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DIFFERENTIAL CHEMOSENSITIVITY OF LOCAL AND METASTATIC HUMAN GASTRIC CANCER AFTER ORTHOTOPIC TRANSPLANTATION OF HISTOLOGICALLY INTACT TUMOR TISSUE IN NUDE MICE

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We have established a metastatic model of human gastric cancer using orthotopic transplantation of histologically intact tissue in nude mice, and have used this model to evaluate the effects of immunochemotherapy using OK-432, 5-fluorouracil (5-FU) and mitomycin C (MMC) against SC-1-NU, a human stomach cancer line. One-quarter or one-half maximum tolerated doses (MTDs) of 5-FU or MMC resulted in a significant reduction of stomach tumor growth, while liver metastases were not reduced, possibly due to suppression of natural killer (NK)-cell activity by both drugs. On the other hand, when combined with OK-432, half MTDs of 5-FU and MMC significantly reduced liver metastases, with synergistic reduction of stomach tumor growth, possibly reflecting a rescue of NK-cell activity by treatment with OK-432. This metastatic model of human stomach cancer shows that locally growing and metastatic tumors may have different chemosensitivities, and provides the opportunity to test both with various treatment regimens.

Although a number of regimens using combinations of chemotherapeutic drugs have been used against advanced and metastatic stomach cancer, most have demonstrated limited rates of efficacy. Moreover, even in the chemoresponsive cases, such effects are generally partial, and long-term survivors have only occasionally been reported, with death often being caused by treatment failure at metastatic sites (The Gastrointestinal Tumor Study Group, 1979, 1984, 1988). Therefore, it is essential to prevent or eliminate metastases in the treatment of stomach cancer. However, the lack of relevant animal models has impeded the development of effective treatment strategies.

Various types of human cancer have been implanted s.c. in nude mice and, after growth, usually resemble the original tumors both morphologically and biochemically. However, metastasis rarely occurred after s.c. transplantation in nude mice, even when the xenografted tumors were derived from patients with extensive metastasis, but it did occur more frequently after orthotopic transplantation of tumor-cell suspensions (Fidler, 1990). Recently, we have established metastatic models of human colon cancer (Fu *et al.*, 1991*a*; Furukawa *et al.*, 1993*c*), bladder (Fu *et al.*, 1991*b*; Fu and Hoffman, 1992), lung (Wang *et al.*, 1992), pancreas (Fu *et al.*, 1992*a*), prostate (Fu *et al.*, 1992*b*) and stomach (Furukawa *et al.*, 1993*a*) using orthotopic transplantation of histologically intact tumor tissue in nude mice. A much higher incidence of local tumor growth and metastasis was observed after orthotopic transplantation of histologically intact tissue than after orthotopic transplantation of tumor-cell suspensions from tumors of the bladder (Fu *et al.*, 1991*b*), colon (Furukawa *et al.*, 1993*c*) and stomach (Furukawa *et al.*, 1993*a*). In this orthotopic model the metastatic behavior of the transplanted gastric tumor reflects the metastatic pattern of the patient donor (Furukawa *et al.*, 1993*b*). In the present study, we utilized this metastatic model to study the efficacy of experimental immunochemotherapy against metastatic human gastric cancer.

MATERIAL AND METHODS

Mice

Male BALB/c nu/nu mice, which originated from the Central Institute for Experimental Animals (Kawasaki, Japan), were obtained from CLEA (Tokyo, Japan). They were 6–8 weeks old and weighed 20–22 g when used.

Drugs

We purchased 5-fluorouracil (5-FU) and mitomycin C (MMC) from Kyowa Hakko Kogyo (Tokyo, Japan), and OK-432, a streptococcus preparation (Okamoto *et al.*, 1967), from Chugai Seiyaku (Tokyo, Japan).

Human stomach cancer xenograft

SC-1-NU, a poorly differentiated human stomach adenocarcinoma xenograft established at Nagoya University, was provided by the Central Institute for Experimental Animals and maintained by serial transplantation into nude mice at Keio University School of Medicine.

Orthotopic cancer-tissue transplantation

Gastric tumor tissues were transplanted orthotopically in nude mice as reported previously (Furukawa *et al.*, 1993*a*, 1993*b*). Tumors in the exponential growth phase in nude mice were resected aseptically, necrotic tissues were cut away and the remaining healthy tumor tissues were scissor-minced into pieces of approximately 3 × 3 × 3 mm in Hanks' HBSS. Each piece was weighed and adjusted to 50 mg with scissors.

