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Brazilin Inhibits Mitogen Induced Cell Proliferation Despite of Augmentation of T Cell Growth Factor(TCGF) Production and Expression of IL-2 Receptors

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Abstract □ The present work was designed to investigate the effects of brazilin on ConA-induced TCGF release, responsiveness to standard IL-2, and mitogens-induced proliferation of splenocyte when administered intraperitoneally to 8 week-old C57BL/6 mice for 2 consecutive days. Immunological tests were performed 72 hours after the treatment of brazilin. The administration of 50 mg/kg brazilin caused a noticeable increase in TCGF release and responsiveness to standard IL-2, but inhibited mitogens-induced proliferation of splenocyte. These results indicated that brazilin is able to modulate immunological functions despite of its inhibitory effect on mitogen induced cell proliferation.

Key words □ Brazilin, immunomodulation, TCGF, responsiveness to IL-2, lymphocyte proliferation.

It was reported that many flavonoids act as biological response modifier affecting various cell systems involved in immunity, inflammation and allergic reaction[1-7].

In our previous experiments, brazilin was also found to have immunomodulating activities; brazilin increases delayed type hypersensitivity (DTH) against bovine serum albumin (BSA) and decreases the circulating leukocyte counts, but showed no significant effects on IgM/IgG plaque forming cells *in vivo* and *in vitro*. In addition, brazilin recovered the decreased DTH in alloxan diabetic mice to normal physiological levels and reversed immunological tolerance induced by high dose antigen, through the potentiation of TCGF release and the suppression of nonspecific suppressor activity of splenocytes[8,9].

From these results, it was motivated to undertake this study to elucidate the more detailed immunomodulating mechanism of brazilin in normal C57B/6 female mice.

EXPERIMENTAL METHODS

Experimental animals

8 week-old C57BL/6 female mice were purchased from the Animal Breeding Center of Seoul National University. These mice were maintained under controlled environmental conditions (air filtered room, 21-24℃, lighting; 7:00-19:00 H) and allowed free access to food and water.

Treatment of animals

100 mg brazilin (Aldrich) was suspended in 20 m*l* saline and sonicated for one hour. 10 m*l* of cyclophosphamide(CY, Sigma, 4 mg/m*l*) was prepared just before use. Mice were given intraperitoneally 50 mg/kg brazilin per day for 2 consecutive days. Controls received vehicle alone. Immunological tests were performed 72 hours after the last administration of brazilin. CY (40 mg/kg) was administerd intraperitoneally 48 hours before the immunological tests.

Preparation of spleen cell suspension

Spleens were removed and placed in RPMI 1640 media (Sigma) supplemented with 100 U/ml penicillin and 100 μg/ml 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-0 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 (260 g, 6 min) and red blood cell (RBC) was lysed by hypotonic shock. After 3 times washing with fresh medium, cell viability was determined by trypan blue exclusion test.

Lymphoproliferative responses to mitogens

Spleen cells from 3 mice in each group were plated in triplicate at 4×10^5 cells in 200 μl K-0 media supplemented with 10% heat inactivated FBS, in a 96 well flat-bottomed microplate (Falcon) and stimulated with either 10 μg/ml phytohemagglutinin (PHA, Gibco), 5 μg/ml ConA (Sigma) or 5 μg/ml lipopolysaccharide from *E. coli* (LPS, 055 : B5, Difco). Cultures were incubated at 37℃ 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 by scintillation spectrometry (LKB). Results were expressed as mean counts/minute± SD in triplicate cultures.

Table I. Effects of mitogen doses on the proliferation of splenocytes from C57BL/6 female mice

Mitogens (μg/ml)	^{3}H-Thymidine incorporation(cpm×10^{-3})		
	ConA	PHA	LPS
0	9.90± 0.47	9.90± 0.47	9.90± 0.47
1.25	26.72± 0.22	ND	ND
2.5	75.36± 2.58	12.63± 0.17	ND
5.0	136.49± 0.62	25.39± 1.84	118.27± 4.58
10.0	171.34± 4.18	40.84± 0.52	141.25± 3.30
20.0	166.51± 3.05	58.46± 0.48	150.84± 1.62
40.0	111.87± 0.34	42.51± 1.08	117.71± 2.92
80.0	ND	28.22± 0.59	88.64± 3.00
160.0	ND	12.26± 0.47	

[a] Representative mean ± SE from 3 separate triplicate cultures

[b] ND; not detected

TCGF production

Spleen cells from 3 mice in each group were cultured at 8×10^6 cells in 1 ml K-0 media in the presence of 10 μg/ml ConA (Sigma, type III), in a 24 well microplate (Falcon). After 24 hours of incubation at 37℃ in a humidified 5% CO_2 incubator, supernatants were harvested and stored at −20℃ until assay. Brazilin was added to medium at the starting time of culture.

