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Tadashi Ofuji*

*Okayama University,

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Abstract

1) OX substance showed marked cytotoxicities in cell suspension culture of Yoshida sarcoma cells, celothelioma cells, and Ehrlich ascites tumor cells. It has become clear that the cytotoxicities have two aspects; one, nuclear shrinkage and karyolysis as seen with Carzinophilin and the other, cytoplasmic swelling as seen with Nitromin. 2) OX substance was effective by its contact action on patients with peritonitis carcinomatosa, celothelioma and rectal carcinoma. 3) Esterified OX substance was injected intravenously or intraperitoneally into CBA mice with ascites leukemia. The substance prolonged their life span and inhibited the progression of leukemia. As it was possible to give the substance repeatedly into mouse tail veins in this experiment, in the future, OX substance might become intravenously injectable for the treatment of patients with leukemia and solid malignant tumors.

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EXPERIMENTAL AND CLINICAL STUDIES ON AN ANTI-TUMOR ACTIVITY OF OX SUBSTANCE

Tadashi OFUJI

*Department of Internal Medicine, Okayama University Medical School,
Okayama Japan (Director: Prof. K. Hiraki)*

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Today it may be said that x-ray therapy is most generally used for cancer. As for its fundamental experiments PERTHES⁸ tried irradiation on chromosomes of a rabbit skin carcinoma, HERTWIG¹ on frog eggs and sperms, and LACASSAGNE and MONAD² on a dog sarcoma; all observed the inhibition of mitosis though no clear explanation was hitherto given to the inhibitory mechanism of mitosis by irradiation.

In 1958, YAMAMOTO⁹ of Okayama University Medical School extracted unsaturated fatty acid from livers removed 24 hours after irradiation of rabbits with 3,000 γ and named it OX substance. This substance was shown to have a strong anti-tumor activity against rabbit testicle tumor and Yoshida sarcoma cells. Furthermore, confirming that the anti-tumor activity was exactly the same as that of irradiation, he thought that the essential mechanism of cell destruction by irradiation was an indirect action of the substance produced in living organisms after irradiation.

In 1958, the author observed an extreme destruction of Yoshida sarcoma cells and celothelioma cells which were cultured *in vitro* in a medium containing OX substance^{8,10}.

An anti-tumor activity against Ehrlich carcinoma cells cultured *in vitro* was also found⁵. Good therapeutic effects were obtained by injecting the substance into the peritoneal cavity of patients with celothelioma⁴ and peritonitis carcinomatosa⁶ and also by injecting it into the rectum of patients with rectal carcinoma⁷.

Lately as good results were obtained in ascites type leukemia of mice, when the substance was given intravenously or intraperitoneally, the authors intend to make a brief report on various therapeutic experiments tried thus far.

1. The influence of OX substance on cell suspension culture of Yoshida sarcoma cells

Roller tube culture of Yoshida sarcoma cells was done in medium consisting of 44% Hank's solution, 19% chick embryo juice, 29% horse serum, 0.8 mg/cc RNA, and 0.8/cc vitamin B12. As soon as the culture began 0.1 cc/cc and

0.4 cc/cc of 2% OX substance were added to see the effects of the drug on the number of cells and their morphology with the phase contract microscope in 6, 24, and 48 hours (Fig. 1).

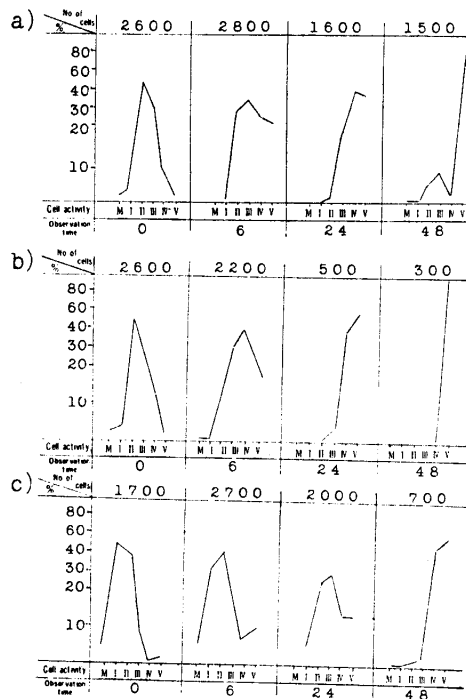


Fig. 1 Activity of Yoshida sarcoma cells cultured in OX substance containing medium

a) Addition of 2% OX substance at 0.1 cc/cc.

