Acta Medica Okayama

Volume 16, *Issue* 5 1962 *Article* 4

OCTOBER 1962

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Abstract

Biological effect of the unsaturated fatty acid fraction from the X-ray irradiated rabbit liver (OX) on HeLa cells has been observed in vitro comparing with the effect displayed on the same strain cells by the unsaturated fatty acid fraction from the non-irradiated rabbits, which is extracted by the same method as in OX, The observations have proven that OX is a powerful cytotoxin in a concentration of 0.05-0.025 per cent and induces a severe cell degeneration and cell death, resulting in a marked arrest in the growth of the cells. The similar effect has been observed by unsaturated fatty acid fraction from the nonirradiated rabbits, but the effect was much less comparing to that of OX. Possible mechanism of the cell damage by OX has been discussed.

*PMID: 14002319 [PubMed - OLDMEDLINE]

Acta Med. Okayama **16**, 265—274 (1962)

GROWTH INHIBITION AND MORPHOLOGIC CHANGES OF HELA CELLS EXPOSED TO UNSATURATED FATTY ACID FRACTION FROM THE LIVER OF X-RAY IRRADIATED RABBITS (OX)

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Received for publication, September 18, 1962

Through the studies on the animals suffering from X-ray injury it has been suggested that some hemolytic substance should be produced in the animal body irradiated with X-ray. Extracting the organs of X-ray irradiated rabbit with several solvents a powerful hemolytic substance was obtained from the alcoholic extract, and chemical analysis proved these substances to be lysolecithin and unsaturated fatty acids[1,2]. Of these components the unsaturated fatty acid fraction inhibited the spermatogenesis of rabbit by intravenous injection resulting in the abnormal mitosis followed by cell death[1]. The morphologic changes were very similar to that induced by X-ray damage. The intravenous application to the tumor (Brown-Pearce) bearing rabbits caused the disappearance of the tumors[3]. Thus it has been suggested that this substance may have a prosperous antitumor activity at least for some kinds of tumors. A few clinical observations on some human skin cancer painted with this substance gave favorable results showing the marked reduction in the size of tumors[4]. In this paper morphologic changes with the suppression of growth of HeLa cells exposed to OX are demonstrated comparing with those exposed to the unsaturated fatty acid fraction from the non-irradiated rabbit, which was obtained by the same method as in OX.

MATERIALS AND METHODS

HeLa cell strain from Department of Pathology of University Institute for Infectious Diseases, Tokyo, served as materials. They were cultured in a culture vessel (200 ml.) with about 10 ml. of YLE culture medium containing 20 per cent of bovine serum. After one week culture, changing the medium every 3 days, the cells were harvested by the following way; the cells proliferated adhering to the vessel wall were washed with YLE medium containing no bovine serum 3 times by changing the liquid and shaking vigorously. After washing 10 ml. of

This work was supported by a grant from National Institutes of Health, (CY-6146 CY,) Bethesda, Maryland.

the same YLE medium was added to the vessel and the cells were detached from the vessel wall by rubbing gently with a soft gum cleaner. The cell suspension thus obtained was used for the inoculation. For the observation of morphologic changes, which would be induced by OX, the cell suspension was diluted with the same medium so that 150,000 to 200,000 cells were contained per ml. Each ml. of this diluted cell suspension was transferred into TD tube having a slender cover slide (6×40 mm) and 5 tubes were used for one observation. These tubes were incubated at 37°C for 24 hours. After confirming the healthy cell growth these tubes were supplemented with 1 ml. of OX colloidal solution in the concentration of 0.1 per cent, 0.05 per cent, 0.025 per cent, and 0.0125 per cent, and as the control the cells cultured in the tubes by the same method but supplemented with the same amount of the unsaturated fatty acid fraction from rabbits received no irradiation, prepared in the same way as in the extraction of OX, were observed. Besides these, one tube added with one ml. of culture medium alone was observed. Observations were carried out on the cells growing on cover-slides under a phase contrast microscope at 37°C at the intervals of 3, 6, 9, 12 and 24 hours after transfer of the cells.

