

# Inhibition of Human Colon Carcinoma Development by Lentinan from Shiitake Mushrooms (*Lentinus edodes*)

MAH-LEE NG, Ph.D., and ANN-TECK YAP, B.Sc. (Hons.)

## ABSTRACT

**Objectives:** Lentinan was extracted from shiitake mushrooms (*Lentinus edodes*) via a new cost-effective procedure that resulted in high purity (88%) and yield. Unlike previous reports whereby the lentinan was given parenterally, in this study the emphasis was on the oral administration of lentinan. The goal is to document whether the efficacy of the antitumor property is still expressed through this route of administration.

**Design:** Initial study on the action of lentinan was conducted using murine lymphoma (K36) cells in a AKR mouse model. Further investigation on the effectiveness of the extracted lentinan was then performed using human colon-carcinoma cell lines in mice. Six established human colon-carcinoma cell lines segregated into three groups of different degrees of differentiation were used in this study. One group was not fed (control) and the second group was prefed with lentinan for 7 days prior to inoculations with the cancer cells. The size of the tumors that developed was rated after 1 month.

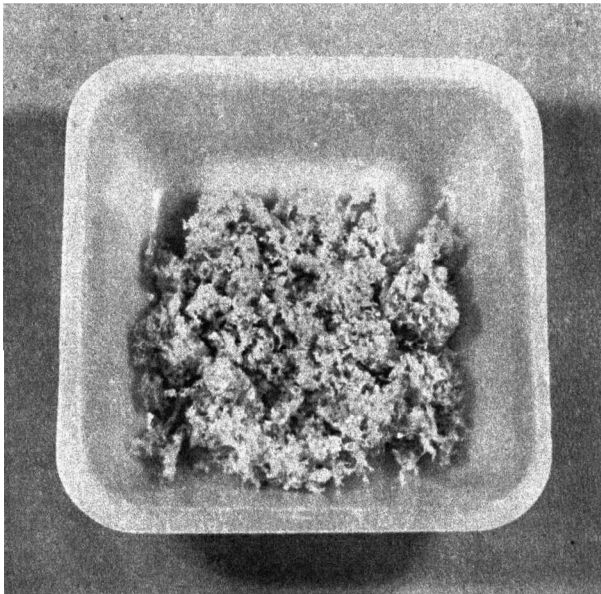
**Results:** Significant regression in tumor formation was observed in prefed mice compared to control (unfed) mice when K36 or human colon-carcinoma cells were used. Significant reductions in the size of the tumors were observed in mice prefed with lentinan. Follow-up investigation proceeded with the use of nude mice (athymic). Lymphocytes extracted from AKR mice prefed with lentinan for 7 days were inoculated into the nude mice. This was then followed by inoculation of the human colon-carcinoma cell lines into these mice. Much smaller tumors were formed in nude mice inoculated with lymphocytes, in contrast to the larger tumor formed in nude mice without lymphocytes inoculation.

**Conclusion:** This study showed that the antitumor property of lentinan was maintained with oral administration. In addition, “primed” lymphocytes, when given passively to immunodeficient mice, were able to retard the development of tumors in these mice.

## INTRODUCTION

Since ancient times in the East, shiitake mushrooms (*Lentinus edodes*) have been—and still are—one of the most edible mush-

rooms shown to have medicinal properties (Jong and Birmingham, 1993; Mizuno, 1995). The glucan component, especially lentinan, a purified  $\beta$ -1,3-D-glucan with  $\beta$ -1,6 branches and a triple helical structure has been proven



**FIG. 1.** Purified lentinan extracted from shiitake mushroom. The preparation is highly soluble and was used for oral administration (3 mg per mouse per day) to the mice for all experiments presented in this paper.

illustrate the importance of cell-mediated immunoresponse in the tumor development.

## MATERIALS AND METHODS

### *Source of lentinan*

Lentinan was extracted from fresh 2-day-old fruiting bodies of shiitake mushrooms (*Lentinus edodes*) using a more productive procedure (Yap and Ng, 2001). A purity test of the extract was performed using both the sugar-pack column chromatography and high-performance liquid chromatography (HPLC).

