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Suppression of Macrophage-Dependent T-Lymphocyte Function(s) by Gallic Acid, a Food Additive Metabolite (39958)

DOUGLAS L. ARCHER, JOANN A. BUKOVIC-WESS, AND BENNETT G. SMITH[1]

U.S. Department of Health, Education and Welfare, Public Health Service, Food and Drug Administration, Division of Microbiology, Cincinnati, Ohio 45226

Introduction. In the Mishell-Dutton system (1), interaction among antigen, macrophages (MØ), "helper" thymus-derived cells, and antibody-forming precursor cells results in a primary anti-sheep erythrocyte (SRBC) plaque-forming cell (PFC) response (2). The thymus-derived cells (T lymphocytes) function as regulatory cells that can suppress or enhance (3) the PFC response and are called suppressor and helper T lymphocytes, respectively. The antibody-forming precursor cell is the B lymphocyte, a bone marrow-derived cell (2). MØ are involved in antigen presentation (4) and in promotion of the viability and well-being of T and B lymphocytes (5), and are thought to have other functions that are not yet clearly defined. This report concerns the effect of gallic acid (GA) (3,4,5-trihydroxybenzoic acid), a metabolite of the food additives propyl gallate and tannic acid, on the murine primary antibody response. The data indicate that GA is immunosuppressive for the PFC response to thymus-dependent antigens, interferes with MØ-dependent T-lymphocyte function(s), and has no effect on B-lymphocyte function.

Materials and methods. Animals. C57Bl/6 female mice, 8-10 weeks old, were obtained from Jackson Laboratories, Bar Harbor, Maine. Female athymic nude mice (Balb/C inbred background), 4-5 weeks old, were obtained from ARS/Sprague Dawley, Madison, Wisconsin.

Antigens. SRBC from a single sheep (No. 446) were obtained from Colorado Serum Co., Denver, Colorado. Cultures were immunized *in vitro* with *E. coli* 0127 by adding 1×10^6 cells, previously boiled for 1 hr, to each culture dish (6).

Cultures. Dissociated mouse spleen cells of both C57Bl/6 and athymic nude mice were cultured exactly as described by Mishell and Dutton (1). Anti-*Esherichia coli* responses were determined using SRBC sensitized with soluble 0127 lipopolysaccharide (LPS) as previously described (6). Appropriate controls, including unsensitized SRBC, were included in all experiments to monitor for polyclonal or nonspecific anti-SRBC responses. All PFC responses were determined on Day 5.

Reagents. GA was obtained from ICN Pharmaceuticals, Cleveland, Ohio, and added to cultures in modified Eagle's minimal essential medium (MEM) (1). The percentage of carbon and hydrogen and the melting point range of the GA used in these studies agree with literature values. GA from Matheson, Coleman and Bell, Norwood, Ohio, gave equivalent results. 2-Mercaptoethanol (2ME), 99% pure, was obtained from Matheson, Coleman and Bell, Norwood, Ohio, and added to designated cultures to a final concentration of 5×10^{-5} *M*.

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

[^{3}H]Thymidine incorporation. The addition of 1 μCi of [^{3}H]thymidine (New England Nuclear, Boston, Massachusetts) to Mishell-Dutton cultures for the final 18 hr of a 72-hr culture period was used to determine DNA synthesis.

Cells were collected on glass fiber pads by vacuum filtration, washed twice with normal saline, and once with 0.5% trichloroacetic acid. The pads were placed in scintillation vials, and 10 ml of Dimilume-30 (Packard Instrument Co., Downers Grove, Illinois) was added to each vial. Counts were per-

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formed in a Packard liquid scintillation counter (Model 5385). The results are expressed as the mean of triplicate plates.

Mitogens. Staphylococcal enterotoxin A (SEA), a T-lymphocyte mitogen (7), 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 (8). *E. coli* 0127:B8 LPS and *E. coli* 055:B11 LPS were obtained from Difco, Detroit, Michigan.

Adherent cell supernatant (ACS). ACS preparation was patterned after the method of Hoffman and Dutton (9). Briefly, peritoneal cells (PC) were removed from normal C57Bl/6 mice by washing peritoneal cavities with 2 ml of MEM. PC were then washed, restored in culture medium, and cultured in plastic dishes for 4 hr. Cultures were then washed three times with MEM to remove nonadherent cells, and culture medium was added. After 24 hr, the adherent PC supernatants were removed and centrifuged at 1000*g* for 20 min, filtered through a 0.45-μm bacterial filter, and used immediately.

