



The 3rd ICMBMP

October 1999

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SHIITAKE MUSHROOM
(*LENTINUS EDODES*) STILL RETAIN
ANTITUMOUR ACTIVITY**

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PARTIALLY PURIFIED LENTINAN FROM SHIITAKE MUSHROOM (*LENTINUS EDODES*) STILL RETAIN ANTITUMOUR ACTIVITY

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ABSTRACT

A more efficient procedure emerged from several methods available was used in this study. The β -D-glucan isolated through ethanol precipitation and freeze-drying in liquid nitrogen was found to be lentinan. Purity tests that were carried out using carbohydrate analysis column, a chromatographic method, had shown a significant lentinan purity of 87.50%. Three experiments were designed to compare the efficacy of this extract. The pre-treatment cohort was administered this lentinan for 7 days before inoculation with the K36 cells (murine leukemic cell line). The simultaneous-treatment cohort had lentinan administered together with the inoculation of K36 cells for 7 days. Lastly, the post treatment cohort was administered lentinan 7 days after tumours have been inoculated. Highest anti-tumour activity was observed with the pre-treatment cohort. The tumour inhibition rates (TIR) of 94.44%, 88.59% and 83.14% were observed with pre-treatment, simultaneous-treatment and post-treatment respectively. On comparison, crude mushroom homogenate showed TIR of 55.20%, 45.32% and 43.06% respectively for the three experiments. This indicated that lentinan was much more effective in the prevention of tumour formation. Ultrastructural studies revealed the number of retrovirus present in the tumour cells were drastically reduced and many of those present were defective. In addition, significant induction of IFN-gamma, TNF alpha and IL-2 was observed using ELISA tests in the mouse model. Thus, host T-cell stimulations by lentinan could well have a role in the anti-tumour activity observed.

INTRODUCTION

Lentinan, a fully purified beta-1, 3-D-glucan, isolated from shiitake mushroom (*Lentinus edodes*), composed mainly of D-glucose units with beta (1-3) linkages as the main chain and beta (1-6) as branched chain, has strong antitumour activity against various tumours, and prevent chemical and viral carcinogenesis (Chihara *et al.*, 1970). Its antitumour action is considered to be host-mediated since it does not have any direct cytotoxicity against tumour cells (Maeda and Chihara, 1973). Lentinan can be isolated from shiitake mushroom (*Lentinus edodes*) via several methods.

Three methods were performed in this study. The resulting extracts were compared according to total yields and purity. YAT's method, a biochemical method that was design for the preparation of lentinan was shown to be the most efficient method among all three. The partially purified lentinan obtained from the method was then evaluated for its antitumour activity by orally feeding the AKR white male mice via three experiments based on the time of administration of lentinan, namely pre-treatment, simultaneous-treatment and post-treatment. Tumour cells were inoculated into the mice and the sizes of the tumour developed were scored after 14 days. The morphologies of the tumour cells and retroviruses were also studied under electron microscope, to determine the underlying mechanism. A series of immune responses that might be stimulated by lentinan were then measured by ELISA techniques.

MATERIALS AND METHODS

Shiitake mushrooms

8-10 weeks old buds of shiitake mushrooms (*Lentinus edodes*) were used.

Preparation of beta-D-glucan (lentinan) from shiitake mushroom (*Lentinus edodes*)

Lentinan, a glucan type polysaccharides with an average molecular weight of 50 kDa were isolated from the edible shiitake mushrooms, *Lentinus edodes*. Three methods

were available for the preparation of lentinan, namely modified Chihara's method (1970), Tan's method (offered by Dr. K.K. Tan) and YAT's method. In modified Chihara's method, fresh mushrooms were boiled for 8-16 hours before a series of extraction procedures with ethanol as well as removal of other components via different techniques. In Tan's method, fresh mushrooms were boiled first and lentinan was then precipitated by using ethanol. In YAT's method, freezing with liquid nitrogen was included in one of the step to remove water content and the partially purified lentinan, was then precipitated out by using ethanol. The extracts obtained from the three methods were compared according to the total polysaccharide yields and purity using phenol-sulfuric acid method (Kobert G., 1978) and carbohydrate analysis column respectively.