Mice were anesthetized with a 2.5% solution of a mixture of 2,2,2-tribromoethanol (Aldrich, Milwaukee, WI) and *tert*-amylalcohol (Wako, Osaka, Japan) (1:1). An incision was then made through the left upper abdominal pararectal line and peritoneum. The stomach wall was carefully exposed and a part of the serosal membrane, about 2 mm in diameter, in the middle of the greater curvature of the glandular stomach was mechanically injured with scissors. A tumor piece was then fixed on each injured site of the serosal surface with a 6-0 Dexon (Davis-Geck, Manati, PR) transmural suture. The stomach was then returned to the peritoneal cavity, and the abdominal wall and the skin were closed with 6-0 Dexon sutures. Animals were kept in a sterile environment.

Experimental immunochemotherapy

On day 7 after orthotopic transplantation, mice were randomized into control and treated groups. 5-FU and MMC, dissolved in 0.2 ml of physiological saline solution, were administered intraperitoneally (i.p.) as a bolus. The doses of the drugs used in this study were 180, 90 and 45 mg/kg for 5-FU and 6, 3

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and 1.5 mg/kg for MMC, which were equivalent to maximum tolerated doses (MTDs), half MTDs and quarter MTDs in nude mice, respectively, as determined in our previous studies (Kubota *et al.,* 1988). OK-432 was administered i.p. every day for 5 days, from day 5 to day 9, at a dose of 1 Klinische Einheit (KE) per mouse. The different treatments were randomized in all the transplanted animals at the same time. Mice were observed every day and killed on day 28 after tumor transplantation. The tumors growing on the stomach and the liver were removed from each mouse, weighed, and then examined histologically after careful macroscopic examination.

Natural killer (NK)-cell activity assay

NK-cell activity was determined by a short-term ^{51}Cr-release assay using spleen cells as effector cells and YAC-1, a murine lymphoma cell line, as the target. Exponential growth of YAC-1 cells was maintained in RPMI-1640 containing 10% FCS (GIBCO, Grand Island, NY), 100 U/ml penicillin and 100 μg/ml streptomycin. Two million target cells were labeled by incubation in 100 μCi/ml of ^{51}Cr for 90 min at 37°C. After 3 washings and incubation for 1 hr at 37°C, cells were suspended in RPMI-1640 (Nissui, Tokyo, Japan) containing 10% FCS at a concentration of 2×10^5 cells/ml. Mice were treated as described above, and killed at intervals. The spleen was removed from each mouse, weighed and minced as finely as possible in HBSS, then filtered through a stainless-steel mesh (400 m/s). After centrifugation, filtrates were incubated in 0.83% NH_4Cl-Tris buffer at 37°C for 5 min to eliminate red blood cells, and the residual spleen cells were suspended in RPMI-1640 containing 10% FCS, following 2 washings in HBSS. Viable splenic cells were counted using the trypan-blue assay and re-suspended to known cell concentrations to yield ratios of the number of effector cells to target cells (E/T ratios) of 200/1, 100/1, 50/1, 25/1, 12.5/1 and 6.25/1. Then, 100 μl of the target-cell suspension and 100 μl of the effector-cell suspension were mixed into each well of 96-well microplates and incubated for 4 hr. After centrifugation, the radioactivity in 100 μl of supernatant of each well was measured with an ARC-300 gamma counter system (Aloka, Tokyo). Spontaneous release was defined as cpm released from target cells incubated without effector cells. Total release was determined as cpm released from target cells, all of which were lysed by incubation in 5% Triton X-100 (Nacalei Tesque, Kyoto, Japan). The percentage of cytotoxicity was calculated as ER-SR/TR-SR, where ER is experimental group release, SR is spontaneous release and TR is total release. The assay was performed in triplicate wells at each E/T cell ratio and the mean was used as the result for each mouse.

