (*9, 10*) could contribute to differences in bioactivity reported in various systems.

In the present study, we evaluated possible artifacts in cell-based studies resulting from the reactivity of polyphenolics. We addressed methods for determining cell viability in the presence of phenolics, and we examined changes in the bioactivity of phenolics as a consequence of spontaneous oxidation during the course of the experiment. We measured the viability and the inflammatory response of rabbit peripheral blood mononuclear cells (PBMCs), a mixture of monocytes and other lymphocytes prepared from fresh blood. We chose to use this rabbit model both for its consistency and convenience. Although

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human monocytes are widely used as models of inflammation, donor gender, age, and physiological status affect the response of the cells, introducing undesirable variability that is easily controlled in our system, which uses blood from a laboratory animal housed under controlled conditions.

In PBMCs, the inflammatory cascade is initiated by a bacterial endotoxin such as lipopolysaccharide (LPS). One of the first events in the inflammatory cascade is the increased production of reactive oxygen species, known as the respiratory burst. In subsequent steps, PBMCs express the pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), which then serve as signaling molecules for the remainder of the inflammatory cascade (*11*). It is plausible to speculate that polyphenolic compounds are anti-inflammatory because of their ability to scavenge free radicals (*9*). However, the ease of oxidation of polyphenolics may introduce artifacts associated either with monitoring cell viability or with spontaneous oxidation of the active compound during testing.

Many different assay systems are currently available to measure cell viability and proliferation. The lactate dehydrogenase (LDH) assay is based on the reduction of NAD^+ to NADH by LDH that is released from dead or dying cells (*12*). The NADH converts a tetrazolium salt into a colored product that can be measured spectrophotometrically. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay relies on the ability of mitochondrial dehydrogenases found in viable cells to cleave a tetrazolium salt, producing a colored formazan product that can also be measured spectrophotometrically (*13*). A third method uses the luciferin/luciferase assay to quantitate the ATP found in live cells (*14*). Because phenolics are known to have potent redox activities (*9*), including reactivity with nitroblue tetrazolium (*15*), we speculated that tannins may interfere with either the LDH or the MTT assay, making it impossible to use these methods to evaluate cell viability.

We examined the bioactivity of polyphenolics by measuring the production of TNF- α by LPS-induced rabbit PBMCs. The concentration of TNF-α was determined using a sandwich ELISA assay. We studied nine different polyphenolics, including representatives of the major classes of tannins (**Figures 1** and **2**). Our model proanthocyanidin was a dimer comprising nearly equal amounts of B-1 and B-3, our model ellagitannin was oenothein, and our model gallotannin was β-1,2,3,4,6-pentagalloyl-*O*-D glucose (PGG). We included the mixed-type tannin EGCG from green tea. We tested methyl gallate and the flavan-3-ol monomers (catechin, epicatechin, and epigallocatechin). We used apigenin, a nontannin flavone with well established anti-inflammatory activity (*16*), as a positive control. Three of the compounds were also examined after spontaneous oxidation at pH 7 to evaluate whether exposure to biological fluids might affect the activity of polyphenolics.

We hypothesized that the polyphenolic compounds would cause problems in some toxicity tests, such as the MTT assay, by interfering in the redox chemistry. We also hypothesized that spontaneous oxidation of the phenolics would alter their bioactivity in cell-based assays so that the oxidized compounds would be either more or less active than the native, reduced phenolics.

