# THE COOPERATIVE RESEARCH ON GANODERMA LUCIDUM (Ling Zhi / Reishi )

The problem clarified by the cooperative research work of Shanghai Medical University, Shanghai, China and Wakan Shoyaku Botany Institute, Tokyo, Japan is that Ling Zhi (Reishi) has the effect of improving microcirculation and enhancing hemodynamics. However, it was saying "Eating Reishi for a long period might make person comfortable and prolong the life without senility" in "Sheng Nong Ben Cao Jing" and "Ben Cao Gang Mu".

* Comfort means no symptom.
* Prolonging life without senility means having a long long life.

Certainly, it would be impossible if there were no such basis of improving microcirculation. The data collected in this book seems in expectation. It has proved that Reishi (LZ) increased hemodynamics in microcirculation by decreasing the blood viscosity. There are not only the data in medical research but also that of clinical investigation. In addition, with the efforts of the teachers of Shanghai Medical University, it has also proved that Reishi (LZ) has the effect of clearing radicals and the effect of two-way immune modulation. I have paid cordially attention for the past 17 years. The fruitful result form the "Cooperative Research" makes me very excited.

Reishi could only be a food not a botany because of no scientific certificate in Japan. However, as a few kind of drug Reishi might be used as a botany according to the past experience in Eastern Medicine without any side effect.

"The Research on Ganoderma Lucidum, Part One" is not only a symbol to which it had made a great effort, but also a basis of more understanding the traditional and modern usage of Reishi (LZ) in the continuing research in future.

The great significance and rich contents of experimental medicine and pharmacy were listed by this book. The efforts and instruction of the researchers in Shanghai Medical University would be appreciated very much.

I would like to thank Professor and President Zhaoyou TANG, Professor and Vice President Shineng ZHU, Shanghai Medical University again for their enhancing and strengthening of the search work. I also say many thanks again to Mr. Masaru KOBAYASHI, President, Wakan Shoyaku Botany Institute for his kindness of providing the big financial support for research work.

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# 關於實驗所使用的靈芝材料

1989 年起中國上海醫科大學和日本和漢生藥研究所開始了“生藥靈芝的合作科學研究”。研究使用的材料全部來自日本國和漢生藥研究所的靈芝栽培農場生產的子實體和由子實體提取出來的精制粉末。實驗使用的制品從該研究所的靈芝制品和原料中隨機抽取，而不是爲此研究使用作特別處理和調整的原料。

栽培靈芝農場概要:

和漢生藥研究所嬬戀村農場

第一農場　9,900$m^2$　　第二農場　13,000$m^2$

共計　22,900$m^2$

(位於群馬縣吾妻郡嬬戀村今井地內)

和漢生藥研究所是日本最大的靈芝研究制造家，包括 60 幢栽培溫室、原木培養樓、果實干燥樓，還有專門配備有組織培養、擴大培養，以及無菌設備的培養樓、工作樓，附有儲設備的淨水裝置，大型滅菌設備以及其他成套設備。

農場的栽培環境是位於海拔 1030 m 的高原，常年氣候很少變化，且可以取得在同一環境條件下山林的原本，水質好，空氣新鮮，是一個有利於自然培養和處理的天然環境，具備生產出質量穩定的靈芝原材料的條件。此外和漢生藥研究所經過多年研究創造的栽培法，具有能充分保護原種菌活性的管理方法和栽培手段的專利，因而能確保質量的穩定。

# REGARDING TO THE MATERIAL LING ZHI ( REISHI ) USED IN EXPERIMENTS

"The Cooperative Scientific Research on Botany Ling Zhi (Reishi)" accomplished by Shanghai Medical University, Shanghai, China and Wakan Shoyaku Botany Institute, Tokyo, Japan started in 1989. The total material Ling Zhi (Reishi) including seeds, powder and its extract was produced in the special Reishi cultivated farm and was provided by Wakan Shoyaku Botany Institute. It should be declared that the material Reishi used in the experiments were taken randomly from the original material produced in the Institute without and special ways.

The brief introduction of Reishi cultivated farm:

Tumagoi-Mura Farm, Wakan Shoyaku Botany Institute

The first farm 9 900m² The second farm 13 000m² Total 22 900m²

(Located in Imai-Sennoiri, Tumagoi-Mura, Agatuma-Gun, Gunma, Tapan)

Wakan Shoyaku Botany Institute is the biggest Reishi (LZ) produced and researched organization in Japan. It includes 60 cultivated warm rooms, original wood cultivated building, seeds dried building. Besides, there are general cultivated building and office building with water cleared apparatus, big sterilized equipment and other instruments.

The cultivated farm is located at the altitude of 1 030 meters above sea level. It is a natural environment with little changed whether, good quality of water, fresh air and getting the original wood at that place. It is benefit to the cultivation and treatment of Reishi (LZ). The condition of original Reishi (LZ) produced with stable quality is ensured in the farm. In addition, with the cultivated technique researched and created in many years Wakan Shoyaku Botany Institute had the patent of management and cultivation of sustaining the activity of original bacterial strain. All these might guarantee the stable quality.

# Effects of Ling Zhi on Lymphocyte Proliferation

## — Immunopharmacological Study (1)

**XU Weimin ZHANG Luoxiu MIAO Honghua**

**Abstract** The study was to evaluate the process of lymphocyte proliferation induced by the hot water extract of Ling Zhi (LZ, Ganoderma Lucidum, Fr. Karst) cultivated in Tokyo, Japan. It was examined by assessing the effect of LZ on $^{3}$H-TdR uptaken by cultured spleen cells alone or in the presence of Con-A or Lps. LZ 300 mg/kg po. qd. x 10 in vivo or 10 ~ 500 μg/ml alone in vitro stimulated lymphocytes proliferation directly, but 1 000 μg/ml of LZ inhibited. The activity of frozen thawed part was stronger than that of soluble part. The effect of LZ on lymphocyte proliferation induced by Con A resulted in diverse regulatory activity. It was mainly related to both concentrations of Con A and LZ. In general, LZ stimulated lymphocyte proliferation in the presence of suboptimal concentration of Con A (0.625 μg/ml) and depressed when the cells were highly activated by optimal concentration of Con A (2.5 μg/ml) in a concentration dependent fashion.

LZ antagonized the activity of cyclophosphamide and promoted the lymphocyte proliferation in the immunosuppressed mice induced by cyclophosphamide. However, LZ had no stimulative activity on lymphocyte proliferation induced by Lps in vitro or in vivo. It implied that B cells may have different mechanism from T cells to interact with LZ.

**Key words** Ling Zhi (LZ, Ganoderma Lucidum, Fr. Karst); Lymphocyte proliferation

The fungus Ling Zhi (LZ, Ganoderma Lucidum, Fr. Karst) exhibits wide pharmacological effects and has been used as a health protective drug for a long time. However, the investigation of LZ on immune system was rare and the mechanism was poorly understood. The aim of this study was to evaluate the effect of LZ on lymphocyte proliferation.

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# Materials and Methods

## Animals

Kunming mice, male, 18 ~ 22 g, were supplied by the Animal Center, Shanghai Medical University.

## Reagents

The hot water extract of LZ was provided by Wakan Shoyaku Botany Institute, Tokyo, Japan. A certain amount of LZ extract was grinded and dissolved in normal saline containing 0.2% carboxymethyl cellulose sodium (CMC) shaken in 80ºC water bath for 4h. This suspension was used for in vivo study. For in vitro study, a little amount (about 10mg) of LZ extract was dissolved in normal saline and rotated slowly in 80ºC water bath for 2h then centrifugated 1 600 rpm 10 min. The supernatant was kept at 4ºC until study. Before experiment this solution was diluted to desire concentration. It was named as soluble part (Part A). When LZ was dissolved and shaken in 80ºC water bath then rapidly frozen (-30ºC) and thawed (37ºC) 3 times, it was named as frozen thawed part (Part B).

PRMI-1640 was purchased from GIBCO Co. It contained Hepes 15 mM, Penicillin 100 U/ml, Streptomycin 100 μg/ml, NCS 10%, 2-Mercaptoethanol (2-ME) $5 \times 10^{-5}$ mmol, pH 7.3.

Con A, S were purchased from Sigma Co.

$^{3}$H-TdR was provided by Shanghai Institute of Nuclear Research, Chinese Academy of Sciences and the specific activity was 1 100 GBq. Mmol.

## Lymphocyte proliferation in vitro (1)

Mice were sacrificed by cervical dislocation and the spleens were removed with sterile technique. The spleen cells were collected after passing through a nylon screen and red blood cells in the cell suspension were hemolyzed with distilled water for 30 s, then osmoregulated. After washing with RPMI-1640 for 3 times the cells were seeded into each well of 90-well microplate and various concentrations of LZ and Con A or LPS were added in alone or combination. The plates were cultured at 30ºC with 5% $CO_2$ in a humidified atmosphere for 48 h. For the last 6 h each well as pulsed with 9.25 kBq $^{3}$H-TdR. The cells were harvested using a multiple automatic sample harvester onto a fibroglass filter paper which was then dried and the radioactivity incorporated were counted by a liquid scintillation counter. All counts/min values shown were the mean of triplicate sample ± SD. Statistical analysis was carried out by student's test.

## Mitogenic activity of LZ in vivo[(2)]

Mice were randomly divided into 2 groups. The mice in control group were given 0.2% CMC 0.5 ml po. qd. x 10 and treated group with LZ 300 mg/kg po. qd. x 10. The spleen cells were prepared as mentioned above.

**The effects of LZ on immunosuppressed mice**

40 mice were divided into 4 groups randomly. The mice in control group were given 0.2% CMC 0.5 ml po. qd. x 10. In cyclophosphamide (CYA) group, mouse were taken CYA 10 mg/kg ip. qod. x 2, q3d x 2. The mice of LZ groups were given LZ 150, 300 mg./kg, po. Combined with CYA 10 mg/kg, ip. qd. x 2, qod. x 2 respectively. The spleen cells were prepared as the same technique mentioned above.

# Results

**Effect of LZ on lymphocytes proliferation in vitro**

In vitro, LZ alone stimulated the $^{3}$H-TdR uptake at 1 ~ 500 μg/ml (Part A) and at 1 ~ 10 μg/ml (Part B) without mitogen. And turned to inhibition at right concentration. The activity of Part B was stronger than that of Part A. Part B inhibited lymphocyte proliferation at less concentration (Tab. 1-1).

**Tab. 1-1 Effect of LZ alone on proliferation of lymphocytes in vitro**

| LZ (μg/ml) | cpm / 1 x $10^6$ cells | |
|---|---|---|
| | Part A | Part B |
| 0 | 2 991 ± 445 | 2 991 ± 445 |
| 1 | 3 744 ± 529 | 11 208 ± 554** |
| 10 | 5 997 ± 699** | 10 532 ± 1 301** |
| 100 | 8 054 ± 1 013** | 1 595 ± 18* |
| 500 | 7 668 ± 1 181** | 1 552 ± 264* |
| 1 000 | 1 126 ± 156** | 499 ± 48** |

Part A: soluble part; Part B: frozen thawed part; $\overline{X}$ ± SD; n = 3;
*P<0.05, **P<0.01

The effect of LZ on lymphocyte proliferation induced by Con A was closely related to the concentration of Con A. LZ both Part A and Part B (1 ~ 500 μg/ml) promoted the lymphocyte proliferation induced by 0.625 μg/ml of Con A in a concentration dependent fashion. Higher concentration (>599 μg/ml) resulted in inhibitive effect (Tab. 1-2, Fig. 1-1).

When the lymphocytes were highly activated by optimal concentration of Con A (2.5 μg/ml) LZ mainly expressed an inhibitive activity.

The activity of LZ was in the middle between two conditions mentioned above when lymphocytes were stimulated by Con A (1.25 μg/ml).

**Tab. 1-2 Effect of LZ on ConA-induced lymphocyte proliferation in vitro**

| | LZ (μg/ml) | cpm / 1 x $10^6$ cells | | |
|---|---|---|---|---|
| | | ConA 0.625 μg/ml | ConA 1.25 μg/ml | ConA 2.5 μg/ml |
| Part A | 0 | 3 589 ± 44 | 19 559 ± 3 087 | 69 991 ± 1 894 |
| | 1 | 5 886 ± 3 020 | 35 293 ± 9 642 | 58 956 ± 12 175 |
| | 10 | 13 366 ± 5 955* | 19 954 ± 8 656 | 38 487 ± 2 523** |
| | 100 | 19 788 ± 4 285** | 16 944 ± 6 768 | 28 640 ± 10 150** |
| | 500 | 9 375 ± 1 677* | 4 948 ± 2 044** | 15 496 ± 1 254** |
| | 1 000 | 984 ± 117** | 764 ± 166** | 4 732 ± 832** |
| Part B | 1 | 16 546 ± 3 217** | 20 509 ± 4 701 | 26 034 ± 7 193** |
| | 10 | 15 944 ± 4 801* | 61 059 ± 5 666** | 35 337 ± 13 165* |
| | 100 | 7 407 ± 585** | 56 432 ± 7 227** | 17 476 ± 2 351** |
| | 500 | 4 196 ± 460 | 4 512 ± 470** | 16 996 ± 4 123** |
| | 1 000 | 685 ± 28** | 1 918 ± 98** | 7 347 ± 825** |

Part A: soluble part; Part B: frozen thawed part; $\overline{X}$ ± SD; n = 3; *P<0.05, **P<0.01

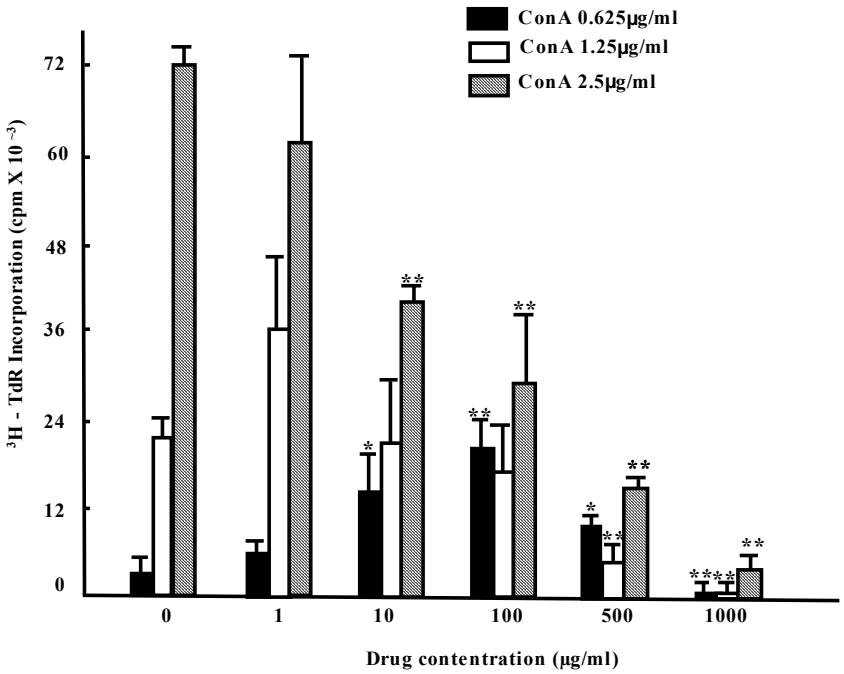


**Fig. 1-1 Effect of LZ (soluble part) on proliferation of ConA-induced lymplocytes in vitro ±SD; n= 3; *P<0.05, **<0.01**

On the other hand, LZ apparently suppressed lymphocyte proliferation induced by Lps (10 μg/ml) in vitro in a dose dependent fashion. It was shown that Lps was a stimulator of B cells and Con A was the T cells mitogen. That means LZ has different action on T and B cells (Tab. 1-3, Fig. 1-2).

