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Effects of triptonide on mouse immune functions

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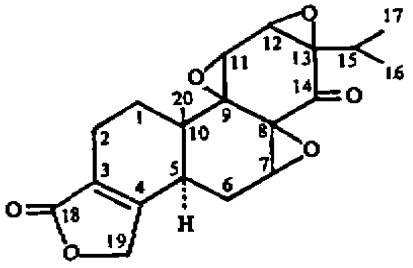
ABSTRACT Triptonide (Tri) extracted from *Tripterygium wilfordii* Hook inhibited the proliferation of mouse splenocytes induced by suboptimal concentration of concanavalin A or lipopolysaccharide at concentrations of 0.02, 0.1, and 0.5 $\mu g \cdot ml^{-1}$. It showed a suppressive effect on one way mixed lymphocyte culture (MLC) at 0.1—0.4 $\mu g \cdot ml^{-1}$. The lymphocytes harvested from the first Tri (0.4 $\mu g \cdot ml^{-1}$)-containing MLC inhibited the second MLC after being washed and irradiated with ^{60}Co source (30 Gy). Serum anti-sheep red blood cell antibody (hemolysin) formation and clearance of charcoal particles were also suppressed by Tri *in vivo*. Although delayed hypersensitivity (DH) reaction to dinitrofluorobenzene (DNFB) was inhibited by Tri 1.2, 2.5, and 5.0 mg $\cdot kg^{-1}$ (ip, qd×5 d), the spleen cell interleukin-2 secretion activities of these mice were not suppressed. Graft *vs* host reaction (GVHR) was not inhibited by Tri 2.5 and 5.0 mg $\cdot kg^{-1}$ (ig, qd×5 d). Helper T cells (T_h)/suppressor T cells (T_s) ratio was reduced at 2.5 mg $\cdot kg^{-1}$. The effects of Tri on mouse thymus and spleen weight were related to the age. Tri (1.2, 2.5, 5.0 mg $\cdot kg^{-1}$) had no effect on thymus and spleen weights in 8-wk-old mice. However, it increased the thymus and spleen weights in 12-wk-old mice at doses of 1.2 and 2.5 mg $\cdot kg^{-1}$. The data indicated that Tri had extensive suppressive effects on mouse immune function and its mechanism may be related to the reduction of T_h/T_s ratio and the induction of T_s cells.

KEY WORDS triptonide; T-lymphocytes; mixed leukocyte culture test; suppressor cells; interleukin-2; hemolysins; delayed hypersensitivity; graft *vs* host reaction; spleen

As a Chinese traditional medicine *Tripterygium wilfordii* Hook has been used to treat various autoimmune diseases. Although its effect has been confirmed, the toxic reactions are serious. Many components extracted from this herb have been investigated in China. We extracted triptonide and studied its effects on immune functions in normal mice.



Triptonide ($C_{20}H_{22}O_6$, M_r 358, mp 252—3℃)

MATERIALS AND METHODS

Animals ICR mice, BALB c mice, ♀, aged 8—10 wk were purchased from Shanghai SIPPR/BK Laboratory Animal Ltd. Mice were housed 4—5 per cage and were given rodent chow and tapwater *ad lib*.

Agents and chemicals Tri extracted from *Tripterygium wilfordii* Hook grown in Taining County of Fujian Province was provided by Department of Phytochemistry of our hospital. Cyclosporine A (Cs A) was produced by Fujian Microbiology Research Institute. Indian ink was produced by Shanghai First Reagent Factory. [^{3}H]Thymidine (TdR) was made in Shanghai Institute of Nuclear Research, Chinese Academy of Sciences, specific activity: 814 TBq $\cdot$ mol^{-1}. Concanavalin A (Con A), lipopolysaccharides (LPS), recombinant human interleukin-2 (rhIL-2), RPMI-1640 medium were bought from Sigma Chemical Co. New-born calf serum (NCS) was produced by Second Military Medical University. Dini-

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trofluorobenzene (DNFB) was synthesized by Xingta Chemical Factory in Jingshan, Shanghai. DNFB solution was prepared just before use with acetone-sesame oil (1 : 1, vol : vol). Anti-L3T4, anti-Lyt-2 and fluorescein isothiocyanate-conjugated (FITC) rabbit anti-rat IgG were purchased from Department of Immunology, Beijing Medical University. Lymphocyte separation medium (Ficoll-Hypaque) was produced by Shanghai Second Reagent Factory.

