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Immunomodulatory activity of the aqueous extract from rhizome of *Smilax glabra* in the later phase of adjuvant-induced arthritis in rats

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Abstract

Our previous paper has reported that the aqueous extract from *Rhizoma Smilacis Glabrae* (RSG) remarkably inhibited the primary inflammation of adjuvant arthritis (AA) in rats. In the present study, we further examined the activity of RSG and its mechanism on the secondary inflammation of AA. The administration of RSG (400 and 800 mg/kg) during the later phase significantly inhibited the swelling of the adjuvant-non-injected footpad of AA rats. The lipopolysaccharide-induced production of IL-1, TNF and NO by peritoneal macrophages was significantly reduced. In contrast, the extract significantly recovered the decrease in weight gain of the AA rats and Concanavalin A-induced T lymphocyte proliferation and IL-2 production by their splenocytes, while prednisolone (10 mg/kg) showed a significant aggravation. Furthermore, RSG significantly recovered the picryl chloride-induced delayed-type hypersensitivity to almost normal levels from the higher or lower levels induced by different treatments of cyclophosphamide with a normalization of CD4/CD8 ratio. These results suggest that RSG exhibit an improvement on AA through down-regulating over-activated macrophages and up-regulating the dysfunctional T lymphocytes during the later phase of arthritis. Such characteristics of RSG on AA may be advantageous to the long-term treatment of clinical rheumatoid arthritis.

Keywords: *Rhizoma Smilacis Glabrae*; Immunomodulatory activity; Adjuvant arthritis; T lymphocytes; Macrophages

1. Introduction

Rheumatoid arthritis (RA) is a kind of chronic immunological disease. Investigations on the pathogenesis have revealed the primary involvement of cellular immune response (CIR), such as an increase in IL-1 production by macrophage, a decrease in T lymphocyte proliferation and IL-2 production, as well as a decrease in functions of suppresser T cells (Firestein et al., 1987; Jasin and Dingle, 1981; Miossec et al., 1987; Sakane et al., 1982). For the treatment of RA, non-steroidal anti-inflammatory drugs (NSAID), steroidal agents and immunosuppressants are usually used. However, these drugs are known to produce various side effects including gastrointestinal disorders, immunodeficiency and humoral disturbances. Efforts need to be made to seek therapeutic agents that can be used for long-term administration.

One of the most widely used models for studying the anti-inflammatory/anti-rheumatic properties of compounds is adjuvant-induced arthritis (AA) in rats. It is an experimental immunopathy that is thought to share many features with human rheumatoid arthritis (Billingham and Davies, 1979). It has been accepted that the adjuvant injection to the rat may not only cause arthritic inflammation in the injected site (primary inflammation) but also in the non-injected hind paw (secondary inflammation). In comparison with the primary non-immune inflammation, the secondary reaction usually occurs with an obligate latent interval to reach a peak and has been indicated to be immunologically mediated by a T lymphocyte-mediated delayed-type hypersensitivity (DTH) reaction (Cohen, 1991). Such characteristics of AA are useful for evaluating anti-inflammatory and immunomodulatory remedies for immunologically related inflammations.

By using this model, our previous study (Jiang et al., 1997) has found that the administration of the aqueous extract from *Rhizoma Smilacis Glabrae* (RSG, the rhizome of the *Liliaceae* plant, *Smilax glabra* Roxb.) from the day of adjuvant injection remarkably inhibited both primary and secondary inflammations of AA. Such activity of RSG included a direct anti-inflammatory activity and a selective anti-CIR mechanism, different from the non-selective activity of steroid and immunosuppressors. These characteristics of RSG, in addi-

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tion to its low toxicity to organs, may be highly advantageous to its long-term use for treating CIR-mediated chronic inflammatory diseases, such as human hepatitis and RA. The present study, therefore, is designed to examine the effect of the administration of RSG during the later phase of AA and to clarify its immunological mechanisms.