### *Cell lines*

*Murine lymphoma (K36) cell line.* The K36 cells were used in the preliminary study to gauge the antitumor property of the extracted lentinan. This cell line was derived from a murine T-cell lymphoma in AKR mice and was infected with a murine leukemia retrovirus.

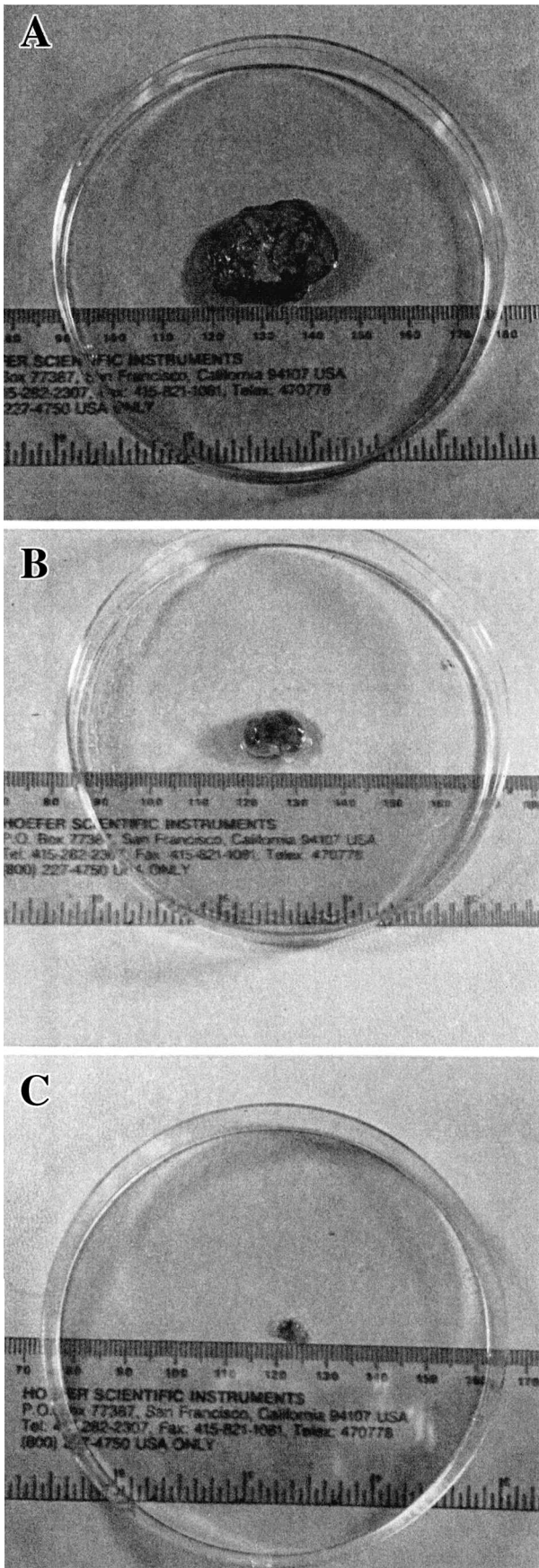
*Human colon carcinoma cell lines.* Further investigation was carried out using six established human colon-carcinoma cell lines. These cell lines represented cancers at three stages of cell differentiation. These six cell lines were divided into three groups based on the degree of differentiation (Trujillo et al., 1991). Group 1 consisted of LoVo and SW48 cells. These two cell lines were the most differentiated cells among the three groups and possessed well-defined gland and signet ring formation. Group 2 consisted of SW480 and SW620 cells that are moderately differentiated. Group 3 consisted of SW403 and SW1116 cells, both of which are poorly differentiated and have no gland or signet ring formation.

to have marked antitumor activity (Ikekawa et al., 1969). Lentinan is also shown to be an immunopotentiator and appears to stimulate macrophage and T-cell proliferation with no direct cytotoxic effect against tumor cells (Maeda and Chihara, 1973).