OX used for this experiment, donated by Toshiba Seiyaku Co., Kawasaki, was the unsaturated fatty acid fraction extracted from the liver of rabbits irradiated with X-rays. With this fraction a colloidal solution of OX was prepared by adding the culture medium and a trace of Tween 80 and by stirring in Waring Blender at 20,000 r. p. m. for 5 minutes.

For the observation of inhibitory effect of OX on the cell growth the original cell suspension was diluted with the culture medium so that it contained 70,000 cells per ml. Each one ml. of this suspension was transferred into TD tubes. Thirty-six tubes, 4 groups and 9 in each, were used for one observation for OX. Each of 9 tubes was added 1 ml. of 0.1 per cent OX in 1st group, 0.05 per cent OX in 2nd group, 0.025 per cent OX in 3nd group and 0.0125 per cent OX in 4th group so that the final concentration of OX would be 0.5, 0.025, 0.0125 and 0.0062 per cent in respective group. These were incubated at 37°C and observed 2, 4 and 6 days after the start of the incubation, three tubes in each group for one observation. As the control, another series of 36 tubes of culture cells growing under identically the same conditions as in the above experiment but added with the unsaturated fatty acid fraction from normal rabbit were observed. Each observation was accompanied also by the one on the cultured cells growing in the culture medium containing no fatty acid. After checking the morphologic change by phase contrast microscope the cell number was calculated by using Toma's counter plate for blood cell count. Three counts were taken for one sample and nine counts for one observation of three tubes. Then the mean value of the nine counts was recorded.

OBSERVATIONS AND RESULTS

HeLa cells grown in the medium containing 0.05 per cent OX show a marked picnosis of the nuclei or blister formation already after three-hours incubation (Plate 1, Ca). After six hours the degenerative changes of cells become more marked and almost all the cells form blisters (Cb). After nine hours many cells crumble down and become detached from the vessel wall (Cc), and after 12 and 24 hours many cells, floating in the medium, show a marked cytolytic change (C d, e). The cells incubated for 3～6 hours with the colloid of unsaturated fatty acid fraction from non-irradiated rabbit liver show hardly any degenerative change excepting the appearance of some lipid granules in cytoplasm (Plate 1, Ba, b, c, d, e).

In the control group (Plate 1, Aa～e) cells are found to be always proliferating actively, showing no degenerative change.

In the medium containing 0.025 per cent OX the bubbling of cytoplasm having lipid granules can be seen in the cells incubated three hours (Plate 2, Ca). A similar change is also observed after 9 hours (Plate 2, Cc) but appearance of gross lipid granules and blister formation occur with a complete disappearance of mitotic picture. At 24 hours of culture the cells become completely detached from the vessel wall, showing granules distinctly (Plate 2, Ce). In the group treated with the fatty acid fraction from the normal rabbits, however, no marked change is observable by three-hours' incubation, (Plate 2, Ba), and a few granules appear by six-hours' incubation (Plate 2, Bb) showing mitotic figure even 24 hours later (Plate 2, Be).

In the group treated with 0.0125 per cent OX, lipid granules begin to appear by 3 to 6 hours' incubation and thereafter the appearance of the granule becomes marked with lapse of time, showing also bubbling. In the group treated with the same concentration of the fraction from non-irradiated rabbit hardly any marked change appears up to the ninth hour, excepting some lipid granules and even after 24-hours many of the cells are still undergoing cell division.

In the group treated with 0.0062 per cent OX at about three-hours incubation some of the cells show bubbling. After about six-hours incubation the appearance of lipid granules can be observed and it becomes more marked with lapse of time. On the other hand, the group treated with the substance from non-irradiated rabbit hardly shows any change, which is about the same as observable in the group cultured in the medium without addition of any substance.

