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Abstract

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COMBINATION CHEMOTHERAPY WITH ANTICANCER AGENTS AND OK-432

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Abstract: Antitumor effects of the combination chemotherapy with hemolytic streptococcus preparation, OK-432, and various anticancer agents were observed on experimental tumors and human cancers. Experimental studies revealed that combined use of OK-432 with Mitomycin C, Nitrogen mustard N-Oxide or Bleomycin was remarkably effective on rodent transplantable tumors such as Ehrlich carcinoma, sarcoma-180 and rat ascitic hepatoma AH-66. As for the mode of action of OK-432, besides a direct action on cancer cells, a host-mediated action appears to be also involved. Clinical trials were made on 14 cases with various advanced cancers, and favorable response was obtained in 5 with lung cancer. Fever was the major side effect of OK-432 and there was no evidence of bone marrow suppression.

Major efforts have been exerted to increase anti-tumor effect and to minimize adverse effects in cancer chemotherapy. Multiple combined therapy is one of the approaches which have been devised for this purpose and this therapy has achieved a considerable progress in recent years.

OK-432, developed by OKAMOTO *et al.* (1) as an anti-tumor agent derived from hemolytic streptococcus, has been the subject of reports indicating its effectiveness in topical as well as systemic administration, although there are still unanswered questions concerning its mechanism of action (2, 3). Our experience with this drug in a small number of clinical cases has not yet established any definitive effectiveness, but it did not cause bone marrow suppression, which is a major problem in cancer chemotherapy.

Combination with OK-432 and available anticancer agents may produce desirable anticancer effect with minimal toxicity, since they were qualitatively different in toxicity and also may be different in mechanism of action.

Initially, experimental studies involving combined use of OK-432 with Mitomycin C (MMC) were carried out, and then this combination was extended to clinical use.

MATERIALS AND METHODS

OK-432: OK-432 was prepared from a low virulent Strain *Su* of *streptococcus haemolyticus* established by OKAMOTO *et al.* One KE (Klinische Einheit, i.e.,

unit) contains 0.1 mg of prepared hemolytic streptococci by dry weight.

Animals: Six-week-old male ICR mice were employed for Ehrlich Carcinoma and Sarcoma-180, and six-week-old male Donryu rats for ascitic hepatoma AH-66 and AH-109A. Each group consisted of 10 animals unless the number of animals is indicated.

Tumors: Ascitic Ehrlich carcinoma and sarcoma-180, and ascitic hepatoma AH-66 and AH-109A were harvested six to seven days after intraperitoneal (i. p.) transplantation. As for intravenous (i. v.) inoculation, tumor cells were washed three times with ice cold phosphate buffered saline (PBS) solution and the cell number was adjusted to a repuired concentration. One tenth ml of the cell suspension was inoculated *via* tail vein.

Administration of agents: Agents were dissolved in isotonic saline solution (ISS) at required concentrations and were administered i. p. for the period and at the dosage as indicated in the results.

To control group in each experiments, ISS was given for equivalent periods.

In vitro-in vivo test: Tumor cells were washed and suspended in PBS solution (pH 7.2) at a concentration of 5×10^7/ml. Adding the agents in concentrations shown in the results, the cells were incubated at 37°C for 60 minutes. The cells were subsequently washed once, and inoculated to mice i. p. at a count of 5×10^6. Survival thereafter was evaluated as an indicator of cytocydal effect of agents on tumor cells *in vitro*.

Nitrogen mustard N-Oxide (Nitromin) incorporation into tumor cells: One milliliter of packed cells of sarcoma-180 was suspended in two ml of PBS solution, to which Nitromin and OK-432 were added at dosage of 100 μg and 2KE respectively, followed by incubation at 37°C. The Nitromin concentration in the cells was determined as a function of time by the NBP method (4) after washing three times with ice cold saline solution.

RESULTS

The effect of combined use of OK-432 with MMC on Ehrlich carcinoma and sarcoma-180 was first studied in *in vitro-in vivo* test (Figs. 1, 2).