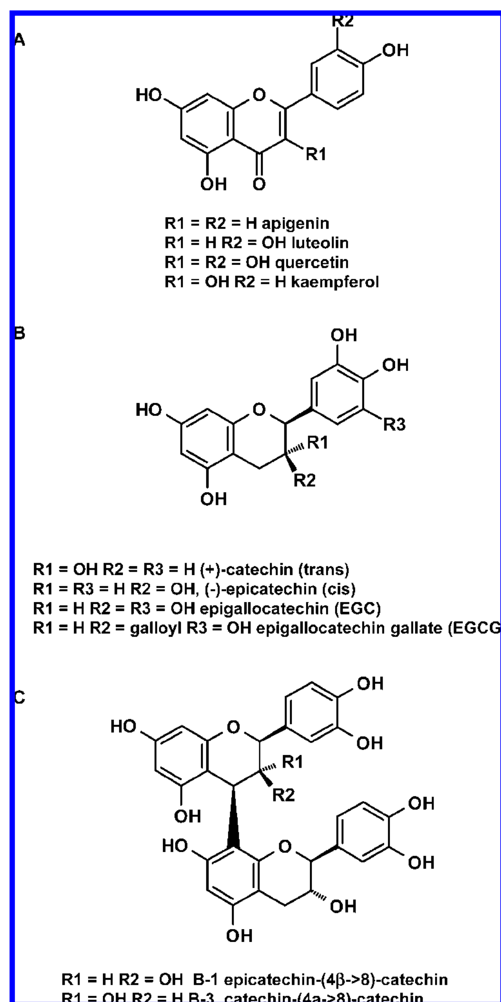

Figure 1. Proanthocyanidins and related flavonoids. (**A**) Flavones. (**B**) Flavan-3-ols and esters. (**C**) Procyanidin dimers B-1 and B-3.

MATERIALS AND METHODS

Chemicals. Solutions used for isolation of PBMCs were purchased sterile from Sigma-Aldrich (St. Louis, MO). All water used to make solutions was purified using a Barnstead (Dubuque, IA) nanopure system. Histopaque with a density of 1.10 g/mL was prepared by mixing equal volumes of sterile Histopaque 1.083 g/mL (Sigma 10831) and Histopaque 1.119 g/mL (Sigma 11191). RPMI 1640 media was purchased from Caisson Laboratories (North Logan, UT) (RPMI-013P), and was reconstituted in water and filter-sterilized. Before using the medium, it was supplemented with 2000 mg/L sodium bicarbonate, 10% fetal bovine serum, and a mixture of 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma L- G1146). LPS was purchased from Sigma (L-2880) and reconstituted in phosphate buffered saline (PBS), 0.1 M phosphate, 0.8% NaCl, pH 7.8, with 0.1 mM EDTA.

The following polyphenolics were used: apigenin (Aldrich 46,074−5), catechin (Sigma C-1251), procyanidin dimer composed of 55% B1, epicatechin−catechin, and 45% B3, catechin−catechin (provided by Herbert Kolodziej, Free University of Berlin, GDR), EGCG, EC, and EGC (all provided by Douglas Balentine, Lipton Tea, Englewood Cliffs, NJ), kaempferol (Sigma K-0133), luteolin (Sigma L-9283), methyl gallate (Aldrich 27,419-4), PGG (*17*), oenothein (*18*), and quercetin (Sigma Q-0125). All of the polyphenolics were at least 97% pure based on HPLC.

Isolation of PBMCs. All glassware were sterilized by baking in an oven for 24 h, while all other materials were sterilized by autoclaving under standard conditions and sterile filtering. An adult female New Zealand White rabbit was housed in a standard sized rabbit cage with a 12 h light/12 h dark cycle. All animal procedures were approved by the Miami University IACUC committee. Standard methods were used to collect up to 50 mL of blood from the rabbit's ear into a tube containing EDTA to prevent clotting. Histopaque (1.10 g/mL) was layered under the fresh blood, and the sample was centrifuged (1800 rpm, 15 min) to collect PBMCs. The top layer containing plasma was removed and discarded. The second layer, containing mononuclear cells, was carefully collected using a fine tip sterile pipet. The mononuclear