A series of experiments was performed in this study using lentinan purified from a simpler and more cost-effective procedure (Yap and Ng, 2001). The yield obtained was also much higher than the more established procedure performed by Chihara and colleagues (1970). The antitumor activity was initially studied using murine lymphoma (K36) cells and subsequently, human colon carcinoma cells were used. Nude mice were also used to

**TABLE 1.** TUMOR INHIBITION RATES OF MURINE LYMPHOMA IN MICE FED WITH CRUDE MUSHROOM EXTRACT OR LENTINAN

	<i>Experimental animal cohorts</i>		
	<i>Lentinan-fed mice</i>	<i>Crude mushroom extract-fed mice</i>	<i>Control mice (unfed)</i>
Average weight of tumor in mice (g)	~0.124	~0.997	~2.226
Tumor inhibition rate (%)	94.44	55.20	—



### Animal model

Inbred male AKR mice, 5 to 6 weeks old, were used. These mice were bred as a strain highly susceptible to leukemia in 1928. Nude (athymic) mice (5–6 weeks old) were also used in this study.

### Oral administration of lentinan

Male AKR mice were given solubilized lentinan by gavage daily for 7 days (3 mg per mouse per day). Control mice without feeding were also included as controls.

### Evaluation of antitumor activity of lentinan on murine lymphoma

Preliminary investigation was performed using murine lymphoma (K36) cells in AKR mouse model. Optimal dosage as previously determined (Yap and Ng, 2001) was 3 mg per mouse per day. Mice were prefed with lentinan or crude mushroom (equivalent volume) extracts for 7 days prior to subcutaneous inoculation with K36 cells. Size of tumors developed were scored at 14 days after tumor cell inoculations.

### Ultrastructural studies on the progeny retrovirus from lymphoma tissues

From the formed tumors, there was still a continuous production of the murine retrovirus. The morphology of these virus particles was studied to investigate if lentinan had an effect on their morphogenesis. The tissues were processed as described in Kumar and Ng (2000).

←

**FIG. 2.** Tumors excised from AKR mice. Mice were orally fed with 3 mg per mouse per day of lentinan or crude mushroom (equivalent volume) extract for 7 days prior to inoculations of murine lymphoma cells (K36). The control cohort was not fed with any mushroom extracts. **A:** Tumor excised from a mouse that was not fed with either crude mushroom extract or lentinan. The average diameter of tumors were between 2.5 and 3 cm. **B:** The tumors observed from mice that were fed with crude mushroom extract were much smaller than tumors in the unfed mice. The average tumor diameter was approximately 1.5 cm. **C:** Most mice that were fed with lentinan did not develop visible tumors. In the small proportion in whom tumors have been sited, the average diameter was approximately 0.5 cm.

*Evaluation on the antitumor effect of lentinan on human colon carcinoma*

Further work was carried out using six established human colon-carcinoma cell lines (as described above). Male AKR mice were prefed with lentinan (3 mg per mouse per day) by gavage for 7 days before subcutaneous inoculation of each of the cell lines. Control mice without feeding were included in each of the groups. The size of the tumors that developed was rated after 1 month.

*Evaluation of antitumor effect of lentinan on human colon-carcinoma in nude (athymic) mice*

Nude (athymic) mice were used for this study. Two cohorts of male AKR mice were prefed with lentinan or crude mushroom extract by gavage for 7 days. One cohort was left unfed. Lymphocytes extracted from the spleens of unfed and fed mice were reinoculated into nude (athymic) mice via their tails. Nude (athymic) mice without lymphocytes inoculation were also included in the experiment as controls for comparison with the other three groups (i.e., lymphocytes from unfed mice, mice fed with crude mushroom extract or lentinan). The size of tumors that developed were scored after 1 month.