In the observations on cell proliferation it has been demonstrated that the group of the cells treated with OX at the concentration of 0.05 per cent shows the cell count of 24,000 on the second day of culture, 26,000 on the fourth day,

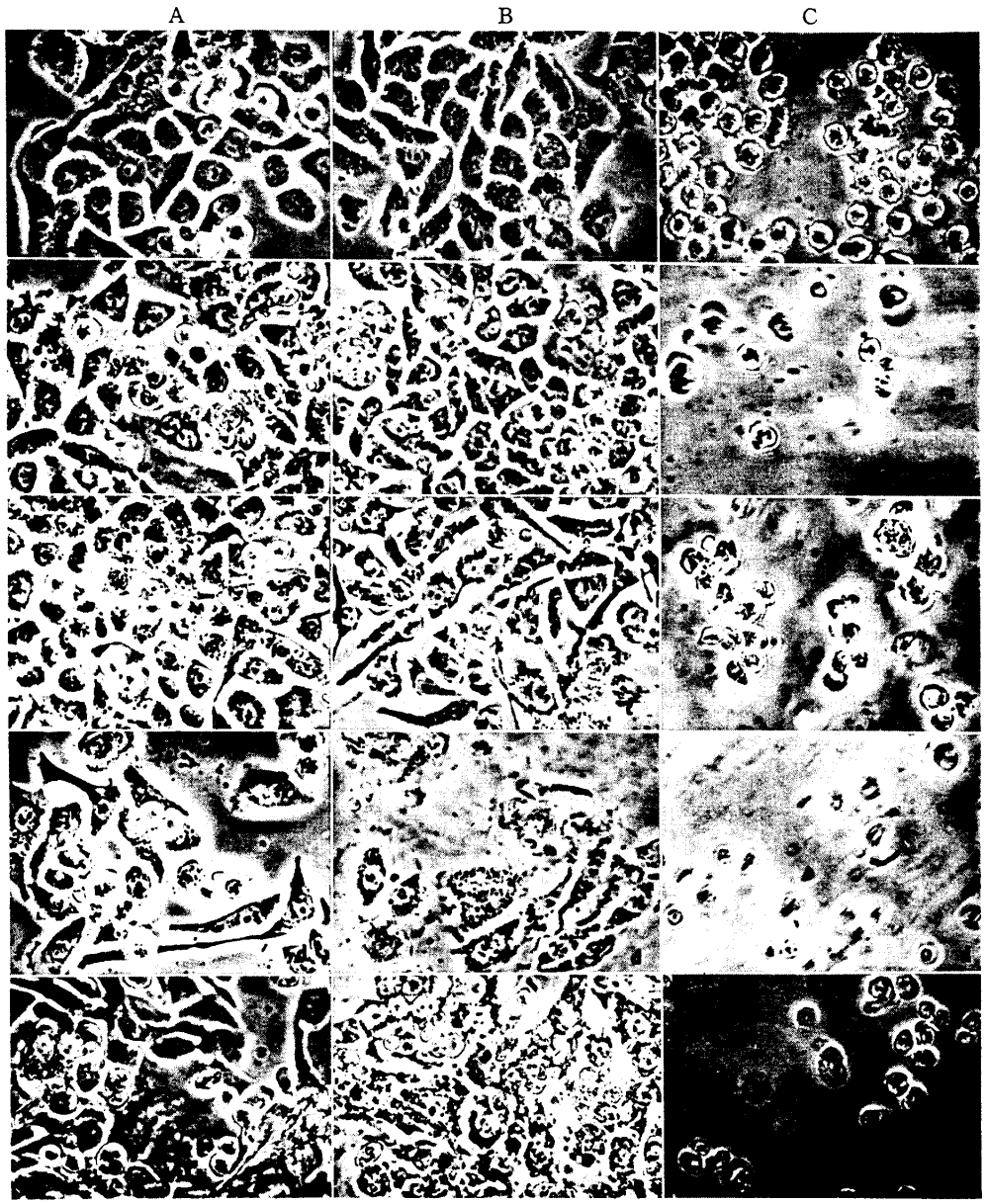

Plate 1. Showing HeLa cells exposed to 0.05 per cent emulsion of the unsaturated fatty acid fractions from the liver of rabbits irradiated and non-irradiated with X-rays.

A. stands for the control, cultured in YLE medium at 37°C.

B. the cells treated with the fatty acid fraction from normal rabbits.
The pictures show the morphologic damages seen after, three (a), 6 (b), 9 (c), 12 (d), and, 24 (e) hours of incubation.