TCGF assay

Supernatants were assayed for TCGF by their ability to maintain proliferation of ConA-activated T cell blasts as described by Coutinho *et al*[10], 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-0 media supplemented with 10% heat inactivated FBS (Gibco) and 100 mM α-methylmannopyranoside (Sigma). 2×10^4 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 with human recombinant IL-2 (Gift from Dr. K.S. Ham, KIST). TCGF activity was expressed as equivalent potency of IL-2 .

Table II. Time course of response of splenocytes from C57BL/6 female mice to mitogens

Culture time(h)	^{3}H-Thymidine incorporation(cpm×10^{-3})			
	ConA 5 μg/ml	PHA 20 μg/ml	LPS 10 μg/ml	
26	17.08± 1.38	35.36± 0.97	42.37± 0.25	
34	99.72± 1.64	66.96± 0.80	98.99± 1.03	
42	148.87± 1.05	66.41± 2.68	124.30± 1.18	
50	168.94± 0.85	53.61± 1.80	133.14± 2.90	

[a] Representative mean ± SE from 3 separate triplicate cultures

Table III. Effects of Brazilin on mitogen induced lymphocyte proliferation in normal C57BL/6 female mice[a]

Mitogens (μg/ml)	^{3}H-Thymidine incorporation(cpm×10^{-3})		
	Control	Brazilin	CY
Experiment 1			
0	3.43± 0.17	3.08± 0.26	2.83± 0.18
ConA			
2.5	47.75± 5.97	33.70± 4.29[b]	37.46± 0.55[b]
5.0	77.06± 1.51	60.90± 3.36[b]	63.39± 3.78[b]
PHA μg/ml			
5	13.38± 0.26	12.76± 0.78	12.33± 0.12
10	20.97± 0.95	23.07± 0.26	22.42± 0.76
LPS μg/ml			
2.5	36.51± 4.16	20.42± 1.44[b]	24.49± 0.94[b]
5.0	46.76± 3.73	32.77± 2.13[b]	33.77± 2.40[b]
Experiment 2			
0	8.26± 0.63	7.37± 0.18	8.59± 0.17
ConA			
2.5	65.77± 0.93	49.18± 2.32[b]	52.52± 3.26[b]
5.0	136.95± 1.48	101.98± 1.38[b]	100.82± 2.19[b]
PHA μg/ml			
5	26.54± 0.50	13.95± 0.61[b]	25.21± 0.47
10	46.31± 1.08	33.40± 0.56[b]	38.58± 2.91[b]
LPS μg/ml			
2.5	106.48± 1.33	79.80± 3.25[b]	79.62± 2.97[b]
5.0	125.78± 0.83	102.64± 1.47[b]	90.82± 0.73[b]

[a]Representative mean ± SE from 3 separate triplicate cultures ofspleens from group of 3 mice. [b]Significantly different from controlgroup ($p<0.01$)

Responsiveness to standard IL-2

Responsiveness of splenocyte to IL-2 was measured as described[20], with slight midification. In brief, spleen cells from 3 mice in each group were cultured in triplicate at 4×10^5 cells in 100 μl K-0 containing 10% FBS, in the presence of a grade amount of standard IL-2, in a 96 well round-bottomed microplate for 3 and 5 days at 37℃ in a humidified 5% CO_2 incubator. 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). Results were expressed as mean counts/minute ± SD of triplicate cultures.

Assay of nonspecific suppressor cells activity

8×10^6 spleen cells in 1 ml K-0 medium, were treated with 25 μg/ml mitomycin C (MMC, Sigma) for 30 min at 37℃. After 3 times washing, MMC-treated cells were counted and used as suppressor cells. Suppressor activity of MMC-treated cells was determined by ConA induced lymphocyte proliferation[9]. Briefly, 1×10^5, 2×10^5, 4×10^5, or 8×10^5 MMC-treated suppressor cells were added to freshly prepared cultures containing 2×10^5 or 4×10^5 C57 BL/6 splenocytes (responder) and 5 μg/ml ConA. The cultures were incubated for 62 or 44 hours at 37℃ in 96 well round-bottomed microplate. ^{3}H-thymidine incorporation was determined during the last 18 hours of incubation.