2. Materials and methods

2.1. RSG preparation and reagents

RSG was purchased from Nanjing Medicinal Material Co. Ltd. (Nanjing, China) and identified as *Smilacis glabra* Roxb. by Dr. Boyang Yu, Department of Chinese Medicinal Prescription, China Pharmaceutical University. A voucher specimen (Jieyun Jiang 950220) was deposited at the Herbarium of China Pharmaceutical University. The aqueous extract from RSG was made by a common method. Briefly, the RSG material was extracted twice with distilled water (1:10, w/v) at 100 °C for 1 h. Then, the supernatant, after centrifugation at 1700 × *g*, was pooled and lyophilized to obtain a powder with 11% yield. This powder was used for the dosage of RSG used in this study.

Other drugs and reagents employed in this study were as follows: Injectio Prednisoloni (Pred, Shanghai 9th Pharmaceutical Factory, Shanghai, China), Cyclophosphamide (Cy, Shanghai 12th Pharmaceutical Factory, Shanghai, China), Bacillus Calmette Guérin (BCG, Shanghai Institute of Biological Products, Shanghai, China). Lipopolysaccharides (LPS), Concanavalin A (Con A) and 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT) were from Sigma. Anti-CD4 (clone GK 1.5) monoclonal antibody (mAb) and anti-CD8 (clone 53-6.7) mAb were obtained from the Department of Immunology, Beijing Medical University (Beijing, China).

2.2. Animals

Male and female Sprague-Dawley rats weighting 145–170 g and ICR mice weighing 22–24 g were obtained from the Experimental Animal Center of China Pharmaceutical University (Nanjing, China) and BALB/c mice weighing 20–22 g were obtained from Sino-British SIPPR/BK Lab Animal Ltd. (Shanghai, China). All the animals were maintained in plastic cages at 20 ± 2 °C with free access to pellet food and water and were kept on a 12 h light/dark cycle. This study complied with current ethical regulations on animal research of this institute and all animals used in the experiment received humane care.

2.3. AA in rats induced by Freund's complete adjuvant (FCA)

AA was induced as previously described (Jiang et al., 1997). Briefly, rats were injected intradermally with 100 μl of FCA (10 mg/ml heat-killed BCG in sterilized liquid paraffin) into the left hind paw. The injection day was regarded as day 0. The thickness of the contralateral (non-injected) hind paw (secondary lesion) was pre-measured before the injection and measured with an engineer's micrometer (Mitutoyo Co., Tokyo, Japan) at 2-day intervals after the administration of drugs. The magnitude of inflammatory response was evaluated by the increase in hind paw thickness after FCA injection. Weight gain, a further parameter of the severity of arthritis (Newbold, 1963), was also recorded at the same time.

2.4. PCl-induced DTH reaction (PCl-DTH) in the ear of mice

PCl-DTH was performed as previously described (Xu et al., 1996). In brief, mice were sensitized by painting 0.1 ml of 1% PCl dissolved in ethanol on the skin of their abdomens. Five days later, they were challenged by painting 30 μl of 1% PCl dissolved in olive oil on each right ear lobe. The DTH reaction was evaluated by the increase in ear thickness measured with the engineer's micrometer 22 h after the challenge.

2.5. Preparation of splenocytes, thymocytes and peritoneal macrophages

Splenocytes and thymocytes were obtained from rats and mice. Briefly, the spleen or thymus was removed sterilely and the cells were dissociated in 5 ml RPMI-1640 (Gibco BRL) containing 100 U/ml of penicillin, 100 μg/ml of streptomycin and 10% FCS (1640 medium). The cell suspension was centrifuged at 200 × *g* for 5 min and after removing the supernatant and washing twice, cells were resuspended in 1640 medium. In the case of spleen cells, 0.17 M tris (hydroxymethylaminomethane)–0.75% NH_4Cl solution was used to remove erythrocytes before washing.

At the same time, rat peritoneal macrophages were also collected by washing the cavity with 15 ml cooled sterile phosphate-buffered saline (PBS, pH 7.2). Cells were washed twice and resuspended (2×10^6 cells/ml) in 1640 medium.

