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Immunological Studies on the Antitumor Components of the Basidiocarps of *Agrocybe cylindracea*

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The effects of cylindan, a polysaccharide isolated from the basidiocarps of *Agrocybe cylindracea*, on murine sarcoma 180 tumor and murine immune cells were examined after intraperitoneal administration. Cylindan exhibited a marked life extension effect in mice against ascite forms of sarcoma 180 and Lewis lung carcinoma at a dose of 50 mg/kg/day, although it did not show any direct cytotoxicity against sarcoma 180, X5563, and MM46 murine tumor cells. Cylindan increased numbers of bone marrow stem cells as well as peritoneal exudate cells in flow cytometry using monoclonal antibodies. The tumor bearing mice group apparently showed the increase of macrophages and cytotoxic T lymphocytes in mouse spleen cells during the early stage of tumor growth. But during the later stage, the control group decreased immune cells and cylindan restored the decreased immune cells in the tumor bearing mice to the normal level. In non-specific immune response, cylindan stimulated the bacterial phagocytosis and acid phosphatase production in macrophages. It also activated components of the alternative complement pathway and natural killer activity against YAC-1 lymphoma. In humoral immunity, cylindan had a mitogenic effect against splenocytes and increased the number of plasma cells as token of stimulation of the differentiation of B lymphocytes. In cellular immunity, cylindan restored the depressed response of delayed type hypersensitivity in the tumor bearing mice to 60% of the normal level and increased the interleukin-2 (IL-2) responsiveness in the IL-2 dependent CTLL-2 cells. These results suggest that cylindan did not show direct cytotoxic effects on tumor cells but restored the decreased immune response of the tumor bearing mice.

Key words : *Agrocybe cylindracea*, Cylindan, Polysaccharide, Monoclonal antibody, Flow cytometry, Phagocytosis, Cellular immunity, Humoral immunity

INTRODUCTION

There are many studies on biological response modifiers derived from microorganisms, especially polysaccharides of basidiomycetes (Chang *et al.*, 1988 ; Cho *et al.*, 1988; Kim *et al.*, 1987), and on restoration of the decreased immune response because of tumor burden. These polysaccharides of basidiomycetes are being actively studied on mechanisms of their action as an immunomodulator (Mizoguchi *et al.*, 1987; Sakagami *et al.*, 1988; Tadafumi *et al.*, 1987). *Agrocybe cylindracea* is an edible mushroom which was newly cultivated by Agricultural Science Institute. We had isolated the antitumor polysaccharides from its basidiocarps supplied by the Institute, and named it "cylindan" in the previous reports (Hyun *et al.*, 1996; Kim *et al.*, 1991). Since this polysaccharide is an attractive component in view of biological response modifiers, we examined its antitumor and immunomodulatory activity in mice. In this study we describe effects of cylindan on murine tumor cells and immune cells.

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MATERIALS AND METHODS

The reagents, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), fluorescin isothiocyanate (FITC), conjugated goat anti-mouse polyclonal immunoglobulin, ferricytochrome C IV, human serum, zymosan A, carboxy fluorescein diacetate (c'FDA), concanavalin A (Con A), interleukin-2 (IL-2) were purchased from Sigma Chemical Co. (St. Louis, Mo). Lewis lung carcinoma, Ehrlich carcinoma, EL-4 leukemia, MM46 carcinoma, X5563 plasmacytoma, YAC-1 lymphoma, and CTLL-2 cell lines were supplied by Dong-A Pharmaceutical Co., Choongnam Univ., Osaka Univ., and Hospital of Radiotheraphy. Anti Mac-1, Anti CD4, and Anti CD8 antibodies were purchased from ATCC. FITC conjugated goat $F(ab')_2$ anti-rat Ig antibody from TAGO Immunological Co., fetal calf serum (FCS), fetal bovine

serum (FBS) from Flow Lab. Co., lymphocyte separation medium (LSM) from ICN Biochemical Co., lipopolysaccharide (LPS) from Difco Co., [methyl-^{3}H]thymidine from Amersham Co., and lipoluma from Luma Co. were used.

Antitumor assay of polysaccharide

After collection of the ascitic fluid from the mice bearing tumor cells, such as sarcoma 180, mouse Lewis lung carcinoma, Ehrlich carcinoma, and mouse EL-4 leukemia tumor cells were washed and diluted to 5×10^6 cells/ml and each 0.1 ml was inoculated into mouse ascite. Each 0.1 ml of cylindan solution was intraperitoneally injected 24 hr after inoculation, once daily for 10 days, and then mortality for 60 days was observed. The survival time and the percentage were compared to those of the control group.

Cytotoxic activity *in vitro*

Trypan blue dye exclusion test: Sarcoma 180 cells (1×10^5 cells/ml) were cultured in RPMI medium containing 10% fetal calf serum in a CO_2 incubator for 24, 48, 72 hr with or without cylindan. After stainning with 0.2% trypan blue in phosphate buffered saline (PBS), cell counting was carried out using a hemocytometer (Richert Jung Co., USA).

MTT assay: It was done according to the method described previously (Mosmann *et al.*, 1983; Carmicael *et al.*, 1987). Mouse MM 46 adenocarcinoma, X5563 plasmacytoma, and Sarcoma 180 cells were cultured in a 96-well plate in RPMI 1640 medium containing fetal calf serum in a CO_2 incubator for 48 hr with 0, 50, 200, 500 g/ml of cylindan, and 20 μl of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) in PBS was added, then washed after the mixture was incubated. Cell viability was measured with a microplate reader (Flow Lab. Co., USA) at 540 nm after adding 150 μl of dimethylsulfoxide and vortexing.