Preparation of splenocyte suspension Mice were killed by cervical dislocation. The spleen was minced and suspended. Erythrocytes were lysed with 0.83% NH_4Cl buffered solution, washed twice in Hank's solution and then adjusted to a desired concentration with RPMI-1640 medium (containing HEPES 25 mmol · L^{-1} and glutamine 2 mmol · L^{-1}) supplemented with penicillin 100 U · ml^{-1}, streptomycin 100 μg · ml^{-1}, 2-mercaptoethanol 50 μmol · L^{-1}, and 10% NCS. Cell viability was usually more than 95%, as determined by trypan blue dye exclusion.

Lymphocyte proliferation reaction BALB c mice were used. Splenocytes (5×10^6 · ml^{-1}) were seeded in 96-well microplates (Corning) in the presence of Con A (5 μg · ml^{-1}) or LPS (10 μg · ml^{-1}) and varying concentrations of Tri. The plates were cultured at 37℃ in a 5% CO_2 incubator (1815-TC) for 72 h. For the final 16 h, each well was pulsed with [^{3}H]TdR 18.5 kBq. The cells were harvested and the radioactivities incorporated were counted by a liquid scintillation count (FJ-2107). Our previous work revealed that Tri had no cytotoxic effect on lymphocytes at the concentrations used in this article.

Mixed lymphocyte culture (MLC) Normal ICR splenocytes and irradiated (30 Gy, ^{60}Co source) BALB/c splenocytes were mixed in 96-well plates (Corning) at equal concentration (2.5×10^6 cells · ml^{-1} for each) in the absence (control) or presence of varying concentrations of Tri. The cultures were incubated for 96 h and pulsed with [^{3}H]TdR (18.5 kBq/well) 16 h before harvesting. The cells were harvested and the radioactivities were determined.

Suppressor T cells (T_s) induction and measurement of T_s activity *in vitro* The methods reported previously[1] were adopted with some modifications. The cells harvested from the first 72-h MLC were washed 3 times and viable cells were counted. These cells were irradiated (30 Gy) and added into the 2nd MLC cultures to measure the T_s activity. The proliferative response was measured by [^{3}H]TdR (18.5 kBq/well) incorporation.

Delayed hypersensitivity (DH) The method of murine wet ear weight measurements[2] was used.

Generation and assay of IL-2 Splenocytes (5×10^6 · ml^{-1}) were mixed in 24-well plates with Con A (5 μg · ml^{-1}). The cultures were incubated for 24 h and the supernatants were collected after centrifugation ($1000\times g$, 10 min) and then stored at −20℃ until use. IL-2 was assayed using the activating mouse spleen cells[3]. The method of AUC (area under the curve)[4] was adopted to calculate IL-2 U in the samples. The proliferative response of the activating mouse spleen cells to Con A (5 μg · ml^{-1}), LPS (20 μg · ml^{-1}) and IL-1 supernatants were determined at the same time. No responsiveness was noted.

Clearance of charcoal particles[5] Mice were injected iv with indian ink 10 ml · kg^{-1}. One minute (T_1) and 10 min (T_2) later, 20 μl of blood were taken from retro-ocular venous plexus and resolved in 0.1% Na_2CO_3 solution. The absorbance (A) value at 680 nm was determined by 722 grating spectrophotometer. The clearance index K value was calculated: $K=(\lg A_1-\lg A_2)/(T_2-T_1)$.