Results. Table I shows that 10 μg of GA/culture (5×10^{-5} *M*) inhibited the C57Bl/6 mouse spleen cell PFC response to SRBC. The addition of 2ME to cultures at the time of antigen addition did not enhance the PFC response in controls, probably because of the high PFC response without 2ME. Complete restoration of the PFC response resulted from the addition of 5×10^{-5} *M* 2ME to GA-inhibited cultures at the time of antigen addition. GA, in amounts as low as 1 or 2 μg/culture ($5 \times 10^{-6} - 1 \times 10^{-5}$ *M*), can suppress the anti-SRBC PFC response >90% (data not presented). Five days of treatment with GA did not interfere with lymphoid cell viability as determined by trypan blue dye exclusion. GA concentrations as high as 50–100 μg/culture ($2\text{–}5 \times 10^{-4}$ *M*) had no significant effect on the number of viable cells recovered per culture. Cultures of noninduced adherent peritoneal cells exposed to GA at 10 and 25 μg/culture for 24 hr remained fully viable by trypan blue dye exclusion (10).

The effect of delayed addition of 2ME to the GA-inhibited anti-SRBC PFC response is shown in Table II. When 2ME was added to GA-inhibited cultures at 0, 24, or 48 hr, relative to antigen addition, the PFC response was restored, but 2ME added at 72 hr failed to restore the PFC response.

In this laboratory, delayed addition of 2ME resulted in enhancement of the anti-SRBC PFC response. It is unlikely that the enhancing effect is responsible for the reversal of PFC suppression at 24 and 48 hr, as reversal of GA-induced suppression was not observed when 2ME was added at 72 hr, although some 2ME-induced enhancement of control culture PFC responses was appar-

TABLE I. INHIBITION OF THE ANTI-SRBC PFC RESPONSE BY GA AND RESTORATION OF THE RESPONSE BY 2ME.

Culture content	Direct anti-SRBC PFC/culture	PFC/10^6 viable cells
Control (SRBC stimulated)	22,533 ± 1,671[a] (400)[b]	2,448 ± 182
2ME added at same time as antigen	19,200 ± 1,973	2,462 ± 253
10 μg of GA added at same time as antigen	<50	<5
10 μg of GA + 2-ME added at same time as antigen	21,467 ± 2,794	2,094 ± 273

[a] Means ± SEM of triplicate determinations; background corrected.
[b] Background PFC/culture.

TABLE II. THE EFFECT OF THE ADDITION OF 2ME AT VARIOUS TIMES TO GA-INHIBITED CULTURES ON THE DIRECT ANTI-SRBC PFC RESPONSE.

		Time of addition of 5×10^{-5} *M* 2ME relative to antigen addition			
Culture content	No 2ME added	0 time	24 hr	48 hr	72 hr
Control (SRBC stimulated)	16,200 ± 490[a] (<50)[b]	16,200 ± 1,143 (<50)	37,550 ± 816 (250)	39,800 ± 814 (775)	29,700 ± 1,469 (875)
10 μg of GA/culture added at time of antigen addition	475 ± 143	14,000 ± 327	40,150 ± 327	23,800 ± 4,735	975 ± 266

[a] Values are the means ± SEM of triplicate determinations; background corrected.
[b] Background PFC/culture.

ent. To further clarify the suppression kinetics of GA, the converse experiments were done. GA (10 μg/culture) added at 0, 24, or 48 hr relative to antigen addition resulted in >95% PFC inhibition; GA added at 72 hr exerted no significant suppressive effect (data not presented).

Soluble factors from MØ (9) and T lymphocytes (11) can replace their corresponding cell types in the generation of antibody-producing cells. Table III shows that cultures exposed to GA for 24 hr, washed twice with medium, and cultured in fresh medium remain suppressed. ACS partially restored the anti-SRBC PFC response of the washed cultures but had no effect on cultures in which GA remained throughout the 5-day culture period. ACS caused neither an increase in the PFC response in cultures not exposed to GA, nor an increase in background PFC/culture. Cultures of 2×10^5 AC could also restore the anti-SRBC PFC response to GA-exposed, washed spleen cells, but the response was suppressed if GA was added to these cultures (data not presented).

TABLE III. EFFECT OF ADHERENT CELL SUPERNATANT (ACS)[a] ON THE PFC RESPONSE TO SRBC IN GA-TREATED CULTURES AND CULTURES FROM WHICH GA HAS BEEN REMOVED.