For both tumors, the cells were treated and inoculated as described above, and survival thereafter revealed that OK-432 was not cytotoxic on Ehrlich carcinoma as well as sarcoma-180 *in vitro*. MMC showed a fairly strong effect on Ehrlich carcinoma and a moderate effect on sarcoma-180. When both were used together, even with a concentration of MMC which had been reduced to one half, a still stronger effect—one that may even be regarded synergistic—was observed.

In the same way, we examined the effect of combined use of OK-432 with Nitromin on sarcoma-180, and completely the same effect as with MMC was observed (Fig. 3).

Next, we studied in the same way the minimal effective concentration

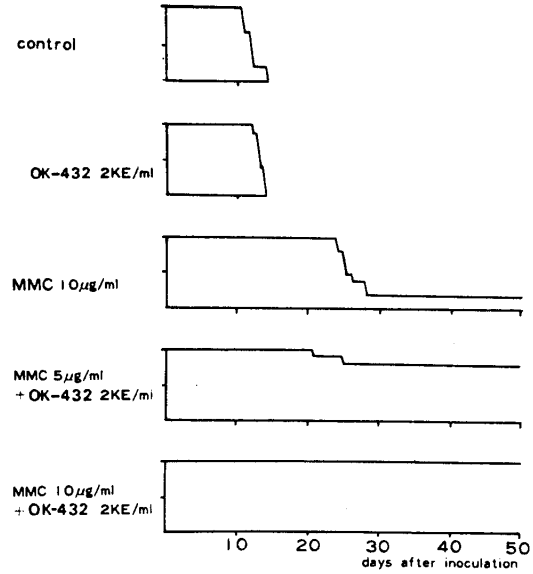

Fig. 1. Effect of combined use of OK-432 with MMC on sarcoma-180 in *in vitro-in vivo* test

Tumor cells were incubated together with agents at a concentration as indicated above. 5×10^6 cells were inoculated i.p., followed by survival. The vertical line indicates percent surviving.

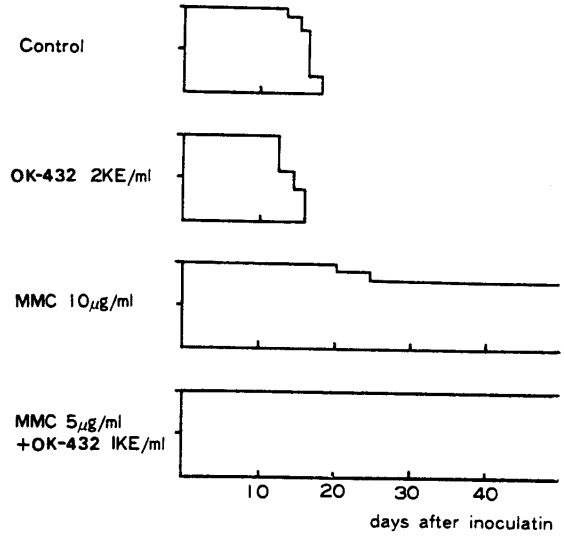

Fig. 2. Effect of combined use of OK-432 with MMC on Ehrlich carcinoma in *in vitro-in vivo* test.

Experiments and symbols are the same as in Fig. 1.

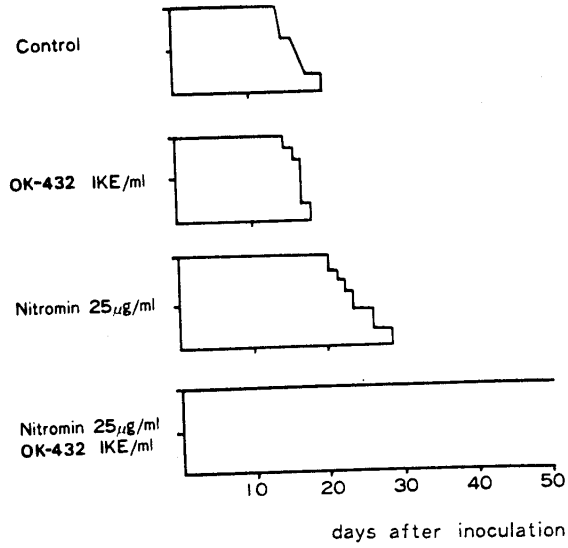

Fig. 3. Effect of combined use of OK-432 with Nitromin on sarcoma-180 in *vitro-in vivo* test.
Experiment and symbols are the same as in Fig. 1.

