行政院國家科學委員會專題研究計畫　成果報告

靈芝菌絲體胞內和胞外多醣體對腸體液免疫反應之影響

計畫類別：個別型計畫
計畫編號：NSC93-2320-B-041-004-
執行期間：93 年 08 月 01 日至 95 年 07 月 31 日
執行單位：嘉南藥理科技大學保健營養系

計畫主持人：夏彩蘭
共同主持人：吳明娟

報告類型：精簡報告

處理方式：本計畫可公開查詢

中　華　民　國 95 年 10 月 30 日

中文摘要

本研究是探討靈芝菌絲體胞內多醣對腸體液免疫反應之影響。五週齡雌性小鼠餵飼含多醣量相當於飼料含 0,、1、3、5 和 10%靈芝菌絲體之 AIN-93G 飼料四週期間，在第七天及第二十一天以管餵之方式給予 5 μg cholera toxin (CT)。餵飼含靈芝菌絲體多醣體飼料之小鼠糞便、小腸內容物、血清的 anti-CT-IgA 和 anti-CT-IgG 抗體與對照組比較均沒有顯著差異。實驗結果顯示，靈芝菌絲體胞內多醣對腸體液免疫反應並沒有調節作用。

關鍵詞：IgA，靈芝菌絲體，腸道免疫，多醣體

Abstract

This study was to investigate the effect of polysaccharide from *Ganoderma lucidum* mycelium on gut humoral immunity in mice. Five-week-old female C57BL/6J mice were fed AIN-93G diet containing different level polysaccharide from *Gandoerma lucidum* mycelium (GLP) which were equivalent to diet containing 0, 1, 3, 5 and 10% (wt/wt) mycelium of *Ganoderma lucidum* for 4 weeks. Mice were orally immunized with 5 μg cholera toxin (CT) on day 7 and 21 of the 4-week feeding period. The total IgA and specific anti-CT IgA level in luminal washes of small intestine, serum and fecal pellets as well as serum total IgG and anti-CT IgG were determined by ELISA. Mice fed the AIN-93G diet containing different levels GLP did not affect the total food intake and showed growth comparable with that for the control mice fed AIN-93G diet only. The total IgA and specfic anti-CT-IgA antibody responses in small intestinal washes and fecal pellets as well as serum total IgG and anti-CT IgG were not affected mice fed GLP compared to the control group mice. The results indicate that the *Ganoderma lucidum* mycelium polysaccharide does not have the immunomodulatory effect in mice orally immunized with CT.

Keywords：*Ganoderma lucidum* mycelium, polysaccharide, gut immunity

一、前言

本研究室曾餵飼五週齡雌性小鼠含靈芝菌絲體的 A1N-93G 飼料四週，在飼養的第 7 天和第 21 天以管餵方式給予 5μg cholera toxin (CT)，飼以含 0.5, 1, 3 和 5%靈芝菌絲體的小鼠的小腸內容物、糞便和血清的 anti-CT IgA 與對照組比較均有顯著減少(9)。為了進一步探討靈芝菌絲體調節腸體液免疫反應之機制，用同一實驗模式研究靈芝菌絲體對皮耶氏班和腸繫膜淋巴結免疫細胞分泌細胞激素之影響，靈芝菌絲體並沒有抑制 anti-CT IgA 之反應。兩次實驗結果不一致可能是不同批發酵之靈芝菌絲體，其中有效成份含量不同而造成。由於靈芝多醣體被認為是具有免疫調節活性，抽取靈芝菌絲體多醣體進行實驗, 探討靈芝菌絲體多醣體是否有調節腸體液免疫反應的效應。

二、研究目的

本研究是探討口服靈芝菌絲體胞內多醣體對小鼠腸特異性抗體反應之影響。計畫的目的是評估靈芝菌絲體多醣體對經口服 CT 免疫的小鼠產生 anti-CT IgA 抗體反應之影響。

三、文獻探討

靈芝對免疫功能的研究包括對自然殺手細胞的活性(18)，對人類血中淋巴球的 mitogenic effect (10)，經γ射線處理小鼠脾臟 T cell subset 和細胞免疫功能的回復(2,3)，和抗腫瘤的作用(6,13,15,16,17)。其中研究靈芝抗腫瘤為最多，特別是靈芝中多醣體的抗腫瘤作用。研究方法普遍以菌絲體或子實體的抽出物對實驗動物給予腹腔注射，結果顯示靈芝抽出物可增強免疫功能。

