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Effects of Brazilin on Induction of Immunological Tolerance by Sheep Red Blood Cells in C57BL/6 Female Mice

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Brazilin was examined for its effects on the induction of immunological tolerance. Brazilin was administered to C57BL/6 female mice for 2 consecutive days before the immunization with high dose SRBC (10^9 cells) which can produce immunological tolerance. Delayed type hypersensitivity, IgM plaque forming cells, ConA induced IL-2 production and mitogen- or antigen-induced proliferation of lymphocytes were measured as evaluation parameters. Administration of brazilin prior to immunization could keep the DTH and IL-2 production almost optimaly immunized levels. Brazilin also inhibited the elevation of non-specific suppressor cell activity. ConA induced proliferation of splenocytes in high dose SRBC immunized mice was significantly decreased by pretreatment of brazilin. And this might be one of the reason for augmentation of DTH by brazilin. However, IgM plaque forming cells were not affected by the treatment of brazilin. These results indicate that brazilin prevents the induction of immunological tolerance caused by high dose SRBC by suppressing the elevation of suppressor cell activity and by inhibiting the decrease in IL-2 production in C57BL/6 female mice.

Key words : Brazilin, *Caesalpinia sappan*, Immunological tolerance, Delayed type hypersensitivity, Interleukin-2, Nonspecific suppressor activity

INTRODUCTION

It has been suggested that antigen induced tolerance may be closely related to elevation of suppressor cell activity or clonal anergy (Bach *et al.*, 1978). The suppressor cells act via the secretion of suppressive cytokines such as TGF-β or IL-4 to T cell-mediated immune reactions after being triggered by tolerogens. Although tolerance is induced by specific tolerogen, it is thought that the suppressor cells may be able to suppress immune responses in antigen nonspecific fashion (Gershon, 1975). Clonal anergy is thought to be induced when T cells are stimulated with antigen via non-professional antigen presenting cells which are lack of secondary signals, that is, expression of adhesion molecules and production of cytokines. As non-specific suppressor cell plays a key role in the induction of immunological tolerance, any chemicals that can affect the suppressor cell activity may be able to modulate immune functions in immunological tolerance. As the immune responses in immunological tolerance are very similar to those of immunodeficient diseases, immunological tolerance has been used as an experimental model for the investigation of immune dysfunction.

Brazilin, an active principle of *Caesalpinia sappan*, has been previously reported to show immunomodulating activities. Recently we reported that brazilin modulate the early abnormal immune dysfunction in some experimental animal models (Choi *et al.*, 1997a, Choi *et al.*, 1997b). From these studies, we found that the effects of brazilin are mainly based on the modulation of T cell functions through affecting nonspecific suppressor cell activities. These immunomodulating activities led us to investigate the effects of brazilin on immune functions in C57BL/6 female mice treated with high dose SRBC which could produce immunological tolerance.

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MATERIALS AND METHODS

Experimental animals and treatment

8 week-old C57BL/6 female mice were purchased from the Animal Breeding Center of Seoul National University. These mice were maintained on a 12/12 hrs day/night cycle with ad libitum access to standard rodent diet and water in air-filtered room (21~24°C). Brazilin monohydrate (Aldrich) was suspended in saline and sonicated.

Mice were intraperitoneally given brazilin for 2 consecutive days from the indicated day. Controls received saline alone. For *in vitro* experiments, brazilin was suspended in normal saline (1 mg/ml) and diluted with culture media on use. Cyclophosphamide (CY, Sigma) was dissolved in normal saline just before use and 40 mg/kg b.w. of CY was intraperitoneally administered to mice. Sheep red blood cells (SRBC) in Alsever's solution (Korea Media) were washed three times with saline. Mice received either 10^6 or 10^9 SRBCs in 0.2 ml via the tail vein.

Assay of delayed type hypersensitivity (DTH)

10^8 SRBC were injected into one hind footpad 4 days after immunization. 24 hrs after the immunization, DTH was assessed by measuring the increase in dorsoventral thickness of the test group over that of the control group using micrometer (Mitsutoyo, Japan). All measurements were conducted by the same individual, and the results were expressed as specific increases in footpad thickness.