**Tab. 1-3 Effect of LZ on proliferation of Lps-induced lymphocyte proliferation in vitro**

| | LZ (μg/ml) | cpm / 1 x $10^5$ cells | |
|---|---|---|---|
| | | Lps 5 μg/ml | Lps 10 μg/ml |
| Part A | 0 | 37 420 ± 2 780 | 63 123 ± 6 025 |
| | 1 | 27 369 ± 665** | 34 113 ± 820** |
| | 10 | 25 348 ± 1 950** | 33 771 ± 6 324** |
| | 100 | 25 901 ± 2 187** | 30 656 ± 4 311** |
| | 500 | 22 080 ± 675** | 24 727 ± 3 412** |
| | 1 000 | 3 942 ± 733** | 11 528 ± 2 865** |
| Part B | 1 | 30 051 ± 3 862 | 37 491 ± 8 736* |
| | 10 | 35 341 ± 5 173 | 44 201 ± 1 813** |
| | 100 | 34 057 ± 4 299 | 30 985 ± 6 037** |
| | 500 | 20 221 ± 3 601** | 26 271 ± 1 357** |
| | 1 000 | 7 288 ± 778** | 8 983 ± 1 413** |

Part A: soluble part; Part B: frozen thawed part; $\bar{X}$ ± SD; n = 3; *P<0.05, **P<0.01

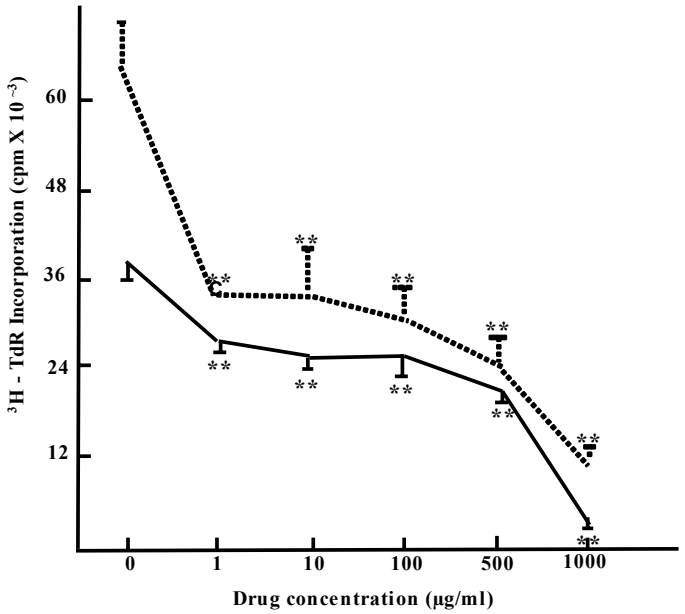


**Fig. 1-2 Effect of LZ (soluble part) on proliferation of Lps-induced lymphocytes in vitro**
**- Lps 5 μg/ml - - -Lps 10 μg/ml**
**±SD; n=3; **P<0.01.**

**Effect of LZ on lymphocytes proliferation in vivo**

It was shown that LZ 300 mg/kg po. qd. x 10 significantly stimulated lymphocyte proliferation of mice without mitogen and enhanced the activity of con A (0.625 ~ 1.25 μg/ml). However, on the same condition LZ statistically inhibited lymphocyte proliferation induced by 5 or 10 μg/ml of Lps (Tab. 1-4).

**Tab. 1-4 Effects of LZ on lymphocytes proliferation in vivo**

| Mitogen | Mg/ml | cpm / 1 x $10^6$ cells | |
|---|---|---|---|
| | | 0.2% CMC | 300 mg/kg LZ |
| — | — | 1 338 ± 94 | 2 676 ± 621** |
| Con A | 0.625 | 4 254 ± 802 | 10 168 ± 1 876** |
| | 1.250 | 21 471 ± 1 563 | 42 946 ± 1 001** |
| | 2.500 | 33 685 ± 1 727 | 42 031 ± 9 645 |
| LPS | 5 | 5 427 ± 996 | 420 ± 186** |
| | 10 | 5 963 ± 30 | 339 ± 4** |

Drug po. qd. x 10; $\bar{X}$ ± SD; **P<0.01

**LZ enhanced the immune function suppressed by CYA in mice**

Tab. 1-5 showed that CYA reduced body weight during the first 5d after treatment and got weight later. LZ could protect the mice from CYA without loss of body weight during the first 5d after treatment. In addition, CYA significantly reduced the size and weight of thymus, but LZ 300 mg/kg could increase the weight of thymus with significant difference when compared with CYA alone group (P<0.05).

**Tab. 1-5 Effects of LZ combined with CYA on the body weight, thymus and spleen of mice**

| Agents | (mg/kg) | Body Weight (g) | | | Thymus (mg) | Spleen (mg) |
|---|---|---|---|---|---|---|
| | | D0 | d5 | d10 | | |
| CMC | — | 24.0 ± 1.5 | 28.0 ± 1.6 | 29.7 ± 1.9 | 79.1 ± 25.2 | 89.5 ± 18.2 |
| CYA | 10 | 24.2 ± 1.8 | 23.3 ± 2.1*** | 28.8 ± 2.0 | 40.8 ± 17.4*** | 113.7 ± 43.9 |
| CYA + LZ | 10 150 | 24.3 ± 1.8 | 28.0 ± 2.1$^{\Delta\Delta\Delta}$ | 29.8 ± 1.6 | 54.8 ± 23.2 | 113.2 ± 19.0 |
| CYA +LZ | 10 300 | 24.7 ± 1.4 | 29.8 ± 1.3*$^{\Delta\Delta\Delta}$ | 30.7 ± 1.0$^{\Delta}$ | 66.3 ± 18.4$^{\Delta\Delta}$ | 125.6±30.2*** |

CYA, CMC: 0.2%, 0.5 ml/20g
LZ po. qd. x 10
CYA ip. qod. x 2, q3d. x 2
$\bar{X}$ ± SD, n = 9 ~ 10, *P<0.05, **P<0.01, ***P<0.001 compared with CMC group
$^{\Delta}$P<0.05, $^{\Delta\Delta}$P<0.01, $^{\Delta\Delta\Delta}$P<0.001 compared with CYA group

Spleen cells of each group were determined for lymphocyte proliferation test. The results showed that CYA markedly inhibited lymphocyte proliferation no matter with or without Con A or Lps. When LZ combined with the same dosage of CYA, it restored the suppressed lymphocyte proliferation. Compared with CYA alone group, LZ 150 ~ 300 mg/kg significantly increased the $^{3}$H-TdR incorporation ($P < 0.01$) (Tab. 1-6).

**Tab. 1-6 Effects of LZ combined with CYA on lymphocyte proliferation in vivo**

| Mitogens | μg/ml | Cpm / 1 x $10^6$ cells | | | |
|---|---|---|---|---|---|
| | | CMC (0.2%) | CYA | CYA+150mg/kg LZ | CYA+300mg/kg LZ |
| — | | 4 598 ± 620 | 1 992 ± 342** | 1 527 ± 190 | 1 533 ± 217 |
| Con A | 0.625 | 61 403 ± 10 172 | 1 475 ± 159** | 7 126 ± 795** | 2 684 ± 58** |
| | 1.25 | 83 372 ± 12 369 | 1 169 ± 337** | 16 231 ± 5 368** | 6 655 ± 2 250 |
| | 2.5 | 84 917 ± 23 495 | 3 565 ± 435** | 56 274 ± 12 847** | 6 559 ± 1 915 |
| LPS | 5 | 14 003 ± 3 094 | 87 ± 12** | 4 142 ± 214** | 763 ± 43** |
| | 10 | 15 845 ± 2 175 | 99 ± 24** | 3 098 ± 612** | 460 ± 27** |

CYA 10mg/kg, ip. qod. x 4, The rest po. qd. x 10;
CYA compared with 0.2% CMC; LZ + CYA compared with CYA: *P<0.05, **P<0.01
$\overline{X}$ ± SD

# Discussion

The experiments both in vitro and in vivo showed that optimum dosage of LZ directly stimulated lymphocyte proliferation but high dosage might inhibit it. In this study, it was demonstrated that the effect of LZ on T lymphocyte proliferation dependented on two major factors. One was the situation of cells and the another was the concentration or dosage of LZ. To the rest T cells or the T cells activated by suboptimal concentration of Con A, LZ enhanced the activity of Con A and promoted the lymphocyte proliferation. However if T cells were highly activated by optimal concentration of Con A, LZ might inhibit it. Lps was a mitogen mainly stimulating B cells. In this study, LZ expressed an inhibitive activity on lymphocyte proliferation induced by LPS in normal mice in vivo or in vitro. CYA was an immunosuppressive agent. It was known that CYA depressed humoral as well as cell mediated immune functions. This study showed that CYA inhibited lymphocyte proliferation induced either by Con A or by Lps. LZ antagonized the effect of CYA and promoted lymphocyte proliferation.

The results presented above implied that LZ possessed immunomodulatory activity and would have therapeutic effects for patients with suppressed immune function.

# Effects of Ling Zhi on Antibody Productive Cells and Allergic Reaction

## — Immunopharmacological Study (2)

ZHANG Luoxiu SHENG Jinzhou XUE Yingden YIN Xiaobin

**Abstract** The authors observed the effects of Ling Zhi (LZ, Ganoderma Lucidum, Fr. Karst) on antibody productive cells, rat passive cutaneous anaphylaxis (PCA) test, SRBC induced delayed type hypersensitive reaction (DTH) test, DNCB induced contactive dermatitis and arthus reaction. It was found that LZ inhibited PFC both in vitro and in vivo. Compared with control group it inhibited DTH and contactive dermatitis as well as arthus reaction with significant difference. There was only a slight inhibitive activity on PCA test.

**Key words** Ling Zhi (LZ; Ganoderma Lucidum, Fr. Karst); Plaque forming cells (PFC); Delayed type hypersensitivity reaction (DTH): Passive cutaneous anaphylaxis (PCA); Contactive dermatitis; Arthus reaction.

It is well known that Ling Zhi (LZ, Ganoderma Lucidum, Fr. Karst) has been used as a traditional medicine in China in the treatment of various aliments for more than thousand years. From the Chinese Pharmacopea “Ben Cao”, LZ was recorded as a valuable drug ingredients upper grade of Chinese traditional medicine.

LZ has been generally recognized as a health protective drug. However, the study of its immunological effects was rare and the mechanism was poorly understood. In the present study we specially observed the effects of LZ planted in Japan on plaque forming cells (PFC) and allergic reaction.

# Materials and Methods

## Animal

Kunming mice, 20 ~ 25g; Wister rats, male, 150 ~ 200g; rabbits 2kg of either sex were supplied from the Animal Center, Shanghai Medical University.

## Reagents

The hot water extract of LZ powder was provided by Wakan Shoyaku Botany Institute, Tokyo, Japan. For in vivo study LZ extract was suspended in 0.2% of carboxymethyl cellulose sodium (CMC). For study in vitro, 10 mg of LZ was prepared in 10 ml of NS then the tube was shaken in 80°C water bath for 2h, centrifuged. The supernatant was kept in 4°C until use. The frozen part was prepared as before. Pokeweed mitogen (PWM) and Lipopolysaccaride (Lps) were obtained from Sigma Chemical Co. RPMI – 1640 was purchased from GIBCO Co. It contained 15 mM Hepes 100 U/ml penicillin, 100 μg/ml streptomycin and 10% NCS.

## Plague forming cells (PFC) test in vitro

Mice were sacrificed by cervical dislocation. The spleens were minced and suspended. Cells were washed twice in RPMI – 1640 medium. Spleen cells 5 x $10^6$/ml were seeded in 24 well microplate in the presence of SRBC with or without test samples and incubated at 37°C for 96h in a humidified atmosphere with 5% $CO_2$. After culture termination the cells were harvested and washed twice then adjusted to a desired concentration, 100 μl of spleen cell, 100 μl of complement, 50 μl of SRBC were mixed with 0.25 ml of 0.4% agarose and from this each of 100 μl was taken to add on a glass with cover. All glass were incubated at 37°C in a humidified atmosphere for 3h. The number of PFC was observed with a diaphotoinverted microscope. Group comparison was performed by the student's test.

## PFC in vivo

Mice were sensitized with 2% SRBC 0.5 ml/mouse ip on do, and were randomly divided into 3 groups. Control group and LZ 1, 2 groups. Control group CMC po. LZ groups 125 and 500 mg/kg po. qd. x 4 (d0 ~ d3). On d4 the mice were killed, and the PFC in spleens were assayed.

## Rat passive cutaneous anaphylaxis (PCA)

40 MALE WISTER RATS, 180 ~ 200g, were-divided into 4 groups: control group 0.2% CMC po. qd. x 5, group 2 LZ 125 mg/kg po. qd. x 5, group 3 LZ 500 mg/kg po. qd. x 5 (-d2 ~ d2), group 4 sodium cromoglycates 10 mg/kg iv. x 1. All rats were passively sensitized by intradermal injection of IGE like antibody in different dilution at the back

of rats (anti-T.M. protein serum). T.M. protein isolated from the roots of Trichosanthes Kiriluwli Maxim was used as antigen. After 48h (2h after the last administration of LZ) rats were challenged by iv. 10 mg/kg of antigen in 1% Evans blue cromoglycates group 10 mg/kg iv. 10 mg/kg iv. and challenged with antigen immediately. 30 min later rats were killed and the diameter of blue area were recorded. Furthermore, the blue area were chopped and put into a certain amount of NS: Acetone = 3 : 7 for 48h. After centrifugation the blue solution were measured by photoelectric colorimeter at 620nm.

**Delayed type hypersensitivity reaction (DTH)**

Each mouse was immunized with SRBC 10% 0.5 ml ip. on do. Mice receiving LZ treatment were administered po. qd. x 8 (d0 ~ d7). On d7 50 µl of 10% SRBC was injected into the left footpad of each mouse interdermally. As a control 50 µl of NS was injected into each right footpad. The volumes of right and left footpad were measured with a special measure after 24h. results were presented as $\Delta V = V_L - V_R$.

**2.4 – dinitrochlorobenzene (DNCB) induced contactive dermatitis**

DNCB was used as a stimulus. The dorsal and abdomen of animals were shaved and 20 µl of 50% DNCB was distributed over the hair free region of dorsal on d1 and d2. Control group gave 0.2% CMC po. qd. x 16. Experimental groups were given LZ 250 or 500 mg/kg po. qd. x 16, dexamethasone 10 mg/kg ip. qd. x 3 and qod. x 7 respectively. On d14 challenge was carried out using 20 µl of 10% DNCB glycel solution was distributed on left car. 30 min later 0.5% Evan's blue 0.1 ml/10 g iv. was given. After 30 min the animals were killed by cervical dislocation and pieces of ear were removed from both ears with a hole driller. The pieces of ears were weighted on an analytical balance and the difference between left and right ear was calculated and compared. On the other side the extent of the blue are of abdomen were also compared.

**Arthus reaction**

Rabbit's arthus reaction is a representative pathological model of III type allergic reaction. It was mediated by IgG and IgM. Rabbits were immunized with an intramuscular injection of 10 mg ovalbumin emulsified in 1 ml of complete Freund's adjuvant. 4 times weekly. Ten days after the last injection 0.2 ml of 1% antigen was injected intradermally into 4 sites on the back of the animal where shaved one day before. The dimension of the Arthus reaction was measured for the first 24h after the antigen treatment. Rabbits were divided into 4 groups. One was the control group with NS iv, group 2 dexamethasone 5 mg/kg iv, the other 2 groups with LZ 0.5 and 1 g/kg iv. at 60 min prior to challenging with antigen respectively.

The diameter and extent of reaction were scored as follow:

++++ Significant haemorrage with local-necrosis.
+++ Significant congestion and haemorrage
++ Congestion with sported haemorrage.
+ Mild congestion
– No reaction.

# Results

### Effect of LZ on PFC test in vitro

Tab. 2-1 showed that both Part A and Part B of LZ (10 ~ 1 000 μg/ml) markedly inhibited PFC production in vitro when compared with control group ($P < 0.01$).

### Effect of LZ on PFC test in vivo

**Tab. 2-1 Effect of LZ on PFC in vitro**

| Reagents | μg/ml | PFC/$10^6$ cells $\overline{X} \pm SD$ | Inhibition % |
|---|---|---|---|
| Control | — | 1 890 ± 164 | |
| A LZ | 10 | 1 484 ± 247 | 21.5* |
| LZ | 100 | 1 203 ± 312 | 36.4** |
| LZ | 1 000 | 281 ± 62 | 85.1** |
| B LZ | 10 | 938 ± 265 | 50.4** |
| LZ | 100 | 1 234 ± 416 | 34.7 |
| LZ | 1 000 | 266 ± 94 | 86.0** |

Part A: Soluble part of LZ; Part B: frozen thawed part of LZ;
Control: NS. $\overline{X} \pm SD$, * $P < 0.05$, ** $P < 0.01$

Mice were immunized with 2% SRBC 0.5 ml/mouse on d0 and divided into 3 groups. One was control group. The other two were given LZ 125 or 500 mg/kg, po. qd. x 4 (d0 ~ d3) respectively. On d4 mice were sacrificed and the antibody productive cells were assayed by PFC test. The results showed that LZ 125 and 500 mg/kg inhibited PFC production significantly ($P < 0.01$) (Tab. 2-2).

