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Enhancement of Interferon-γ Production in Glycyrrhizin-Treated Human Peripheral Lymphocytes in Response to Concanavalin A and to Surface Antigen of Hepatitis B Virus (42241)

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Abstract. The effects of glycyrrhizin, a component of licorice (*Glycyrrhiza glabra*) roots, on the production of interferon-γ in human peripheral lymphocyte–macrophage cultures by concanavalin A (Con A) was examined. Interferon-γ production in normal lymphocyte–macrophage cultures treated with 10 to 100 μg/ml of glycyrrhizin at 37°C for 12 hr or longer, and then treated with 10 μg/ml of Con A, was enhanced four to eight times compared to control cell cultures. Lymphocyte–macrophage cultures treated with 10 to 100 μg/ml of glycyrrhizin alone did not produce interferon. No significant difference in the adsorption of [^{3}H]Con A to glycyrrhizin-treated and control lymphocyte–macrophage cultures was found, but RNA and protein synthesis of the treated lymphocytes was increased compared to control cells; DNA synthesis, however, was reduced. Collaboration between enriched T-lymphocytes and macrophages, both treated with glycyrrhizin, was needed for the enhancement of interferon-γ production. A smaller increase in interferon production was also observed in the glycyrrhizin-treated peripheral lymphocyte–macrophage cultures derived from an asymptomatic carrier of hepatitis B virus, in response to Con A and surface antigen of hepatitis B virus.

Glycyrrhizin, a component of licorice (*Glycyrrhiza glabra*) roots, has been extensively studied in relation to varied biological activities, such as anti-inflammatory activity (1–3), suppressive effect on allergic reactions (4), direct and indirect antiviral activity (5), and interferon inducibility (6). The mechanisms of these activities of glycyrrhizin are not yet clear, though a few studies (1, 7) show that glycyrrhizin inhibits the activity of phospholipase A_2 and production of prostaglandin E_2 in cell membranes. Recently many substances such as cell wall components of *Mycobacterium, Corynebacterium,* and *Nocardia,* polysaccharides of plants, interferon and its inducers, lymphokines, monokines and so on, have been shown to induce nonspecific host resistance to cancer and infection, and are referred to as biological response modifiers (8–10).

In the present study, we have found that production of interferon-γ in human peripheral lymphocyte–macrophage cultures in response to concanavalin A (Con A) and surface antigen of hepatitis B virus (HBs antigen) was enhanced by pretreatment of the cells with glycyrrhizin, indicating a function of glycyrrhizin as a biological response modifier.

Materials and Methods. *Reagents.* Con A was purchased from E • Y Laboratories, Inc., California, and glycyrrhizin was kindly supplied from Minophagen Pharmaceutical Company, Tokyo, Japan. Purified HBs antigen (480 μg protein/ml) was generously supplied by Dr. S. Makino and Dr. T. Takahashi, Kitasato Institute, Tokyo, Japan. Ficoll 400 was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden, and Conray 400 (sodium iotalamate) from Daiichi Seiyaku Company, Ltd., Tokyo, Japan. [^{3}H(G)]Con A (53 Ci/mmole) was obtained from New England Nuclear, Boston, Massachusetts, and [methyl-^{3}H]thymidine (60 Ci/mmole), [5-^{3}H]uridine (25 Ci/mmole), and L-[1-^{14}C]leucine (50 mCi/mmole) from The Radiochemical Centre, Amersham, England. RPMI 1640 medium and Eagle's minimum essential medium (MEM) was purchased from Nissui Seiyaku Company, Tokyo, Japan. Anti-human interferon-α, -β, and -γ rabbit sera were generously supplied by Dr. D. C. Burke, University of

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Cells. Human peripheral total lymphocytes, enriched macrophages, enriched B-lymphocytes, and enriched T-lymphocytes were prepared as follows: normal human peripheral blood, supplied by Asahikawa Red Cross Blood Center, was laid over a mixture of 9% (w/v) Ficoll 400 and 33.4% (w/v) Conray 400 at a ratio of 1:2.4, and centrifuged at 1200 rpm for 40 min. The cells at the interface were collected, washed three times with MEM supplemented with 10% new-born-calf serum (MEM–CS10) containing 5 units/ml of heparin, and resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum (RPMI 1640–FS10) at a concentration of 1–2 $\times$ 10^6 cells/ml. This cell suspension was referred to as lymphocyte–macrophage suspension.