Preparation of spleen cell suspension

Spleens were removed 4 days after immunization 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 uM 2-mercaptoethanol (Sigma) (Throughout this work, this formula is referred to as K-0 medium). Pooled splenocytes 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 SRBC were lysed by hypotonic shock. After 3 times washing by centrifugation, cell viability was determined by trypan blue exclusion test.

Production of interleukin-2 (IL-2)

Splenocytes from 3 mice in each group were pooled and cultured at the density of 8×10^6 cells in 1 ml K-0 media containing 10 μg/ml ConA (Sigma, type III), in a 24 well microplate (Falcon). After 24hrs incubation at 37°C in a humidified 5% CO_2 incubator, supernatants were harvested and stored at 4°C until assay. For the investigation of in vitro effect, brazilin was added to splenocytes culture medium at the initiation of culture.

Assay of IL-2 activity

The supernatants were used for quantitation of IL-2 by their ability to maintain proliferation of CTLL-2 cells (mouse T cell line), which proliferate dose dependently responding to the amount of IL-2. 50 μl of CTLL-2 cells (1×10^5 cells/ml) were incubated with 50 μl of the serially diluted supernatant in 96 well round bottomed culture plate for 24 hrs at 37°C in a humidified 5% CO_2 incubator. After ^{3}H-thymidine (0.5 μCi/well) pulsation for next 24 hrs, cells were harvested and subjected to liquid scintillation counting. For the determination of standard curves, serially diluted recombinant IL-2 was used as standard.

Assay of nonspecific suppressor cell activity

Splenocytes (8×10^6 cells/ml) from CBA mice were treated with 50 μg/ml mitomycin-C (MMC, Sigma) for 30 min at 37°C and used as stimulator cells. Splenocytes (8×10^6 cells/ml) obtained from each treated group were treated with 50 μg/ml mitomycin-C (MMC, Sigma) for 30 min at 37°C and used as suppressor cells. Splenocytes obtained from normal C57BL/6 mice were used as responder cells. Suppressor cells, stimulator cells and responder cells were placed in 96 well round-bottomed plate and incubated for 96 hrs at 37°C. After ^{3}H-thymidine (0.5 μCi/well) pulsation for next 24 hrs, cells were harvested and subjected to liquid scintillation counting. Results were expressed as mean counts/minute±SD.

Assay of conA- or SRBC-induced proliferation of splenocytes

Splenocytes from 3 mice in each group were pooled and splenocytes suspensions were prepared. Splenocytes (4×10^5 cells/well) were incubated with ConA (5 μg/ml) or SRBC (0.5%) for 44 hrs (in case of ConA) or for 90 hrs (in case of SRBC) at 37°C in a humidified 5% CO_2 incubator. After ^{3}H-thymidine (0.5 μCi/well) pulsation for next 18 hrs, cells were harvested and subjected to liquid scintillation counting. Results were expressed as mean counts/minute±SD.

Assay of IgM plaque forming cell (PFC)

Splenic IgM PFCs against SRBC were quantified at 4th day after immunization with SRBC (Cunningham and Szenberg, 1968). Reaction mixture was composed of splenocyte suspension (50 μl), 12.5% SRBC (100 μl), 1/5 diluted guinea pig serum (100 μl, Gibco) and BSS (250 μl) and pipetted in to the Cunningham's dual chamber.

Statistical analysis

The significance of the differences between the means was evaluated by Student's *t*-test. Values which differ from control over $P<0.01$ were considered as significant.

RESULTS AND DISCUSSION

To see if brazilin could prevent the induction of immunological tolerance by SRBC in C57BL/6 mice, we investigated the effect of brazilin on non-specific suppressor cell activity in C57BL/6 female mice treated with high dose SRBC.