Statistical analysis

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

RESULTS AND DISCUSSION

^{3}H-thymidine uptake of splenocyte appeared to

Table IV. Responsiveness of ConA blasts and resting splenocytes from C57BL/6 female mice to Standard IL-2

Standard IL-2 (U/ml)	^{3}H-Thymidine incorporation(cpm×10^3)	
	ConA blast	Nonstimulated cell
0	4.21± 0.48	0.37± 0.03
5	15.99± 0.43	0.24± 0.04
10	25.60± 0.72	0.25± 0.01
20	34.18± 0.78	0.33± 0.02
40	45.20± 0.81	0.32± 0.01
80	56.06± 1.02	0.45± 0.03
160	64.39± 0.42	ND

Table V. Effects of ConA concentration and Cell density on TCGF production of splenocytes from C57 BL/6 female normal mice

ConA (μg/ml)	FCS%	TCGF activity(U/ml)		
		2×10^6/well	4×10^6/well	8×10^6/well
1	0	18.73± 0.18	19.63± 0.31	13.73± 0.35
2	0	25.73± 0.79	40.40± 2.08	24.70± 0.05
4	0	28.26± 0.53	40.80± 0.88	63.13± 2.84
8	0	15.10± 0.25	39.16± 0.03	154.90± 9.85
8	5	17.70± 0.35	42.83± 2.09	152.46± 12.70

*Representative mean ± SE from 3 separate triplicate cultures
#TCGF activities are expressed as IL-2 unit

be maximal at the cell density of 4×10^5/well, 42-50 hours incubation time, 5-20 μg/ml ConA, 10 μg/ml PHA and 10 μg/ml LPS as shown in Table I and Table II. The uptake declined after 58 hours incubation. Using these condition we investigated the effects of brazilin on the mitogen-induced cell proliferation and the result were shown in Table III. Brazilin (50 mg/kg) significantly suppressed the mitogen-induced proliferation of splenocytes but its mechanism was not yet understood. The result was consistent with the findings that flavonoids generally suppress mitogen induced cell proliferation[1-7,13].

It is well known that TCGF or IL-2 receptor expression is necessary in mitogen induced cell proliferation[10,11]. Therefore, we assessed the effects of brazilin on TCGF production and IL-2 receptor expression in order to get informations on its suppressive nature of cell proliferation. Total TCGF

Table VI. Time course of TCGF production of splenocytes in C57BL/6 female mice

Culture time (H)	TCGF activity(U/ml)
8	0
16	90.26± 5.30
20	167.46± 8.84
28	82.66± 9.22
48	44.08± 4.00

*Representative mean ± SE from 3 separate triplicate cultures
#TCGF activities are expressed as IL-2 unit

activities were expressed as equivalently potent IL-2 activities because IL-2 is a major factor for mitogen induced T cell proliferation. A logarithmic plot of applied standard IL-2 units versus ^{3}H-thymidine uptake of ConA blasts appeared nearly linear (Table IV). ^{3}H-thymidine uptake by ConA blasts responsive to supernatant containing TCGF was calculated using the standard IL-2 calibration curve and expressed as equivalently potent IL-2 activities. Resting cells were nonresponsive to standard IL-2. It is, therefore, certain that ConA blasts are the cells with IL-2 receptor of high affinity. 100 mM α-methylmannoside was sufficient to remove residual ConA in ConA blasts and supernatant containing IL-2. Table V and Table VI show the optimal conditions for TCGF production of splenocytes. TCGF release was maximal at 8 μg/ml ConA and cell density of 4×10^6/ml. The amounts of TCGF in supernatants decreased after 24 hours incubation, due to the absorption of TCGF (IL-2) by IL-2 receptor expressed or secreted during activation of cells[12,14]. As shown in Table VII, brazilin increased TCGF release despite of the inhibition of mitogen induced proliferation (Table III).