靈芝需為口服的保健食品，除了抗腫瘤的活性外(4,6)，罕見動物實驗以口服的方式來研究靈芝的免疫效應，研究方法普遍以靈芝菌絲體或子實體的抽出物對實驗動物作腹腔注射或以細胞培養方式作體外研究，而且集中探討對系統性免疫系統的影響，對腸免疫系統功能之影響的研究卻甚為缺乏。另一方面，用上述研究方法所得的結果，靈芝多醣體(1,15,17)和蛋白質(10,11,12)被認為是調節免疫功能的有效成份。極微量的巨量分子(macromolecules)能以 transcellular pathway 的方式通過腸的上皮細胞(8)或被 PP 上的 M 細胞攝入(14)。腸上皮細胞屏障可能阻隔足夠能產生免疫調節作用的靈芝菌絲體或子實體的抽出物進入體內，因而無法產生像以注射方式所得的免疫調節功能。這樣以注射方式所得的實驗結果不一定能反映實際情況，更說明口服方式來研究靈芝的免疫效應的重要性。最近的臨床實驗結果顯示，口服靈芝多醣體對癌症病患的免疫力有調節作用(5,7)。

四、研究方法

1. 材料

不含醱酵液的靈芝（*Ganoderma lucidum*）菌絲體由台糖公司提供。

2. 靈芝菌絲體胞內多醣之萃取

10g 靈芝菌絲體加 100ml 的去離子水，以組織均質機在室溫下打破細胞壁(15,000rpm、2min)，步驟重覆 5 次，把均質液收集在一起，再加 500ml 去離子水，以加熱迴流裝置加熱 60 分鐘。於水浴中冷卻後，離心（4°C，14,000 x g，10 分鐘）收集上清液。上清液以冷凍乾燥方式濃縮體積 3-4 倍後，加入 4 倍 95%酒精，並於-20°C 冷凍萃取過夜，沉澱即為多醣體。離心（4°C，9600 x g，10 分鐘）收集沉澱，冷凍乾燥備用。沉澱委托台糖公司作多醣體的定量。

3. 實驗設計

5 週齡的雌性 C57BL/6J 小鼠隨機分為 4 組，飼養期為四週。靈芝菌絲體多醣體的添加量相當於在 AIN-93G 飼料中添加 1%、3%、5%和 10%靈芝菌絲體。小鼠在飼養至第 7 天和第 21 天時以管餵的方式給與 0.1mL 含 5μg cholera toxin (CT)的 0.2M $NaHCO_3$ 溶液。另加一組餵飼 AIN-93G 飼料的小鼠，管餵 0.1mL 0.2M $NaHCO_3$ 溶液作為 negative control 組。每週記錄體重與飼料攝取量，並於犧牲前一天收集糞便。以 CO_2 麻醉小鼠，眼窩採血後，再以 CO_2 使小鼠窒息死亡，移除小腸。小腸內容物以 Phosphate-buffered saline (0.01M, pH7.4)洗出，洗出液在 4℃下以 10,000rpm 離心 10 分鐘，收集上清液。上清液和血清儲藏於-20℃。

4. 分析方法

(1) 糞便抽出物的製備

糞便冷凍乾燥後稱重，貯存於-20℃。糞便置入離心管，每克糞便加入 20mL PBS，在室溫下靜置 30 分鐘。用藥匙把糞便打散後，vortex 震動 20 秒，於 4℃離心(9,700 x g)10 分鐘。沉澱再加入 PBS 作 2 次萃取，最後上清液定體積至 10ml，貯存於-20℃。