2.6. Monitoring of LPS contamination

All reagents and media for tissue culture experiments were tested for their LPS content with a colorimetric Limulus amoebocyte lysate assay (detection limit 10 pg/ml, Whittaker products, Walkersvile, MD). Maintenance of LPS-free conditions was as described (Ding and Nathan, 1987).

2.7. Con A-induced T lymphocyte proliferation

T lymphocyte proliferation was assayed in 96-microwell plates. One million splenocytes or thymocytes per well

were incubated with Con A (final concentration 5 μg/ml) for 68 h at 37 °C in 5% CO_2 humidified air. Twenty microliter of MTT (5 mg/ml in sterile PBS) were then added to each well and the cultures were incubated for an additional 4 h. The plates were centrifuged at 1000 × *g* for 10 min. After aspirating the supernatants, 200 μl of dimethyl sulphoxide was added to dissolve the formazan particles and the absorbance at 540 nm was measured with a microplate reader (Bio-Tek Instruments, Inc., Burlington, VT). The results are expressed as stimulation index, ratio of OD values between Con A-stimulated and non-stimulated cells.

2.8. Macrophage cultures

Rat peritoneal cells were cultured with 1640 medium (final LPS content < 10 pg/ml) in 24-well plates (2×10^6 macrophages per well in a volume of 1 ml) (Falcon) at 37 °C in 5% CO_2/95% air and enriched for macrophages by 2–3 h adherence steps as described (Ding et al., 1988; Bogdan et al., 1992). Then, they were incubated in the absence or presence of LPS (final concentration 6 μg/ml) for 6 h for TNF-α assay and 24 h for NO and IL-1 assay, respectively. Each assay was carried out in triplicate.

2.9. Measurement of NO

In the presence of H_2O, NO is rapidly converted to nitrite and nitrate (Nathan and Hibbs, 1991). Total production of NO therefore may be determined by assaying for nitrite (Stuehr et al., 1989). To assay nitrite, 100 μl of each culture supernatant (corresponding to 2×10^5 macrophage) was incubated at room temperature for 10 min with 100 μl of Griess reagent (0.5% sulfanilamide, 0.05% *N*-(1-naphthyl) ethylenediamine dihydrochloride in 2.5% H_3PO_4). The OD values of the samples were then read at 540 nm. A standard curve using $NaNO_2$ was then used to calculate NO_2^- concentrations.

2.10. TNF assay

TNF activity was carried out according to the methods of Green et al. (1984) with a minor modification. In brief, L-929 cells (10^4 cells/well) were cultured with 1640 medium containing 1 μg/ml of actinomycin D at 5% CO_2, at 37 °C for 24 h. Serial dilutions of tested samples or standard recombinant TNF-α (Genzyme Corporation, Cambridge, MA) were added and incubated for a further 24 h. Then, 50 μl of MTT were added and incubated for another 3 h. After removing the supernatants, 200 μl of dimethyl sulphoxide were added to dissolve the formazan and the absorption at OD_{540} was measured. The cytotoxicity was calculated as follows: % cytotoxicity $= (1 - OD_{sample}/OD_{medium}) \times 100$. A standard curve between the cytotoxicity and TNF-α concentrations was made in each assay with the low limit for detection being 50 pg/ml (2.5 U/ml). The corresponding TNF-α levels in tested samples were calculated according to the standard.

2.11. IL-1 assay

IL-1 activity was performed by modified thymocyte proliferation assay (Foresta et al., 1992). Thymocytes (2×10^6 cells/well) of BALB/c mice and diluted samples or standard rIL-1 were incubated with Con A (final concentration 2.5 μg/ml) at 37 °C in 5% CO_2 for 68 h. MTT 20 μl (5 mg/ml in PBS) was added and the stimulation index was determined as above. A standard curve between the index and IL-1 concentrations was made in each assay with the low limit for detection being 2.5 U/ml. The IL-1 levels in the test samples were calculated according to the standard.