## RESULTS

*Lentinan extracted from shiitake mushrooms (Lentinus edodes)*

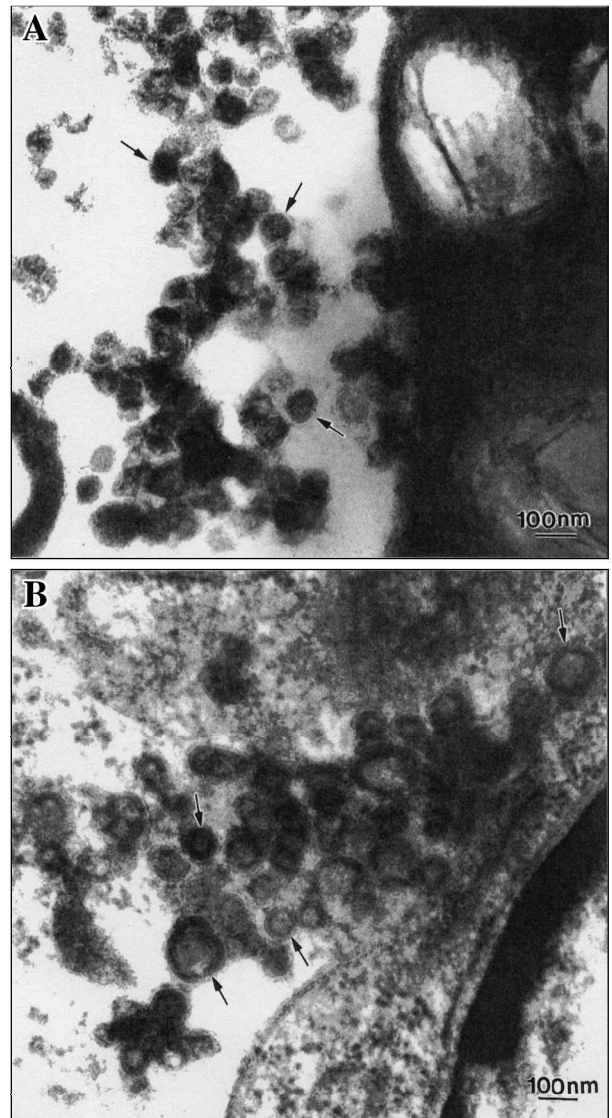
Lentinan was prepared fresh from 2-day-old shiitake mushroom buds. It is a slightly brownish-white powder with a cottony, refined texture as shown in Figure 1 and is highly soluble in water.

A purity test of the extracted lentinan powder was performed using HPLC analysis as well as sugar-pack column chromatography. The purity of the lentinan prepared from the modified procedure (Yap and Ng, 2001) was found to be 88%. The remaining portion (12%) comprised fats (1%) and proteins (11%). Recently, with further modifications to the purification procedure, the fats and proteins were separated from lentinan. The former were then fed by gavage to 10 mice and no antitumor

properties were observed (results not shown) unlike for the lentinan study (see below)

*Evaluation of antitumor activity of lentinan on murine lymphoma*

AKR mice were fed with solubilized lentinan by gavage at an optimal dose of 3 mg per



**FIG. 3.** Electron micrograph of retrovirus produced from murine lymphoma cells. **A:** Clumps of electron-dense retrovirus were visible (arrows) in the extracellular space. The virus particles are spherical and were between 80 and 100 nm in diameter. These viruses are from tumor tissues of mice who were not fed any mushroom extracts. **B:** The progeny retrovirus from tumor tissues of mice who were prefed with lentinan were observed to be defective (arrows). The virus particles lacked the electron-dense centers (no genomes) and were also of pleomorphic shape. The size of the particles also varied widely.

mouse per day for 7 days prior to subcutaneous inoculation of murine lymphoma (K36) cells. Ten (10) mice were used for each cohort (i.e., lentinan-fed, crude mushroom extract-fed, and control unfed). The experiment was repeated three times giving a total of 30 mice for each cohort. Results of the average tumor inhibition rates (TIR) were shown in Table 1. TIR was highest when mice were prefed with lentinan (94.44%). Although the crude mushroom extract also exhibited antitumor activity, the TIR was much reduced at 55.20%. From these studies, it was observed that the tumors present on control mice (unfed, Fig. 2A) were much larger than that of the crude mushroom extract-fed (Fig. 2B) or lentinan-fed (Fig. 2C) mice. The average weight of the tumors obtained from buffer-fed mice was 2.187 g compared to the mice fed with crude mushrooms homogenates (0.97 g with  $p < 0.001$ ) and lentinan-fed mice (0.125 g with  $p < 0.001$ ).

