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BRAZILIN MODULATES THE IMMUNE FUNCTIONS IN NORMAL CBA FEMALE MICE

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ABSTRACT: *Brazilin, the main constitutent of Caesalpinia sappan, was examined for its immunomodulating activities in normal CBA mice. Mitogen induced proliferation and production of ConA induced T-cell growth factors (TCGF) of splenocytes were significantly reduced in brazilin treated group, compared to control group. It was also found that suppressor activities of splenocytes in brazilin treated group was significantly incresed compared to those in normal control group.*

Key Words: *Brazilin, Immunomodulation, Lymphocyte proliferation, TCGF.*

INTRODUCTION

Brazilin, an active principle of *Caesalpinea sappan*, has been previously found to possess diverse biological activities, including antiinflammatory, antihistamic (Gabor and Engi, 1987 and Hikino *et al.*, 1977), cytotoxic and antimicrobial (Aizenman, *et. al.*, 1961, Goncalves, *et. al.*, 1961) and antioxidant effects (Moon *et al.*, 1987). Brazilin also inhibited several enzyme activities such as histidine decarboxylase (Gabor, *et. al.*, 1952), cAMP phosphodiesterase (Nikaido *et. al.*, 1981). It was also reported that brazilin improves the erythrocyte deformability (Moon, *et. al.*, 1989) and inhibited granuloma formation (Hikino *et al.*, 1977). In our previous studies, it was also found that brazilin increased the delayed type hypersensitivity (DTH) against bovine serum albumin (BSA) and decreased the circulating leukocyte counts but no significant effect on IgM/IgG plaque forming cells were observed *in vivo* and *in vitro* (Moon *et al.*, 1988).

Brazilin caused the reversion of immunological tolerance through stimulation of TCGF release and suppression of nonspecific suppressor activity of splenocyte (Mock, 1991) in CBA or C57BL/6 female mice. And also, brazilin inhibits lymphocyte proliferation regardless the augmentation of TCGF relese or the expression of IL-2 receptors (Mock, 1991).

As an attempt to evaluate the immunomodulating actions of brazilin, we determined its effects on some immune parameters in normal CBA female mice.

EXPERIMENTAL METHODS

Mice

CBA female mice (8 week-old) were purchased from the Animal Breeding Center of Seoul National University. The mice were maintanined under the controlled environmental conditions (air filtered room, 21-24°C, lighting; 7:00-19:00) and allowed free access to food and water.

Treatment

Brazilin (Aldrich) was suspended in saline and sonicated. Cyclophosphamide (CY. Sigma) was prepared just before use. CY(40 mg/kg body weight) was administered intraperitoneally 48 hours before the immunological tests. Brazilin (50 mg/kg bdy wt./day) was given intraperitoneally to mice for 2 consecutive days. Controls received vehicle alone. Immunological tests were performed 72 hours after final adminstration of brazilin.

Preparation of Spleen Cell Suspension

Spleens were removed and placed in RPMI 1640 media (Sigma) supplemented with 100 U/m*l* penicillin and 100 μg/m*l* streptomycin (Gibco), 0.2 mM sodium pyruvate (Sigma), 2 mM glutamine (Sigma), 10 mM HEPES (Sigma), 2 g/L sodium bicarbonate (Sigma), 1 mM nonessential amino acids (Gibco) , 50 μM 2-mercaptoethanol (Sigma) (This formula is referred to as K-O medium throughout this work). Pooled spleen cell suspension from 3 mice in each group, obtained by disaggregation of chopped tissue in loosely packed homogenizer, was washed by centrifugation (260g, 6 min) and red blood cell was lysed by hypotonic shock. After 3 times washing by centrifugation, cell viability was determined by trypan blue exclusion test.

Lymphoproliferative Responses to Mitogens

Spleen cells were cultured in triplicate at 4×10^5 cells in 200 μ*l* K-O media supplemented with 10% heat inactivated FBS, in a 96 well flat-bottomed microplate (Falcon) and stimulated with either 10 μg/m*l* phytohemagglutinin (PHA, Gibco), 50 μg/m*l* lipopolysaccharide from *E. coli* 055:B5 (LPS, Difco) or 5 μg/m*l* ConA (Sigma, type III). Cultures were incubated at 37°C in a humidified 5% CO_2 incubator for 44 hours and were pulsed with 0.5 μCi ^{3}H-thymidine (6.7 Ci/mmol, NEN) per well for the last 18 hours of incubation. Cells were collected with an automatic Titertek cell harvester (Flow, UK) and ^{3}H-thymidine incorporation was determined. Results were expressed as mean counts/minute$\pm$ SD in triplicate cultures.

Assay of T-cell Growth Factors (TCGF)

Spleen cells (8×10^6) were cultured in 1 m*l* K-O media in the presence of 10 μg/m*l* ConA in a 24 well microplate (Falcon) for 24 hours and then supernatants were harvested and stored at 20°C until used.