**Tab. 2-2 Effect of LZ on PFC in vivo**

| Reagents | mg/ml | PFC/$10^6$ cells $\overline{X} \pm SD$ | Inhibition % |
|---|---|---|---|
| Control | | 70 400 ± 7 200 | |
| LZ | 125 | 41 000 ± 3 250 | 41.8* |
| LZ | 500 | 26 400 ± 1 720 | 62.5** |

d0 ip. SRBC, drug po. d0 ~ d4; Control: 0.2% CMC; $\overline{X} \pm SD$; **$P < 0.01$

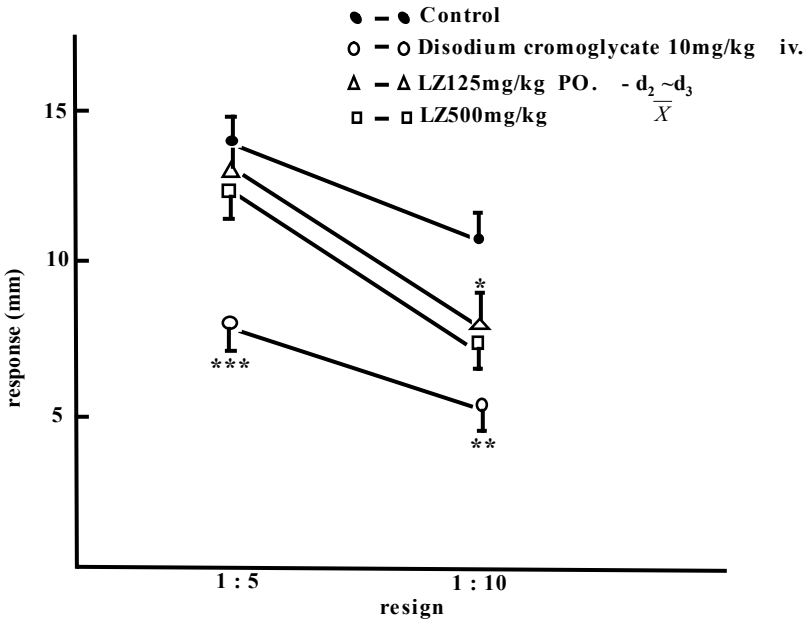


**Fig. 2-1 Effect of LZ on PCA test by diameter ±SD; n=6~7; *P<0.05, **P<0.01, ***P<0.001**

### Effect of LZ on rat PCA test

Fig. 2-1 showed that disodium cromoglycate markedly inhibited rat PCA reaction. LZ showed slight inhibition, but $P > 0.05$ when compared with control.

### Effect of LZ on DTH test

It was found that LZ 500 mg/kg significantly inhibited the SRBC induced DTH reaction at 24 h after antigen challenge. CYA 10 mg/kg ip. qd. x 8 also inhibited DTH reaction ($P < 0.05$) (Fig. 2-2).

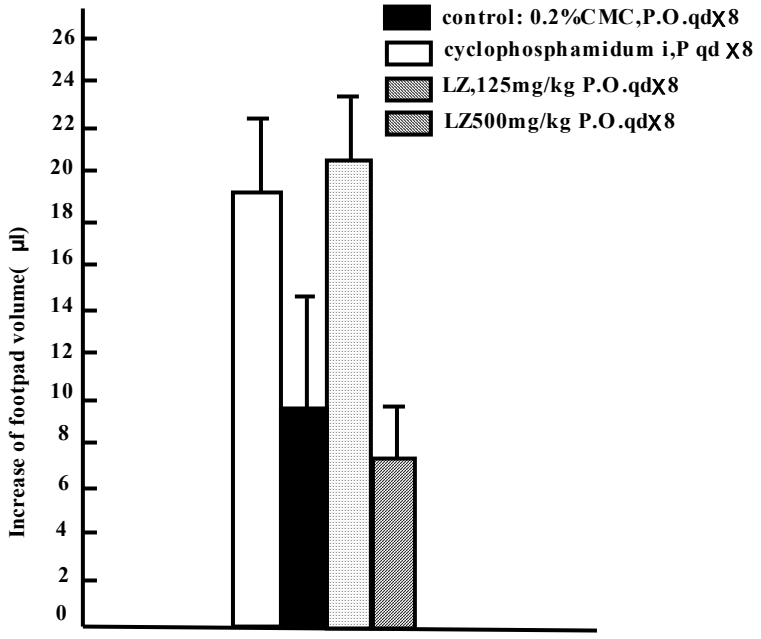


**Fig. 2-2 Effect of LZ on DTH test**
***P<0.05.**

## Effect of LZ on DNCB induced contactive dermatitis

Tab. 2-3 showed that LZ had suppressive effects on DNCB induced contactive dermatitis at 250 and 500 mg/kg, and dexamethasone 10 mg/kg significantly suppressed this contactive dermatitis.

**Tab. 2-3 Effects of LZ on contactive dermatitis reaction**

| | Mg/kg | Animal (n) | Ear weight Increase % | skin blue grade |
|---|---|---|---|---|
| Control | — | 10 | 12.6 ± 9.7 | +++ |
| Dexamethasone | 10 | 10 | 2.5 ± 4.1 | ± |
| LZ | 250 | 9 | 3.7 ± 4.1 | ++ |
| LZ | 500 | 9 | 2.3 ± 3.1 | ++ |

Control: 0.2% CMC, po. qd. x 16; Dexamethasone: ip. qd. x 3, qod. x 7;
LZ: po. qd. x 16, Ear weight increase % = $W/W_B$ x 100%;
$4W = W_L - W_B$; * $P < 0.05$, ** $P < 0.01$

### Effect of LZ on Arthus reaction

As shown in Tab. 2-4, 1-5. The inflammation of the control group was mainly hyperemia appeared after 2 h, haemorrhage area fused and reached a maximum with local necrosis at 12h after the challenge. As compared with the control, the inflammation in rabbits treated by LZ (0.5, 1.0 g/kg) was decreased at 2 ~ 8 h after injection of antigen and as control after 8 h. The effect of dexamethasone prolonged with hyperemia and haemorrhage at 6 h.

**Tab. 2-4 Effects of LZ on Arthus reaction**

| Reagents | mg/kg | Inflammation graded | | | | | |
|---|---|---|---|---|---|---|---|
| | | 2h | 4h | 6h | 8h | 12h | 24h |
| Control | — | +++ | +++ | +++ | +++ | ++++ | ++++ |
| Dex | 5 | — | ± | ++ | +++ | +++ | +++ |
| LZ | 500 | ± | + | ++ | +++ | ++++ | ++++ |
| LZ | 1 000 | + | ++ | ++ | +++ | +++ | ++++ |

Reagents iv. 60 min before antigen challenge

**Tab. 2-5 Effects of LZ on Arthus reaction in rabbits**

| Reagents | Mg/kg | Inflamed dimension (cm) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 2h | 4h | 6h | 8h | 12h | 24h |
| Control | — | 1.52 ± 0.94 | 2.54 ± 0.66 | 2.73 ± 0.65 | 3.40 ± 0.87 | 3.40 ± 0.87 | 3.43 ± 0.52 |
| Dex | 5 | 0 | 0.55 ± 0.50 | 2.29 ± 0.23 | 2.71 ± 0.55 | 2.71 ± 0.55 | 3.01 ± 0.53 |
| LZ | 500 | 0.45 ± 0.48 | 1.74 ± 0.66 | 2.38 ± 0.35 | 2.72 ± 0.15 | 2.72 ± 0.15 | 2.82 ± 0.10 |
| LZ | 1 000 | 0.27 ± 0.07 | 1.54 ± 1.05 | 1.84 ± 0.21 | 2.82 ± 0.49 | 2.82 ± 0.49 | 2.89 ± 0.46 |

# Discussion

The hot water extract from LZ planted in Japan possessed very low toxicity. Mice given LZ 5 g/kg po. had no one head during one week period of observation. It was coincident with the ancient record in Ben Cao. It was described that LZ was non toxic and could be used for a long time.

In the present study, LZ (10 ~ 1 000 μg/ml) inhibited PFC test in vitro and 500 mg/kg po. also inhibited PFC production in vivo. From the result of mitogenic test, it was found that LZ expressed immunomodulatory activity. It inhibited immune function when the organism was in a hypersensitive situation and elevated the immune function when the organism was in an immunosuppressed condition. It also depended on the concentration or dosage of LZ. LZ possessed dual regulative activity according to the situation of host and the dosage.

In the present study LZ 500 mg/kg inhibited DTH reaction and DNCB induced contactive dermatitis as well as arthus reaction. Besides it had a trend to suppress rat PCA test. That means LZ was effective on I, III, IV types of hypersensitive reaction.

# Immune Suppressive Effects of Ling Zhi in Mice

## — Immunopharmacological Study (3)

SUN Bing & ZHANG Luoxiu

**Abstract** Ling Zhi (LZ) 125, 250 mg/kg po. x 8 had an immune suppressive effect on normal KM mice. The formation of PFC was inhibited, the value of $HC_{50}$ and Con A-induced lymphocyte proliferation were also decreased significantly. The inhibition mentioned above was weaker than that of cyclophosphamide (200 mg/kg ip. d1). The $T_H$ and $T_S$ subpopulations were not influenced by LZ in vivo. The results suggested that the immune suppression of LZ on T and B cells directly related to regulate the functions of T and B cells.

**Key words** Ling Zhi (LZ, Ganoderma Lucidum, Fr. Karst); Plaque forming cells; Hemolysin; $T_H$; $T_S$.

Ling Zhi (LZ, Ganoderma Lucidum, Fr. Karst) has been considered as one kind of Chinese traditional herbs which is good in strengthening human defense and keeping benefits against diseases. LZ has a lot of treating actions on different diseases. It is more useful in treating chronic bronchitis, tumor, chronic progressive muscular dystrophy and dermatomyositis. LZ also has the action against senility. The primary study of LZ on immunopharmacology showed that LZ was an immune stimulator[1]. According to the experiment in our department, LZ displayed an immunomodulatory activity and suppressed the humoral immunity in mice [2~3]. In this paper we observed the effect of LZ on Concanavalin A (Con A)-induced T lymphocyte proliferation as well as humoral immunity in normal KM mice. Besides we observed T cells subsets and hemolysin.

## Materials and Methods

### Animals

Kunming (KM) mice, both male and female, 18±2g, provided from the Animal center, Shanghai Medical University.

Dept. of Pharmacology, School of Pharmacy, Shanghai Medical University, Shanghai, China

### Reagents

LZ was provided by Wakan Shoyaku Botany Institute, Tokyo, Japan. Yellow-brown powder was hot water extract of LZ. It was dissolved in 0.2% carboxymethyl cellulose sodium (CMC) with 5% concentration stored at 4°C. Before using, the suspension of LZ would be made in suitable dilution.

Cyclophosphamide was obtained from Shanghai 12$^{th}$ Pharmaceutical Factory.

RPMI 1640 medium was purchased from GIBCO. Fetal calf serum was from Shanghai Academy of Agriculture. Rat-anti-mouse L3T4 (Anti-$CD_4$) and Lyt2 (Anti-$CD_8$) monoclonal antibodies (MAb), sheep-anti-rat IgG fluorescein conjugated MAb were purchased from Department of Immunology, Beijing Medical University. Separated solution of lymphocytes was from Shanghai No. 2 Reagent Co.

**Anti-SRBC PFC assays** performed on d5 by using jerne plaque assay modified by Dresser and Graves [4].

**Hemolysis concentration fifty percent ($HC_{50}$) assay** It was assayed by method described as Xu Xiui yin [5].

**T-cells subsets** measured by indirect immuno-fluorescence [6].

**Lymphocyte proliferation** determined by $^3$H-thymidine incorporation assay [7].

## Results

### PFC and $HC_{50}$ of mice

LZ 125, 250 mg/kg po. x 8 decreased the plaque forming cells (PFC) and the value of $HC_{50}$ in comparison with CMC-treated control (Tab. 3-1).

**Tab. 3-1 Effect of LZ on PFC and $HC_{50}$ of mice**

| Groups | Dose (mg/kg) | Experimental Index | |
|---|---|---|---|
| | | PFC/$10^6$ spleen cells | $HC_{50}$ |
| Control | 0.2% CMC | 67 ± 8 | 312 ± 28 |
| LZ I | 125 | 41 ± 5** | 220 ± 65** |
| LZ II | 250 | 38 ± 3** | 210 ± 77** |
| CYA | 200 | 17 ± 3*** | 222 ± 82** |

** $P < 0.05$, *** $P < 0.01$ Compared with control. n = 10;
LZ. Po. x 8; CYA – 200 mg/kg ip. d1

## T-cells subsets

$T_H$ and $T_S$ subpopulations cells were not influenced by LZ in normal mice. The ratio of T helper cell/T suppressive cell ($T_H$ and $T_S$) were decreased slightly in LZ-treated groups but there was no significant difference in comparison with CMC treated control (Tab. 3-2).

**Tab. 3-2 Mean percentage of T cells subsets in spleen**

| Group | Dose (mg/kg) | Percentage of lymphocyte | | Ratio of $T_H$ / $T_S$ |
|---|---|---|---|---|
| | | $T_H$ (L3T4) | $T_S$ (Lyt2) | |
| Control | 0.2% CMC | 34 ± 3 | 30 ± 2 | 1.13 |
| LZ I | 125 | 35 ± 2 | 33 ± 1 | 1.06* |
| LZ II | 250 | 35 ± 1 | 32 ± 1 | 1.09* |
| CYA | 200 | 35 ± 1 | 32 ± 1 | 1.09* |

* P > 0.05 Compared with control. n = 5

## Lymphocyte proliferation

T lymphocyte proliferation induced by 5 μg/ml Con A was suppressed by LZ in vivo (Tab. 3-3).

**Tab. 3-3 Lymphocyte proliferation in vivo (cpm)**

| Group | Dose (mg/kg) | Con A 5 μg/ml | Without mitogen |
|---|---|---|---|
| Control | 0.2% CMC | 9 166 ± 4 578$^{\Delta\Delta\Delta}$ | 1 116 ± 200 |
| LZ I | 125 | 3 052 ± 1 585** | |
| LZ II | 250 | 4 115 ± 716** | |
| CYA | 200 | 1 626 ± 533*** | |

**P<0.05, ***P<0.01 Compared with control; $^{\Delta\Delta\Delta}$ P<0.01 Compared with without mitogen, n = 10

Under the experimental condition, CYA 200 mg/kg d1, ip. was as a positive control drug to keep the accuracy of the experiment. The results indicated CYA had a strong immune inhibition on PFC, $HC_{50}$ and T lymphocyteproliferation induced by Con A in normal KM mice. LZ also had an immune suppression but the action was weaker than that of CYA.

# Discussion

In this study, the experiment showed that LZ (125, 250 mg/kg po. x 8) suppressed PFC, $HC_{50}$ and T lymphocyte proliferation induced by Con A, but the suppressive actions were weaker than that of CYA. It indicated that LZ inhibited the function of T and B cells of normal mice.

Our department has reported LZ (500 mg/kg) depressed the production of antibody-forming cells in spleen in vivo([3]). We also proved that the action of LZ on Con A-induced lymphocyte proliferation depended upon the concentration of Con A and the function of T cells. When the concentration of Con A was increased from lower to high, the action of LZ varied from stimulation to suppression. When CYA was used to treat normal mice, the suppressed immune function of mice would be enhanced by LZ. For example the decreased Con A-induced T lymphocyte proliferation was increased by LZ. The results suggested that LZ displayed a dual direct immuno-modulator. Both concentration of mitogen and drugs influenced action of LZ on mitogen-induced lymphocyte proliferation. Under the experimental condition, the concentration of Con A was higher (5 μg/ml) with strong stimulation on T cells. LZ suppressed this Con A induced lymphocyte proliferation. This coincided with the results mentioned before.

In this study LZ showed no action on $T_H$ and $T_S$ cells subsets. It suggested the immune suppression of LZ on T and B cells was related to regulation of the function of lymphocyte.

In conclusion, this article repeated the work that LZ showed immune suppression on B cells and also proved that LZ displayed depressive action on Con A induced lymphocyte proliferation. LZ played an immune suppressive role by directly regulating the functions of T and B cells.