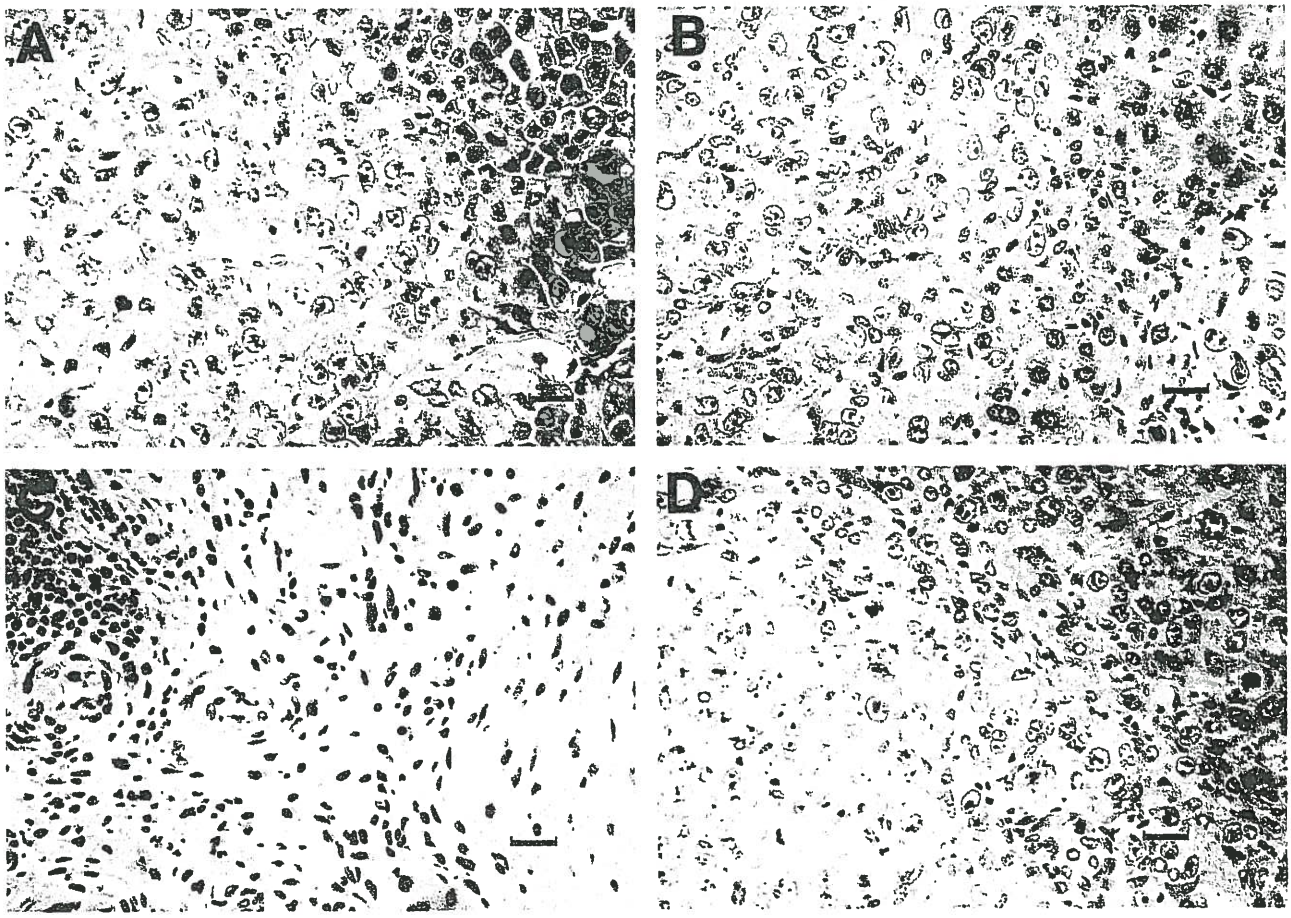

FIGURE 1 – Histological views of the SC-1-NU tumor in a control mouse and in a mouse treated with 5-FU at 90 mg/kg. Stomach tumors and liver metastases were processed for routine histological examination and stained with H. and E. Stomach tumor *(a)* and liver metastasis *(b)* in a control mouse; stomach tumor *(c)* and liver metastasis *(d)* in a treated mouse. The number of viable cancer cells is greatly reduced in *(c)*, demonstrating a significant effect of the chemotherapy (compare with *a*). Cancer cells are viable in both of the liver metastases, demonstrating the ineffectiveness of chemotherapy (compare *b* and *d*). Scale bars: 1 μ.