C. the cells treated with the fatty acid fraction. from the irradiated rabbit (OX).
The symbols, a, b, c, d and e correspond to the respective symbols in B.

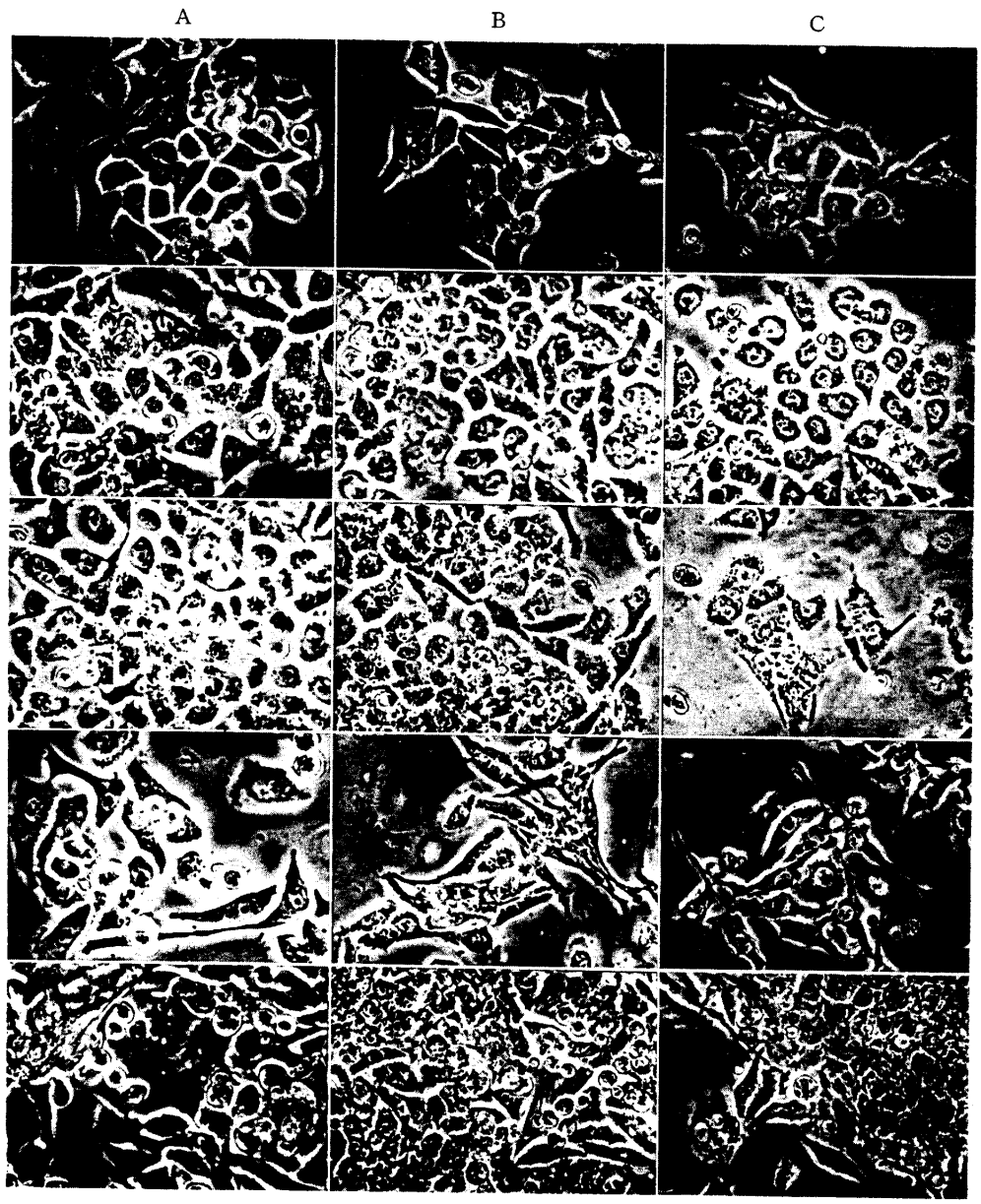

Plate 2. HeLa cells treated with the same substances as those appearing in Plate 1, but in the dilution of 0.025 per cent.