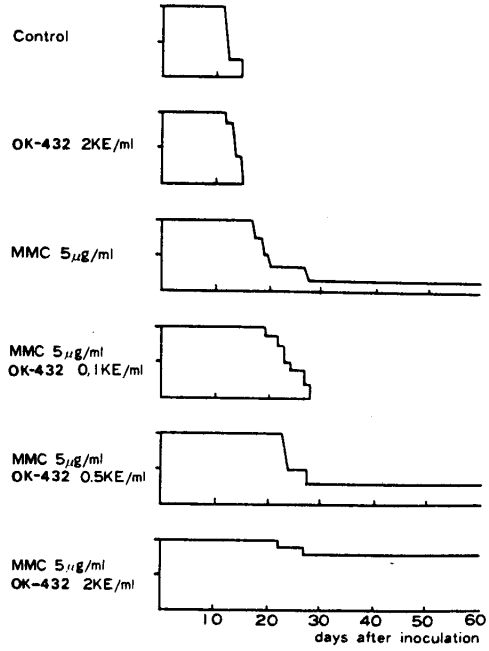

Fig. 4. Concentration of OK-432 and effect on sarcoma-180 in combination with MMC in *in vitro- in vivo* test.
The tumor cells were incubated together with OK-432 in various concentrations and MMC in a concentration of 5 μg/ml and 5×10^6 cells of which were inoculated i. p., followed by survival. The vertical line indicates percent surviving.

of OK-432 on sarcoma-180 when used in combination with MMC *in vitro*. As shown in Fig. 4, OK-432 was thereby found to exhibit a dose-response relationship: and it was also found that, to achieve effective combined use *in vitro*, OK-432 had to be added at a relatively high concentration, 0.5KE/ml.

As mentioned above, the combined use of OK-432 with some anticancer agents definitely proved effective *in vitro*. As one of the attempts to clarify the mechanism of action in combination use, the effect of OK-432 on the incorporation of Nitromin into tumor cells *in vitro* was measured. No difference at all was found between the group to which OK-432 had been added and that to which no OK-432 had been added (Fig. 5).

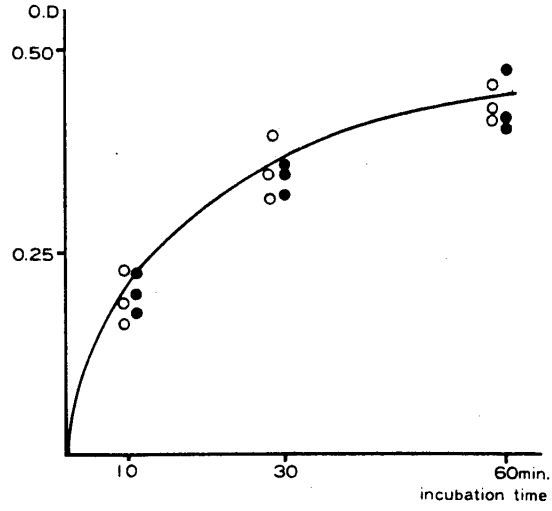

Fig. 5. Nitromin incorporation into sarcoma-180 cells *in vitro*, and the effect of added OK-432.
○ Nitromin alone ● OK-432 added

Subsequently, the effect of combined use of OK-432 with some anticancer agents was examined by i.p. inoculation-i.p. administration (i.p.-i.p.) method.

From day 3 after i.p. inoculation of 5×10^6 cells of sarcoma-180, i.p. administration of OK-432 and MMC were made for five days. The survival thereafter in the group given combined administration of the two agents was considerably longer than in the groups given either of the individual agents alone (Fig. 6). The *in vivo* studies, however, revealed almost the same degree of increased life-span with OK-432 and MMC, differing from the results obtained with the *in vitro* studies.

