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Blockade of Mitogen Induction of the Interferon Lymphokine by a Phenolic Food Additive Metabolite[1] (40122)

DOUGLAS L. ARCHER[2] AND HOWARD M. JOHNSON

Department of Health, Education, and Welfare, Public Health Service, Food and Drug Administration, Division of Microbiology, Cincinnati, Ohio 45226 and Department of Microbiology, University of Texas Medical Branch, Galveston, Texas 77550

The *in vitro* immune response results from a complex sequence of events involving B- and T-lymphocytes and macrophages (1–3). We have recently demonstrated that a phenolic compound, gallic acid (GA) (3,4,5-trihydroxybenzoic acid), suppresses the *in vitro* plaque-forming cell (PFC) response by directly inhibiting a macrophage function required for T-lymphocyte participation in the immune response (4, 5). This has been demonstrated by the following observations: (a) The *in vitro* PFC response to *E. coli* 0127 lipopolysaccharide (T-independent antigen) by spleen cells from athymic nude mice, which lack functional T-lymphocytes, was unaffected by concentrations of GA that blocked the anti-sheep red blood cell (SRBC) response (T-dependent) in C57B1/6 mice (5), and (b) adherent cells, adherent cell supernatant fluids, and 2-mercaptoethanol (2-ME) reversed GA-induced suppression of the PFC response (4, 5).

The effects of GA on the immune response are of particular interest since GA is both an additive metabolite of propyl gallate and tannic acid, and a normal constituent of food. Large amounts are consumed as free GA and as hydrolyzeable tannins in foods such as tea, cocoa, and coffee (6). Further, tea tannins have been causally linked to hepatic and esophageal cancers (7, 8).

Another measure of T-lymphocyte function which might be influenced by GA through its action on macrophages is lymphokine production. The ability of the T-lymphocyte mitogens concanavalin A, phytohemagglutinin, and staphylococcal enterotoxin A (SEA) to suppress the *in vitro* PFC response of mouse spleen cells to SRBC was shown to be proportional to their ability to induce lymphokine interferon (immune interferon) in the cultures (9). Further, dibutyryladenosine 3′,5′-cyclic monophosphate (cAMP) blocked both mitogen induction of suppressor cell activity and production of immune interferon in a manner that suggested a direct relationship between the two (10, 11).

GA was examined for its effect on the induction and action of the quantifiable lymphokine, immune interferon. Because interferon induction is both quantitatively related to suppressor cell activity (11) and dependent on the presence of functional macrophages (12), it is a logical assay system for studying the immunobiological effects of GA and other environmentally encountered compounds that potentially interfere with natural immunoregulation.

Methods and materials. Animals. C57B1/6 female mice, 8- to 10-week-old, were obtained from Jackson Laboratories, Bar Harbor, ME.

Antigens. SRBC were obtained from Colorado Serum Co., Denver, CO. Cultures were immunized *in vitro* with 3×10^6 SRBC.

Cultures. Dissociated mouse spleen cells were cultured exactly as described by Mishell and Dutton (13). Direct PFC assays were performed on microscope slides (14) after 5 days of incubation.

Reagents. SEA was produced by the Microbial Biochemistry Branch, Division of Microbiology, Food and Drug Administration, Cincinnati, OH. Its purity was estimated to be >99% by extinction coefficient (15). GA was obtained from ICN Pharmaceuticals, Cleveland, OH, and added to cultures in culture medium. The percentage of carbon and hydrogen and the melting point range of the GA used in these experiments agree with literature values. The 2 ME, 99% pure, was

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[2] Send reprint requests to Dr. D. L. Archer, Food and Drug Administration, 1090 Tusculum Ave., Cincinnati, Ohio 45226.

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obtained from Matheson, Coleman and Bell, Norwood, OH, and added to designated cultures to a final concentration of 5×10^{-5} *M*.

[^{3}H]-Thymidine incorporation. DNA synthesis was determined by the addition of 1 μCi: ^{3}H-thymidine (New England Nuclear, Boston, MA) to Mishell-Dutton cultures for the final 18 hr of a 72 hr incubation period. Cells were harvested on glass fiber pads by vacuum filtration, washed twice with normal saline, and once with trichloroacetic acid. The pads were placed in liquid scintillation vials and 10 ml of Dimilume-30 (Packard Instrument Co., Downers Grove, IL) was added to each vial. Counts were performed in a Packard Liquid Scintillation Counter (Model 5385).