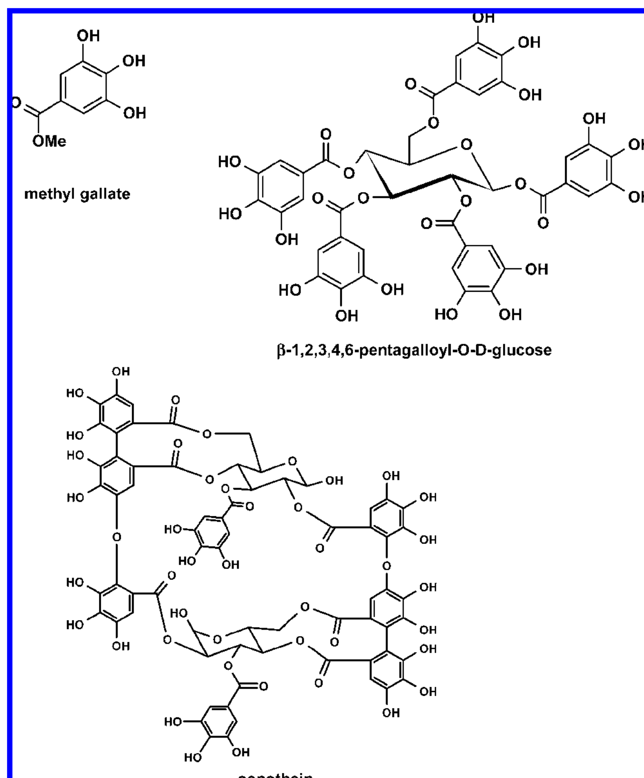

Figure 2. Gallic acid-derived polyphenolics. Methyl gallate represents the monomer unit, pentagalloyl glucose is a representative gallotannin, and oenothein is an ellagitannin.

cells were washed twice with Hank's Balanced Salt Solution (Sigma H4385). The cells were resuspended in the supplemented RPMI 1640 medium. The concentration of cells isolated was determined using a hemocytometer with exclusion of trypan blue used to confirm viability. The mononuclear cells were placed in 24 well tissue culture plates at a concentration of 6.6 × 10^6 cells/mL, using media and the tannin solution to achieve a final volume of 475 μL in each well. After the appropriate tannin solutions were added to the wells, the cells were incubated at 37 °C and 5% CO_2 for 1 h. After 1 h, 25 μL of 0.1 ng/μL LPS or media was added, and the cells were returned to the incubator for 18−24 h. After incubation, the well contents were transferred to microfuge tubes and centrifuged at 10,000 rpm for 15 min. The supernants were collected and stored at −80 °C until further analysis. Within an experiment, each treatment was done in triplicate. Each compound was tested in 2−5 experiments with cells collected on different days.

Preparation of Tannin Solutions. Apigenin, luteolin, kaempferol, and quercetin were dissolved in DMSO. Procyanidin dimer, EGC, EGCG, methyl gallate, oenothein, and PGG were dissolved in water. Catechin and EC were dissolved in methanol and then diluted with water.

Preparation of Oxidized Tannin Solutions. EGCG, PGG, and oenothein were dissolved in 0.08 M Na_2HPO_4 buffer at pH 7. Tannin solutions were incubated at 37 °C for 24 h to allow spontaneous oxidation to occur. Oxidation was confirmed by color change: solutions went from colorless to brownish orange.

ELISA. TNF-α was measured in 96 well plates using a sandwich ELISA assay kit obtained from BD Biosciences Pharmingen (Franklin Lakes, NJ). The primary antibody, purified goat antirabbit TNF polyclonal antibody, was used at a concentration of 8 μg/mL. The detection antibody, a biotinylated mouse antirabbit TNF monoclonal antibody, was used at a concentration of 3 μg/mL. Absorbance was read at 652 nm after 60 min of reaction with streptavidin/horseradish peroxidase, 3,3′,5,5′-tetramethylbenzidine, and hydrogen peroxide. TNF-α standards ranging from 0 pg/mL to 50 pg/mL were used to generate standard curves, and cell supernatants were diluted with media to obtain TNF-α concentrations within the range of the assay. EC_{50} concentrations were calculated by combining individual data points from at least two independent assays and fitting the log-transformed data to a dose−response curve with the bottom set at zero and the top at 100 (GraphPad Prism 4.0, GraphPad Software, San Diego CA).

Cell Viability. To measure cell viability, up to 100 μL of cells were transferred from the 24 well plate to a 96 well plate immediately after the addition of LPS. The cells were incubated as described above for 18−24 h. After the incubation, cell proliferation was measured using one of the following kits: In Vitro Toxicology Assay Kit MTT Based (Sigma), In Vitro Toxicology Assay Kit Lactate Dehydrogenase Based (Sigma), and CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison,WI). For the MTT assay, the kit protocol was followed, in which reconstituted MTT was added to the cells at 10% of the original volume. The cells were incubated for 2 h before the MTT Solubilization Solution was added to dissolve the formazan crystals. A plate reader was used to measure the absorbance at 570 nm. The second assay required a lactate dehydrogenase assay mixture to be added to the cells. The plate was incubated in the dark at room temperature for 20−30 min before the reaction was stopped using 1 N HCl. The absorbance was measured at 490 nm using a plate reader. The luminescent assay (ATP assay) was performed by adding an equal volume of CellTiter-Glo Reagent to each well containing cells and then mixing for 2 min to lyse the cells. The plate was incubated at room temperature for 10 min before measuring the luminescence on the plate reader. LD_{50} concentrations were calculated by combining individual data points from at least two independent assays and fitting the log-transformed data to a dose−response curve with the bottom set at zero and the top at 100 (GraphPad Prism 4.0, GraphPad Software, San Diego CA).

