Arch. Pharm. Res. 15(4), 283-288 (1992)

Immunomodulating Activities of Brazilin *in vitro*

Chang-Kiu Moon, Myung-Soo Mock, Kyung-Mee Yang, Hye-Seung Han, Hyeon-Soon Won, Ji-Young Kim, Jin-Ho Chung and Chang-Hyun Moon*

College of Pharmacy, Seoul National University, Seoul 151-742, Korea

**School of Medicine, Ajou University, Soowon 440-649, Korea*

(Received May 7, 1992)

Abstract □ This work was performed to investigate the effects of brazilin *in vitro* on mitogen-induced proliferation, ConA-induced TCGF release and responsiveness to recombinant IL-2 using splenocytes from C57BL/6 female mice. Brazilin(20-80 ng/m*l*) caused a noticeable increase in TCGF production of splenocytes, but did not affect responsiveness to recombinant IL-2, the expression of ConA-induced high affinity IL-2 receptor and mitogen-induced proliferation of splenocytes.

Keywords □ Brazilin, *in vitro* immunomodulation, TCGF release, responsiveness to IL-2, lymphocyte proliferation

Brazilin, an active principle of *Caesalpinea sappan*, is one of the bioflavonoids and has been previously reported to possess widespread biological activities, including antiinflammatory, antihistamic[1,2], and antimicrobial[3,4] properties. It was also found that brazilin inhibits several enzyme activities such as histidine decarboxylase[5] and cAMP phosphodiesterase[6], and improves the RBC deformability[7]. Brazilin has been also found to have antioxidant properties; it inhibited lipidperoxidation in various animal tissues[8]. Especially potent anti-chronic inflammatory effects of brazilin aroused our special interest to investigate the effects of brazilin on immune functions. Our previous reports indicated that brazilin increases delayed type hypersensitivity (DTH) against bovine serum albumin (BSA) and decreases the circulating leukocyte counts, in normal C57BL/6 female mice[9], and brazilin also inhibits lymphocyte proliferation despite the augmentation of TCGF release or the appearance of IL-2 receptor in C57BL/6 female mice[10], while brazilin inhibits mitogen induced cell proliferation and TCGF production in CBA female mice[11].

The purpose of this study was to assess effects of brazilin *in vitro* on the immunological responses of splenocytes from normal C57BL/6 female mice, to elucidate the acting mechanism of brazilin.

EXPERIMENTAL METHODS

Experimental animals

8 week-old C57BL/6 female mice were obtained 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.

Materials

Brazilin (Aldrich) was dissolved in saline to make 1 mg/m*l* and diluted with culture media to the appropriate concentration before use. Different amounts of brazilin were added to the medium at the beginning of the culture, otherwise mentioned.

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), 10mM HEPES (Sigma), 2 g/L sodium bicarbonate (Sigma), 1mM nonessential amino acids (Gibco),

50 μM 2-mercaptoethanol (Sigma) (This formula is referred to as K-0 medium throughout this work). Spleen cell suspension, obtained by disaggregation of chopped tissue in loosely packed homogenizer, was washed by centrifugation (260 g, 6 min) and RBC was lysed by hypotonic shock. After washing 3 times, cell viability was determined by trypan blue exclusion test.

TCGF production

Spleen cells were cultured at 8×10^6 cells in 1 ml K-0 media in the presence of 10 μg/ml ConA 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 used.

TCGF assay

Supernatants were assayed for TCGF by their ability to induce proliferation of ConA-activated T cell blasts as described by Coutinho, Larsson, Gronvik and Anderson(1979), with slight modification. In brief, blasts cell were obtained by stimulating splenocytes with 30 μg/ml ConA for 72 hours. After washing 3 times with K-0 media supplemented with 10% heat inactivated FBS (Gibco) and 100 mM 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 activities were expressed as equivalently potent IL-2 activities. On the other hand, ConA blasts were cultured with various amounts of IL-2 or brazilin, in order to exclude the effect of brazilin remained in supernatants, since supernatants were not dialysed.

Assessment of high affinity IL-2 receptor expression

To determine whether brazilin affects the expression of high affinity IL-2 receptor, splenocytes were incubated with 30 μg/ml ConA in the presence of brazilin. After 72 hours of incubation, the cells were washed and reincubated with recombinant IL-2, as described previously[12].