With AH-66, to which OK-432 alone was effective to some degree, Bleomycin was found to be definitely effective when used in combination

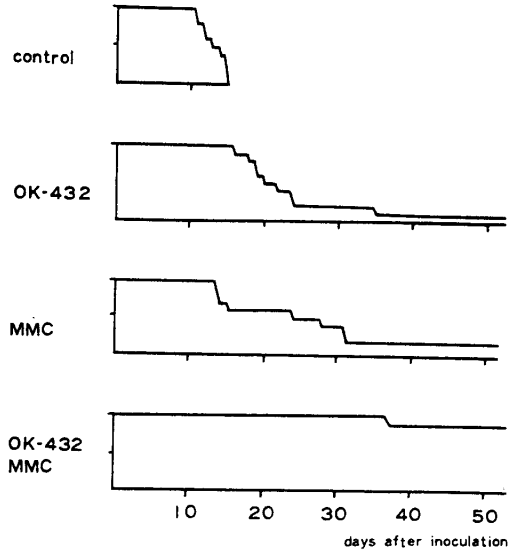

Fig. 6. Effect of combined use of OK-432 with MMC on sarcoma-180.
From day 3 after i.p. inoculation, 1KE/mouse of OK-432 and 0.25mg/kg of MMC were given i.p. for 5 days.
The vertical line indicates percent surviving.

charactor of this agent does not exclude possible host-mediated action. Studies were therefore also made in this respect.

Mice pretreated i.p. with OK-432 were inoculated with Ehrlich carcinoma i.v. *via* tail vein five days after completion of pretreatment, and survival thereafter was studied (Fig. 10). As presented in the figure, the survival rate of the group pretreated with the agents was about twice as high as in the control group at 40 days after inoculation.

We now report the clinical results.

The initial dose of OK-432 was 0.1KE, which was then gradually raised to a maintenance dose with OK-432 (Fig. 7). One the other hand, AH-109A was almost insensitive to OK-432, and the combined use of OK-432 with 864-T or MMC was found not to be more effective than with 864-T or MMC alone (Fig. 8).

Next, we used the i.v. inoculation-i.p. administration (i.v.-i.p.) method to establish whether or not the combined use was effective upon Ehrlich carcinoma inoculated at different sites (Fig. 9). The agents were administered i.p. from day 3 after i.v. inoculation as shown in the figure, and this administration of agents turned out to be completely ineffective.

The results thus far described mainly indicate the direct action of OK-432 against tumor cells. The

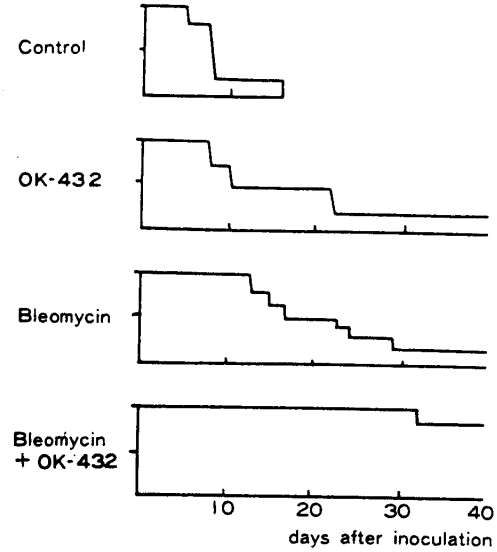

Fig. 7. Effect of combined use of OK-432 with Bleomycin on AH-66.
4mg/kg of Bleomycin and 2KE/rat of OK-432 were administered i.p. from day 3 after i.p. inoculation of 5×10^6 of tumor cells for 10 days.

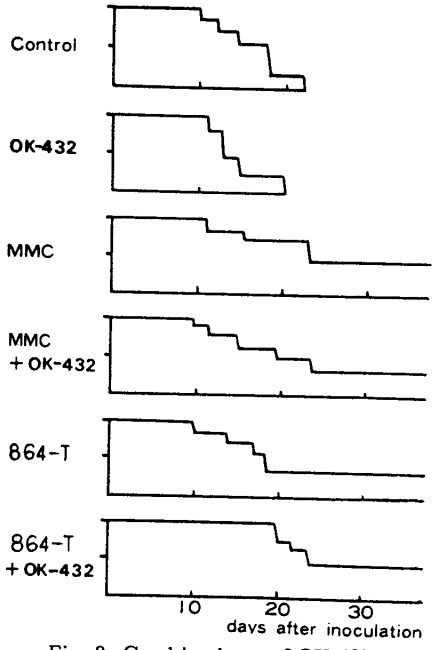

Fig. 8. Combined use of OK-432 with MMC or 864-T on AH-109A.