It was reported that maximal DTH responses to SRBC could be elicited in normal mice by intravenous immunization with optimal dose of SRBC (10^6 cells), while high dose SRBC (10^9 cells) produced minimal responses or unresponsiveness of DTH (Laglange *et al.*, 1974). As seen in Table I, the optimal experimental condition was reproduced in this study. Pretreatment of brazilin was proved to inhibit the decrease in DTH induced by high dose SRBC.

The preventive effects on the induction of immunological tolerance were observed over the range of administration times (-12~-4 days before the immunization) and doses (1~50 mg/kg b.w.) (Table II, III). Particularly, it is of special interest that brazilin exhibited the preventive effect even in the case of administration at 12 days prior to immunization.

To elucidate the prevention mechanism of brazilin on immunological tolerance, ConA-induced IL-2 release from splenocytes and nonspecific suppressor activity of splenocytes in mice treated with high dose SRBC were observed. As shown in Table I, II and III, the positive relationships were found between the amounts of IL-2 released from splenocytes and DTH responses; the amount of IL-2 was significantly reduced in high dose SRBC treated mice, and IL-2 release was increased by pretreatment of brazilin. In addition, brazilin directly increased the production of IL-2 in vitro (Table IV).

In addition, brazilin significantly reduced non-specific suppressor cell activities of the splenocytes of high dose SRBC treated mice (Table V). The suppression of IL-2 release from splenocytes of tolerated mice might be due to the increased nonspecific suppressor activity of splenocytes (Malkovsky *et al.*, 1982). Therefore, the obtained results hitherto suggest that brazilin could improve the immunological alterations not only by increasing IL-2 production but also by decreasing non-specific suppressor cell activity.

This suggestion could be supported by several reports. IL-2 is one of the determinants which regulate the balance between immunity and unresponsiveness in DTH (Asherson and Colizzi, 1985). The inhibition of IL-2 production might result from either nonspecific suppressor factor produced by T suppressor cells (Kramer and Koszinowski, 1982) or direct inactivation of helper

Table I. Effects of brazilin administration on DTH reactions and IL-2 production from splenocytes of C57BL/6 female mice immunized with SRBC

Group	Foot pad thickness (×0.1 mm)	IL-2 contents (U/10^7 cells/ml)
10^6 SRBC	14.72±0.69	87.45±7.97
10^9 SRBC	3.80±0.77[a]	10.70±8.41[a]
10^9 SRBC+brazilin	10.32±0.51[b]	95.89±18.57[b]
10^9 SRBC+CY	8.62±0.34[b]	59.95±3.17[b]

Mice were immunized by the intravenous injection of 10^6 or 10^9 SRBC into the tail vein. Brazilin (50 mg/kg b.w.) was intraperitoneally administered for 2 consecutive days from 4 days prior to immunization. Cyclophosphamide (40 mg/kg b.w.) was intraperitoneally injected 2 days before the immunization. 4 days after immunization, 10^8 SRBC were injected into one footpad and at 24 hrs after challenge, its thickening was measured using calipers in comparison with another footpad injected with physiological salt solution. Results are expressed as mean±SD obtained from group of 5 mice.
Spleens were removed 4 days after immunization. IL-2 production was induced with ConA (10 μg/ml) in splenocytes from each group. IL-2 activity of supernatants was assessed by using CTLL-2 cell lines. Results are mean Units±SD.
[a]Significantly different from 10^6 SRBC immunized group ($p<0.01$)
[b]Significantly different from 10^9 SRBC immunized group ($p<0.01$)

Table II. Effects of administration time of brazilin on DTH reactions and IL-2 production from splenocytes of C57BL/6 female mice immunized with SRBC

Group	Treatment day	Footpad thickness (×0.1 mm)	IL-2 content (U/10^7 splenocytes/ml)
10^9 SRBC		2.40±0.51	52.33±3.05
10^9 SRBC+brazilin	-4	7.43±0.32[b]	123.33±0.57[b]
10^9 SRBC+brazilin	-8	7.00±0.36[b]	165.00±6.55[b]
10^9 SRBC+brazilin	-12	6.30±1.35[b]	135.33±4.04[b]

Brazilin was intraperitoneally administered on indicated days. DTH and IL-2 production were assessed by same methods as shown in table I.
[a]Significantly different from 106 SRBC immunized group ($p<0.01$).
[b]Significantly different from 109 SRBC immunized group ($p<0.01$).

