The Changing Pattern of the Splenic Lymphocyte Subsets in Tumor-bearing Mice after Oral Treatment with OK-432

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The aim of this study was to investigate the influence of oral administration of OK-432 on the tumor growth of tumor-bearing mice. In addition, the changing pattern of the splenic lymphocyte subsets of tumor-bearing mice was evaluated by flow cytometry. OK-432 at a dose of 0.1, 1 or 10 KE was administered orally every 3 days or every other day for 30 days to subcutaneously Meth A tumor-inoculated mice. The tumor growth was significantly inhibited in the 1 KE every 3 days group, in the 1 KE every other day group and in the 10 KE every 3 days group. In the 10 KE every other day group, OK-432 inhibited the tumor growth on days 10 and 20, while the agent did not show a marked inhibitory effect on day 30. The percentages of splenic L₃T₄-positive cells and splenic asialo GM₁-positive cells were significantly increased in the 1 KE every other day group, while the Lyt2⁺/Thy1.2⁺ ratio was decreased. On the other hand, in the 10 KE every other day group, OK-432 showed no effect on the percentages of splenic L₃T₄-positive cells and Lyt2⁺/Thy1.2⁺ ratio on days 20 and 30. Our results suggest that the antitumor effect of oral administration of OK-432 may be correlated with the changing pattern of L₃T₄-positive cells and Lyt2⁺/Thy1.2⁺ ratio.

Key words: OK-432 — Oral administration — Antitumor effect — Splenic lymphocyte subset — Flow cytometry

A hemolytic streptococcus preparation, OK-432, was developed in 1966 as a biological response modifier (BRM) by Okamoto *et al.*¹⁾ Clinically, the agent has been found to have therapeutic effects on cancer.²⁻⁵⁾ OK-432 is administered by various routes, such as intracutaneously,²⁾ intramuscularly,³⁾ intraperitoneally,⁴⁾ and intratumorally.⁵⁾

OK-432 has recently been reported to be effective orally.⁶⁾ Regarding the mechanisms of action of orally administered OK-432, the agent has been demonstrated to be absorbed by M cells in Peyer's patches in the intestines,⁷⁾ resulting in the activation of gut-associated lymphoid tissue (GALT).⁸⁾ Then, lymphocytes which have received antigen information enter the systemic circulation via the thoracic lymph ducts and again return to the intestines (homing pattern).^{9,10)} During this circulation, the systemic immunity appears to be activated.

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MATERIALS AND METHODS

Experimental animals and tumor Eight-week-old male BALB/c mice (Shizuoka Laboratory Animal Center,

Hamamatsu) were used. The tumor used was Meth A fibrosarcoma of BALB/c origin (passaged intraperitoneally), and 5×10^5 cells were transplanted subcutaneously in the backs of the mice. The effects were evaluated by measuring the average tumor diameters ($\sqrt{\text{long} \times \text{short diameters}}$) periodically.

Oral administration of OK-432 OK-432 (Chugai Pharm. Co., Tokyo) was administered for 30 days from the day of the tumor transplantation. Single doses of 0.1 KE, 1 KE or 10 KE were dissolved in 0.5 ml of physiological saline and administered directly into the stomachs of the mice via a stainless steel cannula designed for oral administration to mice. Meth A tumor-bearing mice which were given only 0.5 ml of physiological saline served as the control group. The administration interval was either every 3 days or every other day.

Experimental schedule Inhibitory effects on tumor growth were evaluated for doses of 0.1, 1 and 10 KE in the every 3 days and every other day administration groups. Each group consisted of 20 mice. In the 1 KE and 10 KE every other day groups, five mice were killed on the 10th, 20th and 30th days after tumor transplantation, the spleen was removed and the splenic lymphocyte subsets were analyzed by flow cytometry.