Effects on the number of peritoneal exudate cells

Male mice of ICR, C3H/He, and C57BL/6 strains were injected intraperitoneally with cylindan solution or saline (control). The mice were sacrificed at 1, 2, 4, 7 day after injection, and peritoneal exudate cells (PECs) were obtained with Hank's solution from the peritoneal cavity of the mice. The cells were stained with Turk's reagent, and the number of PECs was counted with a hemocytometer (Maeda *et al.*, 1973).

Effects on mouse bone marrow cells

Colony stimulating factor assay: To investigate effects of cylindan of *A. cylindracea* on mouse bone marrow cells, colony stimulating factor assay was done according to the methods of Kino *et al.* (Kino *et al.*, 1987). After mice of BALB/c strain were sacrificed by cervical dislocation, femur was aseptically removed, and bone marrow cells by flushing the opened end of the femur with Hank's balanced salt solution (HBSS) from a syringe (26 G) were collected and centrifuged. The cells were washed with Eagle's minimum essential medium (EMEM) containing 20% FCS after centrifugation and were suspended (3×10^6 cells/ml). After addition of 50 μl of the cell suspension, 200 μl of cylindan solution and 950 μl of 2× EMEM to 800 μl of 1% agar solution in sterile 5 ml cap tube at 47°C, the mixture was vortexed and placed on the tissue culture dish, and it was incubated at 37°C for 7 days after it was solidified at room temperature. Colony number was counted under an inverted microscope at 40 x.

Effects on mouse splenocytes

Flow cytometry analysis of immune cells with direct stainning method: Sarcoma 180 cells were inoculated to ICR mice. After 24 hrs, cylindan was administered intraperitoneally once daily for 10 consecutive days. The spleen was removed on the day 7, 10, 14, 21, 28, and cut to pieces and squeezed through the screen (Handson *et al.*, 1989). The obtained spleen cells were washed 3 times with 2.5% FBS and PBS, and the cell concentration was adjusted to 1×10^7 cell/ml. Fifty μl of FITC conjugated goat anti-mouse polyclonal immunoglobulin solution was added to 100 μl of the adjusted cell suspension. It was washed 3 times with PBS containing 2.5% FBS after incubation on the ice bath for 30 min. Immunofluorescence stained cells were analyzed by flow cytometry after resuspension in 0.5 ml of PBS containing 2.5% FBS.

Analysis of immune cells with indirect stainning method: Spleen cell suspension was prepared by the above method. Fifty μl of the first antibody (anti Mac-1, anti CD4, anti CD8) was added to 100 μl of cell suspension and incubated for 30 mins at 4°C. After washing 3 times with PBS containing 2.5% FBS, 50 μl of FITC conjugated goat $F(ab)_2$ of anti-rat immunoglobulin solution was added and incubated on an ice bath for 30 min (Janossy *et al.*, 1987). It was washed 3 times and resuspended in 0.5 ml of 2.5% FBS and PBS. Immuno-stainned cells were analyzed by flow cytometry.

Effects on activation of macrophages

Bacterial phagocytosis assay: After C3H/He mice were sacrificed and peritoneal exudate cells were collected, 1 ml of PECs in RPMI 1640 (4×10^6 cells/ml) were added to 24-well plates. The nonadherent cells were removed after incubation at 37°C for 2 hr. Cyl-

indan was added to adherent macrophages and incubated at 37°C for 5 hr and it was further incubated at 37°C for 3 hr. after addition of 100 μl of *Serratia marsecence* suspension (1×10^7 cell/ml). The supernatant was diluted up to $1/10^3$ with PBS and the precipitate was washed and diluted up to 1/20 with PBS. They were plated to agar media and incubated at 37°C for 24 hr and the number of bacterial colony was counted (Ha *et al.*, 1992).

Assay of acid phosphatase activity in macrophages: Normal and tumor bearing mice were administered *i.p.* with cylindan once daily for 5 days. After completion of administration, PECs were collected from the mice with ice cold PBS. The cells were washed twice with RPMI 1640 medium and these cells suspended in RPMI 1640 medium were placed on a 12-well plate, cultured at 37°C for 1 hr in a CO_2 incubator, and non-adherent cells were removed by washing with HBSS. The adherent cells were collected with a rubber policeman, washed twice, and then resuspended in RPMI 1640 medium. The cell suspension in a test tube was centrifuged and the precipitate was added with 0.1 ml of 0.1% Triton X100, 0.5 ml of *p*-nitrophenyl phosphate as a substrate and 0.4 ml of 0.2 M citrate buffer (pH 5.0). The mixture was incubated at 37°C for 1 hr and 1 ml of 0.2 M borate buffer (pH 9.8) was added, then optical density at 405 nm was measured (Suzuki *et al.*, 1989). Activation of macrophages was calculated as follows: Acid phosphatase activity (*p*-nitrophenol μmol/10^6 macrophages/60 min.)=1.15× OD at 405 nm.

Measurement of superoxide anion: Cylindan was administered *i.p.* daily for 5 days. Then peritoneal macrophages on 24 hr after the administration were obtained as described above, and 1.5 ml of PBS containing 10 mM glucose, 80 μM ferricytochrome C and 0.2 mg/ml opsonized zymosan were added into each well of macrophages and the mixture was incubated at 37°C for 30 min. The reaction mixture was centrifuged at 1200 rpm for 10 min and the optical density of the supernatant was measured at 550 nm (Ito *et al.*, 1983) and the amount of SOA from macrophages was calculated as follows: SOA (nmol/10^6 macrophage/90 min.)=15.87× O.D at 550 nm.

Effects on alternative complement pathway

Cylindan in 0.05 M gelatin veronal buffered saline (GVB: gelatin 1 g, NaCl 8.65 g, sodium barbiturate 2.06 g/L, pH 7.4) EGTA-Mg^{++} solution was added to 0.4 ml of human whole serum as a complement source, and then 0.3 ml of 0.01 M GVB-EGTA-Mg^{++} solution and 1×10^7 rabbit RBC were added after incubation at 37°C for 1 hr. After further incubation for 1 hr was done, 2 ml of 0.01 M GVB-EDTA solution was added, and the mixture was centrifuged. Then complement consumption was checked by measuring optical density of the supernatant at 414 nm (Adachi *et al.*, 1990).