Supernatants were assayed for TCGF by their ability to maintain proliferation of ConA-activated T cells blasts as described by Coutinho, *et. al.*, (1979), with

slight modification. In brief, blast cells were obtained by stimulating splenocytes with 30 μg/ml ConA for 72 hours. After 3 times washing with K-O media supplemented with 10% heat inactivated FBS (Gibco) and 100 mM α-methylmannopyranoside (Sigma). 2×10^4 ConA blast cells were cultured in triplicate for 30 hours with 50 μl of supernatants of serial two-fold dilutions, in a 96 well round-bottomed microplate (Falcon). Cells were pulsed with 0.5 μCi ^{3}H-thymidine (6.7 Ci/mmol, NEN) per well for the last 6 hours of incubation. Cells were collected with an automatic Titertek cell harvester (Flow, UK) and ^{3}H-thymidine incorporation was determined by scintillation spectrometry (LKB). Calibration curve was made by using human recombinant IL-2 (Gift from Dr. K.S. Ham, KIST) TCGF-activities were expressed as equivalently potent IL-2-activities.

Assay of Non Specific Suppressor Cells Activity

Spleen cells (8×10^6) in 1 ml K-O medium, were treated with 25 μg/ml mitomycin C (MMC, Sigma) for 30 min at 37°C. After 3 times washing, MMC-treated cells were counted and used as suppressor cells. Suppressor activity of MMC-treated cells was determined using ConA induced lymphocyte proliferation (Hirano, *et. al.*, 1989). In brief, 2×10^5, 4×10^5, or 8×10^5 MMC-treated suppressor cells were added to freshly prepared cultures containing 4×10^5 C57BL/6 splenocyte (responder) and 50 μg/ml ConA. The cultures were incubated for 62 or 44 hours at 37°C in 96 well flat-bottomed microplate. ^{3}H-thymidine incorporation was determined during the last 18 hours of incubation.

Statistical Analysis

The significance of the differences between the means was evaluated by Student's T-test.

RESULTS AND DISCUSSION

Brazilin is one of the bioflavonoids and has been found to possess diverse biological activities, including antiinflammatory and antioxidant properties. And also, brazilin has been previously shown to reverse immunological tolerance in CBA and C57BL/6 mice through stimulation of TCGF release and suppression of nonspecific suppressor activity of splenocyte (Mock, 1991). The present study was conducted to examine brazilin for its immunomodulating activities in normal CBA female mice.

As shown in Table 1, maximum ^{3}H-thymidine uptakes into splenocytes were observed at 20 μg/ml ConA, 10 μg/ml PHA and 40 μg/ml LPS respectively. Cell density (4×10^5/well) and 42 hrs. incubation time were selected in this experiment. Under this optimum condition for the mitogen-induced cell proliferation, experiments were performed to investigate the effects of brazilin on immune function and the results were shown in Table 2. Brazilin (50 mg/kg) showed the statistically significant suppression of the lectin induced lymphocyte proliferation. It is generally known that many flavonoids suppressed mitogen induced cell proliferation (Hirano, *et al.*, 1989), but the mechanism of its suppression of cell proliferation has not yet been clearly understood (Farkas, *et*

Table 1. Effect of mitogen doses on the proliferation of splenocytes from CBA female mice

Dose (μg/ml)	^{3}H-Thymidine Incorporation cpm $\times 10^{-3}$		
	ConA	PHA	LPS
0	13.57±0.30	13.57±0.30	13.57±0.30
1.25	26.54±0.49	16.74±0.12	ND
2	66.15±1.72	19.10±0.48	ND
5	107.83±0.09	38.85±0.46	87.42±0.47
10	156.80±3.66	55.40±1.11	95.79±4.09
20	160.99±1.48	42.26±1.34	109.06±1.56
40	102.91±1.91	26.68±0.81	116.64±1.04
80	ND	ND	100.75±1.25
160	ND	ND	91.95±0.67

Representative means±S.E. from 3 separate triplicate cultures.
ND: Not determined

Table 2. Effect of brazilin on the lectin induced lymphocyte proliferation in normal CBA female mice

Mitogen	Dose (μg/ml)	^{3}H-thymidine Incorporation[a] cpm$\times 10^{-3}$		
		Control	Brazilin	Cyclophosphamide
–	–	9.21±0.31	9.31±0.43	9.24±0.50
ConA	2.5	63.28±2.94	42.06±1.11[b]	39.8±2.21[b]
	5	105.77±1.26	70.12±2.00[b]	78.65±4.33[b]
PHA	5	21.11±0.23	14.74±1.06[b]	18.31±0.81
	10	49.30±1.58	33.37±0.99[b]	43.08±1.37
LPS	2.5	104.20±0.78	62.76±0.98[b]	87.13±3.43[b]
	5	111.87±1.35	69.04±0.19[b]	90.03±9.99

[a] Representative mean±S.E. from 3 separate triplicate cultures of spleens from group of 3 mice.
[b] Significantly different from control ($P<0.01$)

Table 3. Effect of brazilin on TCGF secretion of normal splenocyte in CBA female mice