Analysis of splenic lymphocyte subsets Spleens of five mice from each group were pooled, cut into 2-3 mm slices and passed through a #100 stainless steel mesh. Then, the erythrocytes were removed by treatment with 0.83% NH₄Cl-Tris buffer at 37°C for 5 min. After washing twice with phosphate-buffered saline (PBS) containing 1% fetal calf serum (FCS), cells were suspended

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at a concentration of 1×10^7 /ml in RPMI-1640 with 10% FCS for use. The mouse monoclonal antibodies used were fluorescein isothiocyanate (FITC)-labeled anti-Thy1.2 antibody (Becton Dickinson, California), FITC-labeled anti-Lyt1 antibody (Becton Dickinson), FITC-labeled anti-Lyt2 antibody (Becton Dickinson), phycoerythrin-avidin(PE)-labeled anti-L₃T₄ antibody (Becton Dickinson), and anti-asialo GM₁ antibody (Wako Pure Chemical Industries, Ltd., Osaka). The dilution rates of the antibodies were 50-, 20-, 20-, 20- and 400-fold, respectively. The cell suspension was placed in plastic tubes (100 μ l each), and 100 μ l of mouse monoclonal antibody was added. After mixing well, the mixture was reacted for 60 min at 4°C. The cells were washed twice in PBS with 1% FCS and 0.1% NaN₂. The anti-

bodies were then resuspended in 1.0 ml of PBS with 1% FCS and 0.1% NaN₂. Asialo GM₁ was analyzed by the indirect method. The 2nd antibody, FITC-labeled goat anti-mouse IgG antibody (Cooper Biomedical, New York) was diluted 20-fold, 100 μ l was added to the mixture and the reaction was performed at 4°C for 30 min. After washing twice in PBS with 1% FCS and 0.1% NaN₂, the cells were suspended in 1.0 ml of PBS with 1% FCS and 0.1% NaN₂. The cell suspension was kept in ice water shielded from light, and flow cytometry (Ortho-Spectrum III) was performed within 2 h.

Statistical evaluation Each value represents the mean $\pm$ SD of quintuplicate assays. Statistical significance of differences was assessed by using Student's *t* test, with $P < 0.05$ as the criterion.

Fig. 1. The effect of oral administration of OK-432 on the tumor growth of Meth A tumor-bearing mice. OK-432 was administered for 30 days from the day of the tumor transplantation. The interval of administration was either every 3 days (A) or every other day (B). The control group (●) received only 0.5 ml of physiological saline. Single doses of 0.1 KE (△), 1 KE (○) or 10 KE (□) were administered directly into the stomachs of the mice. Significant differences from the control group: a), $P < 0.05$; b), $P < 0.01$.

RESULTS

Inhibitory effects on tumor growth In the every 3 days group, tumor growth was significantly inhibited by oral administration of OK-432 in a dose-dependent manner (on the 30th day: 1 KE, $P < 0.05$; 10 KE, $P < 0.01$) (Fig. 1-A). In the every other day group, tumor growth was markedly inhibited by OK-432 at the dose of 1 KE (on the 20th day: 1 KE, $P < 0.05$, on the 30th day: 1 KE, $P < 0.01$). The agent at the dose of 10 KE reduced the tumor growth on the 20th day, but showed no inhibitory effect on the 30th day (on the 20th day: 10 KE, $P < 0.05$, on the 30th day: 10 KE, $P > 0.05$) (Fig. 1-B).

Flow cytometric analysis of splenic lymphocytes subsets The percentages of Thy1.2-positive cells, Lyt1-positive cells and L_3T_4 -positive cells among the splenic lymphocytes obtained from tumor-bearing mice treated with saline (control group) were decreased when compared with normal mice treated with saline. In the 1 KE every other day group, the percentages of these cells were increased on the 10th, 20th and 30th days (Thy1.2-, Lyt1- and L_3T_4 -positive cells: on the 10th, 20th and 30th days, $P < 0.01$). In the 10 KE every other day group, the

percentages of these cells were enhanced on the 10th day, while there was no significant effect on the 20th and 30th days (Thy1.2-, Lyt1- and L_3T_4 -positive cells: on the 10th day, $P < 0.01$; on the 20th and 30th days, $P > 0.05$) (Table I-A, B, C).

The percentage of Lyt2-positive cells among the splenic lymphocytes obtained from tumor-bearing mice treated with saline (control group) was reduced when compared with normal mice treated with saline. In both the 1 KE and 10 KE every other day groups, the percentage of this cell type was increased on the 10th, 20th and 30th days (on the 10th, 20th and 30th days, 1 KE and 10 KE, $P < 0.01$) (Table I-D).