To test for the interference of phenolics in the viability assays, the compounds were dissolved in PBS buffer or DMSO. Concentrations ranging from 0 to 200 μg/mL were prepared for each of the compounds used in this study. These solutions were substituted into the cell viability assays in place of the cell cultures, and the assays were completed as described above. All measurements were made in triplicate. Dose−response curves were fit by linear regression.

RESULTS

Measuring Cell Viability. Many authors use the MTT assay to monitor cell viability. In this assay, production of the blue formazan pigment indicates that the cells are alive, with the absorbance proportional to percent viability. We postulated that phenolics would cause interference in this assay because of their potent redox activity. When we attempted to use this assay, we were not surprised to find that addition of 100 μg/mL EGCG to a standard well containing 1 × 10^5 cells gave a very high absorbance, indicating an apparent increase of over 2-fold in cell viability (**Figure 3**). We then tested the MTT assay with phenolics but no cells, and found that many phenolics reacted to yield absorbances proportional to phenolic concentration (**Table 1**). A standard well containing 1 × 10^5 live cells gives an absorbance of about 150 mAU in the MTT assay; therefore, a response slope of less than 0.3 mAU/μg/mL is required to ensure that cells can be treated with ≥5 μg/mL of a given phenolic without significant interference with viability determination by MTT. Unlike the tannins and their subunits, the flavone apigenin was not reactive with MTT. We tested several simple flavones to establish structural features leading to interference in the assay and found that apigenin and luteolin did not react with the MTT but that quercetin and kaempferol were moderately reactive (**Figure 4**).

The LDH assay provides another method for estimating cell viability. In this assay, phenolics interfered by under-estimating cell viability. The integrity of the cell membrane is monitored by evaluating the ability of lactate dehydrogenase that leaks from dead cells to reduce a tetrazolium dye. In the assay, absorbance is inversely proportional to percent viability. As in the MTT assay, many of the phenolics reacted with the tetrazolium dye to yield the pigment (data not shown); therefore,

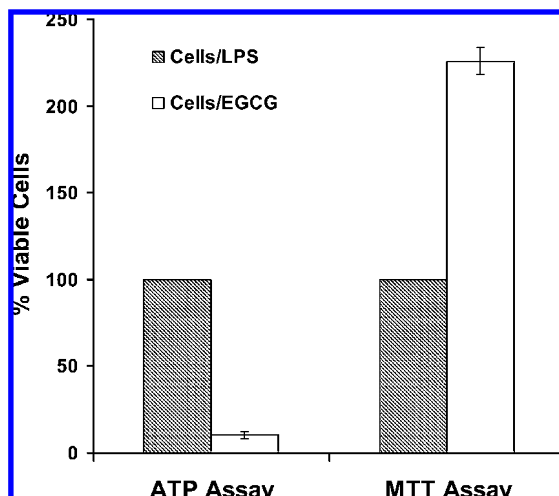

Figure 3. Effect of EGCG on cell viability as assessed by MTT or ATP assay. Monocyte cells (1 × 10^{5} cells) were exposed to LPS after 1 h of treatment with 100 μg/mL EGCG. About 18 h later, viability was measured either with the luciferin/luciferase assay for ATP, or with the MTT assay. Viability was normalized by comparison to control cells not treated with EGCG (100% viable). Each bar represents the average determination for three separate samples of cells from one experiment, and error bars represent the SEM.