Group	Experiment 1(TCGF (U/ml)		Experiment 2(TCGF (U/ml)
	20H	24H	20H
Control	174.7±4.3	221.7±11.5	170.0±10.1
Brazilin(50 mg/kg)	118.0±1.0[b]	107.7± 0.3[b]	104.3± 2.9[b]
Cyclophosphamide 40 mg/kg	ND	175.0±14.4	ND

[a] Representative mean±S.E. from 3 separate triplicate cultures of spleens from group of 3 mice. Responsiveness (cpm) of ConA blasts to standard IL-2(U/ml): 0; 4217, 5; 15996, 10; 25600, 20; 34189, 40; 45204, 80; 56066, 160; 64396.
[b] Significantly different from control ($P<0.01$)
#: TCGF activities are expressed as IL-2 unit
ND: Not determined

al., 1985 and Hirano, *et al.*, 1987). This inhibitory action of brazilin on mitogen induced cell proliferation was similar with the results from the experiments using C57BL/6 normal mice (Mock, 1991) and C57BL/6 mice tolerisated with SRBC (Mock, 1991) or BSA (Mock, 1991). This fact suggested that brazilin might suppress mitogen induced proliferation of B or T lymphocytes. Changes in lymphocyte subsets relatively sensitive to mitogens cannot to be excluded as one of the possible causes of the suppression of the cell proliferation (Ikaezawa, *et al.*, 1989 and Baker, *et al.*, 1987).

TCGF activities were assessed and the results were shown in Table 3. Total TCGF activities were expressed as equivalently potent IL-2 activities because IL-2 is a major factor (Isakow, *et. al.*, 1987) for mitogen induced T-cell proliferation and was not selectively determined in this experiment. Under the consideration of major role of IL-2 in T-cell proliferation, the effects of brazilin on IL-2 receptor expression were also investigated (data not shown). Cells with high affinity IL-2 receptor(ConA blasts) were induced by ConA. A plot of the logarithm of the standard IL-2 unit versus ^{3}H-thymidine uptake of ConA blasts responsive to standard IL-2 appeared nearly linear (data not shown). ^{3}H-thymidine uptake of ConA blasts responsive to supernatant containing TCGF was calculated using this standard IL-2 calibration curve and expressed as equivalently potent IL-2 activities. As shown in Table 3, brazilin suppressed TCGF release in normal CBA female mice, which is not in accord with the results from the experiments using normal C57BL/6 female mice (Moon and Mock, 1991), and SRBC tolerisated C57BL/6 mice(Moon, *et al.*, 1991). Brazilin caused slight increase of TCGF release in normal C57BL/6 mice and also significant improvement of TCGF release in SRBC tolerisated C57BL/6 mice. These results suggested that there might exist interstrain differences in the TCGF production influenced by brazilin, perhaps due to the difference in metabolic activity or MHC restriction, etc (Pevnitsky, *et al.*, 1989). It was also supposed that lymphocyte subsets in spleen might be changed or brazilin might affect IL-2 gene expression directly. The fact that brazilin increased TCGF production of the draining lymph node cells from CBA mie tolerisasted with BSA(Mock, 1991), but not that from normal CBA mice, indicates that brazilin exerts a differential effect on TCGF release depending on experimental conditions, normal or tolerisated state. From the hitherto obtained

Table 4. Effect of brazilin on suppressor activity of splenocyte in normal CBA female mice[a]

Responder/Suppressor Ratio	^{3}H-thymidine Incorporation cpm$\times 10^{-3}$		
	Control	Brazilin	Cyclophosphamide
	64.20±2.88	64.20±2.88	64.20±2.88
1	28.84±0.87	9.95±0.81[b]	4.96±0.73[b]
1/2	15.08±0.51	1.19±0.10[b]	0.59±0.04[b]
1/4	8.34±0.18	0.72±0.09[b]	0.31±0.00[b]

[a] Refer to experimental methods. Representative means±S.E. from 3 separate triplicate cultures spleens from group of 3 mice.

[b] Significantly different from control ($P<0.01$)

results, it is recognized that brazilin could modulate TCGF release and this modulating activity of brazilin on TCGF release might contribute to the improvement of some immunologically abnormal states.

Finally, nonspecific suppressor activity of splenocyte was assayed using ConA induced lymphocyte proliferation. Suppressor cells (MMC-treated cells) were added to freshly prepared cultures containing 5 μg/ml ConA and 4×10^5 normal splenocytes (responder) and incubated for 42 hours. ConA response was significantly suppressed by the addition of suppressor cells (Table 4). Interestingly, unexpected results were obtained from this suppressor system. Since TCGF release was stimulated by the treatment of brazilin in normal and tolerisated C57 BL/6 mice and CBA mice, we expected that suppressor activity of the brazilin treated group should be decreased. But the contrary results were obtained. The suppressor activity was increased in this suppressor system

MMC-treated suppressor cells from both brazilin and CY treated mice intensively inhibited the ConA response compared to those from control group.

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