The lymphocyte–macrophage suspensions were incubated at 37°C for 2 hr in plastic tissue culture flasks (Falcon 3028, 175 cm^2), and the nonadherent cells were collected by pipetting. The adherent cells (enriched macrophage fraction; referred to as macrophages hereafter) were collected by scraping the wall of plastic flasks with a rubber policeman, resuspended in RPMI 1640–FS10 at a concentration of 2 $\times$ 10^6 cells/ml, and stored in ice until use.

The suspensions of nonadherent cells (5 $\times$ 10^6 cells/ml) were mixed with an equal volume of a sheep red blood cell suspension (1 $\times$ 10^8 cells/ml; pretreated with 10 units/ml of neuraminidase at 37°C for 30 min, then washed three times with phosphate-buffered saline solution, pH 7.4), incubated at 37°C for 30 min, and then centrifuged at 1200 rpm for 40 min in a Ficoll 400–Conray 400 mixture. The cells at the interface (enriched B-lymphocyte fraction; referred to as B-lymphocytes) were collected, washed with MEM-CS10 three times, and resuspended in RPMI 1640–FS10 at a concentration of 2 $\times$ 10^6 cells/ml. The cells which were pelleted with sheep red blood cells (enriched T-lymphocyte fraction; referred to as T-lymphocytes) were suspended in 0.14 *M* ammonium chloride–0.017 *M* Tris–HCl-buffered solution (pH 7.4) for hemolysis to occur, washed with MEM–CS10 three times, and then resuspended in RPMI 1640–FS10 at a concentration of 2 $\times$ 10^6 cells/ml.

The same methods were used for preparation of lymphocyte–macrophage suspensions from blood of HBs antigen positive (over 4096 reversed passive hemagglutinating units) asymptomatic carrier of hepatitis B virus, supplied from Asahikawa Red Cross Blood Center.

Interferon induction with Con A or HBs antigen. One of two sets of duplicate cell fractions was pretreated with glycyrrhizin at a final concentration of 100 μg/ml at 37°C for 12 hr and the other set was left untreated. These cells (2 $\times$ 10^6 cells/ml) alone, or mixed with macrophages (2 $\times$ 10^5 cells/0.1 ml) and B-lymphocytes (1.8 $\times$ 10^6 cells/0.9 ml) or T-lymphocytes (1.8 $\times$ 10^6 cells/0.9 ml), were induced with Con A or HBs antigen at a final concentration of 10 or 20 μg/ml, respectively. Glycyrrhizin was left in the culture system during the induction period. After incubation at 37°C for designated periods, culture fluids were sampled for interferon.

Interferon titration. Interferon activity in culture fluids was assayed as follows: FL cell (human amnion cell line; ATCC number, CCL-62) monolayers in 96-well plastic plates (Corning) were incubated with 80 μl of serially diluted interferon samples in duplicate at 37°C for 24 hr, and then challenged with vesicular stomatitis virus at $10^{1.5}$ 50% tissue culture infectious doses per 100 μl. The interferon titers

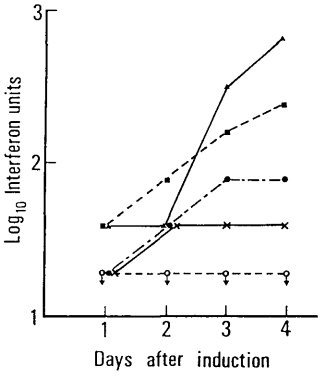

FIG. 1. Effect of glycyrrhizin on interferon production in lymphocyte–macrophage cultures induced with Con A. Cells pretreated with designated concentration of glycyrrhizin for 12 hr at 37°C were induced with 10 μg/ml of Con A. Symbols: ●, untreated; ■, treated with 10 μg/ml of glycyrrhizin; ▲, treated with 100 μg/ml of glycyrrhizin; ×, treated with 500 μg/ml of glycyrrhizin; ○, treated with 100 μg/ml of glycyrrhizin without induction by Con A.

were expressed as the reciprocal of the maximum dilution showing 50% reduction of cytopathic effect.