2.12. Con A-induced IL-2 production by spleen cells from AA rats

Spleen cells from AA rats (10^7 cells per well) were cultured with Con A (final concentration 3 μg/ml) in 24-well plates for 48 h at 37 °C in 5% CO_2 humidified air. The plates were then centrifuged at 1000 × *g* for 10 min and the supernatants were collected for assay of IL-2 activity.

2.13. IL-2 assay

Biological activity of IL-2 was determined by modified activated-splenocyte proliferation assay (Du and Li, 1998). Briefly, spleen cells from BALB/c mice were incubated with Con A (final concentration 3 μg/ml) for 48 h to get activated splenocytes. The cell suspension (2×10^5 cells/well) and diluted samples or human rIL-2 standard (Sino-American Shenyang Sunshine Pharmacy Co. Ltd., Shenyang, China) were incubated at 37 °C in 5% CO_2 for 24 h. MTT, 20 μl (5 mg/ml in PBS) was added and the stimulation index was determined as above. A standard curve between the index and IL-2 concentrations was made in each assay with the low limit for detection being 2.5 U/ml. The IL-2 levels in the test samples were calculated according to the standard.

2.14. $CD4^+$, $CD8^+$ T cell subsets detection

T cell subsets in splenocytes were determined by using a modified cytotoxicity test induced by monoclonal antibodies combined with a complement (Kung et al., 1979). In brief, 10^6 splenocytes were treated with 10 μl of diluted (1:10) anti-CD4 mAb or anti-CD8 mAb on ice for 60 min. After washing twice, cells were then treated with diluted (1:7) complement (fresh serum from guinea pig) at 37 °C for 30 min. The reaction was terminated on ice immediately and washed twice with PBS. The dead (positive) cells among the total population were determined by counting a total of 200 cells. Results were expressed as a percentage of positive cells.

2.15. Statistics

Results were expressed as mean ± S.D. Statistical analysis was evaluated by Student's *t*-test (between naive and control) or by two-way (treatment × time) ANOVA or one-way (between control and drug treatments) ANOVA followed by Dunnett's *t*-test or Student–Newman–Kauls test for multiple comparisons, with the level of significance chosen at $P < 0.05$.

3. Results

3.1. Effects of RSG and prednisolone on the secondary inflammation of adjuvant arthritis in rats

AA rats were divided into several groups. The one serving as the control group was given tap water while others were given either p.o. 400 or 800 mg/kg of RSG or i.m. 10 mg/kg of prednisolone from day 10 to day 24 after FCA injection. In each group of AA rats a single intradermal injection of adjuvant to the left hind paw resulted in a gradual increase in the swelling of the right paw (secondary lesion) with a peak 18 days after immunization and a decrease thereafter. Compared with the control group, the drugs administered from day 10 of the FCA injection to both RSG groups as well as the prednisolone group significantly and strongly suppressed the secondary inflammation in the FCA-non-injected paw (Table 1).

Meanwhile, the increase in body weight of the AA rats was observed. As shown in Fig. 1, AA rats in the control group showed only a mild increase during the observation against naive rats. Compared with the control, both RSG groups showed a gradient increase in a dose-dependent manner after starting the drug administration, while prednisolone significantly decreased the body weights.