# Effects of Ling Zhi on Macrophage Phagocytosis and Carbon Particles Clearance Test

## — Immunopharmacological Study (4)

ZHANG Luoxiu MIAO Honghua SHEN Jinzhou

**Abstract** In the present study we mainly observed the effect of Ling Zhi (LZ) on macrophage phagocytosis. It was found that Ling Zhi (LZ) 10 ~ 50 μg/ml stimulated phagocytosis in vitro, but higher concentration of LZ showed an inhibitive activity. From the experiment in vivo it was observed LZ 100 ~ 500 mg/kg po. qd. x 4 also significantly enhanced the macrophage phagocytosis activity. Carbon clearance test showed that LZ (125 ~ 625 mg/kg, po. qd. x 4) could increase the function of reticular endothelium system.

**Key words** Ling Zhi (LZ, Ganoderma Lucidum, Fr. Karst); Phagocytosis; Carbon clearance test.

Macrophages play an important role in immune system. Activated macrophages are the primary defense mechanism against intracellular bacteria, viruses, fungi and protozoa. It was also evidenced that macrophages play a role in resistance to tumors. Besides, macrophages play a pivotal role in both the initiation and regulation of the immune response by directive action or by secreting some soluble factors.

LZ has been reported to use as a precious drug in the folk to prolong life, to alleviate various chronic diseases. It suggested that LZ may act on the primary defense mechanism. In view of the important role of macrophage, in this paper we mainly reported the effect of LZ on phagocytosis and we will report the effect of LZ on the production of interleukin-1 and tumor necrosis factor later.

## Materials and Methods

### Animals

Kunming (KM) mice, male, 20 ~ 26g, were supplied by the Animal Center, Shanghai Medical University.

Dept. of Pharmacology, School of Pharmacy, Shanghai Medical University, Shanghai, China

**Reagents**

The hot water extract of LZ was supplied by Japan Wakan Shoyaku Botany Institute. For in vivo study, a certain amount of LZ extract was grinded and dissolved in normal saline containing 0.2% of carboxymethyl cellulose sodium (CMC) shaken in 80°C water bath for 4 h. This suspension was used for in vivo study. For in vitro study, a little amount of LZ extract was dissolved in normal saline and rotated slowly in 80°C water bath for 2 h then centrifuged (1 600 rpm x 10 min). The supernatant was stored at -4°C until study. Before use, this solution was diluted to desire concentration. It was named as soluble part (Part A). When LZ was dissolved and shaken in 80°C water bath then rapidly frozen at -30°C and thawed for 3 times. After centrifuged the solution was named as frozen thawed part (Part B).

RPMI – 1640 was purchased from GIBCO. It contained Hepes 15 mmol/L, Streptomycin 100 μg/ml, Penicillin 100 U/ml, 2-Mercaptoethanol (2-ME) $5 \times 10^{-5}$ mmol and 10% of NCS. India ink from Winsaw & Newton, England. Ink: NS = 1:2.

**Macrophage phagocytosis in vitro**

Peritoneal exudate cells PEC were collected from mice which had been stimulated 4 days before by ip. 2.5% thioglycolate sodium medium 1 ml/mouse. PEC were pooled washed and resuspended in RPMI – 1640 medium at a concentration of $3 \times 10^6$ cells/ml. 1 ml of cells was added in a glass dish with a cover lip inside and after adherence for 1 h at 37°C in humidified 95% air -5% $CO_2$ nonadherent cells were removed by washing 2 times with RPMI – 1640 medium. SRBC were washed 3 times in an excess of physiological saline and then centrifuged. The red cells made into 5% v/v suspension by the addition of physiological saline. From the suspension 0.1 ml aliquots were added to the macrophage monolayers on a cover lip inside the dish with or without LZ at final concentration of 1 ~100 μg/ml, triplicate cultures for each condition. The dishes were gently shaken to evenly distribute the red cells and then incubated for 2h at 37°C. After this, the cover lips were rinsed and fixed in a 1:1 mixture of methanol : acetone. They were then stained with Giemsa's method.

**Macrophage phagocytosis in vivo** [(1)]

Mice were stimulated as before then randomly divided into 4 groups. One was control group, others were administered of LZ 125, 250 and 500 mg/kg po. qd. x 4 respectively. 2h after final treatment 1 ml of 2% SRBC ip. to each mouse. 1h later the mice were sacrificed and the peritoneal macrophages were seeded on the cover lip in a glass dish then incubated at 37°C for 1h. After that the cover lips were rinsed and stained as before. The phagocytosis % was calculated as follow:

$$\text{Phagocytosis \%} = \frac{\text{Phagocytored (SRBC) mø}}{\text{200 mø}} \times 100\%$$

**Carbon clearance test**

Mice were divided into 3 groups. One was control group po. 0.2% CMC qd. x 4. LZ groups were administered 125 and 625 mg/kg po. qd. x 14.24 h after final administration India ink (1:2) 0.1 ml/10g was injected through tail vain. Remove samples of 30 μl of blood from the retroocular venous plexus at intervals of approximately 2, 15 min. Transferred the sample to a tube containing distilled water. The absorbance value at 600nm was determined by 721 grating spectrophotometer. At the end of experiment, the mice were killed and the liver, spleen weight were recorded.

The clearance index K value was calculated according to following formula:

$$K - (\log A_1 - \log A_2) / (t_2 - t_1)$$

A corrected phagocytic index *a* is a constant obtained from a formula relating the cube root of K to the ratio of body weight to the weight of the liver and spleen.

$$a = 3\sqrt{k} \times W_b / W_{L.S}$$

For this experiment phagocytic index *a* was used to evaluate the reticular endothelium function.

# Results

**Effect of LZ on macrophage phagocytosis in vitro**

The results expressed in Tab. 4-1 and Tab. 4-2 demonstrated that both soluble part and frozen thawed part of LZ stimulated the phagocytosis at 10 ~ 100 μg/ml in a concentration dependent fashion but the higher concentration (100 μg/ml) of LZ part B exhibited an inhibitive activity (Tab. 4-1, 4-2).

**Tab. 4-1 Effect of LZ (soluble part) on phagocytosis of MØ in vitro**

| Drug | μg/ml | Phagocytosis % | |
|---|---|---|---|
| | | Exp. 1 | Exp. 2 |
| Control | | 22.6 ± 2.8 | 19.5 ± 2.2 |
| LZ | 1 | 23.2 ± 3.2 | 20.0 ± 2.5 |
| LZ | 10 | 29.4 ± 1.8** | 25.9 ± 2.2** |
| LZ | 25 | 35.6 ± 4.6** | 27.5 ± 1.6** |
| LZ | 50 | 37.1 ± 3.6** | 30.0 ± 2.4** |
| LZ | 100 | 40.5 ± 4.2** | 38.3 ± 3.4** |

Control: RPMI – 1640
$\overline{X}$ ± SD; n = 4; **P<0.01

**Tab. 4-2 Effect of LZ (frozen thawed part) on phagocytosis of MØ in vitro**

| Drug | μg/ml | Phagocytosis % | |
|---|---|---|---|
| | | Exp. 1 | Exp. 2 |
| Control | | 23.0 ± 3.9 | 19.4 ± 3.6 |
| LZ | 1 | 22.5 ± 1.8 | 19.3 ± 3.7 |
| LZ | 10 | 29.4 ± 2.6* | 27.0 ± 3.0* |
| LZ | 25 | 30.6 ± 4.0* | 31.1 ± 2.5** |
| LZ | 50 | 34.5 ± 4.8** | 38.7 ± 2.4** |
| LZ | 100 | 20.3 ± 3.8 | 18.3 ± 2.7 |

Control: 1640
$\overline{X}$ ± SD; n = 4; *P<0.05 **P<0.01

## Effect of LZ on macrophage phagocytosis in vivo

It was demonstrated that LZ 125, 250 and 500 mg/kg po. qd. x 4 promoted macrophage phagocytosis in vivo just like the results in vitro (Tab. 4-3).

**Tab. 4-3 Effect of LZ on phagocytosis of Mϕ in vivo**

| Drug | mg/kg | Phagocytosis (%) |
|---|---|---|
| Control | | 73.2 ± 2.68 |
| LZ | 125 | 83.4 ± 3.88** |
| LZ | 250 | 89.1 ± 2.20** |
| LZ | 500 | 93.1 ± 2.38** |

Control: 0.2% CMC
$\overline{X}$ ± SD; n = 5; **P<0.01

**Effect of LZ on carbon clearance test**

Tab. 4-4 showed that the phagocytic index of LZ groups were evidently higher than that of control group.

**Tab. 4-4 Effect of LZ on carbon clearance test**

| Drug | mg/kg | No. of animals | α value ($\overline{X}$ ± SD) |
|---|---|---|---|
| Control | | 9 | 2.11 ± 0.5 |
| LZ | 125 | 8 | 2.92 ± 0.4** |
| LZ | 625 | 8 | 2.53 ± 0.9* |

Control: 0.2% CMC; LZ : 2.5% and 0.5%
Drug po. for 4 days; $\overline{X}$ ± SD; *P<0.05 **P<0.01

# Discussion

From the immunopharmacological study of LZ it was found that LZ exhibited activity as an immunomodulator. The effect of LZ on cells mediated immunity dependented on the dosage of LZ and the function of immune system. It expressed a diverse regulative activity. From the results stated above it was suggested that LZ significantly elevated the function of RES.

It was well known that monocyte macrophage system represents a very important aspect in immune system. Not only involved in specific immune response but also serves as nonspecific defense capacity of organism. LZ exerted a promotive activity on monocyte macrophage system might be one of the mechanism of LZ in the treatment of some diseases especially chronic diseases.

# Influence of Ling Zhi on Natural Killer Cells

## — Immunopharmacological Study (5)

ZHANG Luoxiu YU Mingyan

**Abstract** Ling Zhi ((LZ, Ganoderma Lucidum, Fr. Karst) exhibits various biological activities. In this study we found LZ 0.1 ~ 100 μg/ml caused a slight inhibition of natural killer (NK) cells activity in vitro. The same concentration of LZ had no effect on hydrocortisone inhibited NK cells. Above concentration of LZ had no direct cytotoxic activity on Yac-1 cells in vitro. However LZ 300 mg/kg po. qd. x 6 enhanced NK cells activity in vivo. Hydrocortisone 2 μg/ml inhibited NK activity in vitro, cyclophosphamide 40 mg/kg ip. decreased NK cells activity in vivo. The effect of LZ in combination with hydrocortisone or cyclophosphamide had no significant effect on NK cells activity when compared to the effect of immunosuppressor alone.

**Key words** Ling Zhi (LZ, Ganoderma Lucidum, Fr. Karst); Natural Killer (NK) cells.

It is demonstrated that NK cells play an important role in host resistance against cancer and infections diseases. NK cells have direct cytotoxic effects against target cells and also can produce and release soluble factors that can induce antiviral resistance and cytostacis of tumor cells. Besides, the ability of NK cells to rapidly produce interferon and possibly interleukin-2 provides a mechanism for positive self-regulation.[1]

Recent studies in our lab have revealed that Ling Zhi (LZ) exerts various activity on immune system.[2] In view of the important role of NK cells in immune surveillance we specially observed the influence of LZ and NK cells activity both in vitro and in vivo in this study.

## Materials and Methods

### Animals

$C_{57}$BL/6 mice, male, were used at the age of 6 ~ 8 weeks. Kunming (KM) mice, male, 18 ~ 22g, were supplied by the Animal Center, Shanghai Medical University.

Dept. of Pharmacology, School of Pharmacy, Shanghai Medical University, Shanghai, China

**Target cells**

Yac-1 was originally derived from a moloney virus induced lymphoma in A / Sn mice but maintained in vitro. This cell line was provided by prof. Wang Qiu-da from Shanghai Institute of Cell Biology Chinese Academy of Sciences. The cell line was subcultured with 10% new bovine serum RPMI – 1640 medium in vitro.

**Reagents**

Hot water extract from LZ was provided by Waken Shoyaku Botany Institute in Japan. It was prepared as before. Hydrocortisone (HC) was purchased from Shanghai Sine Pharmaceutical Factory, Cyclophosphamide (CYA) was purchased from Shanghai the Twelveth Pharmaceutical Factory. DNAse from Sigma, Trypsin from Difco. $^{3}$H-TdR was provided by Shanghai Institute of Nuclear Research Chinese Academy of Sciences and the specific activity was 1 110 GBq./mmol. RPMI – 1640 was prepared as before.

**Lable of target cell line** [3~6]

The NK cells activity was assessed against Yac-1 lymphoma cell line which was maintained in RPMI medium supplemented with 10% heat inactivated NCS. Viable cells were counted using the trypan blue dye exclusion method. The percentage of viable cells was 95%. 0.4 ml of target cells (2 x $10^6$ cells/ml) were labeled with 3.7 x $10^5$ Bq of $^{2}$H-TdR for 4 h at 37°C (water bath) and washed 3 times with RPMI – 1640 medium. Finally, the cell concentration was adjusted to 1 x $10^5$ cells/ml.

**Preparation of effect or cells**

Spleen cells from mice were used as effect or cells. The spleen was minced and suspended. Red blood cells were lysed with distilled water. Cells were washed 3 times in RPMI – 1640 medium. Finally the cell concentration was adjusted to 1 x $10^7$ cells/ml.

**Cytotoxicity assay**

$10^4$ isotope labeled target cells in 50 μl were added in 96 well microtiter plates. 0.1 ml of RPMI – 1640 medium in spontaneous release group, 0.1 ml of 0.5 % Triton X – 100 in maximum release group and 0.1 ml of effector cells in experimental group were added with or without test agent in a total volume of 200 μl. Triplicate or quadruplicate cultures for each group were incubated at 37°C in a 5% $CO_2$ humidified atmosphere for 18 h. At the end of incubation the culture cells were treated with 0.24% trypsin and 0.0125% DNAse for 30 min. Then the cells were collected onto glass fiber papers and detected by liquid scientillation. An aliquot of labeled target cells was directly counted by liquid scientillation to determine the total radioactivity.

$$\% \text{ cytotoxicity} = cpm_{com} - cpm_{test} / cpm_{con} \times 100\%$$

$$\% \text{ spontaneous release} = \frac{cpm_{total} - cpm_{con}}{cpm_{total}} \times 100\%$$

$$\% \text{ maximum release} = \frac{cpm_{total} - cpm_{max}}{cpm_{total}} \times 100\%$$

The comparison of different groups was performed by student's test.

1. Influence of tripsin and DNAse on $^3$H-TdR release from target cells: Two groups of maximum and spontaneous release were incubated at 37°C 5% $CO_2$ for 18 h. At the end of incubation the culture cells in one maximum and spontaneous groups were treated with 0.24% trypsin and 0.0125% DNAse. The results were compared with those cells in maximum and spontaneous group which were treated by 50 μl of medium in stead of trypsin and DNAse.

2. Comparison of the NK cells activity of $C_{57}$ BL/6 and Kunming mice: Spleen cells isolated from the inbred $C_{57}$ BL/6 mice and hybrid Kunming mice were assayed for their NK cells activity in the same condition.

3. The direct action of LZ on target cells: In order to observe the direct effect of LZ on target cells activity, 100 μl of 2 x $10^4$ labeled target cells were seeded in each well with or without of various concentration of LZ (0.1 ~ 100 μg/ml). After incubated at 37°C 5% $CO_2$ for18 h the cells were counted as described in the method.

4. Effect of LZ on NK cells activity in vitro: Effector cells and target cells were added in each well in a E : T ratio of 100 : 1 or 50 : 1 with or without LZ (0.1 ~ 100 μg/ml) incubated at 37°C 5% $CO_2$ for18 h. Then the cells were treated as the methods.

5. The direct effect of hydrocortisone (HC), cyclophosphamide (CYA), and cyclosporine A (CsA) on target cells activity and on NK cells activity in vitro: HC 1.1, 2.2 μg/ml, CYA 0.5, 10 μg/ml, CsA 0.2, 0.4 and 4 μg/ml was respectively added in each well with Yac-1 cells alone or with effector and target cells in a E : T ratio of 100 : 1. After incubated at 37°C 5% $CO_2$ for18 h the cells were treated as before.

6. Effect of LZ in combination of HC on NK activity in vitro: LZ (0.1 ~ 100 μg/ml) was added in combination with 2 μg/ml of HC in each well with effector and target cells in a ratio of 50 : 1. After incubation for 18 h the cells were treated as before.