Neutralization test of interferon. Interferon samples (64 IU/ml) were mixed with an equal volume of anti-human interferon-α, -β, and -γ serum at a final concentration of 4 neutralizing units ($t_{1/10}$) (11), incubated at 37°C for 1 hr, and then assayed for residual interferon activity.

Adsorption of Con A. Cell suspensions (5×10^5 cells/ml) were mixed with 2 μCi/ml of [^{3}H(G)]Con A, and incubated at 37°C for designated periods. The cells were washed with phosphate-buffered saline solution (PBS, pH 7.4) three times on a glass-fiber filter paper (Whatman, GF/C), and the radioactivity retained on the filter paper was measured in a Packard 2560 Tri-Carb liquid scintillation spectrometer.

Radioactive labeling experiments. Cell suspensions (5×10^5 cells/ml) were replenished with L-leucine-free MEM supplemented with 5% dialyzed FS, and 2 μCi/ml of [methyl-^{3}H]thymidine, 2 μCi/ml of [5-^{3}H]uridine or 0.2 μCi/ml of L-[1-^{14}C]leucine was used for measuring DNA, RNA or protein net synthesis, respectively. After 30 min of incubation at 37°C the cell suspensions were chilled in an ice bath, washed three times with cold 5% (w/v) trichloroacetic acid (TCA) on glass-fiber filter paper. The radioactivity on filter paper was then measured.

Results. *Effect of glycyrrhizin on interferon production in lymphocyte cultures.* The lymphocyte–macrophage cultures were pretreated with glycyrrhizin at a designated concentration, and then induced with Con A at a final concentration of 10 μg/ml. As shown in Fig. 1, increased interferon production was observed in the cells pretreated with 10 to 100 μg/ml of glycyrrhizin. However, interferon production in the cells pretreated with 500 μg/ml of glycyrrhizin was reduced as compared with controls. Glycyrrhizin itself has no interferon inducing ability.

TABLE I. NEUTRALIZATION OF INTERFERON PRODUCED IN LYMPHOCYTE–MACROPHAGE CULTURES PRETREATED WITH GLYCYRRHIZIN

Interferon	None	Anti-IFN-α	Anti-IFN-β	Anti-IFN-γ
GL–Con A–IFN[a]	32	32	32	<4
Hu–Leu–IFN[b]	64	4	32	64

[a] Interferon was produced in lymphocyte–macrophage cultures pretreated with 100 μg/ml of glycyrrhizin (GL) and then induced with 10 μg/ml of Con A.

[b] Human leukocyte interferon induced by Sendai virus was kindly supplied by Hokkaido Red Cross Blood Center.

TABLE II. EFFECT OF GLYCYRRHIZIN ON THE ADSORPTION OF CON A TO LYMPHOCYTES–MACROPHAGES

Cells treated with glycyrrhizin (μg/ml)	Adsorption of [^{3}H]Con A		
	0.5 min	1 min	15 min
None	4410[a] (1.00)[b]	4896 (1.00)	4950 (1.00)
10	4677 (1.06)	5037 (1.03)	4888 (0.98)
100	4636 (1.05)	4837 (0.98)	5037 (1.02)

[a] Average radioactivity (dpm) per 5×10^5 cells.

[b] Ratio of radioactivity of glycyrrhizin-treated cells to that of untreated cells.

Neutralization of interferon. In order to determine the species of interferon produced in the glycyrrhizin and Con A-treated lymphocyte–macrophage cultures, neutralization tests were carried out with anti-interferon-α, -β, and -γ sera. The interferon was γ type (Table I).

Adsorption of [^{3}H]Con A to glycyrrhizin-treated and untreated lymphocytes. Experiments were performed to determine whether the increased interferon production in the cells pretreated with 100 μg/ml of glycyrrhizin was due to the enhancement of adsorption of Con A. No difference of adsorption rate of Con A was observed between glycyrrhizin-treated and untreated lymphocyte–macrophage cultures (Table II).