It has been reported that IL-2 upregulates its own receptor[15-18]. IL-2 receptor of high affinity was composed of α chain (p70) and β chain(p55, Tac antigen)[18], and the cells with IL-2 receptor of high affinity proliferate in the lower concentration of IL-2 (10^{-12} M). α chain with intermediate affinity, expressed on resting cells, alone requires the more IL-2 (10^{-10} M) for the cell activation and is responsible for IL-2 internalization and probably signal transduction[16]. β chain or Tac antigen with low affinity, is expressed on activated cells. Defects in IL-2 receptor expression may underlie various immunolo-

Table VII. Effects of brazilin on TCGF secretion of normal splenocytes of C57BL/6 female mice

Group	TCGF activity(U/ml)		
	Experiment 1		Experiment 2
	20 H	24 H	24 H
Control	156.67± 1.85	78.00± 3.78	113.70± 5.07
Brazilin 50 mg/kg	214.67± 12.41[b]	117.00± 8.38[b]	140.66± 2.60[b]
CY 40 mg/kg	125.67± 0.21	111.00± 8.00[b]	115.73± 6.06

[a]Representative mean ± SE from 3 separate triplicate cultures of spleens from group of 3 mice.
[b]Significantly different from control group ($p<0.01$)
#TCGF activities are expressed as IL-2 unit

Table VIII. Time course of responsiveness of splenocytes from normal C57BL/6 female mice, to standard IL-2

IL-2 (U/ml)	^{3}H-Thymidine incorporation(cpm×10^{-3}) — Culture time(day) —			
	2	3	5	7
0	2.34± 0.26	3.79± 0.29	3.48± 0.27	5.24± 1.65
200	7.12± 0.49	18.89± 0.17	112.85± 3.85	23.69± 2.64
400	7.84± 0.18	22.04± 0.35	112.11± 4.99	37.18± 4.14
800	12.70± 0.36	34.21± 1.11	149.49± 9.33	29.38± 2.67
1600	20.05± 0.24	52.86± 0.38	171.05± 0.76	33.19± 4.30
3200	30.73± 0.65	79.35± 0.45	140.11± 2.39	25.80± 0.87

*Representative mean ± SE from 3 separate triplicate cultures

gical diseases. Such defects have been described in several experimental models, including systemic lupus erythematosus(SLE) and tumor bearing mice[16]. The responsiveness of splenocytes to exogenous IL-2 was determined in order to evaluate the changes in the expression of functional IL-2 receptor. This method is known to be alternative to the direct determination of total IL-2 receptor using monoclonal anti-Tac antibody[19-23]. Table VIII represents proliferation of splenocytes by exogenous IL-2 at various incubation times. The higher concentration of IL-2 was required for cell proliferation in 3 days' incubation than in 5 days' incubation. Cell proliferation in 3 days' incubation resulted in the increased IL-2 receptor of intermediate affinity or high affinity. On the other hand, only the IL-2 receptor of high affinity was expressed with 5 days' incubation. As shown in Table IX, responsiveness of splenocyte to high unit of IL-2 after 3 days' incubation in the presence of exogenous IL-2 significantly increased in brazilin treated group but after 5 days' incubation it was not changed. Considering

Table IX. Effects of brazilin on responsiveness of splenocytes to standard IL-2 in normal C57BL/6 female mice

IL-2 (U/ml)	^{3}H-Thymidine incorporation(cpm×10^{-3})		
	Control	Brazilin	CY
Day 3			
0	1.13± 0.21	1.23± 0.13	1.08± 0.16
100	8.84± 0.31	11.83± 0.84[b]	6.80± 0.22
200	13.52± 0.26	16.22± 0.19[b]	7.86± 0.17
400	19.40± 0.43	23.74± 0.64[b]	13.84± 0.11
800	23.43± 0.33	34.05± 0.39[b]	19.60± 0.23
1600	28.99± 0.45	47.33± 0.83[b]	26.50± 0.69
DAY 5			
0	1.45± 0.19	1.91± 0.10	1.15± 0.11
100	81.56± 0.47	86.23± 0.88	32.31± 1.68[b]
200	99.78± 3.43	92.65± 1.46	48.19± 5.34[b]
400	110.79± 6.20	124.86± 6.72	90.37± 7.92[b]

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

Table X. Suppressor activities of splenocytes from normal C57BL/6 female mice (Responder 4×10^5/well)

Responder/ Suppressor ratio	^{3}H-Thymidine incorporation(cpm$\times10^{-3}$)	
	ConA 2.5	ConA 5
0	88.77± 2.66	137.02± 4.11
2	57.62± 1.22	66.50± 1.50
1	36.28± 2.67	22.56± 0.43
1/2	6.68± 0.33	2.82± 0.46
1/4	1.07± 0.08	0.61± 0.05