The percentage of asialo GM₁-positive cells among the splenic lymphocytes obtained from tumor-bearing mice treated with saline (control group) was slightly increased on the 10th and 20th days when compared with normal mice treated with saline. In both the 1 KE and 10 KE every other day groups, the percentage of this cell type was increased (on the 20th day: 1 KE, $P < 0.05$; on the 30th day: 1 KE, $P < 0.01$; 10 KE, $P < 0.05$) (Table I-E).

The $L_3T_4^+$ /Lyt2⁺ ratio was increased in the 1 KE every other day group when compared with the control group (on the 20th day, $P < 0.05$; on the 30th day, $P <$

Table I. Analysis of Splenic Thy1.2-positive Cells, Lyt1-positive Cells, L_3T_4 -positive Cells, Lyt2-positive Cells and Asialo GM₁-positive Cells after Oral Administration of OK-432

	Days	Percentages of positive cells (%)			
		Normal mice saline	Tumor-bearing mice		
			saline (control)	1 KE	10 KE
(A) Thy1.2-positive cells	10	52.8 ± 1.4	30.1 ± 2.1	45.3 ± 1.9 ^b	45.7 ± 3.2 ^b
	20	51.9 ± 2.9	16.4 ± 1.8	28.3 ± 2.2 ^b	20.7 ± 3.1
	30	51.5 ± 3.0	10.7 ± 3.1	20.8 ± 3.0 ^b	11.1 ± 2.9
(B) Lyt1-positive cells	10	40.9 ± 2.2	20.4 ± 1.5	32.0 ± 2.1 ^b	29.3 ± 4.6 ^b
	20	41.2 ± 3.2	18.7 ± 3.4	28.9 ± 3.0 ^b	19.1 ± 3.9
	30	40.7 ± 1.9	10.1 ± 2.0	20.3 ± 3.3 ^b	11.2 ± 1.9
(C) L_3T_4 -positive cells	10	30.6 ± 3.1	22.4 ± 2.2	30.1 ± 1.9 ^b	28.3 ± 2.1 ^b
	20	29.4 ± 2.0	15.7 ± 1.5	24.1 ± 2.0 ^b	16.0 ± 1.4
	30	28.8 ± 2.6	14.2 ± 2.1	25.0 ± 1.6 ^b	15.3 ± 1.2
(D) Lyt2-positive cells	10	11.9 ± 2.0	7.2 ± 0.7	10.0 ± 0.3 ^b	10.1 ± 0.4 ^b
	20	12.7 ± 1.2	5.2 ± 0.9	7.5 ± 0.5 ^b	7.0 ± 0.7 ^b
	30	13.1 ± 3.3	4.0 ± 0.7	6.1 ± 0.6 ^b	6.0 ± 0.4 ^b
(E) Asialo GM ₁ -positive cells	10	12.3 ± 1.4	13.4 ± 0.9	15.3 ± 0.8	12.2 ± 1.0
	20	11.2 ± 2.2	12.5 ± 1.3	15.0 ± 0.6 ^a	13.9 ± 0.7
	30	11.0 ± 1.9	9.2 ± 1.5	14.0 ± 0.8 ^b	13.1 ± 0.7 ^a

OK-432 at doses of 1 KE or 10 KE was administered every other day for 30 days to mice inoculated subcutaneously with Meth A tumor. Meth A tumor-bearing mice given only 0.5 ml of physiological saline served as a control group. Normal mice given only 0.5 ml of physiological saline also served. Five mice each were killed on the 10th, 20th and 30th days. The spleens were removed and the percentages of Thy1.2-positive cells, Lyt1-positive cells, L_3T_4 -positive cells, Lyt2-positive cells and asialo GM₁-positive cells were analyzed by flow cytometry. Significant differences from the control group: a), $P < 0.05$; b), $P < 0.01$.