7. Effect of LZ alone and in combination with CYA on NK cells activity in vivo: Kunming mice were randomly divided into 6 groups:

(1) Control group: 0.2% cmc po. qd. x 6
(2) LZ 150 mg/kg po. qd. x 6

(3) LZ 300 mg/kg po. qd. x 6
(4) 0.2% CMC po. CYA 40 mg/kg ip. qd. x 6
(5) LZ 150 mg/kg po. CYA 40 mg/kg ip. qd. x 6
(6) LZ 300 mg/kg po. CYA 40 mg/kg ip. qd. x 6

24 h after the final administration of drug the mice were killed and the spleens of each group were minced. The spleen cells were treated with distilled water in order to eliminate RBC. After washing 3 times the cell concentration was adjusted to 1 x $10^7$ /ml. Effector cells and target cells incubated with Yac-1 cells in E : T ratio of 100 : 1 for 18 h, then assayed as before.

# Results

### Influence of tripsin and DNAse on $^3$H-TdR release from labeled Yac-1 cells

From the results expressed in Fig. 5-1, it was found that enzyme treatment resulted in a very significant increase in the release of $^3$H-TdR from Triton-100 X treated cells in maximum group. However, no apparent influence was found in spontaneous release group. That means enzyme treatment could increase $^3$H-TdR release from disrupted cells but it did not influence the intact undisrupted cells. So the enzyme treatment could increase the sensitivity of this assay method.

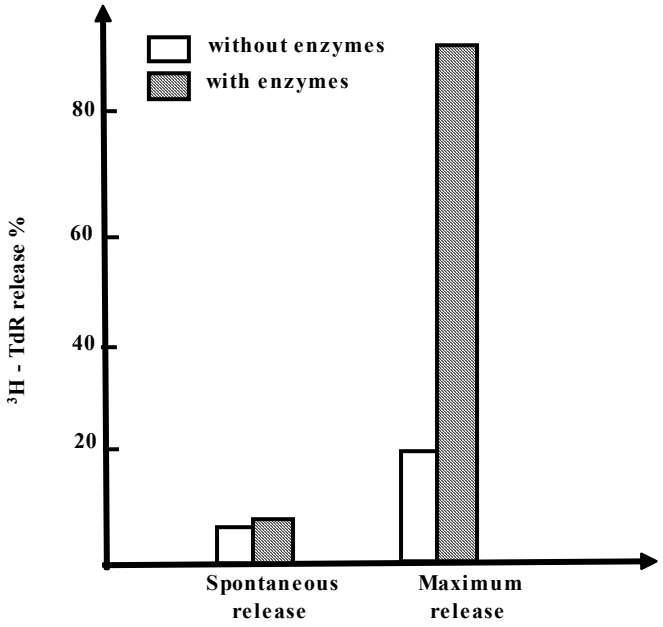


**Fig. 5-1 Effect of enzymes on spontaneous and maximum release**
**Yac-1 cells (1~2 x 10 $^4$/well) were were incubated with RPMI-I640 (10%NCS) or Tritor X-100 for 18 h. The data represents the mean of 3 separate experiments.**

### Comparison of the NK cells activity between $C_{57}$ BL/6 and KM mice

It was shown in Fig. 5-2. That $C_{57}$ BL/6 mice exhibited higher NK cells activity than that of KM mice.

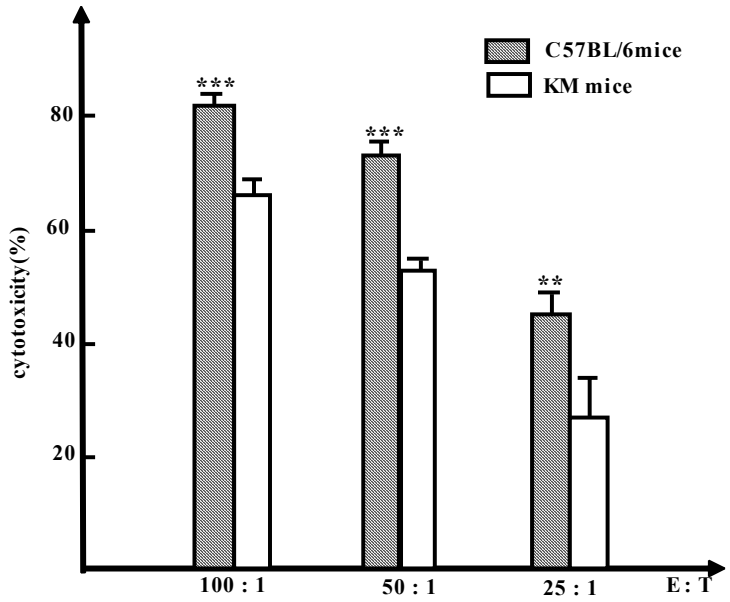


**Fig. 5-2 The comparison of the activity of NK cells between $C_{57}$BL/6 mice and KM mice**
**Effector cells were incubated with target cells for 18h. ±SD; n=3; **P<0.01, ***P<0.001.**

### Direct effect of LZ on Yac-1 cells in vitro

To determine weather LZ produced toxicity directly on target cells. $^3$H-TdR release from Yac-1 cells alone was measured in the absence or presence of LZ. Tab. 5-1 showed that LZ 0.1 ~ 100 μg/ml had no direct effect on the activity of Yac-1 cells. That meant LZ had no direct cytotoxicity on the target cells.

**Tab. 5-1 Effect of LZ on target cells activity in vitro**

| (μg/ml) | Cpm / 2 x $10^6$ cells |
|---|---|
| Control | 1 004 ± 218 |
| LZ 0.1 | 998 ± 29 |
| LZ 1 | 997 ± 49 |
| LZ 10 | 985 ± 10 |
| LZ 100 | 978 ± 83 |

Target cells were incubated with LZ for 18 h. $\overline{X}$ ± SD; n = 4

### Effect of LZ on NK cells activity in vitro

It was observed that LZ expressed slight inhibitive effect on NK cells activity in vitro (Tab. 5-2).

**Tab. 5-2 Effect of LZ on NK cells activity in vitro**

| (μg/ml) | Cytotoxicity % | |
|---|---|---|
| E : T | 100 : 1 | 50 : 1 |
| Control | 76.0 ± 5.4 | 60.7 ± 1.5 |
| LZ 0.1 | 72.3 ± 4.2 | 57.5 ± 1.6* |
| LZ 1 | 70.5 ± 1.7 | 56.5 ± 2.7* |
| LZ 10 | 68.4 ± 3.3* | 55.6 ± 6.1 |
| LZ 100 | 67.4 ± 3.2* | 54.9 ± 6.2 |

Effector cells were incubated with target cells and LZ for 18 h.
$\overline{X}$ ± SD; n = 4; *P<0.05 compared with control

### Effect of HC, CsA and CYA on activity of Yac-1 cells alone and on NK cells activity

The results demonstrated that HC 1.1 and 2.2 μg/ml had no direct influence on Yac-1 cells activity but it significantly inhibited NK cells activity in vitro. CsA in the concentration used in this experiment exhibited apparent direct toxicity on target cells and also significantly inhibited NK cells activity in vitro. CYA had no significant effect on Yac-1 cells and NK cells activity in vitro. Therefore we selected HC as a tool agent in the following experiment (Tab. 5-3).

**Tab. 5-3 Effect of HC, CsA and CYA on Yac-1 cells or on NK cells activity in vitro**

| HC (μg/ml) | $C_S$A (μg/ml) | CYA (μg/ml) | Cytotoxicity % | |
|---|---|---|---|---|
| | | | NK cell | Yac-1 cell |
| 2.2 | — | — | 36.5 ± 3.8*** | 4.0 ± 2.1 |
| 1.1 | — | — | 61.0 ± 9.0* | 0 |
| — | 4 | — | 43.7 ± 11.5** | 55.8 ± 7.3** |
| — | 0.4 | — | 54.6 ± 5.6** | 46.0 ± 5.3** |
| — | 0.2 | — | 50.2 ± 5.1** | 33.7 ± 7.7** |
| — | — | 10 | 71.0 ± 4.1 | 6.4 ± 10.6 |
| — | — | 0.5 | 76.3 ± 4.3 | 19.3 ± 14.7 |
| Control | | | 74.1 ± 5.6 | 0 |

Effect of drug on NK cell activity. Effector target ration 100 : 1. Effect of drug on Yac-1 cells. Target cells were incubated with drug for 18 h. $\overline{X}$ ± SD; n = 4; *P<0.05, **P<0.01, *** P<0.001 compared with control.

**Effect of LZ on NK cells activity inhibited by HC in vitro**

HC 2 μg/ml inhibited NK activity in vitro, LZ in combination with HC had no significant effect on the NK activity which was inhibited by HC (Tab. 5-4).

**Tab. 5-4 Effect of LZ on NK cells activity inhibited by HC in vitro**

| LZ (μg/ml) | HC (μg/ml) | Cytotoxicity % |
|---|---|---|
| Control | — | 42.0 ± 4.9 |
| — | 2.0 | 23.1 ± 6.6** |
| 0.1 | 2.0 | 20.8 ± 3.7 |
| 1 | 2.0 | 20.6 ± 2.5 |
| 10 | 2.0 | 18.8 ± 4.5 |
| 100 | 2.0 | 20.3 ± 3.4 |

Effector cells were incubated with target cells and drug for 18 h effector target ration 50 : 1.
$\bar{X}$ ± SD; n = 3; **P<0.01 compared with control.

**Effect of LZ alone and in combination with CYA on the NK cells activity in vivo**

LZ 150, 300 mg/kg po. qd. x 6 increased NK cells activity in vivo. Expecially 300 mg/kg significantly enhanced the NK cells activity. When LZ administered in combination with CYA 40 mg/kg ip. qd. x 6. It was observed CYA alone decreased NK cells activity in vivo LZ 300 mg/kg slightly enhanced NK cells activity which was reduced by CYA, but no statistical difference when compared with CYA alone group (Fig. 5-3, 5-4).

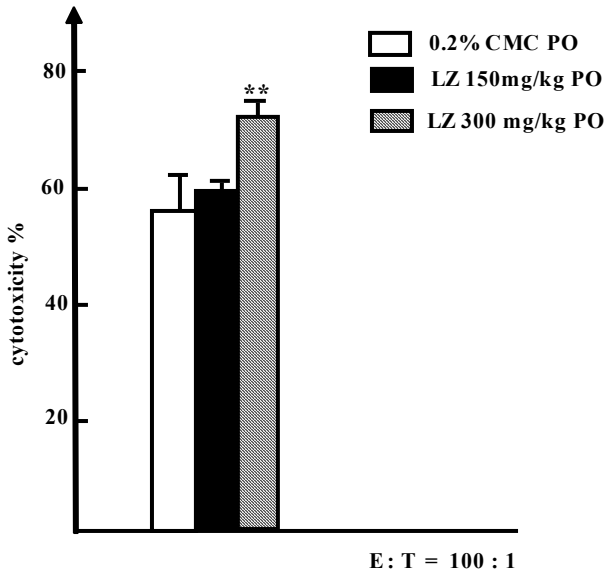


**Fig. 5-3 Effect of LZ on NK cells activity of Kunming mice in vivo**
**Effector cells were incubated with target cells for 18h. ±SD; n=3;**
****P<0.01 compared with CMC control**

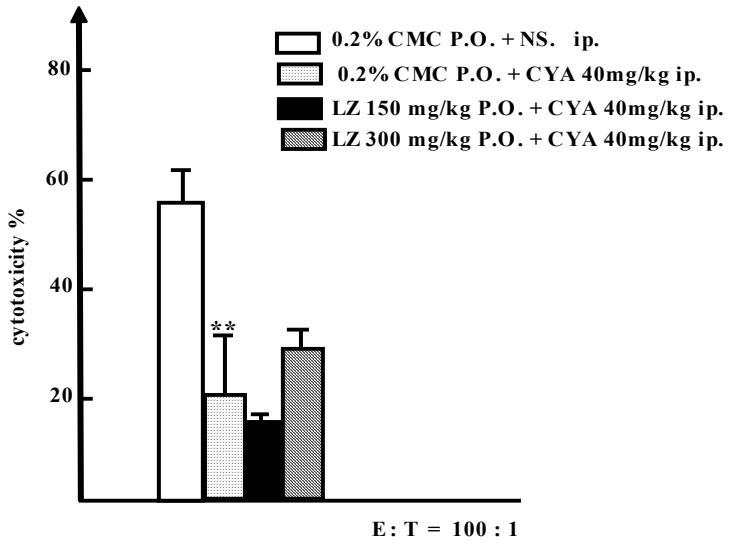


**Fig. 5-4 Effect of LZ and CYA on NK cells activity of Kunming mice in vivo**
**Effector cells were incubated with target cells for 18h. ±SD; n=3;**
****P<0.01 compared with CMC control**

# Discussion

Although NK cells were discovered only about 18 years. Studies in both human and experimental animal models have revealed the important role of NK cells in natural host defense mechanism. It was empathized that the activity of NK cells was related to the incidence, growth and metastasis of tumors. The data on the roles of NK cells in vivo suggested that NK cells were also very important in the first line of defense not only against tumors growth but also against infection by various microbial especially virus. Besides, it might produce IFN and IL-2.

The purpose of this study was to observe the effect of LZ on NK cells activity both in vitro and in vivo. Results from the in vivo experiment suggested the mechanism may be related to the immunoregulatory activity of LZ on immune system, especially may indirectly through increasing IL – 2 production or other soluble factors because LZ had no direct stimulative effect on NK cells in vitro.

Besides, we observed the effect of 3 immunosuppressor HC, CYA and CsA on NK cells activity in vitro. HC had no direct cytotoxic effect on Yac-1 and did inhibit the NK cells activity in vitro. It was able to serve as a tool agent as an immunosuppressor in vitro. CYA did not produce direct effect on target cells as well as NK cells in vitro but significantly inhibited NK cells activity in vivo. This demonstrated that CYA was transferred into active agent in vivo through metabolism. So it is adequate to be selected as a tool agent serve as an immunosuppressor in vivo not in vitro. CsA had direct toxicity on Yac-1 cells therefore it was not a good select in this condition.

# Effects of Ling Zhi on the Production of Interleukin-1 (IL-1)

## — Immunopharmacological Study (6)

JIA Yongfeng XU Weimin REN Jian YIN Xia ZHANG Luoxiu

**Abstract** The hot water extract from Ling Zhi (LZ) was provided by Wakan Shoyaku Botany Institute, Tokyo, Japan. We mainly investigated the effect of LZ on the production of Interleukin-1 (IL-1) from macrophages. The IL-1 activity was measured by the proliferation of BALB/C mouse thymus lymphocytes. The results showed that LZ (125,250,500 mg/kg) in vivo (po. qd. x10) enhanced the production of IL-1 by murine peritoneal macrophages in the presence of Lps (10 μg/ml) and in positive proportion to dosage. Frozen thawed part of LZ (500 μg/ml) in vitro also enhanced IL-1 release from macrophages in the presence of LPS. But LZ both in vitro and in vivo were able to inhibit macrophage to release IL-1 when Lps was absent. It was also shown, no matter with or without Lps, LZ soluble part had no significant effect on IL-1 synthesis by rat Kupffer cells in vitro.

**Key words** Ling Zhi (LZ, Ganoderma Lucidum, Fr. Karst); Interleukin-1; Macrophage; Kupffer cell.

Ling Zhi (LZ, Ganoderma Lucidum, Fr. Karst) as a Chinese medicinal herb can be traced back over 2 000 years. In order to further investigate the immune pharmacological mechanism of LZ, our Laboratory had also studied it in some aspects. It was showed that LZ had immunomodulatory action and had significant inhibitive effects on some pathological conditions such as hypersensitivity I, III, IV. IL-1 was an immunomodulatory factor which regulated body immune reaction and joined pathological process to some diseases. Until now none of effect of LZ on the production of IL-1 had been reported. Our purpose was to investigate the effect of LZ on the production of IL-1 from murine peritoneal macrophage and rat Kupffer cell.

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# Materials and Methods

## Animals

Male Kunming (KM) mice, 18 ~ 22g; male Wistar rat, 170-250g; BALB/C mice, 6 ~ 7 weeks, were provided from the Animal Center, Shanghai Medical University.