*Representative mean ± SE from 3 separate triplicate cultures

these increases in exogenous IL-2 induced splenocyte proliferation at 3 days' incubation in brazilin treated mice, brazilin is thought either to accelerate the expression of IL-2 receptor of high affinity or to increase the number of cells with IL-2 receptor of intermediate affinity. From the data obtained, it was considered that brazilin inhibits splenocyte activation at sites distal to IL-2 and IL-2 receptor pathway; that is, inhibition of splenocyte proliferation by the treatment of brazilin might be due to the interference with post IL-2 receptor pathway or the changes in lymphocyte subpopulations[14,15,23].

Finally, nonspecific suppressor activity of splenocytes was assessed by ConA induced lymphocyte. Suppressor cells (MMC-treated cells) at the indicated ratios were added to freshly prepared cultures containing 5 μg/ml ConA and 4×10^5 normal splenocytes (responder) and incubated for 42 hours. The results were shown in Table X. ConA response was significantly lowered at the responder/suppressor ratios selected in this experiment. MMC-treated suppressor cells from either brazilin or CY treated mice intensively suppressed the ConA response (Table XI). The mechanisms of their suppressive actions are not well understood and these unexpected results need further study. Exogenous IL-2 was found to promote the suppressor activity of MMC treated normal splenocytes (Table XII), suggesting

Table XI. Effects of brazilin on suppressor activities of splenocytes in normal C57BL/6 female mice (Responder 4×10^5 /well)

Responder/ Suppressor ratio	^{3}H-Thymidine incorporation(cpm$\times10^{-3}$)		
	Control	Brazilin	CY
Experiment 1			
0	91.42± 1.41	91.42± 1.41	91.42± 1.41
2	81.39± 1.75	66.17± 1.06[b]	63.05± 0.12[b]
1	55.30± 1.39	44.27± 3.66[b]	39.94± 2.33[b]
1/2	22.15± 1.08	17.12± 4.33	19.51± 8.26
Experiment 2			
0	133.04± 0.67	133.04± 0.67	133.04± 0.67
2	123.43± 1.63	110.39± 0.64[b]	117.81± 1.29
1	109.34± 0.06	65.71± 1.95[b]	90.80± 1.45[b]
1/2	82.88± 0.36	2.73± 0.29[b]	22.55± 0.15[b]

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

Table XII. Effects of exogenous IL-2 on suppressor activities of splenocytes from normal C57BL/6 female mice (Responder 4×10^5/well)

IL-2 (Brmu/ml)	^{3}H-Thymidine incorporation(cpm$\times10^{-3}$)		
	Responder/Suppressor ratio		
	2	1	1/2
0	81.20± 4.11	83.59± 2.16	60.34± 2.34
40	78.71± 3.31	81.04± 1.32	52.52± 1.17
80	105.24± 1.18	87.32± 0.61	50.84± 1.36
160	100.56± 0.44	88.05± 0.82	52.94± 1.80
320	87.92± 2.76	78.07± 1.33	42.26± 0.41

*Representative mean ± SE from 3 separate triplicate cultures

Table XIII. Effects of brazilin on suppressor activities of splenocytes in normal C57BL/6 female mice (Responder 2×10^5/well)

Responder/ Suppressor ratio	^{3}H-Thymidine incorporation(cpm$\times10^{-3}$)		
	Control	Brazilin	CY
0	67.75± 0.81	67.75± 0.81	67.71± 0.81
2	71.68± 0.83	72.70± 1.14	70.15± 2.39
1	65.42± 1.88	66.16± 1.92	65.16± 2.01
1/2	40.17± 0.82	42.50± 2.64	43.45± 1.69

*Representative mean ± SE from 3 separate triplicate cultures

that increase in TCGF production and/or changes in cell subsets in brazilin treated group could be the most probable factor influencing the suppressor activity. Regarding the fact that CY preferentially inactivated the suppressor T cells[19,20,25], the increase in suppressor activity in brazilin treated group might not be due to the increase in the suppressor T cells. However, brazilin did not show any significant effects on the suppressor system in which 2×10^5 responder and the appropriate amount of suppressor were incubated for 60 hours in the presence of 5 μg/ml ConA (Table XIII).

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