ANTITUMOR CELLS FOUND IN TUMOR-BEARING MICE GIVEN UBENIMEX

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Antitumor effector cells in spleens of tumor-bearing mice given ubenimex were investigated. The administration of ubenimex, starting 7 days after the tumor inoculation, was effective in inhibiting growth of IMC carcinoma. Spleen cells taken from these mice showed a marked suppression of the tumor growth by the WINN assay. The antitumor activity of spleen cells was reduced by treatment to remove T cells or NK cells, whereas spleen cell preparations enriched for T cells showed the strongest antitumor activity. Moreover, NK activity against YAC-1 cells was found in the spleen. These results indicate that the administration of ubenimex to IMC carcinoma-bearing mice generates cytotoxic T cells and NK cells in the spleen.

The antitumor effect of ubenimex was not observed in X-ray-irradiated and in anti-asialo GM1 serum-treated mice.

We have previously reported the immunomodulatory effect and antitumor activity of ubenimex in experimental animals[1~4]. These results indicate that the antitumor activity of ubenimex is manifested through activation of host defense mechanisms. Among antitumor effector cells, NK cells[5] and macrophages[6] were activated by ubenimex *in vitro*. However, the effector cells activated by ubenimex in a tumor-bearing host are not known, although it was shown that NK activity was increased in ubenimex-treated rats having carcinogen-induced stomach cancer[4]. Thus, we examined the effector cells in tumor-bearing mice given ubenimex.

Materials and Methods

Mice

CDF_1 mice (6~8 weeks old, female) were purchased from Charles River Japan Inc. and were maintained under specific pathogen-free conditions at 23±2°C and 55±5% humidity. They were employed for experiments at 8 to 12 weeks of age.

Ubenimex

Ubenimex was prepared and supplied by Nippon Kayaku Co., Ltd. and dissolved in saline or culture medium.

Tumor and Cell Line

IMC carcinoma was maintained in CDF_1 mice by weekly intraperitoneal injection. YAC-1 cells were maintained in RPMI 1640 containing 10% fetal calf serum.

Antitumor Activity and Tumor Neutralization Assay

CDF_1 mice were inoculated subcutaneously with 2×10^6 cells in 0.1 ml of saline and given an oral dose of ubenimex, starting 7 days after the tumor inoculation, every other day for a total of 10

doses. Antitumor activity was determined by measuring tumor size (mm^2) at weekly intervals and by weighing the tumor 30 days after tumor inoculation.

Antitumor activity of ubenimex in immuno-deficient mice was also tested. X-Ray-irradiated mice were prepared as follows: CDF_1 mice were irradiated with 200 rad of X-ray daily for 7 days before tumor inoculation. Mice with impaired NK activity were prepared by intraperitoneal injection of anti-asialo GM1 serum. CDF_1 mice were injected intraperitoneally with 5 μl of anti-asialo GM1 serum, 2 days before, and 4, 10 and 16 days after tumor inoculation. These mice were inoculated subcutaneously with 2×10^6 IMC carcinoma cells and, from 7 days thereafter, given 5 mg/kg of ubenimex every other day for a total of 10 times.

Tumor neutralization assay was performed by the WINN assay[7]. Spleen cells were taken from untreated mice or from those treated with ubenimex 30 days after the tumor inoculation. To destroy T cells or NK cells, spleen cells were incubated with diluted anti-thy 1.2 serum (Cederlane Laboratories, Ltd., Ontario, Canada) or a diluted anti-asialo GM1 serum (Wako Chemicals Co., Ltd., Tokyo), respectively, at 4°C for 30 minutes, after which complement was added and incubation continued at 37°C for 60 minutes. According to the method reported previously[8], spleen cells were incubated in a nylon wool column (Wako Chemicals Co., Ltd., Tokyo) at 37°C for 60 minutes, and non-adherent cells (nylon wool column-passed cells) were used as a T cell-enriched population. These cells were mixed with 1×10^6 IMC carcinoma cells at a ratio of 80:1 and the mixture in 0.2 ml was inoculated subcutaneously into mice. Thirty days thereafter, antitumor activity of each cell population was determined by measuring tumor weight.