## Reagents

RPMI – 1640 medium (GIBCO) Grand Island, NY, with sodium pyruvate 1 μmol, 15 mmol Hepes buffer, 10% new born bovine serum (NBS); 2-mercaptoethanol (2-ME) 5 x $10^{-5}$ mol/L, Lipopolysaccharide (Lps), Concanavalin A (Con A), Collagenase I, Pronase E, were purchased from Sigma Co. $^{3}$H-TdR was provided by Shanghai Institute of Nuclear Research (1110 GBq / mmol.)

LZ, a hot water extract of Ganoderma Lucidum, was provided from Japan. For in vivo study, LZ extract was grined and dissolved in normal saline containing 0.2% carboxy-methylcellulose sodium (CMC), shaken at 80°C water bath for 4 h. On the other hand, LZ extract was dissolved in normal saline and rotated in 80°C water bath for 2 h, then centrifuged 1 500 rpm, 10 min and the supernatant was collected for study. This part was defined as soluble part (Part A). As the same with Part A, LZ extract was dissolved and shaken in 80°C water bath for 2 h, then rapidly frozen (-30°C, 1 h) and thawed for 3 times, centrifuged and this supernatant was called frozen thawed part (Part B). These two part were diluted to desired concentration for in vitro study.

## IL-1 induced from peritoneal macrophage

Resident peritoneal cells were collected by lavage with RPMI – 1640 from mice, centrifuged 1 600 rpm 10 min, washed twice, calculated and resuspended in RPMI – 1640 medium, supplemented with 10% NBS. The peritoneal cells were cultured in aliquots of 1 ml containing 2 x $10^{6}$ cells/ml in 24 - well plates. After incubation at 37°C for 2 h, nonadherent cells were removed by thorough washing and adherent cells were further incubated in 1 ml of RPMI – 1640 medium with 5% NBS with or without the following agents: (a) Lipopolysaccharide (Lps) 10 μg/ml, (b) LZ (1 ~ 500 μg/ml). Following incubation for 24 h with the added agents, the supernatants were centrifuged 1 600 rpm, 10 min and collected, store at 4°C.

**IL-1 induced from Kupffer cells** [1~5]

The rat was anesthetized with pentobarbital sodium. All subsequent procedures were carried out sterilized. The abdomen was opened and the portal vein was cannulated with a plastic tube (ø 1.5mm). The inferior vein cava was cut just above the level of the renal veins. The liver was perfusion in situ with Dulbeccos phosphate-buffered saline (DBS) pH 7.2 at a rate of 40 ~ 50 ml/min by the use of pump and the perfusate flowed out from the inferior vein cava. The first 50 ml DBS was allowed to pass through, through, thereby flushing all blood from the organ. Then the inferior vein cava was ligated and the chest was opened and the thoracic portion of the inferior vena cava was cannulated via the right atrium with a plastic tube. The purfusate was returned to reservoir and recirculated. At the time the DBS containing 0.05% Collgenase I was circulated continuously the liver for 30 min. The liver was minced and passed through a 60 – mesh nylon into solution. The solution were centrifuged at 50 g for 1 min, the supernatant was added with 0.1% Pronase E and incubated in a 37°C bath for 30 min, and centrifuged at 1 400 g for 5 min. The pellets were washed two times with DBS. Nonparenchymal cells (NPCs) were collected and resuspended in RPMI – 1640 medium containing 20% NBS, the adherent cells 4 x $10^5$ NPCs in medium were seeded into 24 – well plates. Kupffer cells (KCs) were isolated from the NPC population by adherence, and incubated containing Lps or/and LZ for 24 h, the supernatants was collected for further studies.

**Assay of IL-1** [6]

Thymus from BALB/C mice was minced and the single thymocyte passed through nylon 60 – mesh. Thymocytes, 1 x $10^7$ cells, were made up to 1 ml in volume with Con A 0.625 μg/ml and 20% NBS. The supernatants were diluted with RPMI – 1640 medium (1 : 64). 100 μl cell suspension, diluted supernatants added in flatbottomed 96 – well microplates. Lps were being mixed at a final concentration of 10 μg/ml. Total volume was 0.2 ml. Cultures were set up by triplicate and incubated for 72 h at 37°C in a humidified atmosphere with 5% $CO_2$. To access DNA synthesis, 0.925 x $10^7$ Bq of $^3$H-thymidine was added to each well 6 h before the end of the incubation period. Thereafter, cultures were collected by suction on fiber glass paper filter, using a multiple automated sample harvester. Radioactivity was measured in a liquid scintillation counter and the results were expressed as mean counts per minute (CPM) values ± standard deviation of $^3$H-TdR incorporated. The t test was used.

# Results

## Effect of LZ on the production of IL-1 from peritoneal macrophages in vitro

LZ Part A was able to inhibit macrophages to secrete IL-1, and had no effect on LPS-induced IL-1 release (Tab. 6-1).

The results showed that LZ Part B (500 μg/ml) could enhance macrophage release IL-1 in the presence of Lps, and when Lps was absent, Part B (100 ~ 500 μg/ml) inhibited IL-1 release (tab. 6-2).

**Tab. 6-1 Effect of LZ (A) on IL-1 production from murine peritoneal macrophages in vitro**

| LZ (μg/ml) | Lps (10 μg/ml) | IL-1 activity (cpm/1 x $10^6$ cells) |
|---|---|---|
| — | — | 11 163 ± 177 |
| 1 | — | 10 370 ± 400* |
| 10 | — | 10 354 ± 549* |
| 100 | — | 6 105 ± 400** |
| 500 | — | 6 597 ± 1 123** |
| — | + | 11 944 ± 1 632 |
| 1 | + | 12 215 ± 1 194 |
| 10 | + | 10 867 ± 1 217 |
| 100 | + | 12 298 ± 3 684 |
| 500 | + | 14 832 ± 2 143 |

Note: A: LZ (soluble part), $\bar{X}$ ± SD; n = 3; *P<0.05, **P<0.01 compared with control

**Tab. 6-2 Effect of LZ (B) on IL-1 production from murine peritoneal macrophages in vitro**

| LZ (μg/ml) | Lps (10 μg/ml) | IL-1 activity (cpm/1 x $10^6$ cells) |
|---|---|---|
| — | — | 11 163 ± 177 |
| 1 | — | 12 099 ± 946 |
| 10 | — | 11 825 ± 2 364 |
| 100 | — | 6 101 ± 1 519** |
| 500 | — | 6 076 ± 262** |
| — | + | 11 944 ± 1 632 |
| 1 | + | 11 550 ± 1 263 |
| 10 | + | 12 867 ± 222 |
| 100 | + | 9 100 ± 642 |
| 500 | + | 15 207 ± 1 252* |

Note: B: LZ (frozen-thawed part), $\bar{X}$ ± SD; n = 3; *P<0.05, **P<0.01 compared with control

### Effect of LZ on the production of IL-1 from murine peritoneal macrophage in vivo

As the same with in vitro, in co-operation with Lps, LZ also enhanced IL-1 release in positive proportion to dosage, and inhibited macrophage to release IL-1 when Lps was absent (Tab. 6-3).

**Tab. 6-3 Effect of LZ on IL-1 production from murine peritoneal macrophage in vitro**

| LZ (μg/ml) | Lps (10 μg/ml) | IL-1 activity (cpm/1 x $10^6$ cells) |
|---|---|---|
| — | — | 8 125 ± 1 424 |
| 125 | — | 4 318 ± 366* |
| 250 | — | 4 277 ± 673* |
| 500 | — | 5 197 ± 783* |
| — | + | 9 565 ± 1 262 |
| 125 | + | 10 181 ± 1 259 |
| 250 | + | 14 982 ± 2 040* |
| 500 | + | 17 469 ± 3 391* |

Note: po. qd. x 10 d; $\bar{X}$ ± SD; n = 3; *P<0.05, compared with control

### Effect of LZ on the production of IL-1 from rat Kupffer cells in vitro

No matter with or without Lps, LZ had no significant effect on rat Kupffer cells release IL-1 (Tab. 6-4).

**Tab. 6-4 Effect of LZ (A) on IL-1 production from rat Kupffer cells in vitro**

| LZ (μg/ml) | Lps (10 μg/ml) | IL-1 activity (cpm/1 x $10^6$ cells) |
|---|---|---|
| — | — | 24 726 ± 1 346 |
| 1 | — | 28 834 ± 4 511 |
| 10 | — | 26 899 ± 7 606 |
| 100 | — | 23 921 ± 4 419 |
| 500 | — | 31 819 ± 4 691 |
| — | + | 31 869 ± 622* |
| 1 | + | 34 299 ± 5 980 |
| 10 | + | 29 463 ± 6 422 |
| 100 | + | 28 560 ± 3 439 |
| 500 | + | 29 755 ± 2 489 |

Note: A: LZ (soluble part), $\bar{X}$ ± SD; n = 4; *P<0.05 compared with without Lps control

# Discussion

Interleukin-1, a cytokine secreted by monocytes had an intensive biological action. In the presence of mitogen, LZ significantly enhanced the release of IL-1 from peritoneal macrophages of mice.

Kupffer cell also was a member of the family of mononuclear phagocytes. Following the development of procedures of the isolation of Kupffer cells, many researches showed that Kupffer cell had the intrinsic capacity to interact with lymphocytes for the induction of immune responses. Our laboratory proved that LZ had the effect on protecting hepatocytes. We hoped to observe the effect of LZ on Kupffer cells. This data clearly demonstrated, under our experiment condition, LZ had no effect on IL-1 secreted by Kupffer cells. It suggested that Kupffer cells released cytokines and their immunoregulatory activity were connected with the special situation to liver.

# Effects of Ling Zhi on the Production of Interleukin-2 (IL-2)

## — Immunopharmacological Study (7)

ZHANG Luoxiu MENG Hong ZHON Xianbiao

**Abstract** The purpose of this study was to determine the effect of Ling Zhi (LZ, Ganoderma Lucidum, Fr. Karst) on the production of interleukin-2 (IL-2) from murine splenocytes. It was observed that hydrocortisone (HC) and cyclosporine A (CsA) inhibited the production of IL-2 in vitro. LZ 10 ~ 100 μg/ml could antagonize the inhibitive activity of HC and CsA therefore increased the production of IL-2 in vitro. In vivo, LZ 300 mg/kg could increase the production of IL-2 when po. LZ alone or in combination with HC or CsA.

**Key words** Ganoderma Lucidum, (Ling Zhi, LZ); Interleukin-2.

IL-2 is one of the most important lymphokines. Due to its well known biological property of maintenance of T cell proliferation in vitro and its important role in cells events of immune regulative net work it has been widely attracted and studied. IL-2 is usually secreted by T cells of helper phenotype. Therefore Il-2 produced by lymphocytes indirectly reflects the function of T cells(1). From the results reported before LZ affects some aspects of immune function. In order to further understand the activity of LZ the major purpose of this paper is to study the effect of LZ on the production of IL-2 from murine splenocytes.

## Materials and Methods

### Animals

$C_{57}$ BL/6 mice 8 ~ 12 weeks old; Kunming (KM) mice, 16 ~ 20 g, were supplied by the Animal Center, Shanghai Medical University.

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**Reagents**

The hot water extract of Ling Zhi was supplied by Wakan Shoyaku Botany Institute in Japan. It was prepared as described before. RPMI – 1640 medium from GIBCO contained Penicillin 100 U/ml, Streptomycin 100 μg/ml, Hepes 15 mmol and 10% NCS. Concanavalin A (Con A) from Sigma. M-methyl mannoside from Shanghai Second Reagent Plant, Cyclosporine from Sandoz (Each ml contained Cyclosporin A 50 mg Polyoxyethylated Castor Oil 650 mg and 33% alcohol by volume). $^{3}$H-TdR was purchased from Shanghai Institute of Nuclear Research, Chinese Academy of Sciences and the specific activity was 1 110 GBq/mmol.

**Production of IL-2 in vitro**

Murine spleen cells 5 x $10^6$ /ml were mixed in 24 well plates with or without of test drug in different concentration and 3 μg/ml of Con A. The cultures were incubated at 37°C 5% $CO_2$ atmosphere for 24 h. The culture supernatants were collected after centrifugation and stored at -30°C until use.

**Effect of preincubation of LZ with splenocytes**

In order to eliminate the influence of drug contained in the test culture supernatants some experiments were performed as follows: Spleen cells preincubated with different concentration of LZ for 10 h then the supernatants were discarded. After washing twice the cells were reincubated with Con A 3 μg/ml for 24 h, then the supernatants were harvested, centrifuged and stored at -30°C until use.

**Effect of LZ on IL-2 production in vivo**

Mice were randomly divided into groups. Control group po. CMC 0.2% qd. x 6, LZ groups po. LZ 150 ~ 300 mg/kg qd. x 6 alone or in combination with Hydrocortisone ip. qd. x 6 or Cyclophosphamide ip. qd. x 3 ($d_4$ ~ $d_6$). On d7 mice were killed and the spleen cells were adjusted and cultured with Con A 3 μg/ml for 24 h. The culture supernatants of different groups were collected and stored at –30°C for assay.

**IL-2 assay** [(2)]

IL-2 assay was performed using the activating mouse spleen cells as described before [(2)]. $C_{57}$ BL/6 mice were sacrificed by cervical dislocation. The spleens were minced and suspended. Red blood cells were lysed with distilled water and washed 3 times in RPMI – 1640 medium. 2 x $10^6$ cells / ml of spleen cells were incubated with Con A 3 μg/ml at 37°C 5% $CO_2$ for 48 h. Then the activated spleen cells were collected and washed twice with RPMI – 1640 medium which contained *a*-mm 20 mg/ml to wash out the remaining Con A. Then the cells were counted with trypan blue dye exclusion. Cell viability was always more than 90% and adjusted to 2 x $10^6$ / ml. The cell suspensions

(100 µl/ml) and serias two fold dilutions of IL 2 supernatants (100 µl/well) were added to 96 well plates. The cultures were incubated at 37°C 5% $CO_2$ for 24 h. For the last 6 h each well was pulsed with $^3$H-TdR 9.25 kBq. The cells were harvested and the radioactivity incorporated were counted.

# Results

### Inhibitive activity of Hydrocortisone (HC)

Hydrocortisone 0.025 µg/ml could inhibit the production of IL-2 by splenocytes in vitro. When the supernatant was in adequate dilution, the inhibitive activity of HC was significant.

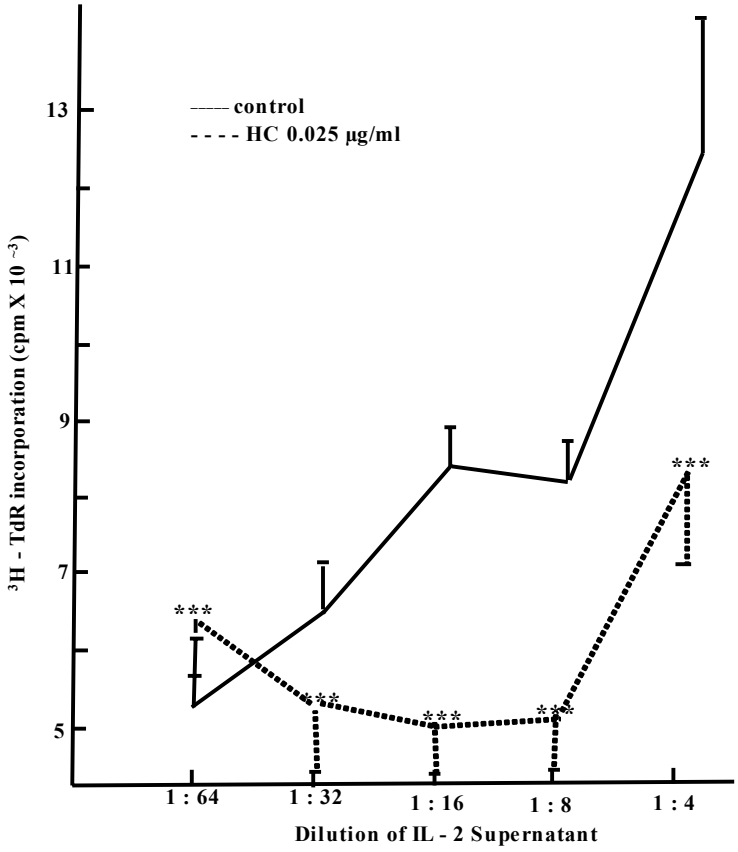


**Fig. 7-1 Effect of HC on IL-2 production of murine splenocytes in vitro**
**Result expressed as cpm/2 x $10^3$ cells; Con A 8µg/ml; ± SD; n=4;**
*****P<0.01 compared with control**

## Effects of LZ in combination with Hydrocortisone on IL-2 production in vitro

Tab. 7-1, 7-2 showed that Hydrocortisone 0.025 ~ 1 μg/ml significantly inhibited IL-2 production however LZ could antagonize the inhibitive activity of Hydrocortisone and elevated IL-2 production when compared with that of Hydrocortisone group.