2KE/rat of OK-432 and 0.2mg/kg of MMC or 10mg/kg of 864-T were given i.p. from day 3 after i.p. inoculation of 5×10^6 cells of AH-109A for 7 days.

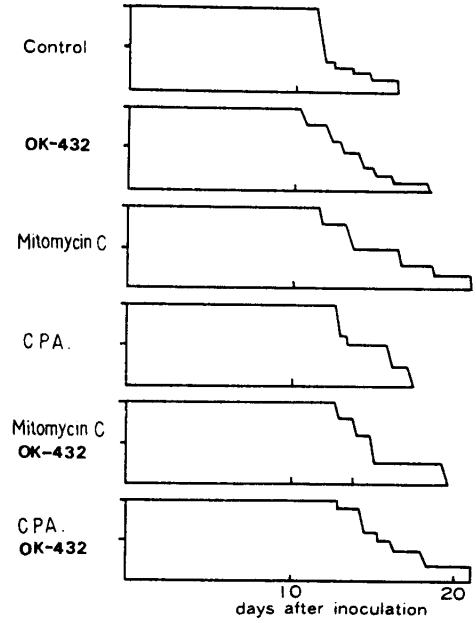

Fig. 9. Combined use of OK-432 with MMC or Cyclophosphamide (CPA) on Ehrlich carcinoma in i.v.-i.p. method.

5×10^6 of tumor cells washed 3 times in ice cold PBS solution were inoculated i.v. *via* tail vein. From day 3 after inoculation, 1KE/mouse of OK-432 and 0.33 mg/kg of MMC or 10mg/kg of CPA were given i.p. for 10 days.

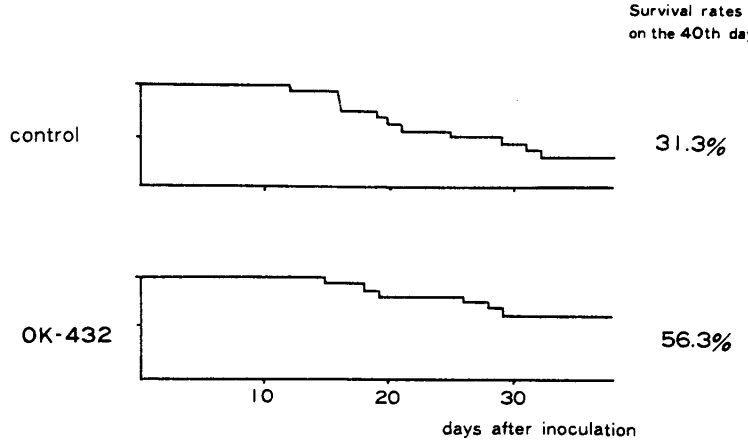

Fig. 10. Effect of pretreatment with OK-432 on survival after i.v. inoculation of Ehrlich carcinoma.

Pretreatment with 1KE/mouse of OK-432 was made for 7 days. Five days later, 2×10^6 of Ehrlich carcinoma were inoculated *via* tail vein, followed by survival. Each group consisted of 15 to 16 mice.

of 0.5 to 2.0KE, through i.v. drip infusion twice a week as a rule. MMC or Cyclophosphamide (CPA) was given at a dosage of 6mg or 400 mg twice a week as a one-shot i.v. infusion.

This treatment was given to 14 cases so far. Eight of these cases had advanced lung cancer, and most of other cases were at a late stage accompanied with ascites or plerual effusion. The treatment proved effective in 5 of 8 cases with lung cancer, but did not bring about any definite evidence of improvement in the cases with liver cancer and other cases (Table 1).

TABLE 1 CLINICAL RERPONSE IN PATIENTS TREATED WITH COMBINED USE OF OK-432 AND MITOMYCIN C OR CYCLOPHOSPHAMIDE (CPA).