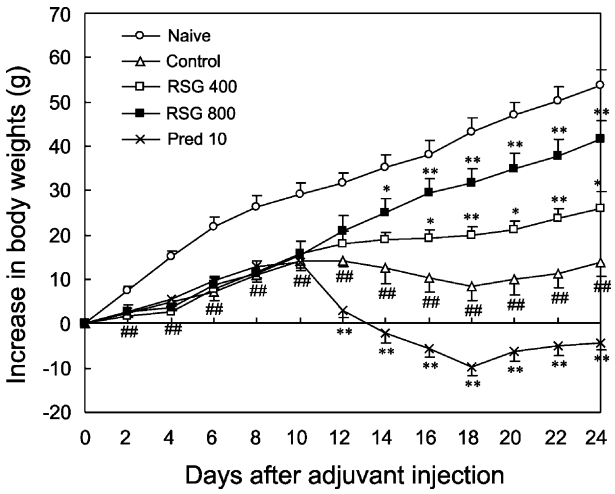

Fig. 1. Effect of RSG and prednisolone (Pred) on body weights of AA rats. The naive and control groups were given tap water and other groups were given RSG (p.o.) 400 or 800 mg/kg or prednisolone 10 mg/kg from day 10 to day 24 after adjuvant challenge. The body weights of rats were measured before the adjuvant injection and then measured again at 2-days intervals. Each point indicates the mean ± S.D. of eight animals. $^{\#\#}P < 0.01$ vs. Naive (Student's *t*-test); two-way ANOVA revealed a significant fluctuation among these groups, except for naive ($F_{11,336} = 12.100$, $P < 0.0001$), and a significant inhibition was also observed by the drug treatments ($F_{3,336} = 159.044$, $P < 0.0001$), $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Control (Dunnett's *t*-test).

3.2. Effects of RSG and prednisolone on LPS-induced IL-1, TNF and NO productions by peritoneal macrophages from AA rats

The LPS-induced IL-1, TNF and NO productions by peritoneal macrophages were evaluated on day 25 of AA induction. As shown in Table 2, the LPS-induced IL-1, TNF and NO productions by peritoneal macrophages from AA control rats were significantly higher than those of naive rats. Compared to the control group, the treatment of RSG significantly decreased the IL-1, TNF and NO productions, recovering them to almost the same level in naive rats (Table 2).

3.3. Effect of RSG and prednisolone on Con A-induced proliferation of T lymphocyte from AA rats

The proliferation of T lymphocytes stimulated with Con A (5 μg/ml) was evaluated on day 25 of AA induction. In comparison with naive rats, the proliferation of T lymphocytes collected from AA rats was significantly reduced. Compared

Table 1
Effects of RSG and prednisolone (Pred) on the secondary inflammation of adjuvant arthritis in rats

Group	Number of rats	Footpad swelling ($\times 10^{-2}$ mm)						
		Day 12	Day 14	Day 16	Day 18	Day 20	Day 22	Day 24
Control	8	40.1 ± 18.9	48.6 ± 14.1	53.4 ± 25.8	57.3 ± 20.6	48.7 ± 23.2	42.7 ± 19.8	39.9 ± 17.3
RSG 400	8	42.3 ± 19.7	40.1 ± 21.3	36.3 ± 12.5	30.3 ± 12.9**	25.1 ± 15.6*	22.0 ± 14.1*	19.7 ± 7.3**
RSG 800	8	41.8 ± 22.6	39.4 ± 17.9	31.6 ± 16.4	29.7 ± 21.6*	21.2 ± 10.7**	19.8 ± 12.4*	16.1 ± 9.4**
Pred 10	8	41.3 ± 26.2	31.2 ± 12.5*	26.1 ± 11.0*	17.2 ± 6.6**	16.0 ± 6.8**	11.2 ± 3.4**	10.6 ± 8.0**

RSG (400 and 800 mg/kg) and prednisolone (10 mg/kg) were given p.o. and i.m. from day 10 to day 24 after FCA injection, respectively. On the swelling in non-injected hind paw, two-way ANOVA revealed a significant fluctuation in these groups ($F_{6,196} = 10.106$, $P < 0.0001$) and a significant inhibition was also observed by the drug treatments at $F_{3,196} = 33.516$, $P < 0.0001$.

$^{*}P < 0.05$ vs. Control (Dunnett's *t*-test).

$^{**}P < 0.01$ vs. Control (Dunnett's *t*-test).