(2) 總 IgA 濃度的測定

96-well flat-bottom plate 以 affinity –purified goat anti-mouse IgA (α-chain specific, 1 μg/well in 100 μl PBS，Sigma Chemical Co., St. Louis, Missouri)在 4℃coating 過夜。Plate 以 PBS 含 0.05% Tween 20 (washing buffer)洗三次後，以 blocking buffer (0.5% gelatin in PBS)在 37℃ blocking 一小時。樣品以 PBS 做適當稀釋後，取 100 μl 加入 well 在 37℃反應 90 分鐘。接著以 washing buffer 洗五次，每一個 well 加入 100 μl peroxidase-conjugated goat anti-mouse IgA (α-chain specific, Sigma Chemical Co.)，在 37℃反應一小時，以 washing buffer 洗五次，每一個 well 再加入 100 μL 含 3,3',5,5'-tetramethylbenzidine tablets (0.1mg/ml, Sigma Chemical Co.)和 0.017 μl H_2O_2 (35%)的 phosphate–citrate buffer (0.05M, pH=5.0)反應 10 分鐘。反應以 50 μl 2N H_2SO_4 終止。在 450nm 讀 peroxidase 的產物濃度。以 mouse myeloma IgA (clone:TEPC 15, Sigma Chemical Co.)作標準曲線。

(3) Specific anti-cholera toxin IgA 和 IgG 的測定

小腸內容物與糞便 specific anti-CT IgA 和血清 specific anti-CT IgA 和 IgG 濃度與總 IgA 濃度的測定相似。96-well flat-bottom plate 加入含 100 μl CT (5 μg/mL)的 0.1 M carbonate buffer, pH 9.6。於 4℃靜置過夜後，以 washing buffer 洗三次，再加入 blocking buffer，37℃靜置一小時。接著以 washing solution 洗三次，加入 100 μL 經適當稀釋的樣品，於 37℃反應 90 分鐘。以 washing buffer 洗五次後，加入適當稀釋的 peroxidase conjugated goat anti-mouse IgA (α-chain specific) 或 IgG (γ-chain specific)。Peroxidase 活性的測定如前述。用小鼠 IgA 和 IgG 作標準曲線以換算樣品中的 anti-CT IgA 和 IgG 的濃度。

5.統計方法

實驗數據以 SAS 統計軟體作統計分析。數據以 one-way ANOVA 分析後，以 Tukey's Studentized Range test 找出各組之間的顯著差異 ($P<0.05$)。

五、結果與討論

靈芝菌絲體水萃取酒精沉澱物含 31%多醣體(不含澱粉)，經計算菌絲體的多醣體含量為 2.5%。靈芝菌絲體含多醣體的抽出物對小鼠的生長和飼料總攝取量在各組間並無顯著差異(表一)。不論飼料中靈芝菌絲體抽出物含量多寡，均對小腸內容物和糞便中 anti-CT-IgA 抗體和 IgA 總量沒有影響的作用(表二)。血清的 anti-CT-IgA、anti-CT-IgG、總 IgA 和 IgG 濃度的結果與小腸內容物和糞便的結果一致(表三)。

實驗結果靈芝菌絲體多醣體對 anti-CT-IgA 免疫反應沒有影響，可能的原因為多醣體添加量不足或多醣體不是有效成份。在預備實驗中，曾先進行小鼠

餵飼添加 1、3 和 5%靈芝菌絲體的飼料四週，發現 anti-CT IgA 反應與對照組比較無顯著差異，因此進行本實驗時設計包括一組多醣體添加量相當於添加 10%的菌絲體，所以多醣體的添加量已相當高。由於醱酵液也含有菌絲體分泌的多醣體，本實驗結果不能推論靈芝胞外多醣體對 anti-CT IgA 反應也沒有影響。

六、成果自評

由於台糖公司未能按照進度提供靈芝菌絲體，使本研究工作較原定計畫延遲一年完成，但研究內容與原計畫完全相同。結果顯示靈芝菌絲體胞內多醣體沒有調控腸體液免疫反應的作用。原計畫第二年探討靈芝菌絲體胞外多醣體對腸體液免疫反應的影響，此部份計畫沒有被國科會通過。菌絲體胞外多醣體對腸之免疫調節作用是值得吾人研究之議題。