*Ultrastructural studies on the progeny virus from lymphoma tissues*

A large number of extracellular retrovirus was observed in tumor tissues extirpated from control (unfed) mice. These viruses (arrows) were infectious with electron dense nucleocapsids, enclosed within virus envelopes (Fig. 3A). In contrast there was, a reduction in the number of virus particles observed from tumor tissues excised from lentinan-fed mice (Fig. 3B). The majority of the virus particles was defective as revealed by the empty (contain no genome) nucleocapsids (arrows). In addition,

these defective virus particles were pleomorphic in shape and size.

*Evaluation of antitumor effect of lentinan on human colon carcinoma*

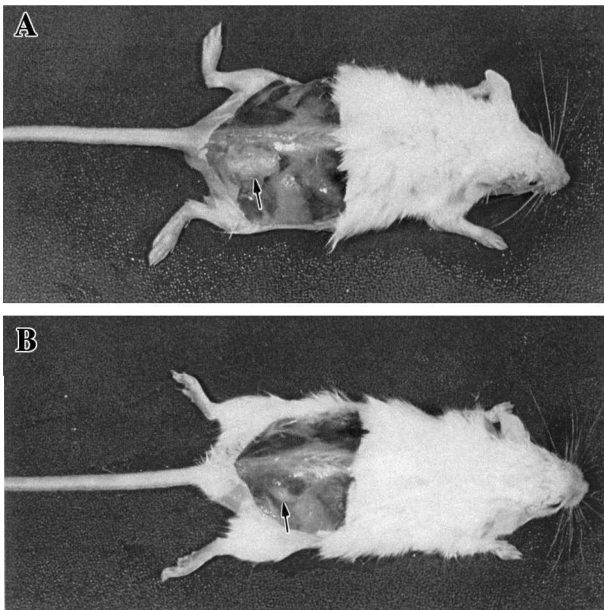
Male AKR white mice were prefed with solubilized lentinan by gavage for 7 days prior to subcutaneous inoculation of the human colon carcinoma cells. Similar to the above, 10 mice were used each time (repeated three times) for each cell line used. Observations of tumor development in the inoculated mice after 1 month showed that regression of tumors occurred in all three groups of cancer cells (Table 2) after feeding. The average TIR for LoVo, SW48, SW620, SW480, SW403, and SW1116 were 90.38%, 91.67%, 90.08%, 90.83%, 92.96%, and 90.32%, respectively. Figure 4 shows an example of the tumor observed in control mice (unfed; Fig. 4A) and in lentinan-fed mice (Fig. 4B) after inoculation with SW 620 cells. The tumors from the unfed mice had an average weight of 2.04g while the tumor from lentinan-fed nude mice was 0.23 g ( $p < 0.001$ )

*Evaluation of antitumor effect of lentinan on human colon carcinoma in nude mice*

Four sets of nude mice were used. The first batch (Fig. 5A) was not inoculated (control) with any lymphocytes. The second batch was inoculated with lymphocytes from AKR mice who were fed with crude mushroom extract by gavage (Fig. 5B). Lymphocytes from AKR mice who were fed with lentinan by gavage