Effects on activity of natural killer cells

Spleen cells were used as an effector cell and YAC-1 lymphoma was used as a target cell. Cylindan was administered *i.p.* daily for 3 days and then the splenocytes on 24 hr after completion of administration were obtained as described above. The obtained splenocytes were suspended at a concentration of 2×10^7 cells/ml and then mixed gently with lymphocyte separation medium at a 2:1 ratio. And also, YAC-1 lymphoma cells were suspended at a concentration of 2×10^5 cells/ml in a 50-ml tissue culture flask after washing with HBSS and centrifugation and incubated in a CO_2 incubator at 37°C for 24 hr. One ml of c'FDA in acetone was added to YAC-1 cell of log phase and the cells were incubated at 37°C for 30 min and washed 3 times. The obtained c'FDA-YAC (1×10^7 cell/ml) suspension was gently mixed at the ratios of 1:50, 1:100 and 1:200 with already prepared splenocytes in lymphocyte separation medium. It was analyzed by flow cytometry after incubation at 37°C for 3 hr. NK cell activity was calculated as follows: NK cell activity (%)=CT-TE/CT×100. CT: number of the target cell labeled with c'FDA at 0 hr after mixing. TE: number of the target cell labeled with c'FDA at 3 hr after mixing.

Effects on B lymphocytes

Morphological changes of cultured splenocytes: The spleen obtained from mice was homogenized with ice cold HBSS and loaded on the lymphocyte separation medium. After centrifugation at 2000 rpm for 20 min the cells on the interface between the lymphocyte separation medium and HBSS were collected. These splenocytes were washed 3 times and counted. Cylindan was added to the splenocytes obtained (10^4 cell/ml) and the mixture was incubated at 37°C for 48 hr and then 100 μl of the cell suspension was transferred to a clean slide glass and centrifuged at 750 rpm for 3 min by cytospin. The cells were stained with 3 drops of Giemsa solution, and rinsed and dried, then morphological changes were observed by microscopy (× 400).

Mitogenicity: Con A, lipopolysaccharide and cylindan respectively were added to the splenocytes (10^4 cell/ml) obtained as described above and incubated at 37°C for 36 hr. Aliquots of 1 μCi of [methyl-^{3}H]thymidine were added to each well and incubated at 37°C for 12 hr. The cells were harvested onto glass membrane filter using a multiple cell harvester. The filtrate was added to a vial containing 5 ml of lipoluma solution. [methyl-^{3}H]Thymidine uptake was measured by β-liquid scintillation counter (Yodomae *et al.*, 1979).

Assay of alkaline phosphatase activity: Cylindan or lipopolysaccharide was added to the splenocyte obtained as described above and incubated at 37°C for 48 hr. 0.02 M *p*-Nitrophenyl phosphate in 50 mM sodium carbonate buffer was added to the splenocyte pellets obtained by centrifugation and incubated at 37 °C for 1 hr. The reaction was stopped by addition of 0.3 N NaOH, and optical density at 405 nm was measured (Ohno *et al.*, 1986). Alkaline phosphatase activity (*p*-nitrophenol μmol/10^6 lymphocyte/60 min.) was calculated as follows: Alkaline phosphatase activity= 1.15×O.D at 405 nm.

Number of plaque forming cells: Cylindan solution was injected *i.p.* to mice once daily for 5 days. At 48 hr after completion of administration, the mice were immunized by injecting 1×10^7 sheep red blood cells (SRBC) *i.p.* into peritoneal cavity. After 5 days, the splenocytes (10^7 cell/ml) were obtained as described above. 50% SRBC in PBS, complement and agar solution at 47°C were added to the splenocytes and well mixed. 150 μl of the mixture was placed on the culture dish. A microscopic cover glass was overlayed on the mixture and incubated at 37°C for 4 hr. Number of the hemolytic plaque forming cells was counted (Cunningham *et al.*, 1973) and calculated by a formula of PFC/10^6 spleen cell=N/C.Vm.A×10^6; PFC/total spleen cell=PFC/10^6 spleen cell×C×Vt/10^6, A= 150/550, N=number of plaques observed in one cover glass, C=count of spleen cells in 1 ml of spleen cell suspension, Vm=volume of mixture filled into one cover glass (ml), Vt=volume of total spleen cell suspension (ml).

Effects on T lymphocytes

Mitogenicity of thymocytes: The thymus of the mice was homogenized with ice cold HBSS and loaded on the lymphocyte separation medium. After centrifugation at 2000 rpm for 20 min, the cells on the interface between lymphocyte separation medium and HBSS were collected. These thymocytes were washed 3 times and counted. Con A, lipopolisaccharide and cylindan respectively were added to the thymocytes (10^4 cell/ml) and incubated at 37°C for 36 hr. Aliquots of 1 μCi [methyl-^{3}H]thymidine were added to each well and incubated at 37°C for 12 hr. The cells were harvested onto glass membrane filter using a multiple cell harvester. The filtrate was added to a vial containing 5 ml of lipoluma solution. [Methyl-^{3}H] thymidine uptake was measured by β-liquid scintillation counter.

Delayed type hypersensitivity: Sarcoma 180 cells were inoculated *s.c.* into the left groin of mice. After 24 hr cylindan was administered *i.p.* once daily for 5 days. After the last sample injection, the mice were immunized by injecting 5×10^5 SRBC *i.v.* into the tail vein. After 5 days, DTH reaction was elicited by injection of 5×10^5 SRBC *s.c.* into the right-hind footpad. Footpad swelling after 24, 48, and 72 hr was measured with a microcalipers (Lagrange *et al.*, 1974).