Responsiveness of normal splenocyte to exogenous recombinant IL-2

Responsiveness of splenocyte to exogenous IL-2 was measured as described by Le Thi Bich-Thuy *et al*[13]. Briefly, spleen cells 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 recombinant IL-2, in a 96 well flat-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 (6.7Ci/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 ± SE of triplicate cultures.

Lymphoproliferative responses to ConA

Spleen cells were cultured 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 5 μg/ml ConA (Sigma, type III). 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 ± SE.

Statistical analysis

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

RESULTS AND DISCUSSION

In the previous studies, it was found that brazilin showed noticeable effects on TCGF release, responsiveness to IL-2, and splenocytes proliferation in mice[10, 12]. The present study was undertaken to determine immunomodulating activities of brazilin *in vitro* for the elucidation of the acting-mechanism. Our first trial was to the effect of brazilin on ConA-induced TCGF production from normal splenocy-

Table I. *In vitro* effects of brazilin on ConA-induced TCGF production of splenocytes from normal C57BL/6 female mice[a]

Brazilin (μg/ml)	TCGF Activity (U/ml)	
	Experiment 1.	Experiment 2.
0	76.66± 10.59	88.30± 7.75
5	92.67± 17.61	ND[c]
10	87.33± 4.50	118.53± 13.27[b]
20	98.00± 5.56	156.06± 9.11[b]
40	135.00± 8.88[b]	151.33± 1.75[b]
80	129.33± 15.88[b]	127.33± 5.51[b]
160	115.33± 14.57	114.73± 13.38
320	107.00± 16.37	113.33± 11.15
625	87.66± 10.26	109.13± 15.48
1250	93.33± 6.34	104.73± 7.12
2500	79.33± 9.29	109.60± 3.65
5000	31.33± 2.51[b]	16.20± 1.11[b]
10000	ND[c]	10.80± 1.12[b]

[a]The results are representative mean ± SD from duplicate experiments.
[b]Significantly different from untreated culture ($p<0.01$).
[c]not determined.

Table II. Effects of brazilin on responsiveness of ConA blasts to standard IL-2[a] (80 U/ml)

Brazilin (μg/ml)	^{3}H-Thymidine uptake (cpm×10^{-3})	
	Experiment 1.	Experiment 2.
0	48.10± 2.13	79.30± 2.23
5	48.04± 0.76	75.13± 2.24
10	47.59± 2.27	77.90± 0.79
20	46.83± 1.64	78.84± 2.41
40	53.18± 0.75	76.86± 1.74
80	47.74± 1.29	78.45± 2.34
160	47.84± 0.51	74.53± 2.49
320	49.78± 1.30	75.37± 2.72
640	44.29± 2.05	56.90± 5.59[b]
1250	43.31± 0.91	54.36± 3.41[b]
2500	39.03± 1.98[b]	45.40± 5.03[b]
5000	29.00± 0.59[b]	13.92± 1.11[b]

[a]The results are representative mean± SE from duplicate experiments.
[b]Significantly different from untreated culture ($p<0.01$).

tes. Normal splenocytes (8×10^6/ml) were incubated at various concentrations of brazilin at 30℃ for 20 hours. But any significant increase in TCGF activity was not observed in the supernatants of splenocytes incubated with brazilin for 20 hrs without Con A treatment. As shown in Table I, brazilin (20-80 ng/ml) significantly increased the TCGF levels in the supernatant from the ConA-stimulated splenocytes, compared to control preparation. This result is consistent with the previous observations in which an increase was found *in vivo* in delayed type hypersensitivity by the treatment of brazilin[10-12]. The precise nature of the enhancing effect of brazilin on TCGF production should be further elucidated. However, TCGF production was inhibited in the concentrations more than 5 μg/ml brazilin. ConA blasts were cultured with various amounts of recombinant IL-2 and/or brazilin in order to exclude the possible effect of brazilin remained in supernatants, since supernatants were not dialysed. As presented in Table II, brazilin itself showed no significant direct effects on proliferation of ConA blasts and recombinant IL-2 induced proliferation of ConA blasts was not interrupted in the concentrations below 640 ng/ml brazilin. Thus the increase in TCGF release by brazilin is ascribed to the stimulation of the synthesis and/or release of TCGF from splenocytes.