Graft *vs* host reaction (GVHR)[5] Newborn hybrid mice of F_1 generation (ICR ♂ × BALB/c ♀) were used. Adult ♂ ICR mice were divided into 4 groups and given relevant drugs once a day for 7 d. One day after the final medication, the mice were killed. Their splenocyte suspensions were prepared in RPMI-1640 complete medium and adjusted to a concentration of 1×10^8 cell · ml^{-1}. The suspensions were injected ip into 9- or 10-d-old hybrid mice (0.1 ml/mouse). After 7 d, the mice were killed. Their spleens were weighed and splenic indices were calculated.

Determination of plasma hemolysin Mice were immunized ip with 1 ml of 10% SRBC suspension (d 0). On d 4, 20 μl of blood were taken from the retro-ocular venous plexus and mixed in 5 ml of normal saline. After centrifugation ($400\times g$, 10 min), 1 ml of supernatant was mixed with 1 ml of 5% SRBC suspension and 1 ml of 1 : 20 diluted guinea pig serum in

a small tube. The tube was incubated at 37℃ in water bath for 20 min and then centrifuged (400 × *g*, 10 min). Supernatant (1 ml) was mixed with 3 ml of Dribkin's solution and the absorbance (*A*) value was determined at 540 nm by 722 grating spectrophotometer.

Determination of T-lymphocyte subpopulations Indirect immunofluorescence assay[6] was used. The isolation of lymphocytes was accomplished by using thymus cell suspensions layered over the lymphocyte separation medium. The neutrophils were proved to be <2% by Wright stain. Cell viability rate was more than 90% by trypan blue dye exclusion method. The labeled cells were examined using a multi-function microscope (HB-2, Olympus). At least 200 lymphocytes were registered and the ratio of T_h/T_s, calculated.

RESULTS

Suppression of lymphocyte proliferation reaction Con A-induced and LPS-induced lymphocyte proliferation were all suppressed by Tri at 0.02, 0.1, and 0.5 $\mu g \cdot ml^{-1}$ (Tab 1).

Tab 1. Effects of triptonide (Tri) and hydrocortisone (HC) on proliferation of mouse splenocytes induced by concanavalin A (5.0 $\mu g \cdot ml^{-1}$) or lipopolysaccharides (10 $\mu g \cdot ml^{-1}$) *in vitro*. $n=4$ experiments in which 1—2 mice were used each time. $\bar{x} \pm s$. $^{***}P<0.01$ *vs* control.

$\mu g \cdot ml^{-1}$	Splenocyte proliferation/ Concanavalin A	[^{3}H]TdR uptake/dpm Lipopolysaccharides
Control	121 965±12 558	6010±887
HC (0.5)	19 526±2 355***	472±62***
Tri (0.02)	76 826±7 908***	3824±396***
Tri (0.1)	59 828±6 368***	2226±288***
Tri (0.5)	8 688±628***	312±64***

Suppression of MLC The [^{3}H]TdR incorporation values in the control group and Cs A (0.4 $\mu g \cdot ml^{-1}$) were 9197±1002 dpm and 1450±72 dpm ($P<0.01$), respectively. The values in group Tri at concentrations of 0.01, 0.1, and 0.2 $\mu g \cdot ml^{-1}$ were 9803±764 dpm ($P>0.05$), 2608±63 dpm ($P<0.01$), and 380±2 dpm ($P<0.01$), respectively ($n=5$). The results showed that at concentrations of 0.1 and 0.2 $\mu g \cdot ml^{-1}$ Tri suppressed MLC and at 0.01 $\mu g \cdot ml^{-1}$ Tri had no significant effects.

Induction of suppressor T-lymphocytes The mixed lymphocytes induced in 1st MLC with Tri at concentration of 0.4 $\mu g \cdot ml^{-1}$ suppressed the [^{3}H]TdR incorporation in 2nd MLC. These results illustrated that Tri might induce suppressor T-lymphocytes (Tab 2).

Tab 2. Effects of triptonide (Tri) and cyclosporine A (Cs A) on murine suppressor T-lymphocytes (Ts). The mixed lymphocytes from the 1st MLC were irradiated and added to the 2nd MLC. $n=3$ experiments in which 4 mice were used each time, $\bar{x} \pm s$. $^{***}P<0.01$ *vs* control.