IL-2 responsiveness: CTLL-2 cells, which were IL-2 dependent cell line, were well washed 3 times with RPMI and suspended to each well (1×10^4 cells). Cylindan and 1 unit/ml of IL-2 were added and incubated at 37°C for 20 hr. Aliquots of 1 μCi of [methyl-^{3}H]thymidine were added to each well and incubated at 37°C for 4 hr. The CTLL-2 cells were harvested onto glass membrane filter using a multiple cell harvester. The filtrate was added to a vial containing 5 ml of lipoluma solution. [Methyl-^{3}H]thymidine uptake was measured by β-scintillation counter.

RESULTS AND DISCUSSION

Antitumor activity

Cylindan showed remarkably high antitumor activity against the ascite form of sarcoma 180, Lewis lung carcinoma, but showed no activity against Ehrlich carcinoma and EL-4 leukemia (Table I).

Cytotoxic activity

Cylindan did not show any cytocidal effect in trypan blue dye exclusion test (Fig. 1) and MTT assay (Table II).

Effects on PECs

When PECs were counted after administration of cylindan *i.p.* at a dose of 50 mg/kg/day, PECs increased 2 fold and lasted for 2-3 days in the mice of ICR, C3H/He strain, compared with those of the control, but had no difference in the mice of C57BL/6 (Fig. 2).

Table I. Effects of cylindan on ascitic forms of sarcoma 180, Lewis lung carcinoma, Ehrlich carcinoma and EL 4 leukemia

Tumor	Group	Dose (mg/kg/day)	Mean survival time (day, mean±S.E.)	T/C (%)*
S 180	Control	---	22.4±2.1[a]	100.0
	Cylindan	20	37.5±5.5[b]	165.6
		50	40.8±3.3[c]	182.3
LLC	Control	---	15.4±4.0[d]	100.0
	Cylindan	50	21.9±1.5	142.3
		100	24.9±4.8[e]	161.8
Ehrlich	Control	---	22.1±3.0	100.0
	Cylindan	50	24.7±2.8	116.0
EL 4	Control	---	21.2±0.7	100.0
	Cylindan	50	21.0±1.4	99.0

$$*T/C\ (\%)=\frac{\text{Mean survival time of treated group}}{\text{Mean survival time of control group}}\times100$$

a vs b, c and d vs e : $p<0.05$ (Ducan's test)

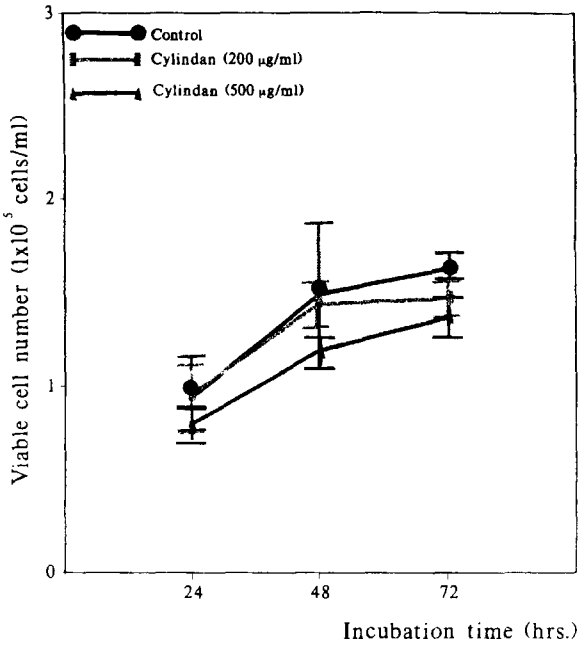

Fig. 1. Effects of cylindan on the viability of sarcoma 180 cells *in vitro*.

Table II. Direct effects of cylindan on viability of tumor cells *in vitro*

Tumor	Dose (μg/ml)	Average viability of tumor cells* (%, mean±S.E.)
Sarcoma 180	50	83.0±12.9
	200	81.8±10.2
	500	79.3±10.4
X 5563	50	94.5±14.7
	200	89.6±13.4
	500	80.5±11.1
MM 46	50	89.3±11.5
	200	87.5±12.4
	500	78.5±11.7
LLC 50	50	99.1±15.5
	200	85.4±12.3
	500	74.9± 9.1

*Average viability of tumor cell (%) =

$$\frac{\text{O.D. of treated group}}{\text{O.D. of control group}} \times 100$$

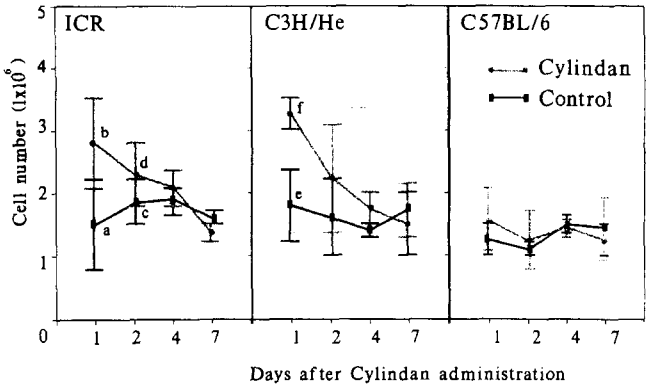

Fig. 2. Effects of cylindan on the peritoneal exudate cells in ICR, C3H/He and C57BL/6 mice strain. a vs b, c vs d, e vs f: $p<0.05$ (Student's t-test).

