Source of Stimulation of Tumor Inocula by Lethally Irradiated Cells.* (32059)

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The addition of supra-lethally irradiated isologous tumor cells to a viable tumor inoculum decreases the latency,‡ increases the percentage of tumor takes,§ and decreases the host survival time(1). This stimulation of tumor growth becomes progressively more pronounced as the number of viable tumor cells in the inocula is decreased. The action is not restricted to irradiated isologous tumor cells; the same effect was observed with the addition of histo-incompatible tumor or isologous liver cells to the tumor inoculum(2). Revesz(2) has postulated the following possible modes of action of the "non-viable" cells on the viable tumor cells: (a) a direct action of the killed cells on the viable tumor cells, such as a transfer of material from the radiation-killed cells to the living cells; (b) an indirect action mediated through the host; or (c) a combination of both. The present study was undertaken to determine if whole lethally irradiated cells are essential, and if not, what cellular components are involved in the enhancement of tumor growth. The results obtained indicate that "nuclear" fractions from lethally irradiated isologous tumor cells are equally capable of producing a similar response on the part of implanted tumor cells. However, subjecting either whole irradiated cells or "nuclear" fractions to sonication before their addition to the viable tumor inoculum eliminated their ability to decrease latency and incrase the number of tumor takes.‖

Materials and methods. Mice and tumor. Both male and female F_1 hybrids of the C3H/Wr and C57/6Wr strains from our breeding colony were used in all experiments. The MTG-B tumor strain was derived from a mammary adenocarminoma that arose spontaneously in an aged breeding C3H female in our colony, and its transplantation history has been described(3). The tumor has been carried in C3H or histocompatible F_1 hybrid mice.

Transplantation. The tumor transplantation procedure used has been previously described (3). Briefly tumor suspensions were prepared in Eagle's basal medium, centrifuged, and serially diluted on the basis of the volume of the centrifugally packed tumor material. Inocula of 0.05 ml of suspensions containing final concentrations of 0.1%, 0.032%, and 0.01% viable cells¶ from untreated tumors, combined with test preparations, were injected subcutaneously in the medial aspect of both hind legs of the recipient mice. These relatively low viable cell levels were employed to increase the ability to detect differences in tumor takes and latency. In each experiment, a minimum of 5 recipient mice, or 10 transplant sites, were used.

Irradiation. Supra-lethally irradiated cells were obtained from animals bearing tumors approximately 10 mm in diameter which had received 11,200 rads of total body irradiation with gamma rays from a 2000 Ci ^{137}Cs source at a dose rate of 46.3 rads/min as previously described(5). In order to insure that no cells capable of producing a tumor remained after

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‡ The term, latency, refers to time between tumor implantation and appearance of a palpable tumor(3).

§ The term, "percentage of tumor takes," refers to percentage of inocula which gave rise to growing tumors.

‖ Data presented in part at Third International Congress of Radiation Research, Cortina, Italy, June 26, -July 7, 1966(4).

¶ The term, "percent viable cells," is used throughout to indicate the viable cells contained in the indicated percent of centrifulgally packed tumor suspension, and does not imply that all the packed material is composed of viable cells.

this dose of radiation, groups of mice were injected with aliquots of the same irradiated cells, irradiated-sonicated cells, or irradiated cell fractions (see below) used in each experiment. None of these controls developed tumors.

Cell fractionation. Supra-lethally irradiated tumor suspensions, prepared in the same manner as transplantation, were homogenized for 10 strokes in sterile teflon Potter-Elvehjem homogenizers. The homogenates were transferred to sterile 15 ml centrifuge tubes and centrifuged at 1800 rpm (600 × *g*) for 10 minutes in a refrigerated centrifuge at 0-4°C (International Equipment Co.). The resulting supernates are referred to below as the "cytoplasmic" fraction, and the packed material, as the "nuclear" fraction. The final concentrations in the tumor inocula of supra-lethally irradiated cells or fractions from them were 31.6% whole cells or the amount of "nuclei" and "cytoplasm" equivalent to 31.6%.