Ts induction (1st MLC) $\mu g \cdot ml^{-1}$	Ts activity, [^{3}H] TdR incorporation in 2nd MLC/dpm $2^{(a)} \times 10^5$	4×10^5	8×10^5
Control	10 561±830	17 248±1 637	26 768±507
CsA (1.0)	6 131±1 396***	6 172±362***	5 123±318***
Tri (0.4)	8 324±248***	4 277±674***	6 008±828***

(a) Number of mixed lymphocytes from the 1st MLC added.

Suppression of DH reaction ICR mice ♀ weighing 21 ± *s* 2 g were divided into 5 groups. Mice were given relevant drugs once a day on d 0—4. Tri had suppressive effects on DH reaction at 1.2, 2.5, and 5.0 $mg \cdot kg^{-1}$ (Tab 3).

Effects on IL-2 production Splenocytes were obtained from the above-mentioned mice which were used for determination of DH. The results showed that Tri had no significant effect on IL-2 secretion activity (Tab 3).

Tab 3. Effects of triptonide (Tri) on delayed hypersensitivity and IL-2 activity secreted by splenocytes in mice *in vivo*. $\bar{x}\pm s$, $^{*}P>0.05$, $^{**}P<0.05$, $^{***}P<0.01$ *vs* **medium.**

$mg\cdot kg^{-1}\cdot d^{-1}$	Route	Mice	Ear swelling/mg	IL-2/$U\cdot ml^{-1}$
Medium	ig	9	8.6±2.9	23.0±12.2
Cyt (100×1)	ip	7	2.3±1.0***	11.8±4.0**
Tri (1.2×5)	ig	7	4.3±1.2***	18.2±7.4*
Tri (2.5×5)	ig	8	2.6±1.3***	23.0±10.1*
Tri (5.0×5)	ig	8	1.4±0.5***	22.0±5.6*

Suppression of clearance of charcoal particles ICR mice ♀ weighing 33± *s* 3 g were medicated once a day for 6 d. The *K* values in group medium, dexamethasone (5 $mg\cdot kg^{-1}$), Tri (1.2 and 2.5 $mg\cdot kg^{-1}$) were 0.053± 0.013, 0.018±0.005 ($P<0.01$), 0.035± 0.008 ($P<0.01$), and 0.020±0.008 ($P<0.01$), respectively ($n=8$). The results showed that Tri 1.2 and 2.5 $mg\cdot kg^{-1}$ suppressed the clearance of charcoal particles. This suggested that Tri had inhibitory effects on the phagocytic function of reticuloendothelial system.

Effect on GVHR ICR mice ♀ weighing 25 ± *s* 2 g were administered with relevant drugs. The splenic indices in group medium, untreated, Tri 2.5 and 5.0 $mg\cdot kg^{-1}$ were 79±20, 56±5 ($P<0.05$), 78±23 ($P>0.05$), and 65±8 ($P>0.05$), respectively ($n=6-7$). The results suggested that Tri had no effects on GVHR.

Effect on hemolysin formation ICR mice ♀ weighing 25± *s* 2 g were divided into 5 groups. Mice were given relevant drugs once a day on d 1—d 4. The results showed that Tri 1.2, 2.5, and 5.0 $mg\cdot kg^{-1}$ inhibited hemolysin formation (Tab 4). The data suggested that Tri had suppressive effects on humoral immunity function.

Effects on thymus and spleen weights

Tab 4. Inhibition of serum hemolysin formation by triptonide (Tri) in mice. $\bar{x}\pm s$, $^{***}P<0.01$ *vs* **medium.**

$mg\cdot kg^{-1}\cdot d^{-1}$	Route	Mice	Absorbance at 540 nm
Experiment 1			
Medium	ig	4	0.247±0.023
Cyt (100×1)	ip	4	0.006±0.004***
Tri (2.5×6)	ig	4	0.094±0.044***
Tri (5.0×6)	ig	4	0.096±0.050***
Experiment 2			
Medium	ig	4	0.705±0.058
Cyt (100×1)	ip	4	0.022±0.002***
Tri (1.2×6)	ig	4	0.394±0.134***
Tri (2.5×6)	ig	4	0.234±0.144***
Tri (5.0×6)	ig	4	0.088±0.077***

ICR mice ♀ used for determination of hemolysin (aged 8 wk) and clearance of charcoal particles (aged 12 wk) served as thymus and spleen donors. After exsanguination, their thymus and spleens were excised. Tri had no significant effects on thymus and spleen weights in 8-wk-old mice, but increased their weights in 12-wk-old mice (Tab 5).