Culture content	Direct anti-SRBC PFC/culture
Control (SRBC stimulated), washed at 24 hr	2700 ± 173[b]
10 μg of GA, washed at 24 hr	<50
10 μg of GA, washed at 24 hr, ACS added after wash	1167 ± 83
Control, washed at 24 hr, ACS added after wash	2333 ± 213
10 μg of GA, washed at 24 hr, ACS plus 10 μg of GA added at 24 hr	<50

[a] Prepared as described in text; 100 μl of final ACS added to designated cultures.
[b] Mean ± SEM of triplicate determinations.

To study the effect of GA on the B lymphocyte in the absence of functional T lymphocytes, a thymus-independent antigen and an athymic nude (Nu/Nu) mouse system were used. Table IV shows that 10 and 25 μg of GA/culture had no effect on the anti-*E. coli* 0127:B8 response. Only slight PFC suppression was observed in cultures containing 40 μg of GA, and 100 μg of GA/culture failed to completely suppress the PFC response. Decreased numbers of viable cells recovered per culture were noted when nude spleen cells were exposed to GA at 40 and 100 μg, but it is interesting that no such decrease was noted when C57Bl/6 cells were exposed to 100 μg of GA. The lack of functional T lymphocytes in the nude mouse was verified by the lack of PFC response suppression or mitogenic stimulation by SEA (7). Incorporating 10 μg of *E. coli* 055:B11 LPS in slides at the time of pouring failed to suppress the anti-*E. coli* 0127:B8 PFC response; incorporation of 10 μg of 0127:B8 LPS did effect suppression, verifying the specificity of the anti-0127:B8 PFC response. The data presented in Table V show that GA did not interfere with B-lymphocyte DNA synthesis induced by a mitogenic dose (100 μg/culture) of LPS.

T-lymphocyte DNA synthesis was induced in C57Bl/6 mouse spleen cell cultures with SEA. Table VI demonstrates that control culture DNA synthesis (Mishell–Dutton culture with no antigen added) is suppressed approximately 50% by GA, as

TABLE IV. EFFECT OF GA ON THE ANTI-*E. coli* 0127:B8 PFC RESPONSE OF ATHYMIC NUDE (Nu/Nu) MOUSE SPLEEN CELLS.

Culture content	Direct anti-*E. coli* 0127:B8 PFC/culture			
	Expt. A[a]	Expt. B	Expt. C	Expt. D
Control	217 ± 27[b]	467 ± 64	238 ± 38	240 ± 23
10 μg of GA[c]	240 ± 25	–	–	–
25 μg of GA	–	700 ± 60	–	–
40 μg of GA	–	–	177 ± 32	130 ± 15
100 μg of GA	–	–	43 ± 26	67 ± 17

[a] Separate lots of Nu/Nu mice used for each experiment.
[b] Means of triplicate cultures ± SEM.
[c] GA added at time of antigen addition in 20 μl of MEM.

compared with slight suppression in T lymphocyte-deficient athymic nude control cultures (Table V). The control culture suppression of C57Bl/6 cultures by GA is reversed by 2ME. SEA exerts a mitogenic effect, and the presence of 2ME with SEA in cultures did not suppress or enhance the effect of the mitogen. GA added to cultures with SEA showed rates of DNA synthesis similar to control cultures with GA added. The GA-induced inhibition of SEA-stimulated cultures was reversed by 2ME, which also reversed the GA-induced suppression of the anti-SRBC PFC response (Table I).

Discussion. The data show that GA suppresses the thymus-dependent PFC response of C57Bl/6 spleen cells to SRBC and that the suppression is not caused by a cytotoxic effect. 2ME can reverse the GA-induced suppression even in the presence of excess GA in the cultures. When the excess GA is removed (or greatly decreased in concentration) by washings, either peritoneal macrophages (data not presented) or ACS can effect partial restoration of the PFC response (Table III). The mechanism of reversing GA-induced suppression by 2ME and ACS may be similar, but 2ME may be more efficient in its function. The restoration of GA-suppressed cultures by 2ME does not involve the promotion of lymphoid cell viability. A direct interaction between 2ME and GA cannot entirely be ruled out, but this seems unlikely since ACS can also restore the PFC response.

TABLE V. EFFECT OF GA ON LPS-INDUCED DNA SYNTHESIS OF ATHYMIC NUDE (Nu/Nu) MOUSE SPLEEN CELL CULTURES.