Table 2
Effects of RSG and prednisolone (Pred) on LPS-induced IL-1, TNF and NO productions by peritoneal macrophages from AA rats

Group	IL-1 (U/ml)	TNF-α (U/ml)	NO_2^- (nmol/l)
Naive	99.8 ± 15.6	168.1 ± 13.8	69.5 ± 6.18
Control	278.1 ± 24.7##	366.6 ± 30.3##	159.2 ± 10.8##
RSG 400	159.0 ± 10.1**	225.4 ± 20.9**	119.7 ± 6.24**
RSG 800	107.8 ± 8.7**	175.5 ± 14.5**	95.3 ± 10.3**
Pred 10	233.4 ± 17.0	248.2 ± 18.1**	115.6 ± 12.5**

The LPS-induced IL-1, TNF-α and NO productions by peritoneal macrophages were evaluated at day 25 in AA rats. Each value represents the mean ± S.D. of three animals and the experiments in each animal included triplicate sets.

$P < 0.01$ vs. Naive (Student's *t*-test); one-way ANOVA revealed significant effects between Control and drug treatment groups ($F_{3,8} = 64.250$, $P < 0.0001$ for IL-1, $F_{3,8} = 41.625$, $P < 0.0001$ for TNF-α, $F_{3,8} = 20.567$, $P < 0.001$ for NO_2^-).

** $P < 0.01$ vs. Control (Dunnett's *t*-test).

with the control group, the treatment of RSG significantly enhanced the proliferation of T lymphocytes, while the treatment of prednisolone significantly aggravated the reduction (Table 3).

3.4. Effect of RSG on Con A-induced IL-2 production by spleen cells from AA rats

The Con A-induced IL-2 production by spleen cells was measured on day 25 of AA induction. As shown in Fig. 2, a significant reduction in IL-2 production was observed in AA control rats. Compared with the control group, both RSG groups significantly increased the IL-2 production. However, in the prednisolone group the IL-2 activity was almost undetectable.

3.5. Regulatory effects of RSG on DTH reaction and CD4/CD8 ratio changed by cyclophosphamide

PCl-DTH was induced in ears of mice. Cyclophosphamide was administered i.p. 150 mg/kg 3 days before PCl

Table 3
Effect of RSG and prednisolone (Pred) on Con A-induced proliferation of T lymphocytes from AA rats

Group	Stimulation index	
	Thymocytes	Spleen cells
Naive	1.63 ± 0.02	2.65 ± 0.31
Control	1.18 ± 0.10#	1.78 ± 0.02#
RSG 400	1.34 ± 0.08	1.99 ± 0.09**
RSG 800	1.48 ± 0.03**	2.55 ± 0.14**
Pred 10	1.01 ± 0.06*	0.98 ± 0.10**

The proliferation of T lymphocytes stimulated with Con A (5 μg/ml) was evaluated on day 25 by using one million spleen cells and thymocytes isolated from AA rats. Each value represents the mean ± S.D. of four animals and the experiments in each animal included triplicate sets.

# $P < 0.01$ vs. Naive (Student's *t*-test); one-way ANOVA revealed significant effects between Control and drug treatment groups ($F_{3,12} = 32.212$, $P < 0.0001$ for thymocytes; $F_{3,12} = 175.323$, $P < 0.0001$ for spleen cells).

* $P < 0.05$ vs. Control (Dunnett's *t*-test).