Table III. DTH reaction and IL-2 production from splenocytes in C57BL/6 female mice treated with various doses of brazilin prior to immunization with SRBC

Group	Footpad thickness (×0.1 mm)	IL-2 content (U/ml)
10^6 SRBC	10.28±0.86	338.00±22.51
10^9 SRBC	3.35±0.35[a]	135.33± 8.32[a]
10^9 SRBC+1 mg/kg brazilin	7.10±0.56[b]	321.66±31.38[b]
10^9 SRBC+10 mg/kg brazilin	7.90±0.32[b]	399.00±56.04[b]
10^9 SRBC+50 mg/kg brazilin	8.28±1.76[b]	358.00±13.85[b]

Mice were immunized by injection of 10^6 or 10^9 SRBC into the tail vein. Indicated doses of brazilin was intraperitoneally administered for 2 consecutive days from 4 days prior to immunization. DTH and IL-2 production were assessed by same methods as shown in table I.
[a]Significantly different from 106 SRBC immunized group ($p<0.01$).
[b]Significantly different from 109 SRBC immunized group ($p<0.01$).

Table IV. *In vitro* effects of brazilin on conA-induced IL-2 production from splenocytes of normal C57BL/6 female mice

Brazilin (ng/ml)	IL-2 contents (U/10^7 splenocytes/ml)	
	Experiment 1	Experiment 2
0	76.66±10.59	88.30± 7.75
5	92.67±17.67	ND
10	87.33± 4.50	118.53±13.27*
20	98.00± 5.56	156.06± 9.11*
40	135.00± 8.88*	151.33± 1.75*
80	129.33±15.88*	127.33± 5.51*
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**	16.20± 1.11
10000	ND	10.80± 1.12

ConA induced IL-2 production from splenocytes of normal mice was measured. Brazilin was added to medium and cells were incubated for 20 hrs. IL-2 activity of supernatants was assessed by using CTLL-2 cell lines. Results are mean Units± SD.
*Significantly different form untreated control ($p<0.01$).
**Significantly different form untreated control ($p<0.01$).
ND: not determined.

Table V. Nonspecific suppressor activities in splenocytes from C57BL/6 female mice treated with brazilin prior to immunization with SRBC

Group	^{3}H-thymidine incorporation (cpm×10^{-3})	
	Experiment 1	Experiment 2
10^6 SRBC	20.30±3.60	32.23±3.32
10^9 SRBC	12.99±1.72[a]	17.77±2.25[a]
10^9 SRBC+Brazilin	23.76±5.41[b]	28.30±3.50[b]
10^9 SRBC+CY	23.88±3.50	ND

Mice were immunized by injection of 10^6 or 10^9 SRBC into the tail vein. Brazilin was intraperitoneally administered for 2 consecutive days from 4 days prior to immunization. CY was intraperitoneally injected 2 days before immunization.
4 days after immunization, spleens were removed from 3 mice in each groups. Non-specific suppressor activity was assessed using one way mixed lymphocyte reaction. Results are represented as mean±SD.
[a]Significantly different from 106 SRBC immunized group ($p<0.01$).
[b]Significantly different from 109 SRBC immunized group ($p<0.01$).

T cell by antigen (Muettler *et al.*, 1989). Reduction of lymphocyte mobility by IL-2 implicates that IL-2 might play an important role *in vivo* in lymphocyte trafficking in antigen exposed or inflammatory sites (Hoon *et al.*, 1986), which might be one of the reason for the increase of DTH by brazilin.