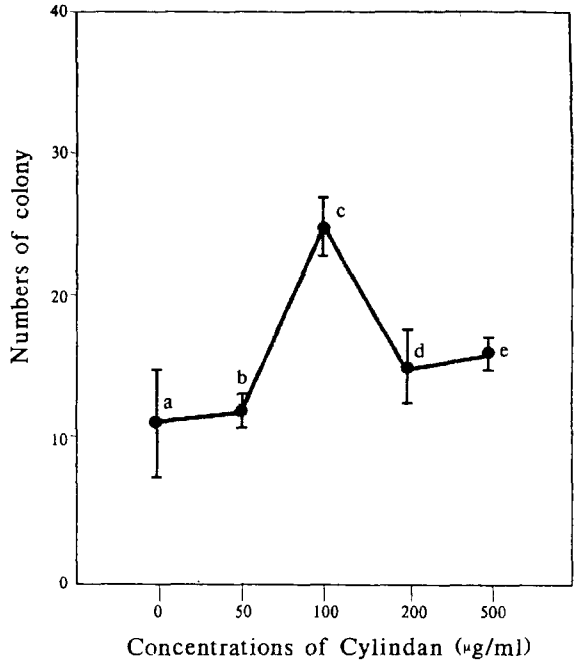

Fig. 3. Effects of cylindan on colony formation of the murine bone marrow cells. c vs a, b, d, e: $p<0.05$ (Duncan's test).

Effects on colony formation of bone marrow cells

Fig. 3 shows the colony formation inducing activities on the bone marrow of the mice injected *i.p.* with cylindan. Colony formation increased 2.5 fold at 100 μg/ml of cylindan, compared with that of the control group.

Effects on mouse splenocytes

To determine effects of cylindan on mouse splenocytes in the tumor bearing mice, the number of Mac-1 positive cells for macrophage, CD4 positive cells for helper T lymphocyte, CD8 positive cells for cytotoxic T lymphocytes, Ig positive cells for B lymphocytes were counted using a flow cytometer. The ratio of Mac-1 positive cells to Mac-1 negative cells was decreased after inoculation of tumor cells for 7 days in the control group, but it was increased to 45% in the treated group (Fig. 4). The ratio of CD4 positive cells was lasted as normal level in the treated group, but decreased once in the control group (Fig. 5). The ratio of CD8 positive cells to CD8 negative cells was decreased after inoculation of tumor cells for 14 days in the control group, but it was slightly increased in the treated group (Fig. 6). The ratio of Ig positive cells to Ig negative cells had no difference to 14 days in the control and the treated group, but it remained at the normal level in the cylindan treated group although it was decreased in the control group without treatment

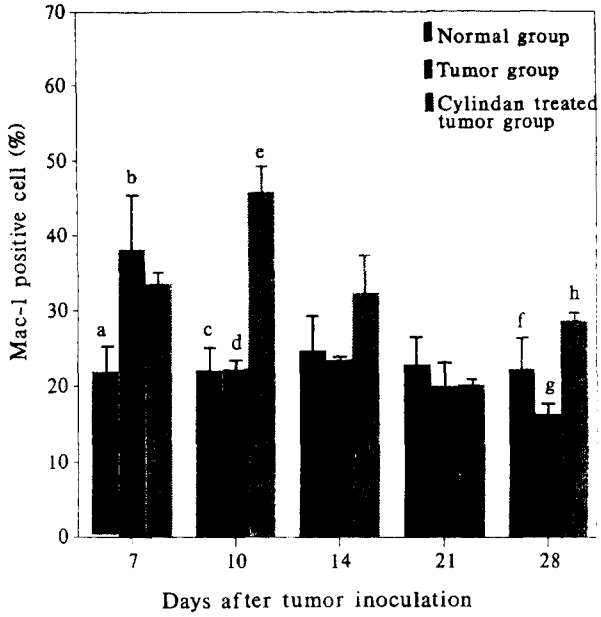

Fig. 4. Effects of cylindan on macrophages in murine splenocytes. a vs b, e vs c, d and h vs f, g: $p<0.05$ (Duncan's test)

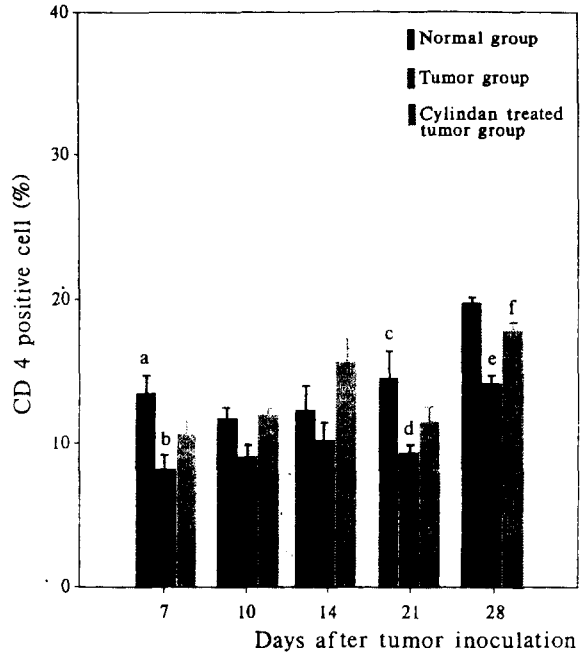

Fig. 5. Effects of cylindan on helper T lymphocytes in murine splenocytes. a vs b, c vs d and e vs f: $p<0.05$ (Duncan's test).

after 14 days (Fig. 7).