Effect of glycyrrhizin on macromolecular synthesis. Two sets of duplicate lymphocyte–macrophage cultures were treated with 100 μg/ml of glycyrrhizin. One set was induced with Con A at a final concentration of 10 μg/ml, and the other set was left uninduced. After a 60-hr incubation, the cells were pulse-labeled for 1 hr with [^{3}H]thymidine, [^{3}H]uridine, or [^{14}C]leucine, and the cold TCA-insoluble radioactivity was measured. Table III shows that glycyrrhizin alone did not influence net DNA, RNA, and protein synthesis; DNA synthesis, however, was inhibited in cells treated with glycyrrhizin and Con A. On the other hand, RNA and protein synthesis in the glycyrrhizin

TABLE III. DNA, RNA, AND PROTEIN SYNTHESIS IN LYMPHOCYTE–MACROPHAGE CULTURES TREATED WITH GLYCYRRHIZIN

Cells treated with glycyrrhizin (μg/ml)	After treatment with glycyrrhizin[a]			After treatment with glycyrrhizin and then with Con A[b]		
	[^{3}H]Thymidine	[^{3}H]Uridine	[^{14}C]Leucine	[^{3}H]Thymidine	[^{3}H]Uridine	[^{14}C]Leucine
None	324[c] (1.00)[d]	208 (1.00)	43 (1.00)	5096 (1.00)	1893 (1.00)	120 (1.00)
10	318 (0.98)	225 (1.08)	50 (1.16)	4139 (0.81)	2118 (1.12)	116 (0.96)
50	375 (1.15)	185 (0.87)	38 (0.89)	3925 (0.77)	2563 (1.35)	146 (1.21)
100	354 (1.09)	192 (0.92)	52 (1.21)	3612 (0.71)	2350 (1.24)	163 (1.36)
500	286 (0.88)	203 (0.97)	48 (1.12)	3300 (0.65)	1975 (1.04)	81 (0.67)

[a] After treatment with glycyrrhizin for 12 hr, the cells were pulse-labeled for 1 hr with radioactive compounds.
[b] After treatment with glycyrrhizin for 12 hr, then induction with 10 μg/ml of Con A for 60 hr, the cells were pulse-labeled for 1 hr with radioactive compounds.
[c] Average radioactivity (dpm) per 5×10^5 cells.
[d] Ratio of radioactivity of glycyrrhizin-treated cells to that of untreated cells.

and Con A-treated cells was elevated compared with the glycyrrhizin-untreated cells.

Interaction between glycyrrhizin-treated macrophages and lymphocytes. In order to obtain some information on the cells producing the augmented levels of interferon, the glycyrrhizin-treated and untreated macrophages, T-lymphocytes and B-lymphocytes were mixed with each other, and then induced with Con A. The results presented in Table IV show that interferon production in glycyrrhizin-treated T-lymphocytes was augmented, and that the mixed culture of glycyrrhizin-treated macrophages and glycyrrhizin-treated T-lymphocytes produced more interferon than the glycyrrhizin-treated T-lymphocytes alone did. No detectable interferon was produced in any mixed cultures of macrophages and B-lymphocytes (data not shown).

Effect of glycyrrhizin on interferon production in lymphocytes derived from asymptomatic carriers of hepatitis B virus in response to Con A and HBs antigen. Lymphocyte–macrophage cultures derived from normal persons and HBs antigen carriers were induced with Con A and HBs antigen after pretreatment with glycyrrhizin. Table V shows that lymphocyte–macrophage cultures derived from HBs antigen carriers had a markedly reduced ability to produce interferon in response to Con A and HBs antigen. However, after the cell cultures were pretreated with glycyrrhizin, they produced detectable interferon in response to Con A and HBs antigen.