b) Addition of 2% OX substance at 0.4 cc/cc.

c) Addition of fatty acid extracted from non-irradiated livers at 0.1 cc/cc.

M: mitosis, I: Kidney shaped nucleus, distinct nucleoli, clear cytoplasm with almost no granules, II: increase of lipoid granules, III: nucleoli rather indistinct, slight vacuolar changes in Golgi area, IV: more definite degenerative changes such as swelling or pycnosis, V: Destructive changes.

0.1 cc-6 hours: Shrinkage of the whole cell and of the nuclei appeared. The cell membrane and nucleoli became irregular. Mitochondria grew small and granular. There was no sign of vacuolar changes in the Golgi area. Thus a slight decrease was seen in the cellular activity.

0.4 cc-6 hours: The effects of the drug was almost as the same as above. A slight destruction of cell margins and a slight decrease in the number of cells could be seen.

0.1 cc-24 hours: Swelling and cytolysis of cell margins, shrinkage and liquification of cells, homogenization of the nucleus because of karyolysis, and nuclear

prolapsus were seen. A small number of cells showed many blunt pseudopods from a side of the cell margin. Also a sharp decrease in the number of cells was noted.

0.4 cc-24 hours: Shrinkage of cells was generally seen. Swelling and cytolysis increased. The nuclear membrane became thick but most cells were completely shrunk and ruptured, and the internal structure could be no longer recognized. The number of cells was also decreased abruptly.

After the 48th hour, the cells were also destroyed in both tubes containing 0.1 cc and 0.4 cc to be recognized.

Untreated control culture could be continued for 12 days. After that period it was successfully back transplanted into the rat's peritoneal cavity, and no cell swelling or cytolysis could be seen within 48 hours. Addition of fatty acid extracted from non-irradiated livers as control induced a remarkable growth promotion, and after six and 24 hours, a great number of mitotic figures was observed and the cell number increased.

In summary, it is clear that OX substance induced two damaging effects against Yoshida sarcoma cells; one swelling action like the one induced by Nitro-min and the other a cytolytic action like the one induced by Carzinophillin. These damage effects against Yoshida sarcoma cells had nothing to do with fatty acid extracted from non-irradiated livers. It should be noticed that it even accelerated mitosis.

2. The influence of OX substance on cell suspension culture of celothelioma cells

Roller tube culture was done of ascites tumor cells obtained from a patient with celothelioma in 1.5 cc of medium consisting of 70 % Hank's solution, 15 % healthy person's serum or serum of various cancer patients, 15 % chick embryo juice, 2 mg/cc RNA and 28/cc vitamin B12. After five days of cultivation, 0.1 cc of 2% OX substance was added and after 6 to 12 hours the shape of tumor cells was observed by phase contrast microscopy. As a result, vacuolar changes were observed in many cells, sometimes involving the entire cytoplasm. The rate of cytolysis was higher than that induced by Carzinophillin. Cell margins showed destruction and partially the cells became amorphous and swollen. Similar changes were noted after treatment with Nitromin. Besides, karyolysis, destruction of the nuclear membrane, change of the shape of nucleoli and loss of the original form were shown.

OX substance demonstrated a karyolytic action stronger than other anti-tumor agents such as Colcemid, Carzinophilin, Glucosamin, Nitromin, and sometimes the cells became homogenous and swollen. In summary, OX substance when acted on celothelioma cells caused karyolysis, pycnosis, and cytoly-

sis. This resembled the mode of action of Carzinophilin but the degree was much greater. On the other hand some cells showed swelling and destruction of the nucleus and cells, as observed after treatment with Nitromin. It has become clear that OX substance has toxic actions as shown by two drugs, Carzinophilin and Nitromin, when used against celothelioma cells as well as against Yoshida sarcoma cells.