Case No.	Age	Sex	Disease	total dosis			Response	Side Effects
				OK-432	MMC	CPA		
1	56	M	Ca. of the lung	90.2KE	64 mg	4800mg	+	fever
2	68	M	Ca. of the lung	5.0	48		++	fever
3	59	M	Ca. of the lung	83.0	88		++	fever
4	58	M	Ca. of the lung	7.4	48		−	fever, leucopenia
5	68	M	Ca. of the lung	57.6	42	3600	−	fever
6	62	M	Ca. of the lung	17.3	42		+	fever
7	72	M	Ca. of the lung	12.5	60		−	fever
8	66	M	Ca. of the lung	5.4	54		+	fever
9	48	M	Ca. of the liver	170.0		3200	−	fever, leucopenia
10	59	F	Ca. of the liver	8.5	64		−	fever
11	68	M	Ca. of the liver	15.0	88		−	fever
12	64	F	Ca. of the stomach	108.5	40		−	
13	48	F	Ca. of the stomach	49.0	54		−	
14	73	F	Ca. of the breast	17.5	48		−	fever, leucopenia

++ : more than 50% reduction of tumor in diameter
+ : more than 25% reduction of tumor in diameter

Figs. 11 and 12 show the changes of peripheral blood counts in the cases during chemotherapy. White blood cell counts tended, if anything, to show a temporary increase following initiation of the treatment. A decline less than 3,000/cmm was seen in only one case. Within one to three hr after i.v. infusion of OK-432, fever with chills were observed in 11 of the 14 cases.

DISCUSSION

We have thus far briefly outlined the results of experimental studies and the clinical application of combined administration of OK-432 with anti-cancer agents. According to OKAMOTO (1), KOSHIMURA *et al.* (5), the anti-

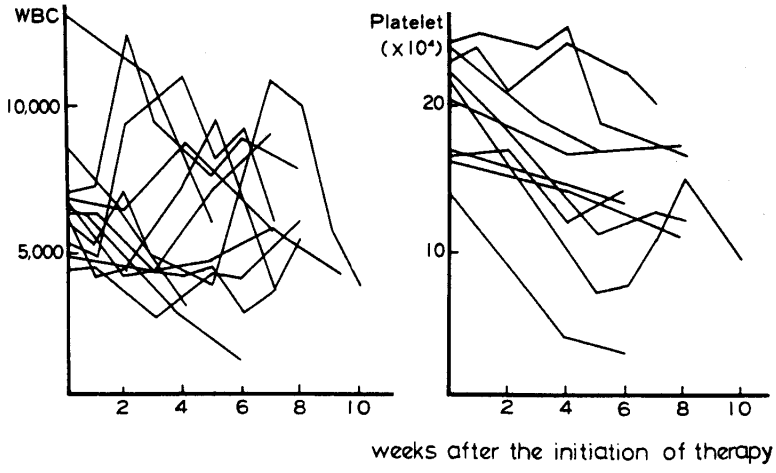

Fig. 11. Changes in white blood cell counts and platelet counts in cases given OK-432 together with MMC or CPA.

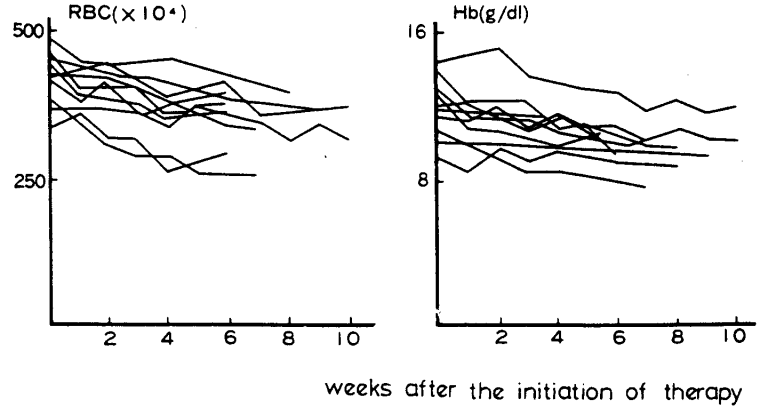

Fig. 12. Changes in red blood cell counts and hemoglobin content in cases given OK-432 together with MMC or CPA.

cancer mechanism of action of the hemolytic streptococcus is understood to be as follows: The hemolytic streptococcus acts upon tumor cells as an enzyme to produce streptolysin S, with the cell RNA functioning as a substrate, resulting in the loss of RNA and the devastation of RNA metabolism.