Table 1. Response of MTT Assay to Phenolics[a]

phenolic	slope mAU/μg/mL
Hydrolyzable Tannin	
PGG	2.8
oenothein	3.1
Proanthocyanidin	
dimer	8.3
Mixed Tannin	
EGCG	12
Monomers	
methyl gallate	40
catechin	13
EC	16
EGC	28
Flavone	
apigenin	0.1
luteolin	0.6
kaempferol	9.0
quercetin	9.3

[a] In the absence of cells, 12 phenolics were tested at 10, 50, and 100 μg/mL. Absorbance (570 nm) was plotted as a function of concentration of phenolic, and data were fit with linear regression. The average RSD was 8.8% for each triplicate determination, and the average R^2 for the data fits was 0.96.

we do not recommend using the LDH assay to assess cell viability in the presence of phenolics. In addition, we found that the RPMI 1640 medium that we used for our cells interfered with the LDH assay.

We found that the viability assay, which relies on the luciferase-catalyzed, ATP-dependent production of luminescence from luciferin, was suitable for measuring cell viability in the presence of phenolics. Live cells contain substantial amounts of ATP, which is released by chemical lysis and then estimated by luminescence. Luminescence is proportional to percent viability. None of the phenolics produced detectable luminescence when added to the luciferin/luciferase reagents, leading us to conclude that phenolics do not positively interfere with the method. To check for negative interference, standard curves were generated using ATP with or without the addition of 100 μg/mL EGCG. The two standard curves were identical, which confirmed that phenolics do not negatively interfere with the method. Using cells, we found that a sample of 1 × 10^{5} cells yields luminescence of around 2000 units, while a similar sample of cells containing 100 μg/mL EGCG produces only about 200 luminescence units or about 10% viability (**Figure 3**).

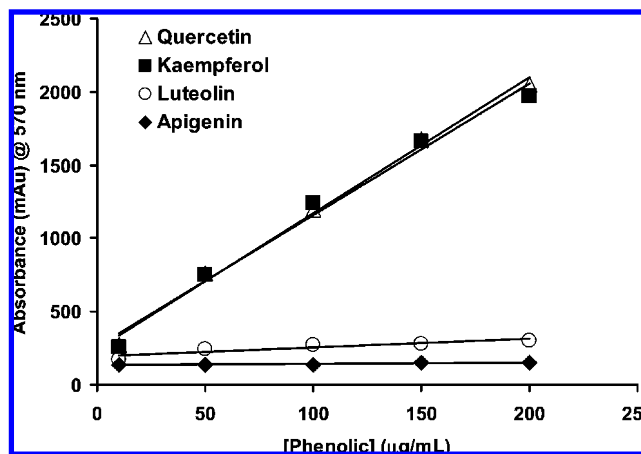

Figure 4. Response curve for flavones in the MTT assay. Each point represents the average determination for three separate samples of cells from one experiment. Standard deviations are smaller than the symbols shown. The slopes for the compounds are quercetin (9.3 mAU/μg/mL), kaempferol (9.0 mAU/μg/mL), luteolin (0.6 mAU/μg/mL), and apigenin (0.1 mAU/μg/mL).

Table 2. Phenolic Toxicity and Anti-Inflammatory Activity[a]

phenolic	LD_{50} (μg/mL)	LD_{50} (μM)	EC_{50} (μg/mL)	EC_{50}(μM)
Hydrolyzable Tannin				
PGG	50	45	85	80
oenothein	90	60	50	30
Proanthocyanidin				
dimer	nontoxic	nontoxic	not inhibitory	not inhibitory
Mixed Tannin				
EGCG	110	240	110	240
Monomers				
methyl gallate	160	870	pro-inflammatory	pro-inflammatory
catechin	nontoxic	nontoxic	pro-inflammatory	pro-inflammatory
EC[b]	nontoxic	nontoxic	pro-inflammatory	pro-inflammatory
EGC	nontoxic	nontoxic	pro-inflammatory	pro-inflammatory
Flavone				
apigenin	170	630	1.0	4.0

[a] Cells were treated with various concentrations of the phenolics for 1 h before adding LPS to stimulate inflammation. Between 18−24 h later, the supernatants were collected, and TNF-α was determined by sandwich ELISA. Toxicity was determined using the luciferin/luciferase ATP assay. Data was log transformed and fit to a dose−response curve to determine LD_{50} and EC_{50}. Compounds that did not affect cell viability at concentrations up to 200 μg/mL were nontoxic, and compounds that increased TNF-α production over the control were pro-inflammatory. The average 95% confidence intervals were ± 23% of the values for LD_{50} and EC_{50}. The average R^2 for the data fit was 0.80. [b] On the basis of cells collected on a single day.