The symbols, A a-e, B a-e and C a-e, mean the same as respective ones in Plate 1. Note the OX is still toxic to HeLa cells even at the concentration of 0.025 per cent.

and 12,000 on the sixth day, while in the group treated with unsaturated fatty acid fraction from non-irradiated rabbit at the same concentration the cell count has been found to be 98,000 on the second day of incubation, 192,000 on the fourth day, and it is 128,000 on the sixth day. In contrast to this, in the untreated control group the cell count is 264,000 on the second day, 492,000 on the fourth day, and it is 884,000 on the sixth day, revealing the cell proliferation 10 to 70 times as much as that in the groups treated with OX substance (Fig. 1). In the group treated with 0.025 per cent OX the growth inhibiting

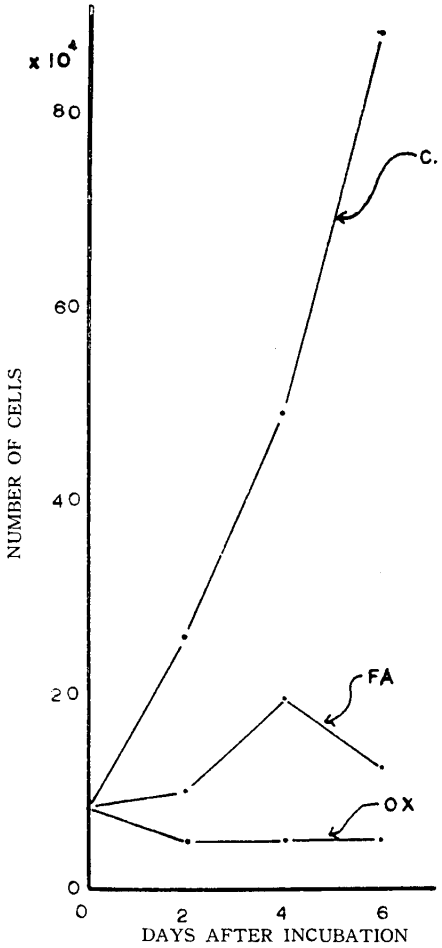

Fig. 1. Inhibitory effect of OX on the proliferation of HeLa cells
OX... incubated with 0.05% OX
FA... incubated with 0.05% FA from non-irradiated rabbit
C.... control

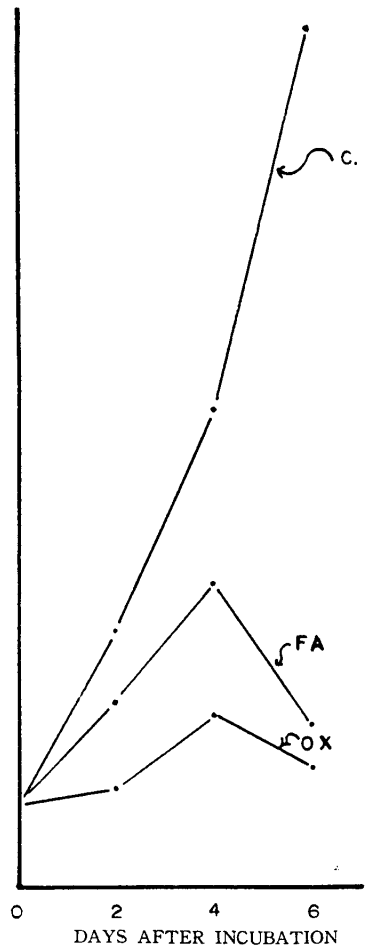

Fig. 2. Inhibitory effect of OX on the proliferation of HeLa cells
OX... incubated wiht 0.025% OX
FA... incubated with 0.025% FA from non-irradiated rabbit
C.... control

effect is rather weaker than the former case, showing the cell count of 99,000 on the second day of culture, 680,000 on the fourth day, and 121,000 on the sixth day. When the fraction in the same concentration from the non-irradiated rabbits is added to the medium, the cell count is 185,000 on the second day, 308,000 on the fourth day, and 160,000 on the sixth day, indicating that this fraction also shows an inhibitory action on the cell proliferation although its effect is weaker than that of OX (Fig. 2).