TABLE I – ANTI-TUMOR EFFECTS OF 5-FU, MMC AND OK-432 ON LOCAL TUMOR GROWTH AND LIVER METASTASES OF SC-1-NU

Treatment	Stomach tumor weight (g)[1]	Inhibition rate on stomach tumor (%)[2]	Mice with liver metastases[3]
Control	5.17 (1.69)		13/22
5-FU 45 mg/kg	2.82** (1.48)	45.4	6/19[4]
90 mg/kg	1.70** (0.63)	67.1	8/20[4]
180 mg/kg	0.270** (0.041)	94.8	2/12*
MMC 1.5 mg/kg	2.17** (0.85)	58.0	5/10[4]
3 mg/kg	1.68** (0.71)	67.5	6/11[4]
6 mg/kg	0.0478** (0.0217)	99.1	0/8**
OK-432 alone	3.98* (1.49)	23.0	1/8*
OK-432 + 5-FU 90 mg/kg	1.16** (0.45)	74.5	2/10*
OK-432 + MMC 3 mg/kg	0.680** (0.150)	86.9	0/6*

Mice were transplanted with SC-1-NU orthotopically on day 0 and treated with 5-FU and MMC on day 7. One KE per mouse of OK-432 was administered i.p. at qd × 5 from day 5 to day 9. Mice were killed on day 28, then the stomach tumors and livers were removed, weighed and examined macroscopically and histologically. [1]Data are shown as mean (± SD in parentheses) of tumors from evaluated mice. *$p < 0.05$; **$p < 0.0001$ difference from control values by Student's t-test.–[2]Data are calculated as follows: (1 − mean stomach tumor weight of the treated mice/mean stomach tumor weight of the control mice) × 100.–[3]Data are shown as number of mice in which liver metastases were observed/number of mice evaluable.–[4]Not significant, *$p < 0.05$; **$p < 0.01$ difference from control values by Chi-square test.

RESULTS

Effects of 5-FU and MMC

Effects of 5-FU and MMC on stomach tumor growth and liver metastases of SC-1-NU are shown in Table I. Stomach tumor growth was inhibited in a dose-dependent manner by both drugs, with a statistical significance even at quarter MTDs. However, the number of treated mice which developed liver metastases was almost equivalent to the number of controls in the case of treatment with one-quarter or one-half MTDs of both drugs. This observation was confirmed by histological examination. For example, although one-half MTD of 5-FU demonstrated an apparent anti-tumor effect on the stomach tumor (Fig. 1*a*,*c*), liver metastases observed in the same mouse were viable (Fig. 1*b*,*d*) reflecting the ineffectiveness of 5-FU. On the other hand, MTDs of both drugs inhibited stomach tumor growth by 95% and significantly reduced the number of mice with liver metastases.

Effects of OK-432 alone and in combination with 5-FU or MMC

Effects of OK-432 alone and in combination with 5-FU and MMC on stomach tumor growth and liver metastases of SC-1-NU are also shown in Table I. OK-432 alone showed a significant anti-tumor effect on stomach tumor growth, while much greater effects were observed in combination with both 5-FU and MMC at half MTDs. Furthermore, the frequency of liver metastases was significantly reduced in the mice treated with OK-432 alone or in combination with half MTDs of 5-FU and MMC.

NK-cell activity

Figure 2 shows NK-cell activity on day 10 in the mice treated with each schedule as determined by ^{51}Cr assay at various E/T cell ratios. As can be seen, NK-cell activity was somewhat increased in the mice treated with OK-432 alone, while it was significantly decreased in the mice treated with half MTDs of 5-FU and MMC. However, when combined with OK-432, NK-cell activity in the mice treated with both drugs returned to the control level, although it was still lower than that observed in the mice treated with OK-432 alone. Similarly, NK-cell activity before transplantation and on days 7, 14 and 28 was determined. Table II shows changes in NK-cell activity with

TABLE II – CHANGE IN NATURAL-KILLER-CELL ACTIVITY WITH TIME IN CONTROL AND TREATED MICE WITH EACH SCHEDULE

Treatment	Days after orthotopic transplantation				
	Before transplantation	Day 7	Day 10	Day 14	Day 28
Control	20.4 (2.2)	6.1 (1.4)	12.6 (1.7)	21.3 (3.7)	14.6 (3.3)
OK-432 Alone			15.3[1] (2.4)	25.4[1] (4.1)	17.3[1] (2.8)
5-FU 90 mg/kg			2.1* (0.4)[2]	3.8* (1.0)[2]	12.6[1] (2.1)[2]
OK-432 + 5-FU 90 mg/kg			10.4[1,3] (2.3)	24.6[1,3] (3.3)	22.6*[3] (2.4)
MMC 3 mg/kg			1.9* (0.5)[2]	4.6* (1.3)[2]	15.3[1] (3.1)[2]
OK-432 + MMC 3 mg/kg			9.7[1,3] (2.7)	18.5[1,3] (2.9)	23.8*[3] (3.2)

Data are shown as mean (± SD) of results from 3 mice. Mice were transplanted with SC-1-NU orthotopically on day 0, treated according to schedule and killed on indicated days. The spleens were removed, weighed and disaggregated mechanically into a cell suspension. NK-cell activity of the splenic cells was determined by ^{51}Cr-release assay against YAC-1 cells. Results at the E/T ratio of 200/1 are shown. [1]Not significant; *$p < 0.05$ difference from control values by unpaired Wilcoxon test.–[2]$p < 0.05$ difference from each other by unpaired Wilcoxon test.–[3]Not significant from OK-432 values alone by unpaired Wilcoxon test.