Induction of interferon and interferon assays. Mouse spleen cell cultures were prepared exactly as described for the *in vitro* PFC response except SRBC were omitted. SEA (0.5 μg/ml) was added to cultures for mitogen induction of interferon. Cultures were incubated for 48 hr under the conditions described for the *in vitro* PFC response (13). Culture supernatants were harvested by centrifugation at (400*g* for 10 min). Supernatants were either assayed for interferon activity on the day of harvest or frozen immediately and stored at −70° for no more than 5 days before assaying. Interferon activity was quantitated in microtiter plates by the method of plaque reduction of vesicular stomatitis virus (40 PFU/challenge dose) in mouse L cells. Methylcellulose was used as an overlay (16) rather than carboxymethyl-cellulose.

Adherent cell cultures. Peritoneal exudate cells were obtained by injecting 8 ml of 37° culture medium intraperitoneally, then withdrawing the injected fluid. The exudate cells were washed by centrifugation and resuspended in culture medium. Sufficient cells were added to culture dishes so that approximately 2×10^5 adherent cells per dish (17) were obtained. Adherent cell cultures were washed three times gently with culture medium before GA-treated spleen cells were added.

Results and discussion. Table I presents data from parallel experiments on the individual and combined effects of GA, SEA, and 2-ME on the direct anti-SRBC PFC response, interferon induction, and DNA synthesis in mouse spleen cell cultures. SEA, an established T-cell mitogen and immune interferon inducer (9, 18), inhibited the PFC response by 90%. Previous studies have shown that this inhibition occurs via suppressor cell activation (9, 11, 19). As shown, the inhibition of the PFC response was associated with induction of immune interferon and stimulation of DNA synthesis (as reflected) by increased ^{3}H-thymidine incorporation into DNA in the cells). GA alone blocked the normal PFC response and inhibited DNA synthesis. These effects were previously shown to be due to blockade of macrophage function (4, 5). GA blocked both SEA-induction of interferon and stimulation of DNA synthesis.

Although 2-ME (5×10^{-5} *M*) alone stimulated DNA synthesis and slightly enhanced the PFC response, it did not induce interferon. This is consistent with its property of replacing some macrophage function(s) (20, 21). Neither the PFC suppressor cell effect of SEA nor SEA-induction of interferon and stimulation of DNA synthesis were blocked by 2-ME. In fact, 2-ME enhanced the interferon-inducing property of SEA, which is known to require macrophage help. Both the PFC suppressive effect and the DNA synthesis inhibitory effect of GA were completely blocked by 2-ME. This is presumably due to 2-ME replacing a blocked macrophage function. The inhibitory effect of GA on SEA-induction of interferon and stimulation of DNA synthesis was blocked by 2-ME, which is able to substitute for macrophage function(s) (20, 21). Thus, as expected, the substitution by 2-ME for the macrophage function(s) inhibited by GA, permitted SEA to suppress the PFC response. Reversal by 2-ME of the interferon lymphokine inhibitory effect of GA indicates that another macrophage-dependent function is blocked by GA.

This blockade of macrophage function by GA, as measured by decreased interferon activity, was further investigated by reconstitution with untreated macrophages. Specifically, 1.5×10^7 spleen cells per ml in culture medium were incubated for 24 hr with 20 μg GA per ml. Excess GA was removed from spleen cells by washing twice with minimal essential medium, followed by the addition of the treated cells to adherent peritoneal cells from mouse peritoneal exudate (17). SEA, 0.5 μg/ml, was added to the cultures and inter-

TABLE I. EFFECT OF GALLIC ACID (GA) ON THE MOUSE SPLEEN CELL ANTI-SRBC PFC RESPONSE, AND SEA INDUCTION OF IMMUNE INTERFERON AND DNA SYNTHESIS.

Treatment	Direct anti-SRBC PFC/culture (Mean ± SEM)	Units interferon/ml (Log_{10})	^{3}H-thymidine uptake, cpm 10^{-2} (Mean ± SEM)
None	3850 + 29[a]	<0.5[b]	187 ± 13[c]
SEA (0.2 μg/ml)	383 ± 192	1.85	814 ± 70
GA (10 μg/ml)	<50	<0.5	98 ± 5
GA + SEA	<50	<0.5	90 ± 7
2-ME[d]	4917 ± 770	<0.5	306 ± 15
SEA + 2-ME	150 ± 150	2.45	683 ± 42
GA + 2-ME	5217 ± 235	<0.5	275 ± 9
GA + SEA + 2-ME	425 ± 25	2.25	507 ± 19