TABLE 2. TUMOR INHIBITION RATES OF HUMAN COLON CARCINOMA IN MICE FED WITH LENTINAN

	<i>Types of human colon carcinoma cells</i>					
	<i>Group 1</i>		<i>Group 2</i>		<i>Group 3</i>	
	<i>LoVo</i>	<i>SW48</i>	<i>SW620</i>	<i>SW480</i>	<i>SW403</i>	<i>SW1116</i>
Average weight of tumor in lentinan-fed mice (g)	0.10	0.10	0.13	0.10	0.05	0.06
Average weight of tumor in control (unfed) mice (g)	1.04	1.20	1.31	1.09	0.71	0.62
Tumor inhibition rate (%)	90.38	91.67	90.08	90.82	92.96	90.32



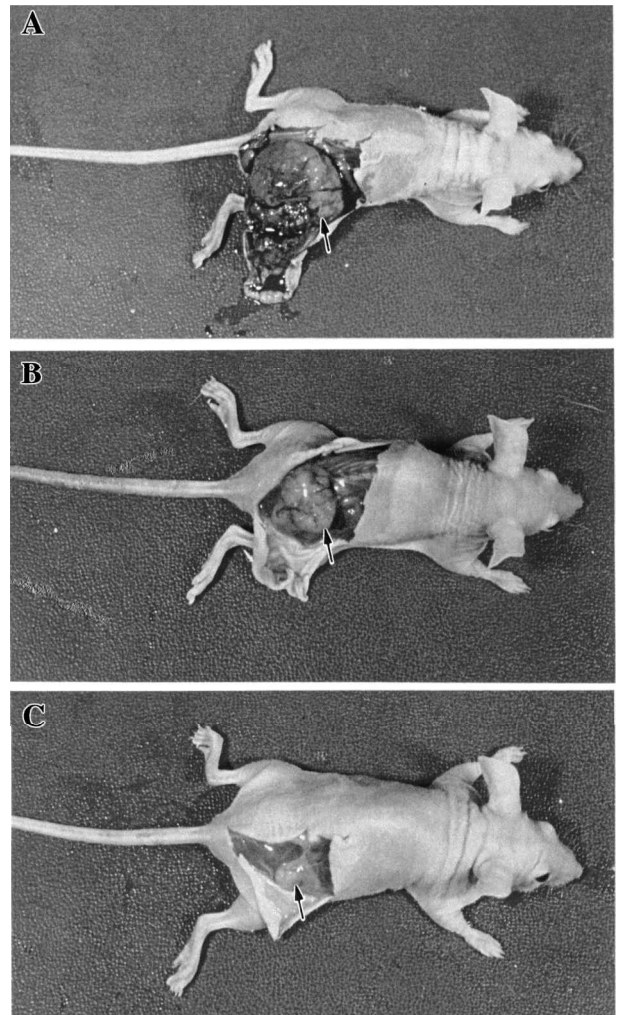
**FIG. 4.** AKR mice inoculated with human colon carcinoma cells. The SW 620 colon-carcinoma cell line was used here. **A:** The mice from this cohort were not fed lentinan prior to inoculation of the carcinoma cells. Tumors were readily observed at the end of 1 month. All of the tumors were large as shown in the figure (arrow). **B:** For the mice that were prefed 3 mg/d of lentinan for 7 days prior to inoculations with SW 620 cells, tumors were either not detected or were a much reduced size. (**B**) shows an example of a small tumor that was seen (arrow).

were inoculated into the third batch before introduction of the carcinoma cells (Fig. 5C). Finally, the fourth batch was given “un-primed” lymphocytes (i.e., lymphocytes from AKR mice not fed with any mushroom products). Ten (10) nude mice were used for each batch.

From Figure 5, it could be deduced that the primed lymphocytes from the fed AKR mice were able to transfer the antitumor properties to the nude mice. Although the tumors were still rather large in the nude mice who received lymphocytes from AKR mice fed with crude extract (Fig. 5B) the size has been reduced considerably when compared to the control mice (Fig. 5A). Dramatic regression in the size of the tumors was seen in nude mice who were inoculated with lymphocytes from AKR mice who were fed with lentinan (Fig. 5C).