Even in a concentration as low as 0.0125 per cent an inhibitory action of

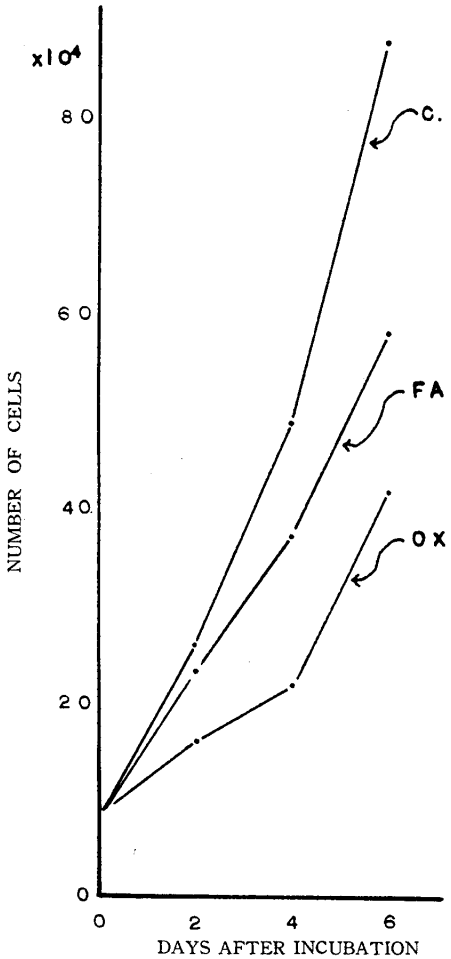

Fig. 3. Inhibitory effect of OX on the proliferation of HeLa cells
OX... incubated with 0.0125% OX
FA... incubated with 0.0125% FA from non-irradiated rabbit
C.... control

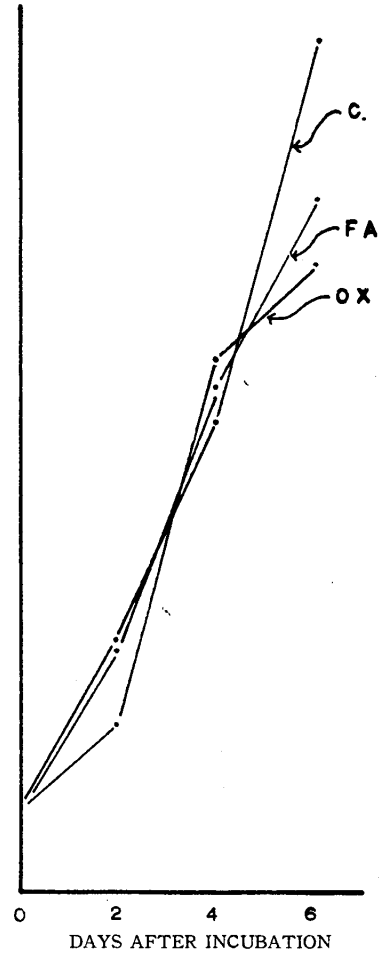

Fig. 4. Inhibitory effect of OX on the proliferation of HeLa cells
OX... incubated with 0.00625% OX
FA... incubated with 0.00625% FA from non-irradiated rabbit
C.... control

OX on the cell proliferation can be recognized. Namely, when OX is added to the medium at this concentration, the cell count on the second day of culture is 164,000, 266,000 on the fourth day and 416,000 on the sixth day, i. e. the cell proliferation on the sixth day is one half that of the control. When the fraction of the same concentration from non-irradiated animals is added to the medium, an inhibitory action can likewise be observed. That is, in this case the cell count on the second day is 236,000, 374,000 on the fourth day, and 584,000 on the sixth day (Fig. 3).