Freshly prepared resting splenocytes are known to proliferate in response to high doses of recombinant IL-2, and the activated splenocytes with high affinity IL-2 receptor proliferate in the presense of physiological concentration of IL-2[15-17]. Therefore, we examined the responsiveness of splenocytes to exogenous IL-2 in order to evaluate indirectly the changes in the expression of functional IL-2 receptor, instead of the direct assay of total IL-2 receptor using monoclonal anti-Tac antibody[18-20]. Table III represents proliferation of resting splenocytes by the addition of exogenous IL-2 for 3 days or 5 days. The higher concentration of IL-2 was required for cell proliferation in 3 days' incubation than that in 5 days' incubation. Three days' incubation resulted in increased number of intermediate or high affinity IL-2 receptors, but with 5 days' incubation, only high affinity IL-2 receptors were expressed. Brazilin did not exhibit any significant changes in the proliferative response of splenocytes to IL-2. This results were not in accordance with the *in vivo* results in which the exogenous IL-2 induced proliferation of splenocytes from brazilin treated mice was increased by 3 days' incubation[11,12]. As shown in Table IV, brazilin did not interfere with the exp-

Table III. Effects of brazilin on responsiveness of resting splenocytes to standard IL-2[a]

Brazilin (μg/ml)	^{3}H-Thymidine incorporation (cpm×10^{-3})			
	Day 3		Day 5	
	800 U/ml	3200 U/ml	50 U/ml	200 U/ml
0	35.87± 0.57	68.26± 1.29	58.97± 7.35	86.58± 8.67
5	35.16± 1.92	68.06± 1.03	53.32± 2.29	82.18± 5.09
10	37.60± 0.75	65.82± 0.30	47.46± 3.73	72.82± 6.39
20	37.08± 1.44	68.89± 0.35	59.07± 2.43	71.87± 10.5
40	34.89± 1.93	71.25± 3.06	64.88± 3.96	90.49± 4.76
80	37.72± 0.54	71.17± 0.99	65.77± 3.21	93.78± 4.31
160	38.01± 1.61	72.39± 1.71	63.44± 1.78	84.50± 3.81
320	38.59± 0.05	66.65± 5.80	52.60± 1.52	81.78± 3.23
640	36.91± 0.64	70.68± 1.76	55.60± 2.17	82.68± 5.02
1250	36.68± 1.00	67.29± 0.58	48.84± 1.80	79.00± 9.45
2500	26.10± 0.29[b]	58.44± 4.46[b]	34.17± 1.56[b]	56.16± 1.98[b]
5000	17.94± 0.54[b]	34.79± 0.86[b]	19.90± 1.00[b]	24.65± 0.26[b]

[a]The results are representative mean± SE from duplicate experiments.
[b]Significantly different from untreated culture ($p<0.01$).

Table IV. Effect of brazilin on ConA induced expression of high affinity IL-2 receptor[a]

Brazilin (μg/ml)	^{3}H-Thymidine incorporation (cpm×10^{-3})		
	Standard IL-2 (U/ml)		
	20	40	80
0	44.88± 1.95	56.02± 0.42	61.88± 0.64
5	46.16± 0.35	54.87± 0.09	65.00± 1.01
10	41.14± 0.42	49.61± 1.04	59.88± 1.20
20	43.06± 0.38	49.88± 4.20	59.67± 0.70
40	42.92± 1.37	51.98± 0.69	60.61± 1.15
80	42.75± 0.72	51.68± 0.76	57.32± 0.63
160	46.45± 0.72	54.28± 1.54	64.18± 0.42
320	37.58± 1.84	44.66± 0.14	57.51± 3.47
640	48.55± 0.95	56.41± 1.86	64.23± 0.80
1250	47.51± 0.58	60.05± 1.52	65.00± 1.21
2500	42.24± 0.76	57.52± 0.62	67.13± 0.77
5000	10.44± 0.47[b]	11.86± 0.24[b]	12.76± 0.25[b]

[a]The results are representative mean± SE from duplicate experiments.
[b]Significantly different from untreated culture ($p<0.01$).

ression of high affinity IL-2 receptor, that is, with the acquisition of the responsiveness to IL-2 of ConA blasts with high affinity IL-2 receptor.

Table V indicates that ConA induced proliferation of splenocytes was not affected by the treatment of brazilin. No explanation was still possible why brazilin did not have any effects on ConA induced proliferation of splenocytes in spite of the augmentation of TCGF release in the concentrations of 20-80 ng/ml.

The results obtained in this *in vitro* study were different from the *in vivo* experimental results that brazilin decreasecs splenocyte proliferation and increases TCGF release and responsiveness to IL-2.