Many immunomodulators showed their effects by affecting the lymphocyte subsets or by acting differentially on particular cell subsets. Immunomodulators, such as methotrexate and cyclosporin A, reversed the elevated Th/Ts ratios and the reduced ConA responses of arthritic rats, toward normal non-arthritic values (Jaffee *et al.*, 1989). Cyclophosphamide (CY)-pretreatment was found to increase the pan T/Ts/c ratios in guinea pig lymph nodes during the development of contact sensitivity (Baker *et al.*, 1987). It was reported that splenocytes from CY treated mice required high dose of ConA for the maximal stimulation than those from untreated mice, while the phenotype of responding cells in ConA response were Lyt1+ T cells (Ikezawa *et al.*, 1987). In this experiment, ConA responses of splenocytes from high dose SRBC treated mice were higher than those of splenocytes from normal mice. This higher response might be mainly due to the proliferation of Ts cells. And ConA-induced proliferation of splenocytes from high dose SRBC immunized mice was significantly decreased by brazilin pretreatment (Table VI). Hence, we suppose that brazilin might prevent the alteration of lymphocyte subsets caused by tolerating antigen. Mice treated with B cell depleting dose (200~300 mg/kg b.w.) of CY suppressed anti-

Table VI. ConA or SRBC induced proliferation of splenocytes from C57BL/6 female mice treated with brazilin prior to immunization with SRBC

Group	^{3}H-thymidine incorporation (cpm×10^{-3})			
	ConA (5 μg/ml)		SRBC (0.5%)	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
10^6 SRBC	62.53±7.98	128.98±9.51	66.12±4.28	42.29±3.30
10^9 SRBC	90.37±8.58[a]	140.81±15.11	75.56±4.73	71.51±7.68[a]
10^9 SRBC+Brazilin	50.21±3.77[b]	79.78± 6.60[b]	78.18±4.21	80.95±0.30
10^9 SRBC+CY	45.35±5.55	90.60± 4.91[b]	85.56±5.12	75.77±5.17

Mice were immunized by injection of 10^6 or 10^9 SRBC into the tail vein. Brazilin was intraperitoneally administered for 2 consecutive days from 4 days prior to immunization. CY was intraperitoneally injected 2 days before immunization.
4 days after immunization, spleens were removed from 3 mice in each group. Splenocytes were incubated with ConA for 42 hrs or incubated with SRBC for 96 hrs. ^{3}H-thymidine pulsation was performed for last 18 hrs of incubation. Results are represented as mean±SD.
[a]Significantly different from 10^6 SRBC immunized group ($p<0.01$)
[b]Significantly different from 10^9 SRBC immunized group ($p<0.01$)

Table VII. Splenic IgM PFCs in C57BL/6 female mice treated with brazilin prior to immunization with SRBC

Group	IgM PFCs/spleen	
	Experiment 1	Experiment 2
10^6 SRBC	340±34	8625±1060
10^9 SRBC	75600±5939[a]	26320±590[a]
10^9 SRBC+Brazilin	81577±6848	38740±4214
10^9 SRBC+CY	89185±5140	34860±593

Mice were immunized by injection of 10^6 or 10^9 SRBC into the tail vein. Brazilin was intraperitoneally administered for 2 consecutive days from 4 days prior to immunization. CY was intraperitoneally injected 2 days before immunization.
IgM PFCs were quantified 4 days after immunization by the method of Cunningham *et al.*
Results are represented as mean±SD.
[a]Significantly different from 10^6 SRBC immunized group ($p<0.01$).

body responses and enhanced DTH to SRBC (Lagrange *et al.*, 1974). CY was also found to augment the DTH without affecting antibody response at the low dose (20 mg/kg b.w.) (Askenase *et al.*, 1975). These results raised the possibility that in the regulation of DTH, antibody feedback and suppressor T cells might be involved. Different from the case of CY, brazilin didn't show any effect on IgM PFC in mice sensitized with high dose of SRBC (Table VII). This fact suggests that the augmentation of DTH by brazilin might not be mediated by the antibody feedback.

In summary, brazilin prevents the induction of immunological tolerance in SRBC-induced immunological tolerant state of C57BL/6 female mice by suppressing the elevation of suppressor cell activity and by inhibiting the decrease in IL-2 production.

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