GA added	[^{3}H]-Thymidine, cpm × 10^{-2}		
	Control	100 μg of LPS[a]	SI[b]
None	261 ± 46[c]	1410 ± 63	5.4
10 μg	188 ± 24	1103 ± 172	5.9

[a] *E. coli* 0127:B8 lipopolysaccharide.
[b] Stimulation index: cpm LPS-stimulated culture/cpm unstimulated control.
[c] Means ± SEM of triplicate determinations.

The athymic nude mouse spleen cell PFC response to the thymus-independent antigen *E. coli* 0l2:B8 LPS is unaffected by GA at concentrations far greater than the concentration necessary for complete suppression of the thymus-dependent PFC response in C57Bl/6 cultures. Also, GA has no effect on LPS-induced (B-lymphocyte) DNA synthesis of athymic nude cultures (Table V). GA did suppress SEA-induced (T-lymphocyte) DNA synthesis, but the suppression was reversible by 2ME (Table VI). These data are strong evidence that the T-lymphocyte function(s) is the target of GA's suppressive activity.

The kinetic data shown in Table II and in the text further suggest that GA suppression of the anti-SRBC response is effected via the T lymphocyte. The fact that GA can be added to cultures 48 hr after antigen addition and still suppress the PFC response is evidence that antigen presentation and lymphocyte induction are not affected. Dutton (12) has recently proposed a model for the induction of the humoral immune response in which a T lymphocyte-derived factor initiates differentiation of B lymphocytes previously "triggered" by antigen. This factor must be added before 48 hr of culture for an optimal PFC response to occur.

Data presented in Table II and in the text indicate that GA affects the anti-SRBC PFC response only for the first 48 hr and that 2ME can restore the PFC response up to, but not later than, 48 hr. Our data support the Dutton model (12) of T-lymphocyte "triggering" of B-lymphocyte differentiation to high-level, antibody-producing cells.

It is well established that 2ME can substitute for some MØ functions (5) and that its

TABLE VI. SUPPRESSIVE EFFECT OF GA ON SEA-INDUCED T-LYMPHOCYTE DNA SYNTHESIS AND RESPONSE RESTORATION BY 2ME.

GA added[a]	[^{3}H]Thymidine, cpm × 10^{-2}			
	Control	2ME	SEA[b]	SEA + 2ME
None	187 ± 13[c]	306 ± 15	815 ± 70	683 ± 42
10 μg	98 ± 5	275 ± 9	90 ± 7	507 ± 19

[a] Reagents added in order: SEA, 2ME, GA.
[b] SEA added at 2 μg/culture.
[c] Means ± SEM of triplicate determinations.

effects are exerted primarily on T lymphocytes (13). GA-induced suppression of the PFC respone to the thymus-dependent antigen SRBC and T-lymphocyte mitogen-induced DNA synthesis were reversed by 2ME. The fact that peritoneal macrophages or peritoneal macrophage supernatants (ACS) also reversed PFC suppression strongly suggests that GA exerts its suppressive effects on T-lymphocyte function(s) via MØ.

It was previously reported that butylated hydroxyanisole, a phenolic antioxidant food additive, was immunosuppressive *in vitro* (14). GA is also a phenolic compound and is a metabolite of the food additives propyl gallate and tannic acid. Furthermore, considerable amounts of GA could be consumed as free GA and hydrolyzable tannins and other polyphenols in foods such as tea, cocoa, and coffee (15). Further work is under way to determine if GA is suppressive *in vivo*.

Other chemical agents such as carrageenan (16) exert an immunosuppressive effect via MØ. GA should provide another chemical probe of MØ function in the immune response.

Summary. At concentrations as low as 5×10^{-6} *M*, gallic acid (GA), a metabolite of the food additives propyl gallate and tannic acid, suppressed the anti-sheep erythrocyte (SRBC) plaque-forming cell (PFC) response of C57Bl/6 mouse spleen cells when added to cultures as late as 48 hr after antigen addition. GA-induced suppression was reversed by 5×10^{-5} *M* 2-mercaptoethanol (2ME) added at the same time as, or up to 48 hr after, antigen and was reversed less efficiently by adherent-cell supernatant (ACS). GA also suppressed mitogen-induced DNA synthesis of C57Bl/6 T lymphocytes, and this suppression was reversed by 2ME. GA had no effect on the response of athymic nude mouse spleen cells to the thymus-independent antigen *Escherichia coli* 0127:B8, and failed to suppress lipopolysaccharide (LPS)-induced B-lymphocyte DNA synthesis. The data suggest that GA selectively suppresses a macrophage (MØ)-dependent T-lymphocyte function(s).

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