Experimental mice

AKR male white mice at the age of 6-8 weeks old, from Laboratory Animal Centre, Sembawang (Singapore) were involved in the experiments.

Cell lines

K36 cells derived from a murine T-cell lymphoma in AKR mice were used. This cell line is a transformed cell line and is naturally tumourigenic in syngeneic mice. It has been infected with a murine leukaemia retrovirus.

Tumour inhibition rate assay

Three experiments were designed to evaluate the anti-tumour effects of lentinan. Lentinan were administered orally at different period of time, namely pre-treatment, simultaneous-treatment and post-treatment. In pre-treatment cohort, lentinan was fed to the mice for 7 days before the inoculation of tumour cells (K36 cells). In simultaneous-treatment cohort, lentinan was fed to the mice at the same time as the inoculation of tumour cells. Lastly, in post-treatment, lentinan was fed to the mice 7 days later from the

time of tumour cells inoculation. The sizes of the tumours developed were scored after 14 days and control mice were included in each of these experiments.

Determination of the levels of cytokines induced by lentinan using ELISA (Enzyme-Linked Immunosorbent Assay) test kits

Blood were extracted from the AKR white mice after treatment via cardiac puncture. The levels of three cytokines were determined by ELISA kits, namely, mouse interferon-gamma (mIFN- γ), mouse tumour necrosis factor-alpha (mTNF- α) and mouse interleukin-2 (mIL-2).

Ultrastructural studies of tumour cells and retroviruses using transmission electron microscopy

The tumours were dissected out from male AKR mice and processed for electron microscopy. The morphologies of the tumour cells and retroviruses observed were compared accordingly for the different period of treatment.

RESULTS

Extraction of lentinan from shiitake mushrooms using various methods

Various parameters were compared with the use of different techniques of extraction, such as the time taken for the preparation, cost effectiveness of method (based on the materials needed for the procedure and recovered yields), and the ease of performance (whether any sophisticated equipment or techniques were required). Table 1 shows the comparison of total yields and purity of the extracts prepared from the three methods available.

Table 1: Comparison of different methods of preparation for β -D-glucan (lentinan) from shiitake mushroom (*Lentinus edodes*)

Factors of comparison	Method of Extraction		
	Modified Chihara's method	Tan's method	YAT's method
<u>Ease of preparation</u>			
Number of days taken to prepare the extract	14 days.	3 days.	5 days.
Requirement of sophisticated equipments or rarely used chemicals	Many.	None.	None except the need for liquid nitrogen.
Cost of preparation	High.	Low.	Low.
Total yields from 100 g of fresh mushrooms	4 mg	42 mg	325 mg
Percentage concentration of lentinan in extract produced (%)	96.03%	71.64%	87.50%
Purity obtained(%)	99.23%	75.40%	87.65%

Evaluation of the therapeutic effects of lentinan using animal model

Each mouse was fed at a dose of 3mg daily. Three different sets of experiments were carried out to determine the effective period of oral administration of lentinan. The inhibition ratios of tumour growth are calculated by the formula:

$$\text{Inhibition ratio (\%)} = [(A - B)/A] \times 100$$

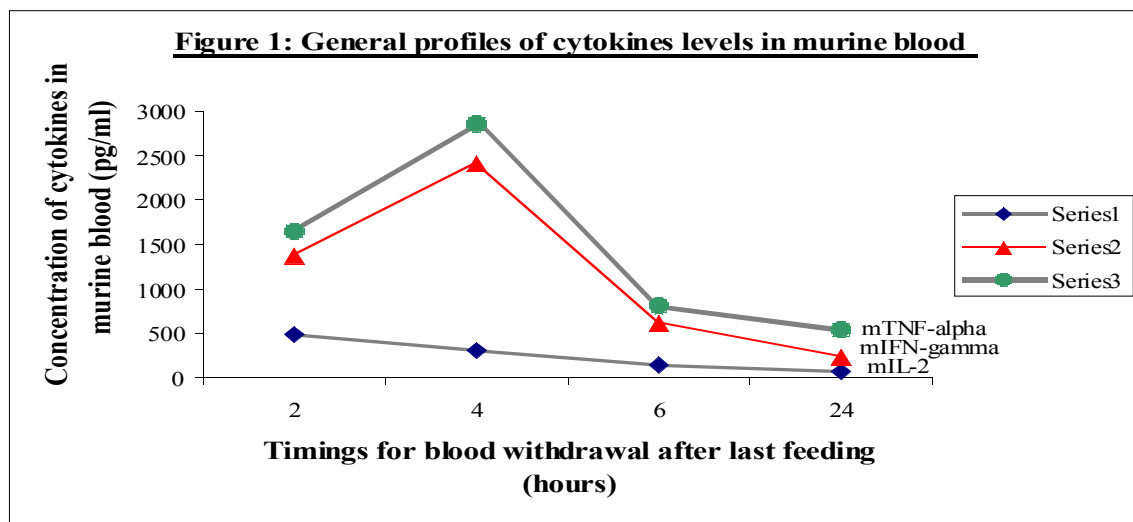
Table 2 shows the tumour inhibition rate from the various period of feeding.