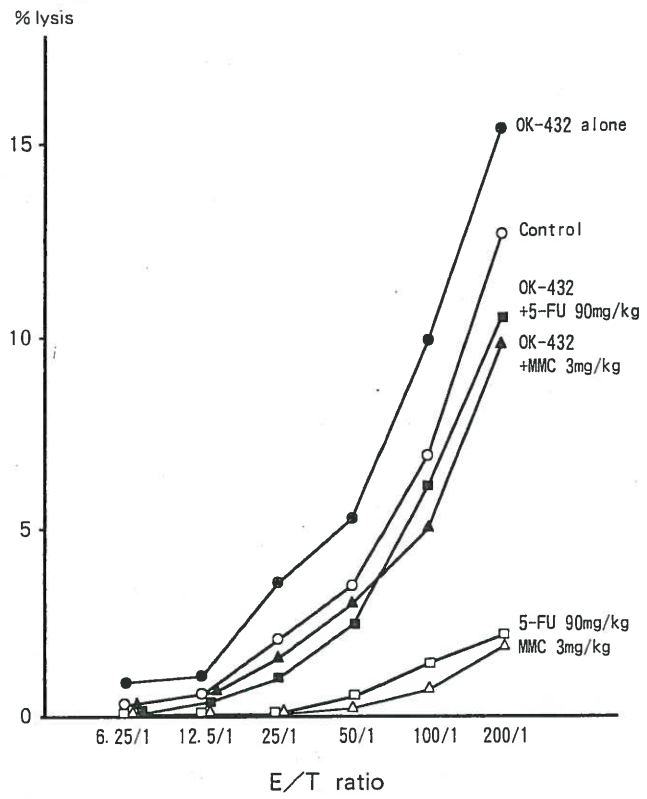

FIGURE 2 – NK-cell activity on day 10 in the mice treated with each schedule determined by ^{51}Cr assay at various E/T cell ratios. Mice were transplanted SC-1-NU orthotopically on day 0, treated according to each schedule and killed on day 10. The spleens were removed, weighed and disaggregated mechanically to cell suspension. NK-cell activity of the splenic cells was determined by ^{51}Cr assay against YAC-1 cells. Each point represents the mean of results from 3 mice.

time in the mice treated with each schedule at the E/T ratio of 200/1. On day 7 after transplantation, NK-cell activity was found to be suppressed from 20.4% before transplantation to 6.1%, apparently due to surgical stress (Pollock and Lotzova, 1987). From days 10 and 14, NK-cell activity gradually recovered in the control mice while, in mice treated with OK-432, NK-cell activity increased beyond control values. On the other hand, NK-cell activity in the mice treated with half MTDs of 5-FU and MMC was further reduced to less than 5%. However, NK-cell activity was approximately at the control level in the drug-treated mice when combined with OK-432. On day 28, NK-cell activity of the control mice and of those treated with OK-432 alone decreased, while that of the mice treated with half MTDs of 5-FU or MMC in combination with OK-432 was maintained, to reach significantly higher levels than that of the control mice and of those treated with OK-432 alone.

Number of spleen cells

Table III shows changes in the number of spleen cells with time in the mice treated with each schedule. Mice treated with OK-432 had greater numbers of spleen cells than the control mice, regardless of combination with 5-FU or MMC, except that mice treated with OK-432 in combination with half MTDs of 5-FU or MMC on day 10 had smaller numbers of spleen cells than those treated with OK-432 alone. However, mice treated with half MTDs of 5-FU or MMC without OK-432 had significantly smaller numbers of spleen cells than the mice treated with any other schedule.