NK Activity

The NK activity of spleen cells taken from mice given ubenimex 30 days after tumor inoculation was determined against YAC-1 cells according to the method described before[8]. YAC-1 cells were labeled with ^{51}Cr (New England Nuclear, Boston, Mass., U.S.A.), and 2×10^5 cells/ml were incubated with effector cells at ratios of 100:1 for 4 hours. After incubation the supernatant was collected and ^{51}Cr radioactivity was counted in a gamma counter (Aloka Co., Ltd., Tokyo). The maximum counts in target cells were determined after disruption by freezing and thawing. The mean percentage of specific cytotoxicity was calculated as follows:

$$\%\ \text{Cytotoxicity}=\frac{\text{Test count}-\text{Spontaneous count}}{\text{Maximum count}-\text{Spontaneous count}}\times100$$

Results and Discussion

As shown in Fig. 1, the administration of ubenimex resulted in marked antitumor activity against IMC carcinoma. We have previously shown that the antitumor activity of ubenimex against murine transplantable tumors such as IMC carcinoma[1] and leukemia C1498[2] is dependent on dosage and time of administration during the period of tumor growth in mice. In this case, the oral administration of ubenimex at 5 mg/kg starting 7 days after the tumor inoculation was effective in inhibiting growth of the tumor.

Thirty days after tumor inoculation into untreated mice or into those subsequently given ubenimex, spleen cells were taken and their antitumor activity was determined by the WINN assay. As shown in Table 1, spleen cells taken from mice given ubenimex showed a marked suppression of the tumor growth. On the other hand, spleen cells treated with anti-thy 1.2 serum or anti-asialo GM1 serum and complement had little antitumor activity. The spleen cells which had passed through a nylon wool column showed the strongest antitumor activity. All mice inoculated with a mixture of tumor cells and nylon wool column-passed cells were tumor-free. These results indicate that the administration of ubenimex to IMC carcinoma-bearing mice activates cytotoxic T cells and NK cells in the spleen.

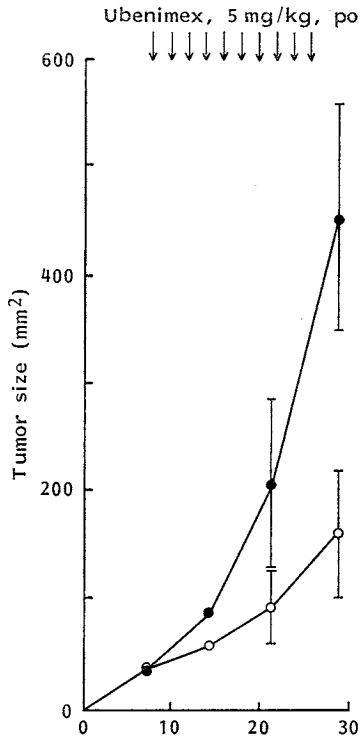
NK activity of spleen cells taken from mice given ubenimex was determined against YAC-1 cells.

Table 1. Antitumor activity of spleen cells from ubenimex-treated mice on IMC carcinoma.

Spleen cells taken from	Spleen cell treatment[c]	Tumor weight (mg±SD)	No. of tumor-free mice
Non-treated mice[a]	None	4,515±1,348	0/5
Ubenimex-treated mice[b]	None	1,996±683	0/5
Ubenimex-treated mice	Anti-thy 1.2 serum and C′	4,094±1,382	0/5
Ubenimex-treated mice	Anti-asialo GM1 serum and C′	3,833±1,864	0/5
Ubenimex-treated mice	Nylon wool-passed	0	5/5

[a] Thirty days after inoculation of 2×10^6 IMC carcinoma cells.
[b] Mice were given ubenimex (5 mg/kg, orally) from 7 days after the tumor inoculation, every other day, for a total of 10 times.
[c] Spleen cells were mixed with 1×10^6 IMC carcinoma cells at a ratio of 80 : 1.