At the concentration of 0.0062 per cent OX shows an inhibitory effect on the cell proliferation but the fraction from the non-irradiated animals at this concentration reveals hardly any marked difference from that of the control group. Namely, in the case with addition of OX the cell count is 170,000 on the second day, 558,000 on the fourth day and 646,000 on the sixth day, whereas with the fraction from the non-irradiated animals it is 249,000 on the second day, 528,000 on the fourth day and 720,000 (884,000 in the control group) on the sixth day (Fig. 4).

DISCUSSION

A few years have elapsed since the specific biological activity of OX displayed on the spermatogonia of rabbit has been found. In the former paper it has been reported that the unsaturated fatty acid fraction from the liver of non-irradiated rabbits has no such an effect as OX on spermatogonia. Since then the antitumor activity of OX has been noticed and several experiments have been done proving this substance to show a favorable effect on some animal tumors and some of human tumors. But the observation by YAMAOKA[7] and others showed no difference in the antitumor activity between OX and the unsaturated fatty acid fraction from non-irradiated rabbit as revealed on contact test by using Yoshida tumor cells aud Ehrlich ascites tumor cells. The present results, however, indicate that the unsaturated fatty acid fraction isolated from the liver of rabbit after X-ray irradiation has a biological effect different from that of non-irradiated animals. Both of them show the cytotoxic activity but the activity is extremely high in the former. It induces a severe morphologic damage of HeLa cells inhibiting the cell proliferation *in vitro* in an extremely low concentration.

Such an inhibitory effect can be recognized most markedly on the cells cultured in the medium supplemented with 0.05 per cent OX. The cells show an intense blister formation already at the three-hour incubation, showing pycnosis of the nuclei and the cells, the cells turn into a spherical form, ultimately fall off from the wall of culture vessel. These changes mean the death of cell after degeneration, and cell count in each tube proved that the cell prolifera-

tion almost completely ceases. As the concentration of OX is decreased the change in the cells becomes less and simultaneously its inhibitory effect on the cell proliferation grows weaker. In this manner with OX at the concentration as dilute as 0.0065 per cent the morphologic change of the cells in culture hardly differs from that of the control group, but even then an inhibitory effect on the proliferation can be recognized though only slightly. The blister formation of the cells affected by OX may be due to the disterbance of the cell metabolism, because it has been proven that OX acts as a powerful uncoupler of oxidative phosphorylation as revealed by using rat liver mitochondria[5]. UENO[4], one of our collaborators, has reported that in the Ehrlich ascites tumor cells treated with OX P^{32}-incorporation into DNA and RNA is impeded and it also inhibits protein synthesis. These effects may be connected with the picnotic change of the nuclei observed *in vitro* or to the formation of giant cells in the former experiment. It is uncertain what is the primary effect and what is the secondary one. But the arrest of energy producing system, the loss of ATP synthetic activity, may be the most important one[5]. SHIAKU[6] showed further that the activity of succinic-dehydrogenase of some tumor cells is markedly diminished by OX. This confirms again that OX disturbs the energy producing system. DNA, RNA and protein synthesis is dependent on the energy metabolism and by the disturbance of the latter the formor is severely affected, but the distubances of the synthesis of DNA etc., may not directly induce the damage of energy producing system. So it is probable that the arrest will primarily be on the reaction related with ATP synthesis. But the final decision requires the further observation as the morphologic change of the cell, especially those in germinal cell *in vivo*, is much similar to that induced by x-ray which will arrest first the mitotic apparatus.

SUMMARY

Biological effect of the unsaturated fatty acid fraction from the X-ray irradiated rabbit liver (OX) on HeLa cells has been observed *in vitro* comparing with the effect displayed on the same strain cells by the unsaturated fatty acid fraction from the non-irradiated rabbits, which is extracted by the same method as in OX, The observations have proven that OX is a powerful cytotoxin in a concentration of 0.05—0.025 per cent and induces a severe cell degeneration and cell death, resulting in a marked arrest in the growth of the cells. The similar effect has been observed by unsaturated fatty acid fraction from the non-irradiated rabbits, but the effect was much less comparing to that of OX. Possible mechanism of the cell damage by OX has been discussed.

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