Cytotoxicity. On the basis of our conclusions that phenolics interfered with both the MTT and the LDH assays, cell viability was measured in all experiments using the luminescence assay for ATP. We categorized the phenolics as either nontoxic (LD_{50} > 150 μg/mL) or toxic (LD_{50} < 150 μg/mL) (**Table 2**). Most of the tannins were toxic, killing up to 80% of the cells at a dose below 150 μg/mL, but the tannin subunits were not toxic to rabbit PBMCs. In addition, the procyanidin dimer was not toxic. All three of the oxidized tannins that we tested were less toxic than their reduced counterparts. For example, at 200 μg/

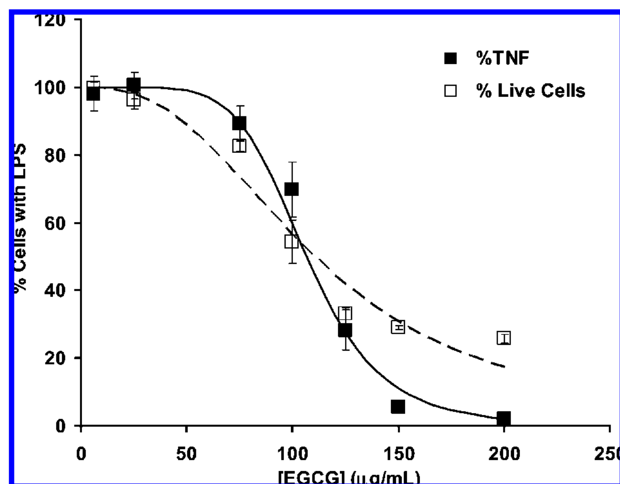

Figure 5. Viability and TNF-α production by cells treated with various concentrations of the mixed tannin EGCG. The viability and cytokine production are affected in a dose-dependent fashion by EGCG, with LD_{50} = 110 μg/mL and EC_{50} = 110 μg/mL. Each point represents the average determination for three separate samples of cells from one experiment, and error bars represent the SEM.

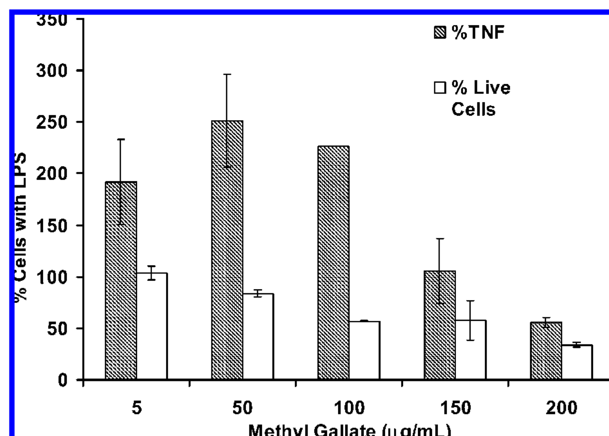

Figure 6. Viability and TNF-α production by cells treated with various concentrations of the tannin monomer methyl gallate. The viability is not affected by low levels of the tannin, with LD_{50} = 160 μg/mL. TNF-α production is stimulated by low levels of the phenolic, suggesting that methyl gallate is pro-inflammatory. Each point represents the average determination for three separate samples of cells from one experiment, and error bars represent the SEM.

mL, cells exposed to oxidized oenothein were 85% viable, in contrast to cells exposed to the same level of reduced oenothein, which were only 20% viable.

Effects of Native Tannins on TNF-α Production. A sandwich ELISA assay was used to measure TNF-α production by rabbit PBMCs. The amount of TNF-α produced by cells is expressed relative to the amount produced by phenolic-free PBMCs in the same experiment. All cells were treated with LPS, which increased TNF-α levels an average of 3- to 5-fold over cells that were not stimulated. We classified the phenolic compounds into three groups: anti-inflammatory compounds reduced TNF-α production, inactive compounds did not change TNF-α production, and pro-inflammatory compounds increased TNF-α production. The only phenolic that was anti-inflammatory was our positive control, apigenin. Apigenin had an EC_{50} value of 1.0 μg/mL, well below its LD_{50} value of 170 μg/mL. The hydrolyzable tannins and EGCG diminished the amount of TNF-α produced by the cells, but the EC_{50} values were similar to the LD_{50} values for these compounds (**Table 2**). For