The TIR was high (above 90%) for all six cell lines tested when the nude mice were primed

with lymphocytes from lentinan-fed AKR mice (Table 3; average tumor size was 0.23 g with  $p = 0.000$ ). This was followed by nude mice who received lymphocytes from AKR mice fed with crude mushroom extract (50% inhibition rate, average tumor size was 1.03 g with  $p =$



**FIG. 5.** Nude mice inoculated with SW 48 human colon carcinoma cells. **A:** The nude mouse was not given any lymphocytes prior to inoculation of the carcinoma cells. A large tumor was visible (arrow). **B:** Lymphocytes from an AKR mouse who was fed crude mushroom extracts for 7 days were given to a nude mouse before inoculation with SW 48 cells. The tumor that was visible (arrow) was smaller than the one in the untreated mouse in (**A**). **C:** The lymphocytes from an AKR mouse who was prefed lentinan appear to be rather effective in curbing the size of the tumor (arrow) that formed in the nude mouse. It could be deduced that the “primed” lymphocytes from crude mushroom extract and lentinan are able to confer some degree of antitumor effect in these nude mice.

TABLE 3. EFFECT OF LYMPHOCYTES ON TUMOR DEVELOPMENT IN NUDE MICE

	<i>Type of human colon carcinoma cells</i>					
	<i>Group 1</i>		<i>Group 2</i>		<i>Group 3</i>	
	<i>LoVo</i>	<i>SW48</i>	<i>SW620</i>	<i>SW480</i>	<i>SW403</i>	<i>SW1116</i>
Average weight (g) of tumor in nude mice inoculated with lymphocytes extracted from lentinan-fed mice (TIR)	0.16 (90.30%)	0.21 (89.39%)	0.23 (88.73%)	0.19 (88.95%)	0.12 (91.6%)	0.08 (93.38%)
Average weight (g) of tumor in nude mice inoculated with lymphocytes extracted from crude-mushroom extracts-fed mice (TIR)	0.84 (49.09%)	0.98 (50.51%)	1.03 (49.51%)	0.91 (47.09%)	0.69 (51.75%)	0.59 (56.69%)
Average weight (g) of tumor in nude mice inoculated with lymphocytes extracted from unfed mice (TIR)	1.41 (14.55%)	1.72 (13.13%)	1.82 (10.78%)	1.49 (13.37%)	1.20 (16.08%)	1.12 (17.65%)
Average weight of tumor in control mice (no lymphocytes)	1.65	1.98	2.04	1.72	1.43	1.36

TIR, tumor inhibition rate.

0.000). Unprimed lymphocytes (mice not fed with any mushroom products had an average tumor size of 2.04 g) also exhibited a low degree of antitumor effect. An average of 15% of the nude mice did not develop tumor and the sizes of the tumor observed were the largest compared to the two groups given the primed lymphocytes.

## DISCUSSION

The antitumor properties of the lentinan extracted using Yap and Ng's (2001) procedure was maintained. Oral administration by gavage of the AKR mice using 3 mg per mouse per day for 7 days prior to inoculations with murine lymphoma (Fig. 2) or human colon carcinoma

(Fig. 4) cells was effective to inhibiting tumor formation (Tables 1 and 2). The important point of this study is that this is the first investigation whereby the lentinan was given orally rather than parenterally as in previous reports. The results presented clearly illustrated that the oral route is a feasible, convenient and viable route of administration of the lentinan without any compromised of efficacy of the product.

Previous work reported by Yap and Ng (2001), also showed that the antitumor property was evident when lentinan was fed simultaneously with the inoculation of the murine lymphoma cells. However, the TIR was lower at 88% compared to 94% in the prefed cohort. Regression of tumors (83%) were also seen in groups that were administered the