DISCUSSION

Although 5-FU and MMC have been used most frequently in the treatment of stomach cancer, clinical response rates to both drugs have been limited, with a range of 10–25% (Comis and Carter, 1974). On the other hand, in experimental chemotherapy using s.c.-growing human cancer xenografts in nude mice which usually do not result in metastasis, the efficacy rates of both drugs have been over-estimated (Kubota *et al.*, 1988; Inaba *et al.*, 1988). In this study, the anti-tumor effects of even quarter and half MTDs of both drugs were effective on local stomach tumor growth, while the effects of the drugs on liver metastases of the stomach tumor were limited. Furthermore, the numbers of metastatic nodules in the individual mice which developed liver metastases become somewhat larger when mice were treated with half MTDs of both drugs (data not shown). Therefore, the results observed in experimental chemotherapy using this metastatic model seemed to reflect clinical chemotherapeutic efficacy more closely than in the s.c.-growing xenografts.

Since nude mice lack functional T-lymphocytes, the main immune defense against human cancer xenografts is thought to be NK-cell activity which seems to be above normal in nude mice (Kindred, 1979). In the present study, NK-cell activity found in the mice treated with half MTDs of 5-FU or MMC was significantly suppressed, which may have influenced the formation of liver metastases observed in the mice which had significantly reduced stomach tumors. This is consistent with the results which showed that, when combined with OK-432, both 5-FU and MMC at half MTDs reduced liver metastases as well as local stomach tumor growth. Since NK-cell activity in the mice treated with OK-432 was significantly rescued from the suppression due to the chemotherapeutic drugs, it appears that maintenance of NK-cell activity may be important in preventing metastases in the nude-mouse model described here. This observation was supported by the finding that, when anti-asialo GM1 antibody was administered to the mice treated with OK-432 to suppress NK-cell activity, liver metastases were almost equivalent to those seen in the mice treated without OK-432 (data not shown). In addition, NK-cell activity found in the control and treated mice with OK-432 alone on day 28 was considerably reduced, possibly due to extensive stomach tumor growth, while that found in the mice treated with OK-432 in combination with half MTDs of 5-FU or MMC was not reduced, possibly reflecting the high efficacy of treatment on local and metastatic stomach tumors. Suppressed NK-cell activity was also observed in patients with various advanced solid malignancies (Trinchieri and Perussia, 1984). Thus, it may be worth while to combine immunopotentiating agents with chemotherapy of human stomach cancer, in particular, to prevent metastases. It should be noted that the effects of OK-432 were not limited to enhancement of NK-cell activity, but included an increase in the number of spleen cells.

This report describes the application of a "patient-like" metastatic model of human stomach cancer to experimental immunochemotherapy. Although i.p. administration is not a routine mode of administration of drugs against liver metastasis in clinical practice, pharmacokinetic parameters in the patient serum after i.p. administration of drugs were reported to be comparable to those observed after i.v. administration (Speyer *et al.*, 1980; Howell *et al.*, 1983). Clinical trials will be carried out in the future to establish whether the system described here is a workable "patient-like" treatment model of metastatic stomach cancer.

TABLE III – CHANGE IN NUMBER OF SPLEEN CELLS WITH TIME IN CONTROL AND TREATED MICE WITH EACH SCHEDULE

Treatment	Days after orthotopic transplantation				
	Before transplantation	Day 7	Day 10	Day 14	Day 28
Control	5.7 (2.1)	6.0 (1.8)	6.3 (2.2)	7.2 (1.8)	8.3 (2.4)
OK-432 alone			14.4* (1.0)	13.4* (2.2)	16.9* (3.3)
5-FU 90 mg/kg			3.2* (0.9)[2]	2.9* (1.3)[2]	3.9* (1.2)[2]
OK-432 + 5-FU 90 mg/kg			9.6[1,3] (1.7)	13.6*[4] (2.6)	14.4*[4] (2.1)
MMC 3 mg/kg			2.3* (1.1)[2]	4.1* (1.2)[2]	4.6* (2.0)[2]
OK-432 + MMC 3 mg/kg			8.7[1,3] (2.8)	11.4*[4] (1.3)	12.3[1,4] (2.3)

Data are shown as mean (± SD) of results from 3 mice, expressed as $\times 10^7$. Mice were transplanted with SC-1-NU orthotopically on day 0, treated according to each schedule and killed on indicated day. The spleens were removed, weighed and disaggregated mechanically into a cell suspension. The splenic cell number was calculated from the cell concentration in the suspension determined by trypan-blue assay and the weight of the spleen. [1]Not significant; *$p < 0.05$ difference from control values by unpaired Wilcoxon test.–[2]$p < 0.05$ difference from each other by unpaired Wilcoxon test.–[3]$p < 0.05$; [4] not significant from OK-432 values alone by unpaired Wilcoxon test.

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