In our *in vitro-in vivo* studies, OK-432 alone was not found effective. Yet, when OK-432 was used together with MMC or Nitromin, the *in vitro- in vivo* tests established a considerably higher effectiveness than by the use of those drugs alone, a difference is so remarkable that it might even be regarded as synergistic. OK-432 is thereby inferred to have an action which, if not

cytocidal, still produces fairly substantial damage within the cells, and, together with the action of the anti-cancer antibiotic or alkylating agent, may be presumed responsible for lethal damage to the tumor cells.

OK-432 consists of the bacterial cell itself, deprived of its ability to multiply. If OK-432 has a direct action on tumor cells, the first presumption must be that bacterial cells come into contact with the tumor cell membrane, with the subsequent occurrence of some interference phenomenon or others. From this viewpoint, it was conjectured that this drug might affect the incorporation of anticancer agents into the cells. The concentration of Nitromin incorporated into the cells following the addition of the present drug was therefore determined. The findings obtained tended to exclude this possible effect on the incorporation of anti-cancer agents into the cells.

The effect of OK-432 *in vivo* has been reported to be highest in Ehrlich carcinoma, followed by some rat ascitic hepatoma and Yoshida sarcoma resistant to Nitromin. These results were mostly obtained in i.p.-i.p. studies, in which the effect appeared most readily (6, 7).

In the present studies, the dosage of OK-432 was 1KE/mouse (about 30 KE/kg and 2 KE/rat (about 13 KE/kg). The above dosage was approximately equivalent to the minimal effective dosis on sarcoma-180, Ehrlich carcinoma, and AH-66 by the i.p.-i.p. method. When used simultaneously with effective dose of MMC and Bleomycin, OK-432 produced a remarkable effect closely paralleling the results of the *in vitro* studies. In AH-109A that failed to respond to OK-432 alone, the use of this drug in combination with an effective dose of MMC or 864-T did not show any benefit. The marked effectiveness of combined administration, already indicated, should be regarded as obtainable only when used on tumors which are sensitive in some degree to OK-432 and the combined agents. In Ehrlich carcinoma that proved amenable by i. p.-i. p. method, no effect of OK-432 alone or in combination with another drug was observed even at the same dosage in the case of i. v.-i. p. method, which gave reduced accessibility of the drugs to the tumor focus.

The above-described results support the interpretation that the effect of OK-432 arises from its direct action on the tumor. Since the drug is of bacterial origin, the possibility of host mediation in addition to the direct action should also be fully considered. SAKURAI (8) showed the results with AH-13 suggesting the presence of some host-mediated action. In our present studies, also, OK-432 when tested alone showed different results for *in vitro* and *in vivo* studies. Survival rate after tumor inoculation was evidently higher in the group given pretreatment with the present drug. These findings by no means exclude the possibility of host mediation.

The number of clinical cases treated with the present drugs is still small.

In addition, many of the cases were at an advanced stage of the disease. Thus, the sujects in the present studies were hardly ideal. An attention should, however, be drawn to the fact that in the cases of lung cancer where the evaluation of drug effects is easier, the tumor was found to diminish in five out of 8 cases, although MMC was, of course, used at more than the minimal effective dose. Further studies appear to be worthwhile.

The OK-432 dose per kg, body weight, was varied with the clinical cases, but was considerably less than that used on animals. As pointed out by KUROKAWA *et al.* (3), there was no relationship observed between the clinical response and the total dosage. This is another apparent reason for assuming the presence of host-mediated action.

The combined administration of OK-432 and MMC at least does not aggravate hematological side effects, but gives, on the contrary, the impression of lessening them in comparison with cases MMC alone. This, however, is a matter that remains to be clarified with many more clinical cases.

As stated above, at the present stage of our knowledge about OK-432, we have to postulate a direct action on the tumor and an indirect acton *via* the host. If both these factors should really prove to be involved together in this drug, its combined administration with anticancer agents may constitute a special type of combined chemotherapy.

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