** $P < 0.01$ vs. Control (Dunnett's *t*-test).

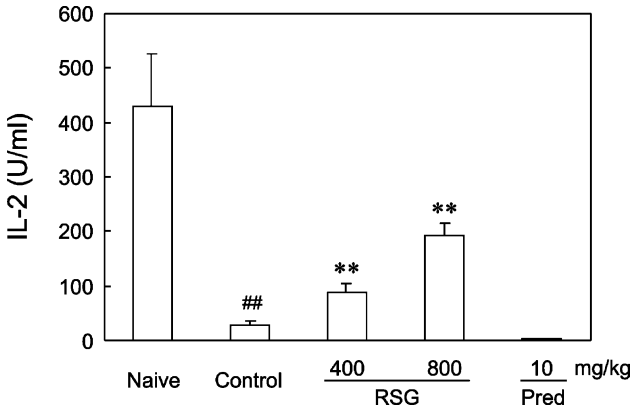

Fig. 2. Effect of RSG and prednisolone (Pred) on Con A-induced IL-2 production by splenocytes from AA rats. The IL-2 production was measured on day 25 of AA induction. Each column represents the mean ± S.D. of three animals and the experiment in each animal included triplicate sets. ## $P < 0.01$ vs. Naive (Student's *t*-test); one-way ANOVA revealed a significant effect between Control and drug treatment groups ($F_{2,9} = 95.599$, $P < 0.0001$), ** $P < 0.01$ vs. Control (Dunnett's *t*-test).

Table 4
Regulatory effect of RSG on increased or decreased PCl-DTH reaction and the CD4/CD8 ratio induced by cyclophosphamide (Cy)[a]

Group	Dose (mg/kg)	Number of mice	Ear swelling ($\times 10^{-3}$ mm)	CD4/CD8 ratio
Control	–	9	91.0 ± 27.7	1.15 ± 0.24
RSG	200	8	52.6 ± 16.0**	1.02 ± 0.15
Cy (day −3)	150	8	151.3 ± 39.8**	2.12 ± 0.55**
Cy (day −3) + RSG	150 + 200	8	107.5 ± 25.1#	1.07 ± 0.12##
Cy (day 0)	80	8	61.9 ± 15.8*	0.61 ± 0.03**
Cy (day 0) + RSG	80 + 200	9	120.0 ± 30.0†	0.98 ± 0.13†

PCl-DTH was induced in ears of mice. Cyclophosphamide was administered i.p. at a dose of 150 mg/kg three days before PCl sensitization (day −3) or at 80 mg/kg just after PCl sensitization (day 0). RSG 200 mg/kg was co-administered 0, 5, and 10 h after the PCl challenge (effector phase). Eighteen hours after the challenge, ear thickness and T subsets in splenocytes were measured. The DTH reaction was evaluated by the increase in ear thickness.

[a] Each value indicates the mean ± S.D. of eight to nine animals. One-way ANOVA revealed a significant effect on ear swelling ($F_{5,44} = 14.964$, $P < 0.0001$) and the CD4/CD8 ratio ($F_{5,44} = 30.461$, $P < 0.0001$).

* $P < 0.05$ vs. Control.

** $P < 0.01$ vs. Control.

# $P < 0.05$ vs. Cy (d −3).

$P < 0.01$ vs. Cy (d −3).

† $P < 0.01$ vs. Cy (d 0) (Student–Newman–Kauls test).

sensitization (day −3) or 80 mg/kg just after PCl sensitization (day 0). RSG (200 mg/kg) was co-administered with cyclophosphamide 0, 5 and 10 h after the PCl challenge. As shown in Table 4, cyclophosphamide given on day −3 significantly enhanced the DTH reaction and increased the ratio of $CD4^+$ T cells to $CD8^+$ T cells. Against such enhancement, the co-administration of RSG during the effector phase showed an almost complete blockade. In contrast, the administration of cyclophosphamide on day 0 significantly lowered both the DTH reaction and the CD4/CD8 ratio, while the co-administration of RSG showed complete recovery from the inhibition.

4. Discussion

The present study examined the therapeutic effects of aqueous extract from RSG and its immunological mechanisms on AA in rats. First, the administration of RSG during secondary inflammatory response significantly inhibited the swelling of non-stimulated footpads in AA rats (Table 1) and recovered the lower level of weight gain of the rats (Fig. 1). These results reflected an effect of RSG on AA different from those when RSG was given during primary inflammation (Jiang et al., 1997). Considering the similarities in pathological features of AA to human RA and the sensitivity of this model to anti-inflammatory and immunosuppressing agents (Baimgartner et al., 1974; Walz et al., 1971), the efficacy of RSG on both primary and secondary inflammations of AA may be useful for the treatment of clinical rheumatoid arthritis.