Effects on activation of macrophages *in vitro*

To determine activation of macrophages, the phagocytic activity of peritoneal macrophages against *Seratia marsecense*, and acid phosphatase and superoxide anion (SOA) release in peritoneal macrophages were

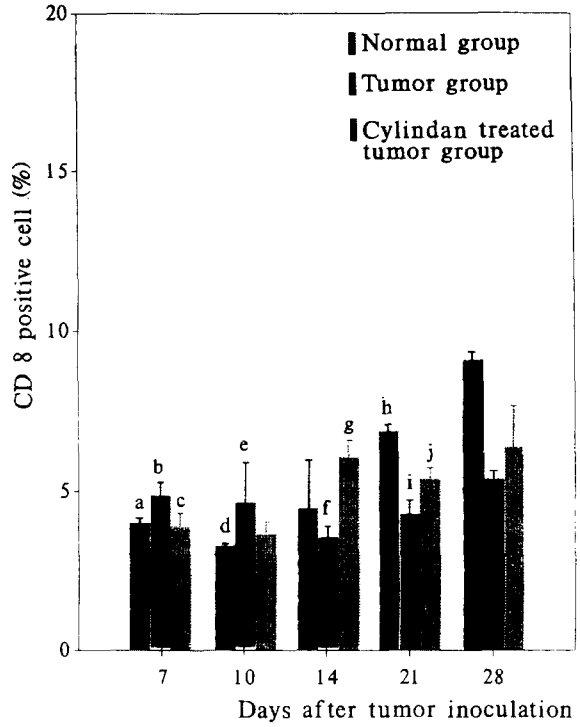

Fig. 6. Effects of cylindan on cytotoxic T lymphocyte in murine splenocytes. b vs a, c, d vs e and f vs g, h vs i, j: $p<0.05$ (Duncan's test).

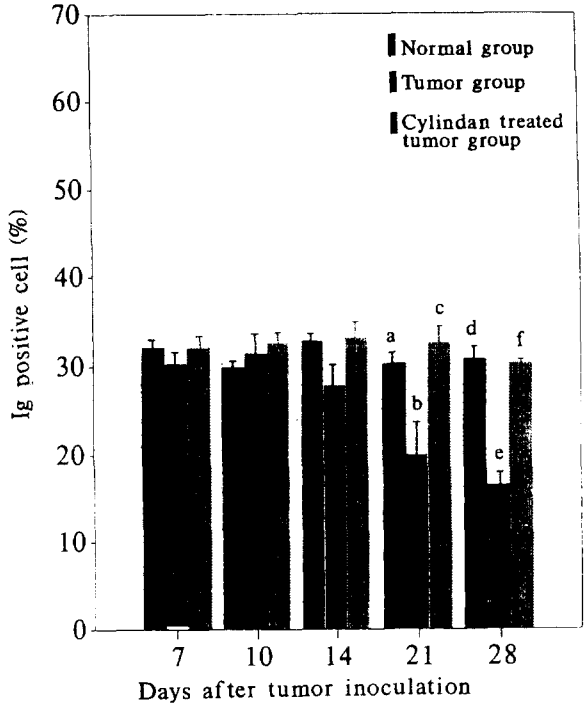

Fig. 7. Effects of cylindan on B lymphocyte in murine splenocytes. b vs a, c and e vs d, f: $p<0.05$ (Duncan's test).

measured. The phagocytic activity was increased twice, compared with that of the control, by treatment with 50 μg/ml of cylindan (Fig. 8). And cyl-

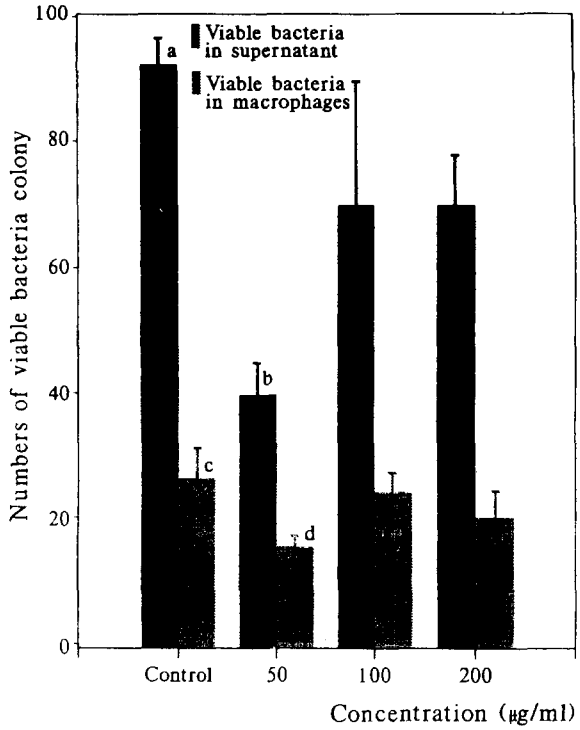

Fig. 8. Effects of cylindan on the phagocytic activity of peritoneal macrophages against *Seratia marcescens*. a vs b and c vs d: p<0.05 (Duncan's test).

Table III. Effects of cylindan on release of acid phosphatase in peritoneal macrophages of normal and tumor bearing ICR mice

Mice	Group	Enzyme activity[1] (mean ± S.E.)	Stimulation[2] index
Normal	Saline	0.93±0.04[a]	1.00
	Cylindan	1.98±0.01[b]	2.13
	Saline	0.80±0.19[c]	0.86
Tumor	Cylindan	1.99±0.01[d]	2.14

[1] *p*-nitrophenol μmol/10^6 macrophages/60 mins.=1.15×O.D. at 405 nm

[2] Stimulation index = $\frac{\text{Enzyme activity of treated group}}{\text{Enzyme activity of control group}}$

a vs b, d and c vs b, d: p<0.05 (Duncan's test)

indan enhanced the release of acid phosphatase about twice, compared with that of the normal group (Table III). And cylindan did not show any significant effect on the release of SOA, although the release of SOA in peritoneal macrophages was decreased in the tumor bearing mice compared with the normal group (Table IV).

Effects on alternative complement pathway

Activation of alternative complement pathway was shown at the concentrations of 2.5 and 5.0 mg/ml of cylindan (Fig. 9).