Table V. Effects of brazilin on ConA induced T cell proliferation[a]

Brazilin (μg/ml)	ConA (μg/ml)	^{3}H-Thymidine incorporation (cpm×10^{-3})		
		Culture time (Hour)		
		16	24	32
Experiment 1				
0	2.5	15.99± 0.51	37.55± 0.98	45.01± 0.98
5	2.5	14.60± 0.24	33.68± 1.05	44.92± 0.97
10	2.5	17.52± 0.07	33.29± 0.93	40.50± /0.15
20	2.5	16.89± 0.16	31.64± 0.91	42.40± 2.37
40	2.5	18.34± 0.13	34.98± 0.12	43.87± 1.13
80	2.5	17.08± 0.24	34.32± 0.60	43.74± 0.71
160	2.5	17.18± 0.13	34.80± 1.09	42.20± 1.06
320	2.5	17.25± 0.18	36.56± 1.81	47.24± 1.31
640	2.5	14.87± 0.09	29.84± 0.89	40.11± 0.62
1250	2.5	18.48± 0.48	29.55± 0.71	39.74± 0.53
2500	2.5	4.17± 0.07[b]	9.12± 0.21[b]	9.23± 0.46[b]
5000	2.5	1.32± 0.07[b]	0.52± 0.06[b]	0.34± 0.04[b]
Experiment 2				
0	5	75.84± 1.52	119.44± 0.40	126.59± 0.23
5	5	68.53± 0.41	113.86± 3.41	114.12± 0.84
10	5	82.03± 1.17	100.14± 4.13	111.98± 1.98
20	5	85.94± 1.01	98.50± 0.80	110.12± 2.96
40	5	87.39± 0.36	104.29± 0.68	102.37± 0.84
80	5	82.76± 0.73	101.58± 2.20	105.75± 1.30
160	5	81.63± 2.21	105.99± 1.78	102.92± 0.71
320	5	79.23± 0.56	118.54± 0.82	109.34± 0.83
640	5	74.57± 0.21	105.11± 1.73	109.66± 0.66
1250	5	64.78± 1.04	95.30± 2.29	108.38± 0.15
2500	5	12.13± 0.69[b]	27.36± 0.55[b]	43.78± 1.92[b]
5000	5	2.58± 0.03[b]	0.38± 0.05[b]	0.52± 0.14[b]
Experiment 3				
0	10	155.32± 0.48	184.68± 4.11	163.25± 0.48
5	10	149.70± 2.42	172.66± 1.51	150.64± 0.15
10	10	164.21± 1.29	164.07± 2.35	147.18± 2.20
20	10	159.59± 0.92	166.96± 0.92	147.68± 2.37
40	10	170.44± 0.08	164.79± 1.23	150.58± 0.72
80	10	162.83± 2.09	169.57± 1.27	141.78± 1.92
160	10	164.74± 2.47	167.40± 1.35	135.43± 1.72
320	10	157.08± 2.09	157.64± 2.72	138.09± 0.53
640	10	159.60± 3.53	157.58± 3.61	144.15± 0.88
1250	10	136.95± 1.93	147.04± 0.57	149.17± 0.98
2500	10	32.92± 0.97[b]	63.15± 2.46[b]	88.10± 3.67[b]
5000	10	2.92± 0.03[b]	0.38± 0.03[b]	0.54± 0.03[b]

[a]The results are representative mean± SE from duplicate experiments.
[b] Significantly different from untreated culture ($p<0.01$).

This difference might be resulted from the changes in cell subsets rather than from those in activites of the cells themselves[10-12].