[a] Mean ± SEM of triplicate determinations, background corrected; data are representative of several experiments.
[b] Mean of duplicate determinations for each dilution; data are representative of at least two experiments.
[c] Mean ± SEM of triplicate determinations; data are representative of several experiments.
[d] Added to final concentration of 5×10^{-5} *M*.

feron concentrations were determined after 48-hr incubation (9). The adherent cells restored the ability of SEA to induce immune interferon in GA-treated cultures (compare line 5 with line 4, Table II). The combined interferon output of the GA-treated spleen cell control and untreated adherent cell control was only one-fourth that of the macrophage-reconstituted cultures. This pattern of interferon induction was shown in repeated experiments. Cultures that did not receive SEA contained less than 0.5 units of interferon per ml. The data are consistent with previous studies showing that macrophages are required for optimal production of immune interferon in humans (12).

An alternate interpretation of the above findings is that GA inactivated the induced interferon rather than blocked its induction. To test this possibility, immune interferon was incubated with various concentrations of GA at 37° for 1 hr and titrated for residual antiviral activity (Table III). At concentrations as high as 10 μg per ml, GA had no inhibitory effect on already induced interferon. Thus, GA blocked the induction of immune interferon but did not block the establishment of antiviral activity of already induced interferon.

The interactions of immunoregulatory cells are poorly understood; however, loss of suppressor cell function in the gut could contribute to autoimmune disorders (22, 23), altered mucosal penetrability, and increased IgE immunoglobulin formation (25). The data show that GA is capable of blocking a macrophage-dependent T-cell function (induction of immune interferon) that may be associated with suppressor cell activity. Since GA and related metabolites occur in highest concentration in the gastrointestinal tract and liver, their effects should be greatest in these organs.

TABLE II. SEA INDUCTION OF INTERFERON IN MOUSE SPLEEN CELLS; ADHERENT CELL REVERSAL OF GA SUPPRESSION.

Spleen cells	Treatment: SEA	Treatment: GA[a]	Treatment: Adherent cells[b]	Units interferon per ml (Log_{10} ± SEM)
+	+	−	−	3.00 ± 0.00 (1000)[c]
+	+	−	+	2.63 ± 0.18 (427)
−	+	−	+	1.43 ± 0.18 (27)
+	+	+	−	1.87 ± 0.09 (74)
+	+	+	+	2.63 ± 0.32 (427)

[a] Cultures incubated 24 hr with 20 μg/ml GA, then washed and restored in culture medium.
[b] Approximately 2×10^5 adherent mouse peritoneal cells.
[c] Concentrations of interferon expressed numerically; data are presented as the mean ± SEM of duplicate determinations; patterns of interferon production are representative of at least two experiments.

TABLE III. EFFECT OF GA ON THE ESTABLISHED ANTIVIRAL ACTIVITY OF SEA-INDUCED IMMUNE INTERFERON.

GA[a] (μg per ml)	Residual interferon activity (Log_{10} mean ± SEM)
0	2.45 ± 0.05
2	2.40 ± 0.00
5	2.35 ± 0.15
10	2.45 ± 0.05

[a] GA and SEA-induced interferon were incubated together at 37° for 1 hr and the residual antiviral activity was determined as previously described (9). Data presented as mean ± SEM of duplicate determinations; replicate experiments showed GA had no effect on established antiviral activity of immune interferon.

We have previously shown and have further demonstrated here that GA is capable of blocking a macrophage-dependent helper cell function (PFC response to SRBC) (4, 5). GA provides a chemical probe for specifically blocking at least two macrophage-dependent T-cell functions. Another food additive, carrageenan, affects various macrophage-dependent immunologic events *in vitro* (26, 27) and depresses resistance to virus-induced tumors in mice (28). Because GA is a normal and additive-type dietary component consumed in large amounts in various countries, this and related phenols and polyphenols should be further studied to determine possible biological consequences of ingestion.

Summary. Gallic acid (GA), a food constituent and food additive metabolite, blocked macrophage-dependent mitogen-induced interferon production and suppressor T-lymphocyte function. Interferon production was restored by adherent peritoneal macrophages or 2-mercaptoethanol, a macrophage substitute. GA failed to block the antiviral activity of preformed mitogen-induced interferon. Possible consequences of loss of lymphokine production and suppressor T-lymphocyte function(s) are discussed.

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