References

1. Bao, X.F., Wang, X.s., Dong, Q., Fang, J.N. and Li, X. Y. (2002) Structural features of immunologically active polyscaccharides form Ganoderma lucidum. Phytochemistry 59: 175-181.
2. Chen, W.-C., Hau, D.-M., Wang, C.-C., Lin, I.-H. and Lee, S.-S. (1995) Effects of *Ganoderma lucidum* and Krestin on subset T-cell om spleen of γ-irradiated mice. Am. J. Chinese. Med. 23:71-80
3. Chen, W.-C., Hau, D.-M. and Lee, S.-S. (1995) Effects of *Ganoderma lucidum* and Krestin on cellular immunocompetence in γ-irradiated mice. Am. J. Chinese. Med. 23:71-80
4. Cheng, H.H., Tung, Y.C. and Tung, T.C. (1985) The anti-tumor effect of cultivated Ganoderma lucidum extract. II. The effect of Ganoderma lucidum extract by oral administration on Sarcoma-180 tumor growth in mice. J. Chinese Oncol. Soc. 1:118-122.
5. Chen, X., Hu, Z.P.,Yang, X.X., Huang, M., Gao, Y., Tang, W., Chan, S.Y., Dai, X., Ye, J., Ho, P.C., Duan, W., Yang, H.Y., Zhu, Y.Z. and Zhou, S.F. (2006) Monitoring of immune responses to a herbal immunomodulator in patients with advanced colorectal cancer. Int. Immunopharmacol. 6:499-508.
6. Gao, Y., Gao, H., Chan, E., Tang, W., Xu, A., Yang, H., Huang, M., Lan, J., Li, X., Duan, W., Xu, C. and Zhou, S. (2005) Antitumor activity and underlying mechanisms of ganopoly, the refined polysaccharides extracted from Gaoderma lucidum, in mice. Immuno. Invest. 34:171-198.
7. Gao, Y., Tang, W., Dai, X., Gao, H., Chen, G., Ye, J., Chan, E., Koh, H.L., Li, X. and Zhou, S. (2005) Effects of water-soluble Ganoderma lucidum polysaccharides on the immune functions of patients with advanced lung cancer. J. Med. Food 8:159-168.
8. Gardner, MLG. (1988) Gastrointestinal absorption of intact proteins. Ann. Rev. Nutr. 8:329-350.
9. Ha, C.-L. (2003) The inhibitory effect of the Chinese herb *Ganoderma lucidum* mycelium on gut immunoglobulin A responses to cholera toxin in mice. Nutr. Res. 23:691-701.
10. Haak-Frendscho, M., Kino, K., Sone, T. and Jardieu, P. (1993) Ling Zhi-8: A novel T cell mitogen induces cytokine production and upregulation of ICAM-1 expression. Cellular Immunol. 150:101-113.
11. Kino, K., Yamashita, A., Yamaoka, K., Watanabe, J., Tanaka, S., Shimizu, K. and Tsunoo, H. (1989) Isolation and characterization of a new immunomodulatory

protein, Ling Zhi-8, from Ganoderma lucidum. J. Biol. Chem. 264:472-478.
12.Kino, K., Sone, T., Watanabe, J., Tsuboi, H., Miyajima, H. and Tsunoo, H. (1991) Immunomodulator, LZ-8, prevents antibody production in mice. Int. J. Immunopharmacol. 13:1109-1115.
13.Lee, S.-S., Wei, Y.-H., Chen, C.-F., Wang, S.-Y. and Chen, K.-Y. (1995) Antitumor effects of *Ganoderma lucidum*. J. Chin. Med. 6:1-12.
14. Owen, R.L. (1977) Sequential uptake of horseradish peroxidase by lymphoid folloicle epithelium of Peyer's patches in the normal unobstructed mouse intestine: an ultrastructural study. Gastroenterology 72:440-451.
15.Song, Y., Okuda, R., Wada, N., Kishida, E. and Misaki, A. (1985) Structures and antitumor activities of the polysaccharides isolated from fruiting body and the growing culture of mycelium of Ganoderma lucidum. Agric. Biol. Chem. 49:2641-2653.
16.Wang, S., Zhang, J., Mizuno, T., Zhang, C., Ito, H., Mayuzumi, H. and Li, J. (1993) antitumor active polysaccharides from the Chinese mushroon Songshan Lingshi, the fruiting body of Ganoderma tsugae. Biosci. Biotech. Biochem. 57:894-900.
17.Wang, S.-Y., Hsu, M.-L., Hsu, H.-C., Tzeng, C.-H., Lee, S.-S., Shiao, M.-S. and Ho, C.-K. (1997) The anti-tumor effect of *Ganoderma lucidum* is mediated by cytokines released from activated macrophages and T lymphocytes. Int. J. Cancer 70:699-705.
18.Won, S.-J., Lin M.-T. and Wu, W.-L. (1992) *Ganoderma Tsugae* mycelium enhances splenic natural killer cell activity and serum interferon production in mice. Japan. J. Pharmacol. 59:171-176.