Discussion. Recent studies have focused on the development of biological response modifiers which augment the specific and nonspecific resistance of the host (8). Our present results show that glycyrrhizin increased the activity of T-lymphocytes to produce interferon-γ in response to Con A. The mechanisms of this enhancement are not yet clear; however, an interaction between T-lymphocytes and macrophages, both cells pretreated with glycyrrhizin, was required for this enhancement of interferon-γ production. Sonnenfeld *et al.* (12), Arbeit *et al.* (13), and Ratliff *et al.* (14) also showed that interferon-γ was produced by T-lymphocytes in interaction with macrophages stimulated with lectins,

TABLE IV. INTERACTION BETWEEN MACROPHAGES AND T-LYMPHOCYTES TREATED WITH GLYCYRRHIZIN

Cells	Interferon units 72 hr after Con A induction
Untreated M[a]	<2
GL-treated M[a]	<2
Untreated T[b]	8
GL-treated T[b]	18
Untreated M + untreated T[c]	10
GL-treated M + untreated T[c]	11
GL-treated M + GL-treated T[c]	27

[a] Macrophages (2×10^6 cells/ml) untreated and treated with 100 μg/ml of glycyrrhizin.
[b] T-lymphocytes (2×10^6 cells/ml) untreated and treated with 100 μg/ml of glycyrrhizin.
[c] Untreated or glycyrrhizin-treated macrophages were mixed with untreated or glycyrrhizin-treated T-lymphocytes at a ratio of 1:9.

TABLE V. EFFECT OF GLYCYRRHIZIN ON INTERFERON PRODUCTION IN LYMPHOCYTE–MACROPHAGE CULTURES DERIVED FROM HBs ANTIGEN CARRIER

Sources of lymphocyte–macrophage cultures[a]	Glycyrrhizin treatment (100 μg/ml)	Inducers[b]	Interferon units (days) 3	4	5
HBs antigen carrier	+	HBs	<5	<5	10
HBs antigen carrier	−	HBs	<5	<5	<5
HBs antigen carrier	+	Con A	10	5	ND[c]
HBs antigen carrier	−	Con A	<5	<5	ND
Normal person	+	HBs	<5	<5	<5
Normal person	−	HBs	<5	<5	<5
Normal person	+	Con A	80	640	ND
Normal person	−	Con A	20	40	ND

[a] Lymphocyte-macrophage cultures were prepared from the blood of asymptomatic carriers of HBs antigen over 4096 reversed passive hemagglutinating units/0.1 ml, and HBs antigen negative persons.

[b] The cells were induced with HBs antigen and Con A at a final concentration of 20 and 10 μg/ml, respectively.

[c] Not done.

though in our experiment enriched T-lymphocytes alone produced interferon-γ in response to Con A (Table IV), indicating that this cell fraction might not be pure. The interferon titers in fractionated T-lymphocyte cultures in the present studies were lower than that in unfractionated cell cultures, probably because the activity of T-lymphocytes was reduced by several steps of fractionation treatment.

Marcucci *et al.* (15) showed that short-term culture of murine spleen cells in T-cell-growth-factor-conditioned medium resulted in the production of high titers of interferon-γ in response to Con A, and suggested that the lectin receptor on the surface of cells cultured in T-cell-growth-factor-conditioned medium might undergo quantitative changes. In the present studies, it is not probable that the enhancement of interferon-γ in the glycyrrhizin-treated lymphocyte–macrophage suspension is due to the increased adsorption of Con A on these cells (Table II). On the other hand, DNA synthesis in glycyrrhizin-pretreated and Con A-stimulated lymphocytes was remarkably inhibited. It is not clear whether the reduced DNA synthesis relates to the elevated interferon-γ production; however, recent reports showed that an inhibition of cellular DNA synthesis by 5-bromodeoxyuridine or sodium butyrate led to increased production of interferon (16–18), enzymes (19), and hormones (20). Further studies are needed to analyze the relationship between the enhancement of interferon production and the inhibition of DNA synthesis of host cells.

Recently, glycyrrhizin has been widely used for treatment of chronic active hepatitis with hepatitis B virus in Japan, and the efficacy of this drug has been reported (21, 22). However, the mechanism of action of glycyrrhizin on chronic active hepatitis is not known. Earlier reports have shown that interferon production is apparently deficient in hepatitis B virus infections (23, 24), and that lymphocytes from children with chronic hepatitis B have decreased ability to produce interferon when induced *in vitro* (25). The present results showed that interferon production in lymphocyte–macrophage cultures derived from asymptomatic carriers of hepatitis B virus in response to HBs antigen was augmented by pretreatment of the cells with glycyrrhizin. The enhancing effect of glycyrrhizin on interferon-γ production in lymphocytes may participate in the efficacy of glycyrrhizin on chronic active hepatitis.

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