3. The influence of OX substance on cell suspension culture of Ehrlich ascites tumor cells

Ehrlich ascites tumor cells were maintained in Cb mice and the tumor cells were removed on the fifth day, they were cultured in roller tubes with medium of 60 cc of Hank's solution, 40 cc of horse serum, 0.05 g of yeast extract, 0.25 g of lactalbumin, and 100/cc of vitamin B₁₂. The number of cells were adjusted to 6,000-17,000/mm³ at the time the culture began. Every tube contained 2 cc and all the tubes were divided into five groups. They were divided as follows:

- a) non-added group
- b) 0.2 cc of 0.1 % OX substance added group
- c) 0.1 cc added group
- d) 0.04 cc added group
- e) 0.02 cc added group

The number of cells were counted at the 24th hour, 48th hour, 72th hour, and 99th hour from the beginning of culture. At the same time morphological changes were observed by phase contrast microscopy (fig. 2).

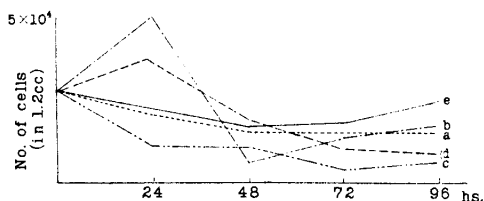


Fig. 2 Changes in the number of cultured Ehrlich ascites cells.

a : untreated controls, b - - - : with 0.2 cc of 0.1 % OX substance, c - . - . : with 0.1 cc of 0.1 % OX substance, d - - - - : with 0.04 cc of 0.1 % OX substance, e — : with 0.02 cc of 0.1 % OX substance.

The result showed that with (b) and (d) groups, the cell number once increased when compared with the non-added group at the 24th hour but began to decrease after that. After all, excluding (e) group which had the least concentration, the number of cells decreased in (b), (c) and (d) groups compared with the non-added group 48 to 72 hours after the culture began. Morphologically, the non-added group did not show any particular change throughout the 96 culture hours, but the added group showed cell vacuolization, cell swelling,

nuclear shrinkage, karyolysis, and destruction of the nuclear membrane as the culture time went on. These changes appeared earlier in groups with high concentrations of the drug.

As a whole it can be said that OX substance possessed the same type of inhibitory action against the nucleus and cytoplasm of Ehrlich ascites tumor cells as against Yoshida sarcoma cells and celothelioma.

4. Cases treated with OX substance

a. Celothelioma: Patient: Age 47, female. In late October 1960, the patient noticed sense of fullness in the lower part of her abdomen. She was diagnosed as having peritonitis carcinomatosa and treated in a certain hospital from November 1960 to January 1961. In the middle of March, because of increasing loss of weight and swelling of the abdomen that she entered our hospital on April 24. On detailed examination, no primary tumor could be found in the breast, abdomen, intestine, liver, and uterus. Peritoneoscopy revealed numerous nodules, in size of a rice to a bean, in the peritoneum, and there also many adhesions between the abdominal organs. The specific gravity of the ascites was 1023, protein 5.0g/dl, Rivalta positive, TPT reaction positive, and erythrocytes were also present.

Observing tumor cells in ascites with the phase contrast microscope or after May-Giemsa staining, the majority of cells were 25 to 40 μ . Each cell had one or two large nuclei with one or two large nucleoli. Large and small vacuoles were seen in the cells. What interested us most was that part of the cell margin was protruding in a hill shape and from this point thin needle or beard-shape substance of 2 to 10 μ , which stained purple pink with May-Giemsa stain, was radially projecting. The figure of this tumor cell agreed with that of celothelioma cells formerly reported by ASAKA of our department. 2.5% OX substance was injected intraperitoneally, 20 cc each, two times a week for five weeks totalling ten times.