Table 2: Tumour inhibition rate (TIR) resulted from the three sets of experiments

Period of oral administration of lentinan	Pre-treatment	Simultaneous-treatment	Post-treatment
Tumour inhibition rate of lentinan fed mice (%)	94.44%	88.59%	83.14%
Tumour inhibition rate of homogenated crude mushrooms fed mice	55.20%	45.32%	43.06%

Evaluation of cytokine levels in murine blood

Blood extracted from mice fed with lentinan were tested for three cytokine levels, namely mIFN- γ , mTNF- α and mIL-2. The following graph showed the general profile of the levels of these cytokines (Series 1 – mIL-2, series 2 – mIFN- γ and series 3 – mTNF- α) in murine blood at different intervals after the oral administration of lentinan:



Transmission electron microscopy (TEM) of morphologies of tumour cells

Tumours were dissected from AKR mice that were involved in the three regimes of treatment. Tumour tissues were processed and viewed under 208S electron microscope (Philips, Holland). Under TEM, tumour cells derived from lentinan-treated mice were generally highly vacuolated and disintegrating. The number of virus particles was significantly reduced and most of them were defective with empty nucleocapsids.

The following electron micrographs show the morphologies of the tumour cells and retroviruses, extirpated from control mice as well as mice fed with lentinan.

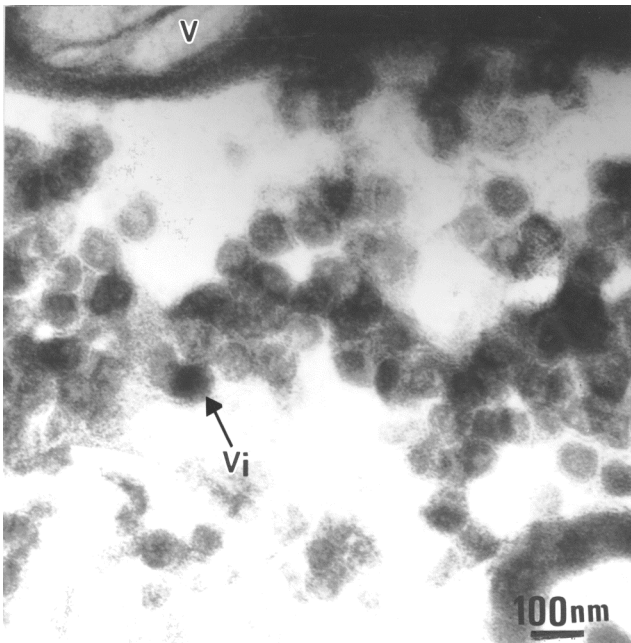


Figure 2. Electron micrograph showing a large number of infectious retroviruses (Vi) in tumour tissues extirpated from control mice.