Table IV. Effects of cylindan on release of superoxide anion in peritoneal macrophages of normal and tumor bearing ICR mice

Mice	Group	SOA release[1] (mean±S.E.)	Stimulation[2] index
Normal	Saline	7.72±0.06[a]	1.00
Tumor	Cylindan	8.10±0.20[b]	1.05
	Saline	6.82±0.30[c]	0.88
	Cylindan	7.61±0.17[d]	0.99

[1] O^{2-} nmol/10^6 macrophages/90 mins.=15.87×O.D. at 550 nm

[2] Stimulation index = $\frac{\text{SOA amount of treated group}}{\text{SOA amount of control group}}$

[c] vs a, b, d: p<0.05 (Duncan's test)

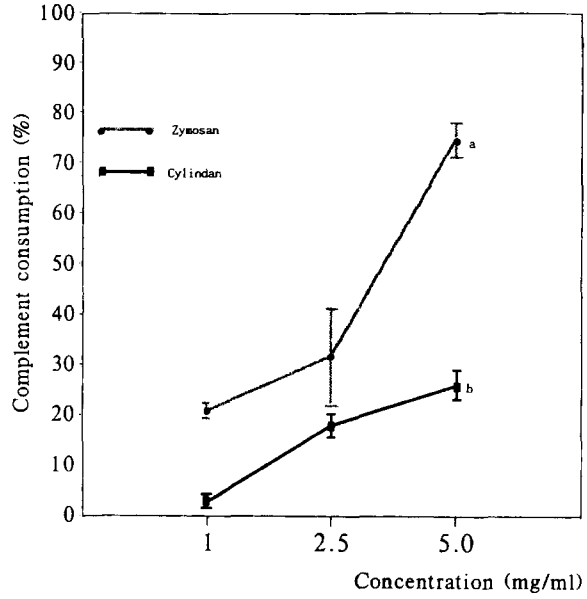

Fig. 9. Effects of cylindan on the alternative complement pathway. a vs b: p<0.05 (Student's t-test).

Effects on natural killer cell activity

When spleen cells as an effector cell and YAC-1 lymphoma as a target cell were used for determination of natural killer cell activity, cylindan enhanced the natural killer activity of spleen cells (Fig. 10).

Effects on B lymphocytes

Effects of cylindan on activation of B lymphocytes was investigated by microscopy, mitogenic activity assay on splenocytes and alkaline phosphatase assay. Size of the cell and the nucleus was significantly increased in the splenocytes by treatment with cylindan, compared with those of the control (data not shown). And DNA synthesis was increased 2.5 fold in the splenocytes treated with 200 μg/ml of cylindan (Fig. 11). Activity of alkaline phosphatase which was an indicator of differentiation of B cells was increased 1.3 fold in the splenocytes treated with cylindan, while

activity of alkaline phosphatase was increased 1.6 fold in the splenocytes treated with LPS as a positive control (Table V). And the number of plasma cell which was increased after differentiation of B lymphocytes was also increased by the treatment with cylindan (Table VI).

Effects on T lymphocytes

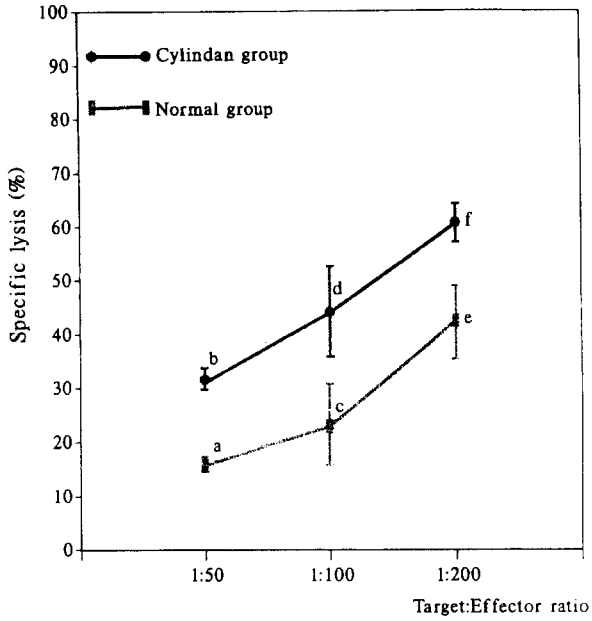

Fig. 10. Effects of cylindan on NK cell activity. a vs b, c vs d and e vs f: $p<0.05$ (Student's t-test).

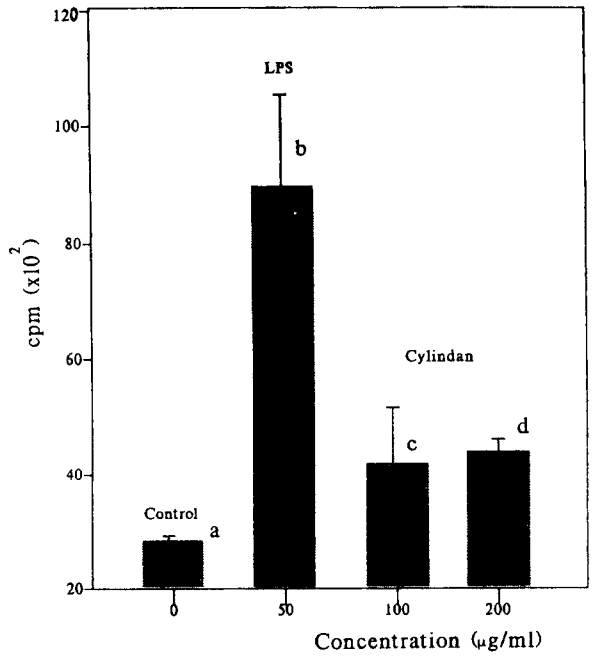

Fig. 11. Effects of cylindan on the blast formation of murine splenocytes. b vs a, c, d: $p<0.05$ (Duncan's test).