**Tab. 7-1 Effects of LZ with HC on IL-2 production of murine splenocytes in vitro**

| LZ (μg/ml) | Con A (μg/ml) | HC (μg/ml) | cpm / 1 x $10^6$ cells |
|---|---|---|---|
| Control | 3 | — | 2 025 ± 167 |
| — | 3 | 0.025 | 1 530 ± 149$^{\Delta\Delta}$ |
| 1 | 3 | 0.025 | 1 409 ± 183 |
| 10 | 3 | 0.025 | 2 005 ± 210** |
| 100 | 3 | 0.025 | 2 205 ± 335** |

Dilution of supernatant was 1 : 16
Results expressed as cpm / 1 x $10^6$ cells. $\overline{X}$ ± SD; n = 4; $^{\Delta\Delta}$ P<0.01 compared with control;
**P<0.01 compared with HC control

**Tab. 7-2 Effects of LZ with HC on IL-2 production of murine splenocytes in vitro**

| LZ (μg/ml) | Con A (μg/ml) | HC (μg/ml) | Cpm / 1 x $10^6$ cells |
|---|---|---|---|
| Control | 3 | — | 8 389 ± 658 |
| — | 3 | 1 | 3 944 ± 262$^{\Delta\Delta}$ |
| 1 | 3 | 1 | 4 348 ± 482 |
| 10 | 3 | 1 | 5 396 ± 260** |
| 100 | 2 | 1 | 5 450 ± 426** |

Dilution of supernatant was 1 : 16
Results expressed as cpm / 1 x $10^6$ cells. $\overline{X}$ ± SD; n = 4; $^{\Delta\Delta}$ P<0.01 compared with control;
**P<0.01 compared with HC control

### Effect of LZ in combination with Cyclosporine A on IL-2 production in vitro

Tab. 7-3 indicated that Cyclosporin A (CsA) 0.05 μg/ml apparently inhibited IL-2 production but LZ could antagonize the action of CsA and promoted IL-2 production.

**Tab. 7-3 Effects of LZ with CsA on IL-2 production of murine splenocytes in vitro**

| LZ (μg/ml) | Con A (μg/ml) | CsA (μg/ml) | Cpm / 1 x $10^6$ cells |
|---|---|---|---|
| Control | 3 | — | 8 389 ± 658 |
| — | 3 | 0.05 | 5 667 ± 450$^{\Delta\Delta}$ |
| 1 | 3 | 0.05 | 5 763 ± 395 |
| 10 | 3 | 0.05 | 6 495 ± 446** |
| 100 | 3 | 0.05 | 6 539 ± 557** |

Dilution of supernatant was 1 : 16
Results expressed as cpm / 1 x $10^6$ cells. $\overline{X}$ ± SD; n = 4; $^{\Delta\Delta}$ P<0.01 compared with control;
**P<0.01 compared with CsA group

### Preincubation of spleen cells with LZ and immunosuppressor Hydrocortisone and Cyclosporine A

In order to eliminate the possible affect of drug contained in the culture supernatants on IL-2 assay we observed the effect of preincubation of spleen cells with LZ alone or in combination with Hydrocortisone or Cyclosporine A for 10 h. After decanted the medium containing drug the cells were thoroughly washed and reincubated with fresh medium and Con A for 24 h. The supernatants were assayed for IL-2. It was demonstrated that pretreated the cells with LZ 10 ~100 μg/ml alone had no pronounce influence on IL-2 production. Hydrocortisone and CsA 0.05 μg/ml suppressed IL-2 production however LZ could antagonize the inhibitive activity of Hydrocortisone or Cyclosporine A and elevated IL-2 level (Tab. 7-4, Fig. 7-2).

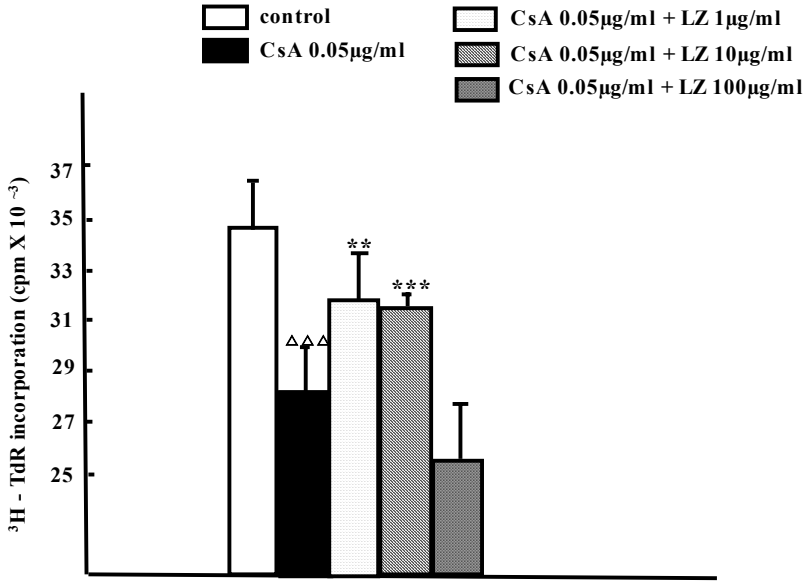


**Fig. 7-2 IL-2 production from murine splenocytes pretreated with LZ and CsA for 10**
**Results expressed as cpm/2 x $10^6$ cells. ±SD, n=4**
**△△△P<0.01 compared with control. ***P<0.01 compared with CsA group**

**Tab. 7-4 IL-2 production from murine splenocytes pretreated with LZ and HC for 10h**

| LZ (μg/ml) | Con A (μg/ml) | HC (μg/ml) | Cpm / 2 x $10^6$ cells |
|---|---|---|---|
| Control | 3 | — | 34 724 ± 1 702 |
| 10 | 3 | — | 35 157 ± 949 |
| 100 | 3 | — | 35 178 ± 1 076 |
| — | 3 | 0.05 | 24 438 ± 2 004$^{\Delta\Delta}$ |
| 1 | 3 | 0.05 | 31 811 ± 1 639** |
| 10 | 3 | 0.05 | 31 751 ± 1 064** |
| 100 | 3 | 0.05 | 33 744 ± 2 850** |

Dilution of supernatant was 1 : 8
Results expressed as cpm / 2 x $10^6$ cells. $\overline{X}$ ± SD; n = 4; $^{\Delta\Delta}$ P<0.01 compared with control;
**P<0.01 compared with HC group

**The effect of LZ on IL-2 production in vivo**

Fig. 7-4 showed that LZ 300 mg/kg po. qd. x 6 increased the IL-2 production from murine splenocytes in vivo. Fig. 7-3 and 7-4 showed that the IL-2 produced in LZ with Hydrocortisone or Cyclophosphamide (CYA) group were much higher than Hydrocortisone and Cyclophosphamide alone groups. But in the conditions expressed above Hydrocortisone 15 mg/kg ip. qd. x 6 and Cyclophosphamide 40 mg/kg qd. x 3 (d3 ~ d6) had no apparent effect on IL-2 production when compared with that of control group. It was possible that in this condition $T_S$ and $T_H$ were both affected and in a balance situation. But anyhow LZ might enhances the function of $T_H$ and promoted IL-2 production.

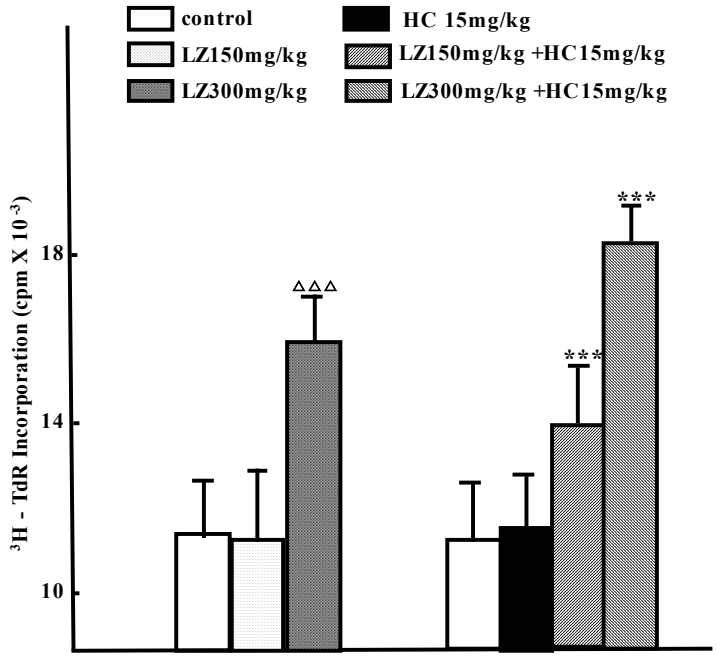


**Fig. 7-3 Effect of LZ with HC on IL-2 production of murine splenocytcs in vivo LZ po. qd. x 6; HC ip. qd. X 6. Results expressed as cpm/1 x $10^6$ cells. ±SD; n=4;$^{\triangle\triangle\triangle}$P<0.01 compared with control. ***P<0.01 compared with HC group**

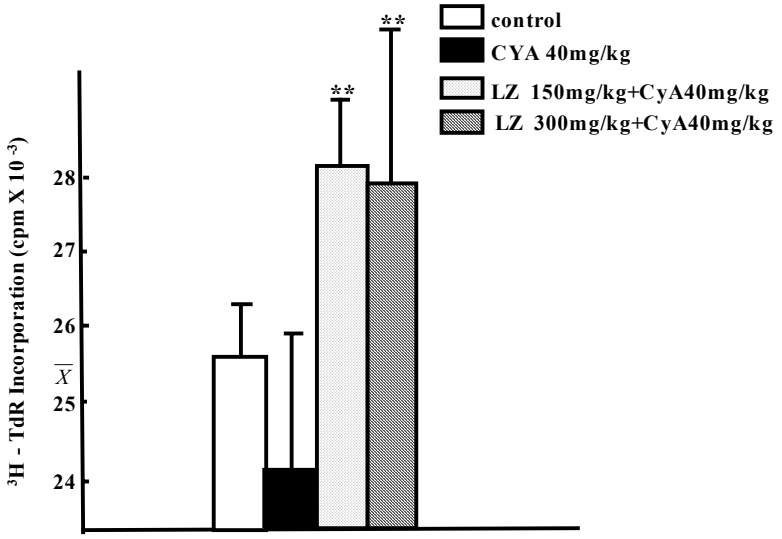


**Fig. 7-4 Effect of LZ with Cyclophosphamide (CYA) on IL-2 production of murine splenocytcs in vivo**
**LZ po. qd. X 6; CYA ip. qd. d4~d6. Results expressed as cpm/1 x $10^6$ cells.**
**±SD; n=4;**P<0.05 compared with CYA group**

# Discussion

In 1976 Morgen reported that supernatants from the primary cultures where lymphocytes had been stimulated by the lectin could maintain the responding cells growth for extended period of time. This factor was named as T cells growth factor (TCGF). TCGF was renamed "Interleukin-2" at the second International Lymphokine Workshop at Ermatingen, Switzerland in 1979[3]. In turns of the important role of IL-2 in regulation of immune function in the stimulation of activate T cells, induce T cytotoxicity, NK cells activity and also mediate the function of activated B cells and macrophages. The discovery of IL-2 emerged a new hypothesis of T cell activation, a new idea to overcome cancer, so it would be important to study the drug that may have effect on the production of Il-2. [4]

As it has been reported before that LZ has selective activity on immune system. We have studied that the effect of LZ on some aspects of immune functions. In the present investigation we observed that LZ augmented the production of IL-2 both in vitro and in vivo not only stimulate the IL-2 production from normal lymphocyte but also from immunosuppressor such as Hydrocortisone, Cyclosporine A or CYA inactivated cells. It was reported that the peripheral lymphocytes from those patients with suppressed T cell function such as primary or acquired immune deficiency such as cancer, aged patients etc. produced less amount of Il-2 than that of normal person. The present study suggested that LZ would be of benefit to these patients.

# Influence of Ling Zhi on the Production of Tumor Necrosis Factor (TNF)

## — Immunopharmacological Study (8)

ZHANG Luoxiu & XIE Xuhei

**Abstract** Effect of Ling Zhi (LZ) on the cytotoxic activity of Tumor Necrosis Factor (TNF) was examined using murine fibroblast cell line ($L_{929}$ cells). LZ 1 ~ 100 μg/ml had no direct influence on the growth of $L_{929}$ cells. The γTNF mediated cytotoxicity on $L_{929}$ cells was also determined in the presence or absence of LZ. It was observed that LZ showed little effect on the cytotoxicity of γTNF. However, LZ 1 ~ 100 μg/ml promoted TNF production from murine peritoneal macrophages in vitro. Marcrophages preincubated with LZ for 8 h in vitro, produced higher TNF activity when induced by Lps after washing. Besides, LZ 100 ~ 300 mg/kg po. qd. x 14 enhanced the level of TNF in the serum of mice primed by BCG and induced by Endotoxin.

**Key words** Ling Zhi; Tumor Necrosis Factor (TNF).

In 1975 Carswell et al described the serum from mice that had been infected with BCG and subsequently injected with Endotoxin caused tumor hemorrhagic necrosis of various tumors in mice whereas normal cell cultures were unaffected [(1)]. The factor in this serum was given the name of Tumor Necrosis Factor (TNF). It was demonstrated that Tumor Necrosis Factor (TNF) was a monocyte derived protein exhibiting its ability to produce hemorrhage necrosis of some animal tumors and direct cytotoxic effect on tumor cells in culture. However recent evidence showed that TNF was a regulatory cytokine with multiple biological activities which included a mitogenic effect in fibroblasts, activation of granulocyte functions, stimulation of cytokine production and modulation of T cell functions. These results suggested that TNF might act as a mediator of some immunological and inflammatory processes [(2~3)].

Ling Zhi (LZ, Ganoderma Lucidum, Fr. Karst) has long been used as an health protective drug. We have reported before that LZ may act as an immunomodulator. It regulates some immune functions stimulates macrophage phagocytosis, promotes the clearance of charcoal particles, enhances the function of reticular endotherial system and increases the production of IL-1 induced by Lps. But on the other side it inhibits PFC and hemolycin production. To further study the immunoregulative activity of LZ in this paper we examined the effect of LZ on the production of TNF.

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# Materials and Methods

## Animals

$C_{57}$ BL/6 mice, male, 8 ~ 10 weeks old, Kunming (KM) mice, male 20 ~ 24 g were supplied by the Animal Center, Shanghai Medical university.

## Cell line

The $L_{929}$ tumorigenic murine fibroblast used in all assays was kindly supplied by Prof. Quian Dinghua (School of Pharmacy, Second Military Medical University). The cell line was subcultured with 10% new bovine serum PRMI – 1640 medium in vitro.

## Reagents

γTNF was kindly provided by Prof. Qian Dinghua. Bacillus Calmette Guerin (BCG) was obtained from Shanghai Institute of Biological Products, Chinese Ministry of Public health. Endotoxin was provided by Department of microbiology, Second Military Medical University. Crystal violet (analytical reagent) from Shanghai Chong-ming Chemical Reagent Factory. RPMI – 1640 from GIBCO was prepared as before. Hot water extract of LZ provided by Japan Wakan Shoyaku Botany Institute was prepared as before.

## Induction of TNF in vitro

Peritoneal exudate cells (PEC) were obtained from the mice peritoneal cavity which had been primed with 5% thioglycolate medium 2 ml/mouse 4 days before. Peritoneal cells 2.5 x $10^6$ cells in 1 ml were added to 24 well plates. After adherence for 2 h at 37°C humidified 95% air – 5% $CO_2$ nonadherent cells were removed by washing two times with RPMI – 1640 medium. This procedure generally resulted in full monolayers of macrophages in the plates. Macrophages were incubated with or without Lps 10 μg/ml and different concentration of LZ. After incubation of 24 h the cell free supernatants containing TNF were harvested and stored at - 30°C until assay.