Fig. 2. The effect of oral administration of OK-432 on the splenic lymphocyte $L_3T_4^+ / \text{Lyt } 2^+$ ratio and $\text{Lyt } 2^+ / \text{Thy } 1.2^+$ ratio of Meth A tumor-bearing mice. The interval of administration was every other day. Meth A tumor-bearing mice given only 0.5 ml of physiological saline served as a control group (●). Normal mice (■) given only 0.5 ml of physiological saline also served. Single doses of 1 KE (○) or 10 KE (□) were administered directly into the stomachs of the mice. Significant differences from the control group: a), $P < 0.05$; b), $P < 0.01$.

0.01). On the other hand, in the 10 KE every other day group, this ratio showed no significant change ($P > 0.05$) (Fig. 2-A).

The $\text{Lyt } 2^+ / \text{Thy } 1.2^+$ ratio was decreased in the 1 KE every other day group when compared with the control group (on the 20th day, $P < 0.05$; on the 30th day, $P < 0.01$). On the other hand, this ratio showed no significant change in the 10 KE every other day group ($P > 0.05$) (Fig. 2-B).

DISCUSSION

The present study has demonstrated that the oral administration of OK-432 inhibits the growth of Meth A tumor and changes the pattern of the splenic lymphocyte subsets. In the every 3 days group, the inhibitory effect on the tumor was dose-dependent; agent at the dose of 10 KE showed the most potent antitumor effect. In both the every 3 days group and the every other day group, OK-432 at dose of 1 KE showed a tumor growth-inhibitory effect that increased in proportion to the increase of the number of administrations. In the 1 KE every other day group, the percentages of $L_3T_4^+$ - and asialo GM_1 -positive cells were maintained at higher values than in the control group. Generally, $L_3T_4^+$ -positive cells are regarded as a

marker of helper/inducer T cells. Asialo GM_1 -positive cells are known to be NK cells and activated macrophages. OK-432 appears to manifest its antitumor effects by inducing various types of cytokines indirectly via the host's immune system.¹¹⁻¹⁵ The increased percentages of $L_3T_4^+$ - and asialo GM_1 -positive cells were considered to be an important mechanism of the antitumor effects of oral administration of OK-432. We have also found that oral administration of OK-432 increases NK activity (unpublished data). Furthermore, the $L_3T_4^+ / \text{Lyt } 2^+$ ratio was increased and the $\text{Lyt } 2^+ / \text{Thy } 1.2^+$ ratio was decreased. These results are likely to suggest a decrease in $\text{Lyt } 2^+$ -positive cells, which are regarded as a marker of suppressor T cells. This seems to be favorable for cancer immunotherapy.

On the other hand, in the group given oral administration of OK-432 every other day, the inhibitory effect on the tumor was not dependent on the dose of OK-432. In the 10 KE every other day group, OK-432 inhibited the tumor growth on the 10th and 20th days, but not on the 30th day. In the 10 KE every other day group, the percentages of $\text{Thy } 1.2^+$ -, $\text{Lyt } 1^+$ - and $L_3T_4^+$ -positive cells among the splenic lymphocytes were not markedly increased on the 20th and 30th days. Furthermore, the $\text{Lyt } 2^+ / \text{Thy } 1.2^+$ ratio was not decreased on the 30th day.

The changing pattern of the splenic lymphocyte subsets seemed to parallel the antitumor effects of OK-432. These results indicate that the antitumor effect of OK-432 is not always correlated with the dose and the interval of administration.

The gastrointestinal immune system includes an immunological tolerance mechanism to prevent excessive immune response to cell components originating from food, which includes many antigens, and the digestive tract.^{16,17)} There is a condition whereby no systematic immune response to orally administered antigens occurs via the gastrointestinal lymph tissue. In 1946, Chase found that when guinea pigs were administered large

amounts of picryl chloride orally, no skin hypersensitivity response to the drug occurred and he called this phenomenon "unresponsiveness."¹⁸⁾ Our results may have some relation to this phenomenon.

The results obtained in this study demonstrated that oral administration of OK-432 has an antitumor effect, which may be correlated with the changing pattern of L₃T₄-positive cells and the Lyt2⁺/Thy1.2⁺ ratio. However, this effect was not always related to the dose and interval of administration. Our study provides further background data for oral OK-432 therapy of cancer.

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