When effects of cylindan on T lymphocytes were investigated by assay of mitogenicity of thymocytes using the same method as used in mitogenicity of B lymphocytes, cylindan enhanced the uptake of [methyl-^{3}H] thymidine 1.6 fold in the treated group (Table VII). And effects of cylindan on delayed type hypersensitivity to sheep RBC were also investigated. There was no difference between the normal and the treated group. The results showed 70% restoration of decreased delayed type hypersensitivity in the group treated with cylindan, compared with that of the control group (Fig. 12). And also IL-2 responsiveness of CTLL-2 cell was increased 1.5 fold by cylindan (Table VIII).

Table V. Effects of cylindan on the alkaline phosphatase activity in the murine spleen cells

Group	Dose (μg/ml)	Enzyme activity[1] (mean±S.E.)	Stimulation[2] index
Saline	---	0.59 ± 0.01^a	1.00
LPS	50	0.94 ± 0.10^b	1.59
Cylindan	50	0.64 ± 0.03^c	1.08
	100	0.74 ± 0.03^d	1.19
	200	0.70 ± 0.04^e	1.25

[1] *p*-nitrophenol μmol/1×10^6 lymphocytes/60 mins.$=1.15\times$O.D. at 405 nm.

[2] Stimulation index = $\frac{\text{Enzyme activity of treated group}}{\text{Enzyme activity of control group}}$.

b vs a, c, d, e: $p<0.05$ (Duncan's test).

Table VI. Effects of cylindan on the hemolytic plaque forming cells in the spleen of normal ICR mice immunized with SRBC

	Control group	Treated group
Spleen index[1]	1.00	1.24
Spleen cells (1×10^6/ml)	32.4±2.70	35.8±3.10
PFC/total spleen cells (1×10^3)	43.5 ± 2.40^a	93.9 ± 5.20^b
Stimulation index[2]	1.00	2.16

[1] Spleen index = $\frac{\text{Spleen weight/body weight of treated mice}}{\text{Spleen weight/body weight of control mice}}$

[2] Stimulation index = $\frac{\text{PFC of total spleen cells in treated group}}{\text{PFC of total spleen cells in control group}}$

a vs b: $p<0.05$ (Studient's t-test)

Table VII. Effects of cylindan on the mitogenicity of murine thymus cells

Group	Dose (μg/ml)	[Methyl-^{3}H] thymidine uptake (cpm, mean±S.E.)	Stimulation* index
Control	---	1612 ± 346^a	1.00
Con A	2	9809 ± 809^b	6.08
LPS	50	2652 ± 638^c	1.60
Cylindan	100	2355 ± 184^d	1.46
	200	2610 ± 682^e	1.62

*Stimulation index = $\frac{\text{Mean cpm of the treated cultures}}{\text{Mean cpm of the control cultures}}$

a vs b, c, e and b vs c, d, e: $p<0.05$ (Duncan's test).

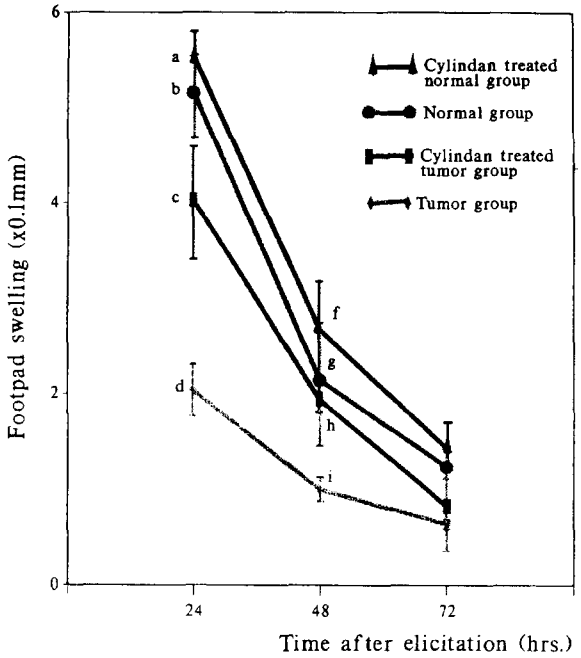

Fig. 12. Effects of cylindan on the delayed type hypersensitivity reaction. d vs a, b, c and i vs f, g: $p<0.05$ (Duncan's test).

Table VIII. Effects of cylindan on responsiveness of IL-2 dependent CTLL-2 cell to IL-2

Group	Dose (μg/ml)	[Methyl-^{3}H] thymidine uptake (cpm, mean±S.E.)	Stimulation* index
Control	---	2856±245[a]	1.00
Cylindan	50	261±287[b]	1.49
	100	3984±539[c]	1.39
	200	3707±542	1.30

*Stimulation index = $\frac{\text{Mean cpm of the treated cultures}}{\text{Mean cpm of the control cultures}}$

a vs b, c : $p<0.05$ (Duncan's test).

We previously reported that the protein-bound-polysaccharide isolated from several basidiomycetes had an antitumor activity in tumor-bearing mice (Kim *et al.*, 1979; 1987). The present study showed that cylindan isolated from *A. cylindracea* enhanced or at least normalized the cytotoxic potential of several immune cells in the tumor-bearing mice. In summary it increased the number of PECs in several tumor-bearing mice, enhanced colony formation in the bone marrow cells, increased the number of several immune cells in splenocytes, enhanced the phagocytic activity of the peritoneal macrophages, activated the alternative complement pathway, enhanced the NK activity of lymphocytes, and activated T and B lymphocytes. These results show that cylindan is an immunomodulatory rather than a direct cytotoxic substance on the tumor cells. A number of polysaccharides has been extracted from mushrooms, including *Coriolus, Lentinus* species (Hirase *et al.*, 1970) and are used to treat the compromised immune state of tumor-bearing hosts (Sakagami *et al.*, 1991). Therefore further investigations on cylindan isolated from *A. cylindracea* as an immunopotentiator, are needed.

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