八、圖表

Table 1. Growth indices for mice fed AIN-93G diet, only, or with different levels of *Ganoderma lucidum* mycelium extracts for 28 days[a]

groups[b]	n	Weight (g/mouse) Initial	Weight (g/mouse) Final	Feed intake (g/mouse for 28 days)
NC	7	16.6±0.9	19.5±0.7	81.5±4.4
C	9	16.4±0.9	20.4±1.2	84.2±7.4
L	10	16.3±1.0	20.5±1.4	79.3±6.5
M	10	16.3±1.0	20.5±1.4	84.6±7.3
H	10	16.4±0.7	20.0±0.9	80.0±8.8
D	10	16.3±0.7	19.9±1.3	84.8±10.0

[a]Mean±SD. No significant differences among treatment groups according to Tukey's Studentized Range test.
[b]NC, negative control; C, control; L, M, H and D, control diet containing 0.17%, 0.51%, 0.85% and 1.7% (wt/wt) *Ganoderma lucidum* mycelium extracts, respectively. Mice were orally immunized with either of cholera toxin or vehicle at day 7 and 21 of feeding period.

Table 2. The immunoglobulin A level of small intestinal washes and fecal pellets for mice fed AIN-93G diet, only, or with different levels of *Ganoderma lucidum* mycelium extracts for 28 days[a]

		Small intestinal washes (μ g)		Fecal pellets (μ g/g fecal)	
groups[b]	n	anti-CT IgA	total IgA	anti-CT IgA	total IgA
NC	7	0.4±0.2[b]	56.4±25.3	0.4±0.2[b]	134.3±30.6
C	9	14.3±12.1[a]	50.7±14.1	30.3±21.3[a]	143.9±17.2
L	10	14.1±9.5[a]	71.0±19.2	19.4±8.8[a]	140.0±30.0
M	10	14.6±10.4[a]	50.5±19.2	19.0±13.0[a]	144.5±52.6
H	10	22.1±13.1[a]	48.7±18.7	20.0±12.8[a]	200.6±83.4
D	10	25.6±20.5[a]	59.0±19.8	19.1±17.2[a]	135.8±57.4

[a]Mean±SD. Within a column, values not sharing a superscript letter are different ($P<0.05$) according to Tukey's Studentized Range test.

[b]NC, negative control; C, control; L, M, H and D, control diet containing 0.17%, 0.51%, 0.85% and 1.7% (wt/wt) *Ganoderma lucidum* mycelium extracts, respectively. Mice were orally immunized with either of cholera toxin or vehicle at day 7 and 21 of feeding period.

Table 3. The concentration of serum immunoglobulin A and G for mice fed AIN-93G diet, only, or with different levels of *Ganoderma lucidum* mycelium extracts for 28 days[a]

groups[b]	n	anti-CT IgA	total IgA	anti-CT IgG	total IgG
			μ g/ml		
NC	7	0.3±0.1[b]	83.0±10.1	0.5±0.1[b]	1264.7±332.7
C	9	16.7±8.8[a]	81.4±12.0	365.8±152.8[a]	1282.8±438.3
L	10	20.4±7.8[a]	99.1±13.9	392.6±107.8[a]	1456.0±604.5
M	10	18.3±10.3[a]	92.4±16.7	302.1±172.6[a]	1426.5±355.6
H	10	20.8±6.5[a]	93.4±13.7	298.5±121.8[a]	1209.8±249.2
D	10	20.2±9.9[a]	94.7±29.6	329.0±121.1[a]	1563.6±443.7

[a]Mean±SD. Within a column, values not sharing a superscript letter are different ($P<0.05$) according to Tukey's Studentized Range test.

[b]NC, negative control; C, control; L, M, H and D, control diet containing 0.17%, 0.51%, 0.85% and 1.7% (wt/wt) *Ganoderma lucidum* mycelium extracts, respectively. Mice were orally immunized with either of cholera toxin or vehicle at day 7 and 21 of feeding period.