After the fifth injection, loss of appetite, general weakness, and sense of fullness in the abdomen disappeared. After the 7th injection, removal of the ascites became difficult and the circumference of the abdomen was reduced to 71 cm from 81 cm before treatment. No ascites accumulation was seen 16 days after the end of injection and the patient was discharged from the hospital with no complaints. Tumor cells after the completion of therapy became remarkably thin. Karyolysis, nuclear shrinkage, cell swelling and vacuoles were seen. Protrusion of the cell margin disappeared. Beard-shape substance became short and sparse. No side-effects attributable to OX substance were seen in the blood picture and liver function tests.

b. Peritonitis carcinomatosa: The first case: Age 61, female. Since Octo-

ber 1960, the patient had had a feeling of general weakness and sense of fullness in the epigastrium. In the late October she was told that she had an abdominal tumor with ascites by her family physician and entered our hospital. On examination here she was diagnosed as having peritonitis carcinomatosa associated with a gastric cancer. Immediately intraperitoneal injection of OX substance was started two times a week, delivering 20 cc of 2.5 % OX substance each time, for a total of 10 times.

After the 6th injection, subjective symptoms such as sense of fullness in the abdomen and loss of appetite decreased and the speed of ascites accumulation slowed down. Phase contrast microscopy revealed thin tumor cells and marked karyolysis and cell swelling. No side-effects of the OX substance were reflected in the blood picture and examination of liver function during and after the injection.

The second case: Age 62, female. In August 1960, the patient had sense of fullness in the abdomen and entered a hospital as having ascites and tumor in the abdomen. She was transferred to our hospital in December. Biopsy of the omentum showed a histological picture of mucoid carcinoma. 20 cc of 2.5 % OX substance was injected twice a week totalling 10 times. The drug effect was marked in reducing the sense of fullness in the abdomen, loss of appetite, general weakness and size of the abdominal tumor. Tumor cells became thin following the injection of OX substance. Nuclear karyolysis and cell vacuolization were also evident. No particular side effects were noted.

c. Rectal cancer: Patient age 76, male. In October 1950, the patient had a difficulty in fecal evacuation. In April 1960, he became unable to evacuate feces and was found to have a rectal tumor in our hospital.

Following admission he received topical application of 10 cc of 2.5 % OX substance every day through a Nelaton's catheter. After the third application, a small amount of diarrhea was seen. After that, evacuation became easier day after day and at the 8th application thumb-sized fecal masses were passed out with a marked decrease of the tumor. Before treatment hemorrhage occurred at the time of palpation but never after the 8th application.

For about 2 months the patient continued to have good fecal evacuation by the daily application of OX substance. Later with growth of the tumor, the patient again showed difficulty in fecal evacuation and the amount of OX substance was raised to 20 cc to 30 cc per day. The tumor became smaller again and the patient became possible to evacuate feces. Thus after the topical application of OX substance for 130 days totaling 1980 cc, the patient continued to have satisfactory fecal evacuation, though on September 4, fistula of the rectum and bladder due to cancer infiltration developed and 2 days later he had to receive an operation for an artificial anus. No side effects of the OX substance

were noted. This is an interesting case of rectal cancer with absolute ileus, in which fecal evacuation became possible for 4 months with OX substance given continuously to the rectum and the tumor became smaller after the third topical application.

5. *Effects on mouse leukemia*

The experiments thus far described were done to observe the direct effect of OX substance on tumor cells.

On the other hand, it had been planned to treat cancer patients by intravenous injection of emulsified OX substance but its repeated injections were found too toxic to blood vessels. There was also a case in which an abrupt fever followed an intravenous injection of emulsified OX substance. But as it was impossible to give it repeatedly, the result could not be evaluated. Lately, OX substance which was esterified and then emulsified with a special emulsifier to make the granule below 1μ was developed and its intravenous injection into mice with leukemia became feasible. The object of this experiment was to examine its inhibitory effect on mouse leukemia and a possibility of repeated intravenous injections of the agent. Ascites leukemia cells maintained in DBA strain mice and the same ascites leukemia cells transplanted in CBA strain mice were used. After transplantation of the leukemia cells into the peritoneal cavity, 0.3 cc of 5%, 0.5%, 0.05% OX substance solutions were injected into the tail vein or peritoneal cavity.