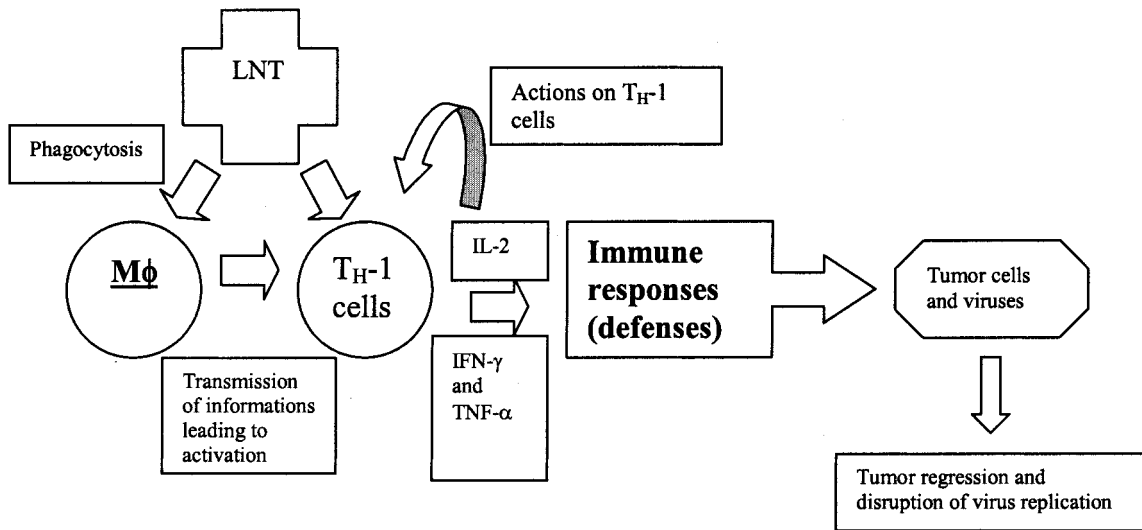


FIG. 6. Proposed hypothesis on the action of lentinan. LNT, lentinan;  $M\phi$ , macrophages; IL-2, interleukin-2; IFN- $\gamma$ , interferon- $\gamma$ ; INF- $\alpha$ , tumor necrosis factor- $\alpha$ .

lentinan 1 week after the inoculation of the tumor cells.

The antitumor properties were transferable to nude mice when primed lymphocytes from ARK who were fed with lentinan or crude mushroom extracts were inoculated into the nude mice before tumor cell inoculation (Table 3 and Fig. 5). The average weight of the induced tumor was highest in nude mice who received no lymphocytes at all. This was followed by the cohort that received unprimed lymphocytes (lymphocytes from mice not fed any mushroom products). Lymphocytes from AKR mice fed crude mushroom extracts further reduced the size of the induced tumor and increased the TIR percentage. Once again, the smallest sized tumor and highest TIR value were observed in the group that received lymphocytes from the lentinan-fed AKR mice.

The action of lentinan was reported to be mainly host-mediated (Chihara et al., 1969) and could probably take the form of either direct impact on macrophages or indirectly via lentinan-stimulated T-helper 1 cells ( $T_{H1}$ ). This resulted in the induction of many immunologic changes in the host.

A proposed hypothesis for the action of lentinan based on the observed results is

shown in Figure 6. Cytokines induction was observed in a previous study conducted by Yap and Ng (2001). An increase in the production of various cytokines such as interleukin-2, interferon- $\gamma$ , and tumor necrosis factor- $\alpha$ , were the results of the activation of  $T_{H1}$  cells. Because  $T_{H1}$  cells required macrophages for their activation, lentinan was considered to be phagocytosed by macrophages that in turn, sent this information to  $T_{H1}$  cells, thereby stimulating them. Activation of  $T_{H1}$  cells led to the increased production of various cytokines, which directed the whole immune system against the tumor cells or virus particles present.

The data from the various experiments discussed above led to the conclusion that oral administration of lentinan was effective in the suppression of tumor development. Compared to crude mushroom extracts (Tables 1 and 3), lentinan was proven to be more effective.

#### ACKNOWLEDGMENT

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Address reprint requests to:

*Mah-Lee Ng, Ph.D.*

*Department of Microbiology  
National University of Singapore*

*5 Science Drive 2  
Singapore, 117597*

*E-mail: micngml@nus.edu.sg*

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