ACKNOWLEDGEMENT

This work was partially supported by Research

Center for New Drug Development, KOSEF

LITERATURE CITED

1. Gabor, M. and Engi, E.: Effect of natural and semisynthetic flavonoids on croton oil induced rabbit ear edema. *Kiserl Orvostud(Hung).* **39**, 433 (1987).
2. Hikino, H., Tarashi, T., Hajime, F. and Yasuzo, H.: The validity of oriental medicine 3. Antiinflammatory principles of Caesalpinia sappan wood and of Hematoxylin campechianum wood. *Planta medica,* **31** 214 (1977).
3. Aizenman, B. Y., Shvaiger, M. O., Mandrik, T. P., Zelepukha, S. I. and Kiprianova, O. A.: Comparison of antimicrobial activity of different dye groups with their *in vitro* activity on cells of Ehrlich adenocarcinoma. Mikrobiol Zh., Akad Nauk Ukr RSR **22**, 52 (1961).
4. Goncalves de Lima, O., Dalia Maria, M. H., Machado, M. and Macial, G. M.: Antibiotic spectra of some flavonoids and of brazilin. *Rev. Inst. Antibiot. Univ. Recife.* **3**, 81 (1961).
5. Gabor, M., Szorady, I. and Dirner, Z.: Inhibiting effects of the members of hematoxylin group on the action of histidine decarboxylase. *Acta. Physiol. Acad. Sci. Hunf.,* **3** 595 (1952).
6. Nikaido, T., Ohmoto, T., Noguchi, H., Kinishita, T., Saito, H. and Sankawa, U. : Inhibitors of cAMP phosphodiesterase in medicinal plants. *Planta Medica* **43**, 18 (1981).
7. Moon, C. K., Chung, J. H., Lee, Y. M. and Lee, S.H.: Effects of brazilin on erythrocyte deformability in streptozotocin induced diabetic rats. *Toxicologist,* **9**, 288 (1989).
8. Moon, C. K., Ha, B. J., Lee, S. H. and Mock, M. S.: A study on the antilipidperoxydative effects of brazilin and hematoxylin (I). *Kor J Food Hygiene.* **2**, 35(1987).
9. Moon, C. K., Mock, M. S., Lee, S. H., Park, K. S., Hwang, G. S. and Ha, B. J.: Immunomodulating activities of brazilin and hematoxylin in normal young mice. *Kor. J. Toxicol.,* **4**, 151 (1988).
10. Moon, C. K., Mock, M. S., Yang, K. M., Lim, C. H., Kim, K. S., Chung, J. H. and Moon, C. H.: Brazilin inhibits the mitogen -induced cell proliferation despite the augmentation of TCGF production and expression of IL-2 receptor. *Arch. Pharm. Res.,* **1**, 275 (1992).
11. Moon, C. K., Mock, M. S., Yang, K. M., Chung, J. H. and Ha, B. J.: Brazilin modulates the immune function in normal CBA female mice. *Kor. J. Toxicol.,* **8**, 1 (1992).
12. Coutinho, A., Larsson, E., Gronvik, K. and Anderson, J.: Studies on T lymphocyte activation II. The target cells for ConA induced growth factors. *Eur. J. Immunol.,* **9** 587 (1979).
13. Le Thi Bich-Thuy, Ducovich, M., Peffer, N. J., Fauci, A. S., Kehrl, J. H. and Greene, W. C.: Direct activation of human resting T cells by IL-2; the role of an IL-2 receptor distinct from the Tac protein. *J. Immunol.* **139**, 1550 (1987).
14. Wang, H. M., Smith, K. A.: The IL-2 receptor; functional consequences of its bimolecular structure. *J. Exp. Med.* **166**, 1055 (1987).
15. Tanaka, T., Saiki, O., Doi, S., Negoro, S. and Kishimoto, S.: IL-2 functions through novel IL-2 binding molecules in T cells. *J. Immunol.* **140**, 470 (1988).
16. Holan, V. and Lipoldova, M.: Low responsiveness of spleen cells from tumor bearing mice to recombinant IL-1 and IL-2. Impaired expression of IL-2 receptors. *Int. J. Cancer,* **45**, 798 (1990).
17. Spitz, M., Gearing, A., Callus, M., Spitz, L. and Thorpe, R.: IL-2 *in vivo*; production and response to IL-2 in lymphoid organs undergoing a primary immune response to heterologous RBCs. *Immunol.* **54**, 527 (1985).
18. Robb, R. J., Greene, W. C. and Rusk, C. M.: Low and high affinity cellular receptors for IL-2; Implications for the levels of Tac antigen. *J. Exp. Med.* **160**, 1126 (1984).
19. Hirono, I., Flavonoids.: in "*Naturally occuring carcinogens of plant origin; toxicology, pathology and biochemistry*". edited by Hirono I, chapter III. 1987.
20. Brache, M. E., De Pestal, G., Castronovo, V., Vynche, B., Foldart, J. M. Vakeat, L. and Mareel, M. M.: Flavonoids inhibit malignant tumor invasion *in vitro. Prog. Clin. Biol. Res.* **280**, 219 (1988).