Tab 5. Effects of triptonide (Tri) on thymus and spleen weights in mice. $\bar{x}\pm s$, $^{*}P>0.05$, $^{**}P<0.05$, $^{***}P<0.01$ *vs* **medium group. Cyt: cytoxan.**

Age/wk	Drug/$mg\cdot kg^{-1}\cdot d^{-1}$	Route	Mice	Thymus index g/kg body weigh	Spleen index
8	Medium	ig	8	1.6±0.6	6.5±1.4
8	Cyt (100×1)	ip	8	0.8±0.4***	4.8±0.6***
8	Tri (1.2×6)	ig	4	1.4±0.4*	5.9±1.6*
8	Tri (2.5×6)	ig	8	1.3±0.3*	6.0±1.7*
8	Tri (5.0×6)	ig	8	1.3±0.3*	6.4±0.6*
12	Medium	ig	8	0.9±0.3	3.3±0.5
12	Cyt (100×1)	ip	8	0.3±0.1***	1.8±0.3***
12	Tri (1.2×6)	ig	8	1.4±0.2***	4.7±1.3**
12	Tri (2.5×6)	ig	8	1.3±0.4**	4.0±0.2***

Effects on T_h/T_s ratio ICR mice weighing 26± *s* 2 g were used. The T_h/T_s ratios of the cytoxan (100 mg×1 d, ip) and Tri (2.5 mg·kg^{-1}, qd×7 d, ig) groups were significantly decreased (0.83±0.13 and 0.84±0.08, respectively) when compared with the medium group (1.27±0.13) (n=10).

DISCUSSION

Our results showed that Tri had extensive suppressive effects on mouse immune function both *in vivo* and *in vitro*. Although Tri had no influence on GVHR and IL-2 activity of splenocytes in mice given Tri for 5 d, the IL-2 activity was suppressed (data not shown) when spleen cells from normal mice were cultured with Con A and Tri. The most conceivable interpretation was that the suppressive effects might be reversible in the absence of Tri when the immune cells supressed by Tri met with stimulating factors such as mitogen (Con A) or major histocompatibility antigen.

The effects of Tri on the weight of immune organs are related to the age of mice. No influence on the thymus weights in 8-wk-old mice but increased the weights in 12-wk-old mice. this feature is not in accordance with the case of ordinary immunosuppressive agents. The suppressive effects of Tri were not realized by reducing the immune cell number. These facts that Tri reduced T_h/T_s ratio *in vivo* and induced T_s cells *in vitro* were in accordance with the effects of Tri on mouse thymus and spleen weight and may be important in its mechanism of action.

In conclusion, Tri had extensive immunosuppressive effects on mouse immune function and the mechanism may be related to the induction of T_s cells and the reduction of T_h/T_s ratio.

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雷公藤内酯酮对小鼠免疫功能的影响

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摘要 雷公藤内酯酮(triptonide，Tri)显著抑制小鼠T，B淋巴细胞增殖和混合淋巴细胞反应(MLC)，诱导抑制性T细胞(Ts)活性. 血清溶血素生成，碳粒廓清和迟发性超敏反应(DH)亦显著受抑，胸腺Th/Ts细胞比值明显降低；但对脾细胞的IL-2分泌活性和移植物抗宿主反应(GVHR)无明显影响. Tri对胸腺和脾脏重量的影响与鼠龄有关.

关键词 雷公藤内酯酮；T—淋巴细胞；混合型白细胞培养试验；抑制细胞；白细胞介素-2；溶血素类；迟发型超过敏；移植物抗宿主反应；脾

T细胞 白细胞介素-2