It has been reported that secondary inflammation occurring in non-stimulated footpads is mainly caused by a typical cellular immune response, delayed-type hypersensitivity (DTH) (Cohen, 1991). In view of the results shown in Table 1, it is reasonable to suggest that the inhibition of RSG on the secondary inflammation of AA is related to its inhibition on DTH reaction that had been reported by us previously (Xu et al., 1993). To show additional mechanisms of the therapeutic effects of RSG, we examined its modulatory activity on the cellular immune response in AA rats. In the rat, we found an increased activity in the production of IL-1, TNF and NO by over-activated macrophages (Table 2) and decreased T lymphocyte proliferation (Table 3) and IL-2 production by splenocytes (Fig. 2). It is obvious that such disorders in the immune response makes curing AA by present immunosuppressants more difficult. In fact, in inhibiting the inflammation in AA rats, prednisolone did not recover the ability of T lymphocytes to proliferate (Table 3), the production of IL-2 (Fig. 2), and caused a strong reduction of weight gain (Fig. 1). Interestingly, the treatment of RSG significantly inhibited the production of IL-1, TNF and NO (Table 2), but enhanced the T lymphocyte proliferation (Table 3) and IL-2 production (Fig. 2). Additionally, our investigation also found that RSG, given in effector phase, showed a significant regulation of the DTH reaction by regulating the CD4/CD8 ratio (Table 4). These results suggest that RSG exhibit its inhibitory activity on AA through down-regulating the function of over-activated macrophages and up-regulating that of the dysfunctional T lymphocytes from AA rats. Such characteristics of RSG are quite different from immunosuppressants and may be advantageous to the treatment of chronic rheumatic arthritis with an immune system disorder.

The activation of macrophages results in the production of several cytokines including IL-1, IL-6, interferon-γ (IFN-γ) and TNF-α which have been implicated in immune arthritis (Arend and Dayer, 1990; Guerne et al., 1989; Hom et al., 1988; Thorbecke et al., 1992). Moreover, the leishmanicidal activity of macrophages correlates with their ability to express NO synthase following activation with TNF-α or IFN-γ (Liew et al., 1991). Macrophage-derived NO is not only cytotoxic against invaded microorganisms and tumors, but may also produce host damage (Kolb et al., 1991; Kroncke et al., 1991). Furthermore, NO may increase vasodilatation and vascular permeability at the inflammatory site, which may aggravate the arthritic process (Ialenti et al., 1993; Farrell et al., 1988). Although the secondary inflammation of AA is mainly induced by T cell-mediated DTH reaction, with the development of the disease, a dysfunction of $CD4^+$ T cells is usually observed as evidenced by decreases in IL-2 production and CD4/CD8 ratio. For treating complicated disorders of immune response such as occurring in AA, a novel therapeutic agent having a different effect from that of present immunosuppressants is required. Based on this need, the present study gives an outline of the activities of RSG which satisfy the requirements.

It is also interesting that the lowered weight gain of arthritic rats was significantly recovered by RSG. The weight loss in AA rats may be linked to the over-production of TNF-α since TNF-α or cachectin has been closely related to the loss in body weight occurring in animals suffering from chronic inflammation and/or infection (Beutler and Cerami, 1989). The effects of RSG on weight gain, which appears to be correlated to its anti-inflammatory actions, may possibly involve a modulatory role on the formation and/or the effects of TNF-α in arthritic rats.

In conclusion, RSG may act as a therapeutic agent of immunoinflammatory diseases through selectively suppressing and modulating CIR involved in inflammation, as opposed to the non-selective activity of steroidal agents, and through a direct anti-inflammatory mechanism like NSAIDs inhibiting prostaglandins (Jiang et al., 1997). These characteristics of RSG in addition to its low toxicity to organs may be highly advantageous to the long-term treatment of chronic immunoinflammatory diseases including AA.

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