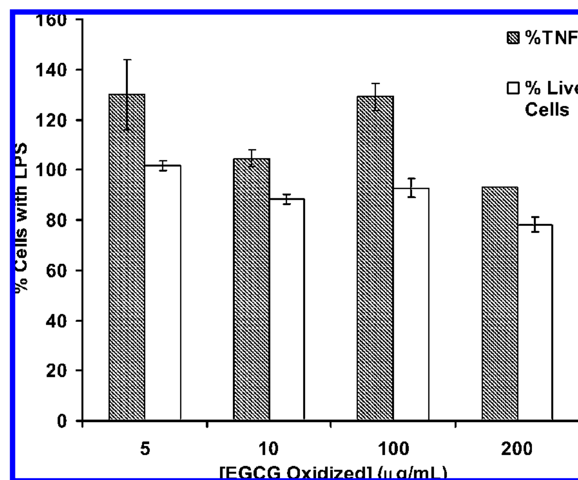

Figure 7. Viability and TNF-α production by cells treated with various concentrations of the oxidized mixed tannin EGCG. Neither viability nor cytokine production is affected by the oxidized tannin. Each bar represents the average determination for three separate samples of cells from one experiment, and error bars represent the SEM.

example, EGCG (**Figure 5**) decreased TNF-α production in a dose-dependent manner, but a very similar concentration dependence was obtained when cell viability was measured instead of TNF-α production, and the EC_{50} and LD_{50} are the same. Similar results were obtained for PGG and oenothein. Therefore, the decrease in production of TNF-α by two hydrolyzable tannins and the mixed tannin EGCG can be attributed to cell death and not anti-inflammatory activity. The procyanidin dimer was inactive and did not decrease or increase TNF-α production by the cells even at the highest concentration (200 μg/mL) that we tested (**Table 2**). The catechins and tannin monomers were pro-inflammatory at low concentrations (**Figure 6**). PBMCs incubated with these phenolics produced more TNF-α than the control cells, while cell viability remained high.

Unlike the native, reduced phenolics, oxidation of the hydrolyzable tannins or EGCG converted them to inactive compounds with little effect on the production of TNF-α at any concentration. We had hypothesized that preincubating the phenolics to allow spontaneous oxidation to occur would alter their activity in the cell system. Our data confirmed this hypothesis. For example, **Figure 7** shows that the TNF-α levels for oxidized EGCG at 200 μg/mL remained at 93%; however, at the same concentration in reduced EGCG, TNF-α levels decreased to about 2%.

DISCUSSION

Recently, there has been much interest in the health benefits of polyphenolics. Individuals have become interested in their antioxidant and anti-inflammatory properties as well as the possible roles phenolics play in preventing certain diseases, such as cancer and cardiovascular disease. Cell-based systems provide an attractive avenue for testing the thousands of phenolic compounds found in plants. However, there are significant differences between activities achieved in cell culture and those noted in humans. For example, the high concentrations often used in cell studies do not accurately reflect the low bioavailability of many polyphenolics (*8, 19, 20*). In addition, little attention has been given to the chemical reactivity of phenolics, which may significantly influence measures of bioactivity in cell culture (*21*).

In this study, we tested polyphenolics in three different assays that are routinely used to assess cell viability. A search of the

literature showed that many research groups routinely use the MTT assay to measure cell viability in the presence of phenolics (*22−24*). The continued use of the assay emphasizes the importance of reporting our findings that phenolics interfere in the MTT assay.

We tested nine different phenolics in the MTT assay. Many of the compounds reacted with MTT, leading to the conclusion that phenolics often give false positive responses in the MTT cell viability assay. However, we found that a few compounds, including the flavone apigenin, did not interfere in the assay. Apigenin does not have the ortho-phenolic groups typical of the tannins and their subunits, and associated with potent redox activity (*21, 25*). We hypothesized that the phenolic substitution pattern of apigenin may dictate its reactivity with MTT and tested the idea by testing three other flavones in the MTT assay: luteolin and quercetin, which have ortho phenolic substitution, and kaempferol, which, like apigenin, is not ortho-hydroxylated. Apigenin and luteolin did not react with MTT at any of the tested concentrations (**Figure 4**). This result suggested to us that substitution on the heterocyclic C ring of the flavone is a more important determinant of activity in the MTT assay than is phenolic substitution. Apiginen and luteolin are 3-deoxy flavones and do not interfere with the MTT assay, regardless of their phenolic substitution pattern. Similar results were obtained in a study of radical scavenging by flavonoids (*26*), although the phenolic substitution pattern appeared to play a larger role in radical scavenging than in the reaction with MTT. The proanthyocanidin dimer and the catechins we examined are 3-hydroxylated and are very reactive with MTT, consistent with our results with the flavone model compounds.