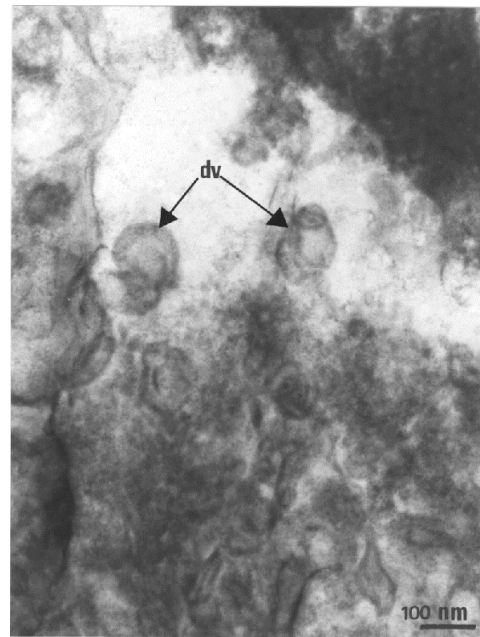


Figure 3. Electron micrograph showing comparatively fewer defective viruses (dv) in tumour tissues extirpated from lentinan fed mice.

DISCUSSION

From the results shown in Table 1, it was observed that modified Chihara's method offered the most purified form of lentinan extract, followed by YAT's method and lastly, Tan's method. However, the cost of preparation was high, and it involved many procedures and the requirement of more expensive equipment in the first method. Tan's method was the easiest to carry out among the three, however, it did not give large amount of lentinan extracts with high purity. Hence, the YAT's method that was specially designed (designed by Ann-Teck Yap in 1998) for the preparation of lentinan from shiitake mushrooms (*Lentinus edodes*) was used in this experiment. This method required a relatively shorter period of time to carry out and produced a large amount of lentinan extract (325 mg from 100 g of fresh mushrooms) of sufficiently high purity (87.50%).

From Table 2, it is clearly shown that pre-treatment with lentinan gives the highest tumour inhibition rate (TIR) of 94.44%, followed by simultaneous treatment with lentinan, giving a TIR of 88.59% and lastly, post-treatment with lentinan, giving a TIR of 83.14%. The enhancing effects on the stimulation of immune system observed must be higher in pre-treated mice than the other two cohorts, with which were also beneficial in the prevention of tumour growth. As compared, the TIR of homogenized crude mushroom fed mice were much lower (ranging from 43% to 55%).

The inhibitory actions on tumour growth by oral administration of lentinan led to the investigation of the underlying mechanism of action. Since it was reported that the actions of lentinan were mainly host-mediated (Chihara, 1969), the levels of various cytokines in murine blood after feeding with lentinan were determined (Figure 1). Critical cytokines such as interleukin-2 (IL-2), interferon-gamma (IFN- γ) and tumour necrosis factor-alpha (TNF- α) were measured using ELISA techniques.

The levels of these cytokines peaked at different hours after feeding. The level of IL-2 was highest at 2 hours, while that of the IFN- γ and TNF- α peaked at 4 hours after

the last fed. There is no rise in the level of cytokines in the blood obtained from the control mice. Similar patterns were observed from mice fed with homogenized crude mushroom but the levels were much lower. These results indicated that the increased cytokines levels were due to the oral administration of lentinan extracts and these cytokines, were produced mainly by T helper-1 cells (T_H-1). Chihara (1969) has also reported that lentinan represents a unique class of immunopotentiator and a T-cell oriented adjuvant. The antitumour action of lentinan is host-mediated, mainly by the actions of T-cells in which macrophages also play some part as demonstrated by the increase production of cytokines.

Production of IL-2 and its receptors is the key step of response by T cell to antigenic stimulation. Activation of T cells resulted in its proliferation and synthesis of new products such as IFN- γ and TNF- α . Several of these probably helped to promote the proliferation and differentiation of T cells. It appears that the overall effect of lentinan is to restore or augment the ability of host cells to response to infectious agents or cancer metastases. The physiological constitution of host defense mechanisms is improved by consumption of lentinan. This in turn restored homeostasis and enhanced resistance to diseases. One fundamental principle in Oriental medicine is to regulate homeostasis of the whole body and to bring the diseased person to the normal state. Thus, lentinan form a bridge linking modern immunology and traditional medicine.

To conclude this study, lentinan has shown to be effective against tumour formation and its effects on host's immune system causes tumour regression when administered orally.

ACKNOWLEDGEMENT

The authors will like to thank the National University of Singapore for funding this project and Dr. Tan Kok Kheng, director of Everbloom Biotechnology (Singapore)

Pte. Ltd., for his sponsorship of shiitake mushrooms. We will also like to thank those who had helped out in the experiments for their commitments and support.

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