Fig. 1. Effect of ubenimex on growth of IMC carcinoma.
○ Ubenimex, ● non-treated control.



As shown in Table 2, the administration of ubenimex at 5.0 mg/kg showed the strongest antitumor effect and resulted in the highest NK activity. The antitumor effect coincided with the NK activity of the spleen cells. As reported previously[4], ubenimex administration inhibited development of MNNG-induced stomach cancer in rats and their NK activity was increased. However, ubenimex alone neither induces interferons nor enhances NK activity in normal mice, although it augments production of interferons in BCG-sensitized mice†. It was reported that ubenimex induced production of interleukin 1 (IL-1) by macrophages[9] and enhanced production of interleukin 2 (IL-2) in spleen cell cultures stimulated with concanavalin A (Con A)[9,10] or allogeneic cells[11]. Thus, it can be considered that the high NK activity induced in tumor-bearing hosts by ubenimex treatment may be due to enhanced production of cytokines, including IL-2, which participate to activate NK cells or to inhibit suppressor factors[12] of NK activity present in tumor-bearing mice. As described above, the effect of ubenimex of NK activity also depends on its dosage level as well as the antitumor activity. It may be due to effects of ubenimex on membrane-associated events of macrophages and/or lymphocytes[13].

The antitumor effect of ubenimex in X-ray-irradiated and in anti-asialo GM1 serum-treated mice was examined, and the results were shown in Table 3. In normal mice, ubenimex showed a marked antitumor effect against IMC carcinoma, whereas the effect was not observed in either kind of im-

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Table 2. NK activity in tumor-bearing mice (IMC carcinoma) after ubenimex treatment.

Mice	Ubenimex[a] (mg/kg)	Mean weight[b] of tumor (g)	Inhibition (%)	Spleen weight (mg)	NK activity[c] (%)
Tumor-bearing	0	8.23	—	400±98	1.4±1.0
Tumor-bearing	0.5	2.09*	74.6	321±112	4.2±0.4*
Tumor-bearing	5.0	0.43**	94.8	183±75	7.7±0.5**
Tumor-bearing	50.0	4.51***	18.6	388±100	3.6±0.9***
Normal	0	—	—	97±3	9.1±2.4

[a] Ubenimex was given orally, days 7, 9, 11, 13, 15, 18, 20, 22 and 25 after tumor inoculation.
[b] Tumor weight was measured 27 days after tumor inoculation.
[c] NK activity was tested against YAC-1 cells at a ratio of 100:1.
* $P<0.01$. ** $P<0.001$. *** $P<0.05$.

Table 3. Reduction of antitumor activity of ubenimex in mice irradiated with X-ray and treated with anti-asialo GM1 serum.

Treatment of mice	Ubenimex[c]	Tumor weight (mg±SD)[d]
None	−	3,085±1,507
None	+	676±173
X-Ray-irradiated[a]	−	7,339±2,411
X-Ray-irradiated	+	6,065±2,107
Anti-asialo GM1 serum[b]	−	5,305±1,227
Anti-asialo GM1 serum	+	5,554±2,371

[a] Mice were irradiated with 200 rad of X-ray daily for 7 days before tumor inoculation.
[b] Mice were injected with 5 μl/mouse of anti-asialo GM1 serum, 2 days before and 4, 10 and 16 days after tumor inoculation.
[c] Ubenimex (5 mg/kg, po) was given a total of 10 times every other day starting 7 days after tumor inoculation.
[d] Tumor weight was measured 30 days after tumor inoculation.

muno-deficient mouse. This result indicates that the antitumor effect of ubenimex is a host-mediated event and that X-ray-sensitive cells and asialo GM1-bearing cells such as NK cells are essential for the antitumor effect in mice.

The results shown in this report indicate that ubenimex treatment activates cytotoxic T cells and NK cells in tumor-bearing mice. These cells may be generated through ubenimex-activated cytokine production and/or inhibition of suppressors present in the tumor-bearing host.

Acknowledgments

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