We recommend against using the MTT assay to determine cell viability in the presence of phenolic compounds, unless they have been shown not to interfere in the assay. Phenolics that are toxic may not be properly identified with the MTT assay since the phenolics themselves give the same response as living cells. We also examined the LDH assay and found that in addition to interference from tannins, there is significant interference from the fetal calf serum-supplemented media. We found that the only reliable method to use if polyphenolics are present in the cell medium is to use a luciferin/luciferase assay to measure the amount of ATP released by chemical lysis of the cells. This provides a dependable and simple method for measuring cell viability in the presence of polyphenolics. Nine different phenolics did not interfere either positively or negatively in this assay. Therefore, this assay was used in all of our experiments to measure cell viability, and we recommend its use in cell-based studies of phenolic compounds.

One of the main goals of this study was to determine if the oxidation of phenolics would alter their bioactivities in cell-based assays. We chose to measure activity by measuring cytokine production in LPS-stimulated rabbit PBMCs. In preliminary experiments, we found that apigenin, a simple flavone, was nontoxic and anti-inflammatory. This is consistent with other literature reports (*27, 28*) and confirms that our PBMC system does respond in the normal fashion to inflammatory agents such as LPS and to anti-inflammatory agents.

We used the PBMC system to assess high molecular weight tannins in their native, reduced form and after spontaneous oxidation. The procyanidin dimer was not toxic and did not affect TNF-α production. Three of the tannins that we tested were gallate esters, representing gallotannins (PGG), ellagitannins (oenothein), and the mixed tannin (EGCG). All three compounds were toxic to PBMCs, with diminished TNF-α production paralleling toxicity. We did note that oxidation of the galloyl ester-derived tannins reduced their toxicity. Consistent with the relationship we noted between high toxicity and inhibition of TNF-α production, the oxidized tannins did not affect TNF-α production. Phenolic oxidation occurs spontaneously at cellular pH, and it is likely that in biological systems such as the gastrointestinal tract, a mixture of phenolics, quinones, and other oxidation products exist. For example, the initial oxidiation products of the tea catechins are either unstable quinones or their dimers (*29*). Our results suggest that very different bioactivities may be recorded for a single phenolic depending on whether the bioassay system promotes polyphenolic oxidation or maintains the compound in reduced form. In living organisms, passage through the gastrointestinal tract subjects a dietary polyphenolic to a wide range of oxidizing conditions, further complicating comparison of cell-based to whole-animal studies.

The nontannin phenolics that we tested represented the phenolic subunits of the high molecular weight tannins. Catechin, EC, and EGC comprise the procyanidins such as the dimer we tested here, and the flavonoid subunit of the mixed tannin EGCG. Methyl gallate represents the galloyl esters common to gallotannins, ellagitannins, and EGCG. These nontannin phenolics were not toxic to the PBMCs (**Table 2**). However, they were pro-inflammatory and stimulated the PBMCs to increase TNF-α production several fold (**Table 2** and **Figure 6**). Similar pro-inflammatory activity of phenolics on cells growing in culture has been linked to metal cycling redox reactions (*10, 21*).

In summary, our results show that some phenolics interfere significantly with the redox chemistry of certain assays widely used to measure cell viability. On the basis of our data, we strongly recommend against the use of the MTT and LDH assays in the presence of tannins or other phenolics. Instead, the luciferin/luciferase ATP assay is suitable. Our results also show that spontaneous oxidation of phenolics changes chemical reactivity. We found that oxidized tannins are less toxic to the cells than their reduced counterparts and consequently do not affect TNF-α production by LPS-stimulated PBMCs. This study emphasizes the importance of understanding the fundamental chemistry of phenolics in cell-based systems in order to better establish their roles as anti-inflammatory agents and antioxidants in humans.

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