The number of transplanted ascites cells was 40 million in CBA strain, and 80 million in DBA strain. The effect of the drug was evaluated by observation of the survival days, peripheral blood pictures, amount of ascites, and changes of the ascites cells. The effect was as follows. A group of CBA mice given intravenous injection of 0.5% OX substance survived 8.2 days compared with the survival days of 5.2 days of the control. The number of leukocytes in the treated group were always 10 to 20 thousands while the control showed 40 to 60 thousand leukocytes after the 5th day. Accumulation of the ascites was delayed and the number of cells in the ascites was decreased than in the control. Degeneration of the cytoplasm and nuclei was marked and mitotic index was low. No difference could be seen in the degree of anemia and body weight. A group of mice given intraperitoneal injection of the drug survived almost as long as a group of mice given intravenous injection of 0.5% OX substance. But it was natural that degeneration of ascites tumor cells in the former was more pronounced than in the latter group (Fig. 3).

In case of a DBA group given intravenous injection, the survival days were 10.4 days compared with 8.2 days of the control when 0.05% OX substance was used. Also comparing with the control, anemia appeared late and

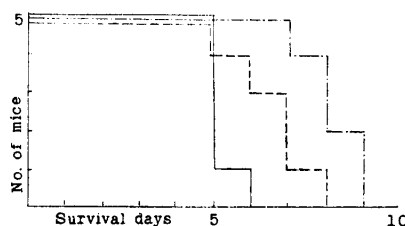


Fig. 3 Ascites leukemia cells in CBA mice. intravenous injections of OX substance.
 —: control, - - -: 0.3 cc of 0.5% OX substance, ·····: 0.3 cc of 5% OX substance.

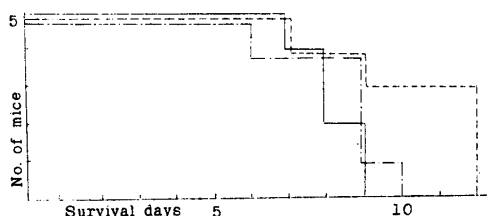


Fig. 4 Ascites leukemia cells in DBA mice. intravenous injections of OX substance.
 —: control, - - -: 0.3 cc of 0.5% OX substance, ·····: 0.3 cc of 0.05% OX substance.

the increase of the number of leukocytes delayed and ascites accumulated slowly (Fig. 4).

Unsaturated fatty acid extracted from non-irradiated rabbit livers was used as a control but failed to prolong the survival time. Conversely it showed a tendency to accelerate the development of leukemia and anemia.

It became clear that OX substance proved to be inhibitory against leukemia cells in mice and prolong their life span. Since unsaturated fatty acid from non-irradiated rabbit livers showed no anti-leukemia action, the importance of irradiation on the production of OX substance should be emphasized.

The experiment showed that OX substance was refined to decrease vascular lesions arising from its toxicity and repeated injections of 0.3 cc to 0.5 cc OX substance into mouse tail veins were possible for 12 to 15 days.

From these results it is suggested that administration of OX substance by the intravenous route might become feasible against human leukemia and other malignant tumors.

CONCLUSION

1) OX substance showed marked cytotoxicities in cell suspension culture of Yoshida sarcoma cells, celothelioma cells, and Ehrlich ascites tumor cells. It has become clear that the cytotoxicities have two aspects; one, nuclear shrinkage and karyolysis as seen with Carzinophilin and the other, cytoplasmic swell-

ing as seen with Nitromin.

2) OX substance was effective by its contact action on patients with peritonitis carcinomatosa, celothelioma and rectal carcinoma.

3) Esterified OX substance was injected intravenously or intraperitoneally into CBA mice with ascites leukemia. The substance prolonged their life span and inhibited the progression of leukemia.

As it was possible to give the substance repeatedly into mouse tail veins in this experiment, in the future, OX substance might become intravenously injectable for the treatment of patients with leukemia and solid malignant tumors.

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