## Production of TNF in vivo

Kunming mice were injected with BCG 0.02% 0.2 ml/mouse into the tail vain then were randomly divided into 4 groups. Control group: CMC 0.5 ml per mouse po. qd. x 14, groups LZ 100, 200, 300 mg/kg po. qd. x 14. Two weeks later Endotoxin 25 μg iv. was administered. 90 mins later blood was collected, centrifuged and the TNF containing serum were stored at - 30°C until use. The serum were diluted just before assay.

**TNF assay**

Because $L_{929}$ cells were quite sensitive to the cytotoxic action of TNF, so TNF activity was assayed by the cristal violet staining of TNF treated $L_{929}$ cell cultures. $L_{929}$ cells 5 x $10^5$/ml (viability > 95%) were seeded in 96 well plates and cultured at 37°C 5% $CO_2$ for 20 h (final 200 μl/well). Triplicate wells were used for each experimental condition. At intervals, plates were removed from incubator and medium was removed by rapid decantation. 100 μl of RPMI – 1640 medium containing actinomycin D yielding a final concentration of 1 μg/ml. Plates were similarly reincubated for 20 h followed by removal of culture supernatants and staining with 0.5% crystal violet for 20 min. Rinsed and dried plates were enumerated by measuring absorbance at 590 nm on Elisa autoreador.

% cytotoxicity was calculated using the formula:

$$\% \text{ cytotoxicity} = \frac{OD_{con} - OD_{test}}{OD_{con}} \times 100\%$$

ODcon = absorbance in control well
ODtest = absorbance at a particular dilution of test sample.
TNF (U/ml): a unit is defined on that amount of cytotoxin necessary to cause 50% destruction of the cell-culture.

# Results

**The effect of LZ on the growth of $L_{929}$ cells**

As shown in Tab. 8-1 LZ 1 ~ 100 μg/ml had no significant influence on the growth of $L_{929}$ cells.

**Tab. 8-1 Effect of LZ on $L_{929}$ cells activity**

| Drug | Dose (μg/ml) | OD |
|---|---|---|
| Control | — | 0.380 ± 0.02 |
| LZ | 1 | 0.357 ± 0.015 |
| LZ | 10 | 0.370 ± 0.036 |
| LZ | 100 | 0.363 ± 0.021 |

Note: Dilution of LZ was 1 : 20 $L_{929}$ cells seeded density was 5 x $10^4$ / well. $\overline{X}$ ± SD; n = 3

### The effect of LZ on γTNF mediated cytotoxicity on tumor cells

Tab. 8-2 indicated that 5U of γTNF resulted in 76.3% of cytotoxicity. Addition of LZ 1 ~ 100 μg/ml to the cultures with 5U of γTNF showed that LZ had no effect on the cytotoxic activity of 5U γTNF.

**Tab. 8-2 Effect of LZ on the reaction of $L_{929}$ cells to γTNF**

| Drug | Dose (μg/ml) | γTNF (U) | γTNF cytotoxicity % |
|---|---|---|---|
| Control | — | 5 | 76.3 ± 1.3 |
| LZ | 1 | 5 | 75.6 ± 0 |
| LZ | 10 | 5 | 74.8 ± 3.4 |
| LZ | 100 | 5 | 77.0 ± 5.6 |

Note: Dilution of drug was 1 : 20. $L_{929}$ collected density was 5 x $10^4$ / well.
Result expressed as cytotoxicity %, $\bar{X}$ ± SD; n = 3

### LZ stimulated the production of TNF from murine peritoneal macrophages

Tab. 8-3, 8-4 and Fig. 8-1 showed that LZ 0.01 ~ 1 μg/ml stimulated murine peritoneal macrophages to produce TNF in a concentration dependent fashion. Higher concentration of LZ (100 μg/ml) resulted in an inhibitive activity. Unfiltered LZ solution showed a stronger stimulative activity to produce TNF than filtered LZ solution in vitro.

**Tab. 8-3 Effect of LZ (unfiltered preparation ) on TNF production**

| Drug | Dose (μg/ml) | OD | | | TNF production (U/ml) |
|---|---|---|---|---|---|
| | | 1 : 40 | Dilution of test supernatant | | |
| | | | 1 : 80 | 1 : 160 | |
| Control | — | 0.137 ± 0.015 | 0.210 ± 0.01 | 0.260 ± 0.01 | 63.4 ± 2.1 |
| LZ | 0.01 | 0.150 ± 0 | 0.170 ± 0.01* | 0.210 ± 0.017* | 89.9 ± 6.01* |
| LZ | 0.1 | 0.133 ± 0.006 | 0.177 ± 0.006* | 0.220 ± 0.01* | 86.7 ± 4.0* |
| LZ | 1 | 0.120 ± 0 | 0.180 ± 0.02 | 0.200 ± 0.026* | 103.8 ± 5.3** |
| LZ | 10 | 0.143 ± 0.025 | 0.173 ± 0.015 | 0.207 ± 0.012** | 93.9 ± 6.0** |
| LZ | 100 | 0.147 ± 0.006 | 0.207 ± 0.015 | 0.247 ± 0.015 | 61.8 ± 2.5 |

Notes: OD of control well (only AntiD.): 0.363 ± 0.006
Control NS. $\bar{X}$ ± SD; n = 3; *P<0.05; **P<0.01, compared with control

**Tab. 8-4 Effect of LZ (filtered preparation ) on TNF production**

| Drug | Does (μg/ml) | OD | | | | TNF production (U/ml) |
|---|---|---|---|---|---|---|
| | | 1 : 40 | Dilution of test supernatant | | | |
| | | | 1 : 80 | 1 : 160 | 1 : 320 | |
| Control | — | 0.347 ± 0.025 | 0.420 ± 0.053 | 0.473 ± 0.015 | 0.493 ± 0.07 | 64.8 ± 6.8 |
| LZ | 0.01 | 0.330 ± 0.017 | 0.473 ± 0.047 | 0.540 ± 0.062 | 0.623 ± 0.03 | 55.2 ± 3.1 |
| LZ | 0.1 | 0.323 ± 0.015 | 0.463 ± 0.02 | 0.503 ± 0.02 | 0.590 ± 0.053 | 60.3 ± 3.8 |
| LZ | 1 | 0.263 ± 0.012* | 0.420 ± 0.035 | 0.493 ± 0.025 | 0.603 ± 0.05 | 81.7 ± 3.9* |
| LZ | 10 | 0.283 ± 0.024* | 0.390 ± 0.035 | 0.517 ± 0.04 | 0.610 ± 0.06 | 79.4 ± 3.8* |
| LZ | 100 | 0.350 ± 0.01 | 0.507 ± 0.04 | 0.650 ± 0.017 | | 48.4 ± 1.7* |

Notes: OD of control well (only AntiD.): 0.787 ± 0.015
Control NS. $\overline{X}$ ± SD; n = 3; *P<0.05, compared with control

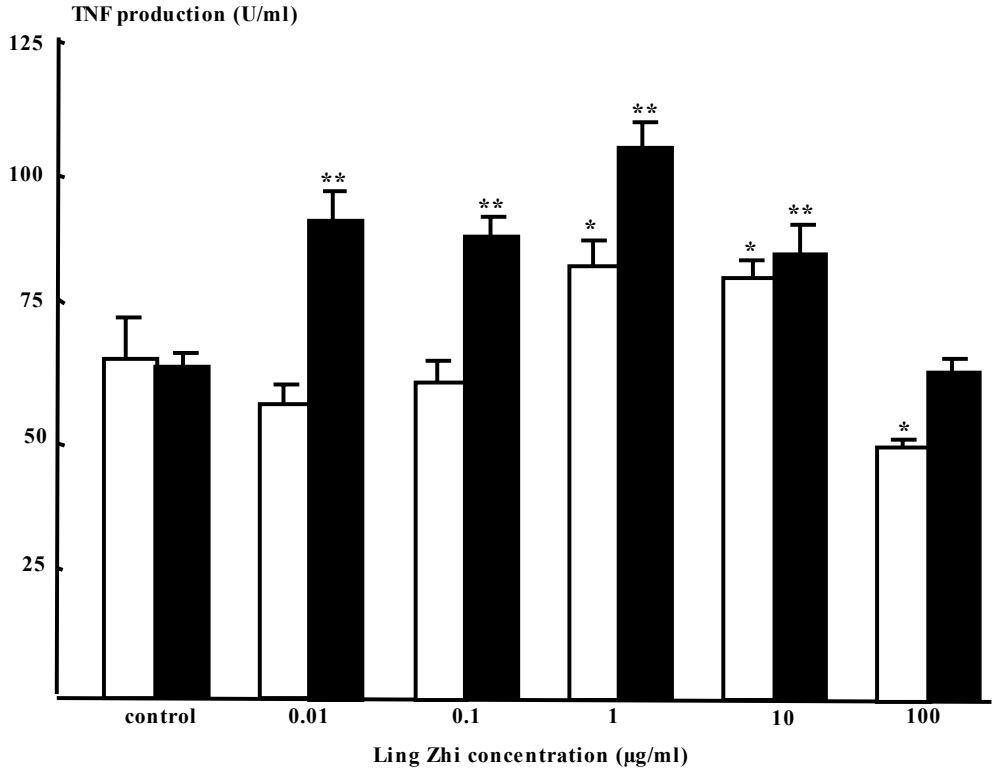


**Fig. 8-1 Effects of unfiltered ( ■■ ) or filtered ( □ ) LZ on the Lps-indused release of TNF from 5% TG-primed macrophages**
**±SD; n=3;*P<0.05; **P<0.01 compared with control**

### The effect of pretreatment of macrophages with LZ

Then we examined the effect of preincubation of macrophages with LZ on the production of TNF. Macrophages were incubated with LZ (0.01 ~ 100 μg/ml) or without of LZ for 8 h, then the medium were removed, cells were washed and reincubated with LPS 10 μg/ml at 37°C for 24 h. The TNF activity of tested supernatants were assayed. Tab. 8-5 showed that preincubation of macrophages with LZ slightly increased the activity of TNF.

**Tab. 8-5 Effect of preincubation with LZ on release of TNF**

| Drug | Dose (μg/ml) | TNF cytotoxicity (%) |
|---|---|---|
| Control | — | 45.6 ± 2.5 |
| LZ | 0.01 | 57.5 ± 5.9* |
| LZ | 0.1 | 47.8 ± 2.5 |
| LZ | 1 | 57.2 ± 2.5** |
| LZ | 10 | 53.9 ± 1.9* |
| LZ | 100 | 51.1 ± 3.8 |

Note: 5% TG-primed macrophages (from 2.5 x $10^6$ /ml PECS) incubation with drug for 8 h. Thereafter, cells were washed to remove drug and fresh medium containing 10 μg/ml Lps was added. Then incubating for 24 hours, tested supernatant diluted to 1 : 160.
$\overline{X}$ ± SD; n = 3; *P<0.05; **P<0.01, compared with control

### Effect of LZ on TNF production in vivo

Tab. 8-6, Fig. 8-2 indicated that LZ 100, 200, 300 mg/kg po. qd. x 14 stimulated the level of TNF in serum of BCG primed and Endotoxin treated mice.

**Tab. 8-6 Effect of LZ on TNF production in vivo**

| Drug | Dose (μg/ml) | TNF cytotoxicity (%) |
|---|---|---|
| Control | — | 2.90 ± 1.2 |
| LZ | 100 | 50.0 ± 7.5** |
| LZ | 200 | 40.0 ± 9.9** |
| LZ | 300 | 26.5 ± 8.9* |

Note: Serum were diluted to 1 : 1000 and tested
LZ: po. qd. x 14; 0.2% CMC: po. qd. x 14
$\overline{X}$ ± SD; n = 3; *P<0.05; **P<0.01, compared with control

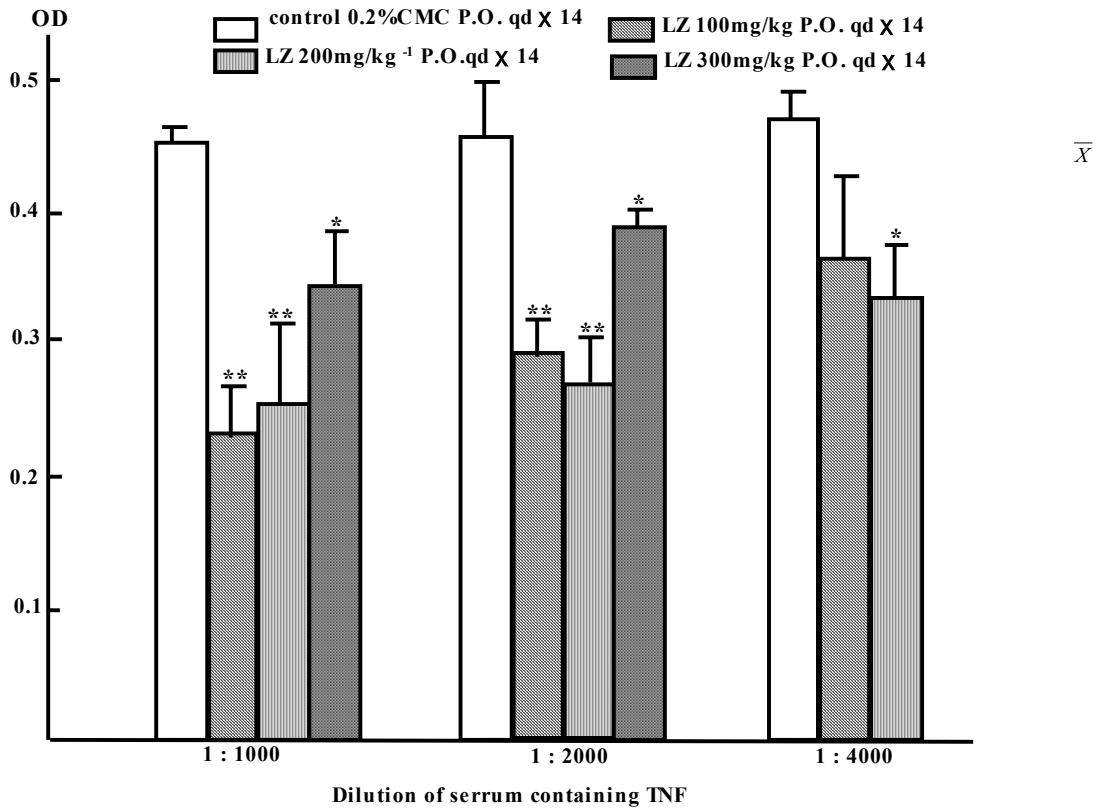


**Fig. 8-2 Effects of LZ on TNF production in vivo**
**±SD; n=3;*P<0.05, **P<0.01 compared with control**

# Discussion

Since TNF has been widely noticed, several investigators have developed in vitro cell cytotoxicity assays for TNF. Flick et al compared four published in vitro assays which measured cell cytotoxicity of TNF[4]. These included determination of residual cell number by crystal violet staining in the presence and absence of Actinomycin D, lack of viability as determined by neutral red uptake and $^3$H-Thymine release in cytotoxin treated $L_{929}$ cells. Flick compared these methods and discovered that treatment of $L_{929}$ cells with Actinomycin D followed by crystal violet staining was the most sensitive method measured. We set up this method of assay TNF activity. We observed various conditions, $L_{929}$ cell density, the amount of TG, the dosage cause of Actinomycin D and the mice strain. We found that crystal violet culture was a sensitive and repeatable assay. Addition of Actinomycin D to the cell cultures greatly enhanced killing and shortens the necessary incubation time to 20 h. We used this method for all the experiments.

From this study it was shown that LZ enhanced the production of TNF in vitro. LZ itself had no direct cytotoxic activity against $L_{929}$ cells. There was less pronounce synergistic activity between LZ and γTNF under this condition. However LZ increased the TNF production both in vitro and in vivo. Even preincubation of macrophages with LZ for 8 h promoted the production of TNF. We have reported that LZ stimulated macrophage phagocytosis and enhanced the carbon particles clearance activity. Now we further demonstrated that LZ elevated macrophage activity to increase the capacity of the host defense mechanism against cancer and infection diseases through indirect mechanism.

One must emphasize that from the in vitro study we can see LZ modulated TNF production form 0.01 ~ 1 μg/ml LZ stimulated TNF release but higher concentration (100 μg/ml) resulted in an inhibitive activity. This further demonstrated LZ played a role as an immunomodulator.