Sonication. Suspensions of supra-lethally irradiated tumor cells or nuclei were sonicated in a Branson Model LS-75 sonicator (Branson Instruments, Inc.) which delivered a nominal frequency of 20,000 cycle/sec for a total of 3 minutes using 15-second pulses, separated by 30-45-second intervals. All sonicated preparations were inspected by phase contrast microscopy to determine the extent of cell or nuclear membrane disruption. Hemocytometer counts indicated that sonication was effective in disrupting 95% of the whole cells, and 97% of the "nuclear" preparations. To increase the efficiency of sonication, the percentages of nuclei or radiation-killed cells were reduced by a factor of 10 from the percentages used in non-sonication experiments, *i.e.,* to 3.2% final concentration.

Results. The changes observed in both tumor latency and number of tumor takes as a result of adding either supra-lethally irradiated tumor cells or their isolated "nuclei" to viable tumor inocula are shown in Table I and Fig. 1. Supra-lethally irradiated cells and "nuclei" were equally effective in reducing tumor latency at all levels of viable cells employed. However, their effect on latency was more pronounced at the lower two concentrations of viable cells (0.032%, 0.01%) used in this study. At the level of 0.01% viable cells, a concentration of 31.6% radiation killed cells or an equivalent amount of "nuclei" in the tumor inocula decreased latency by 6 days (Fig. 1). When the concentration of radiation killed cells or "nuclei" was decreased, the results were similar, but less striking. At a concentration of 3.2% radiation killed cells, a decrease in latency of 2.5 days was observed in the 0.01% viable cell group, whereas, addition of an equivalent amount of "nuclei" decreased tumor latency by 1.5 days (Table II). Similarly, addition of either supra-lethally irradiated cells or "nuclei" to the tumor inocula increased the number of tumor takes, and as noted above, the effect was greater at the lower concentrations of viable cells. At a concentration of 0.032% viable cells and 31.6% of either radiation killed cells or "nuclei," a 3-fold increase in tumor takes was observed (Table I). When the inocula contained 0.01% viable cells and either 31.6% radiation killed cells or its "nuclear" equivalent, an 8-fold increase in tumor takes was observed (Table I). Similar responses were obtained for the 3.2% concentrations of radiation killed whole cells or "nuclei," but they were less marked. There was a 2-fold increase in tumor takes at the 0.01% level of viable cells (Table II). The addition of a "cytoplasmic" fraction decreased the tumor latency and enhanced the number

TABLE I. Effect of Subcellular Fractions on Percent of Tumor Takes.

Viable tumor inocula*	No. inoculation sites	Cell fraction added†	% Takes
.100	98	—	80.3
.100	88	Whole cells	100.0
.100	40	Nuclei	100.0
.100	40	Cytoplasm	100.0
.032	42	—	34.6
.032	42	Whole cells	92.3
.032	20	Nuclei	95.0
.032	20	Cytoplasm	90.0
.010	86	—	10.7
.010	76	Whole cells	80.2
.010	40	Nuclei	75.0
.010	40	Cytoplasm	66.0

* Percentage by volume of centrifugally packed tumor suspension.

† Concentration approximately equal to 31.6% packed whole cells or equivalent percentage of nuclei and cytoplasm.

TABLE II. Influence of Sonication on Added Irradiated Whole Cells and Nuclei on Tumor Takes and Latency.

Viable tumor inocula*	No. inoculation sites	Cell fraction added†	Latency ± S.D.	% Takes
.100	10	——	12.8 ± 1.9	100
.100	10	Whole cells	10.1 ± .9	100
.100	10	" " (sonicated)	12.0 ± 1.4	100
.100	10	Nuclei	10.0 ± .5	100
.100	10	" (sonicated)	13.1 ± 1.6	100
.032	10	——	13.0 ± 2.1	100
.032	10	Whole cells	10.2 ± .7	100
.032	10	" " (sonicated)	14.8 ± 3.5	80
.032	10	Nuclei	10.0 ± 1.3	100
.032	10	" (sonicated)	13.3 ± 2.7	80
.010	10	——	14.2 ± 1.7	50
.010	10	Whole cells	11.6 ± 1.7	100
.010	10	" " (sonicated)	15.8 ± 4.1	40
.010	10	Nuclei	12.7 ± 2.4	100
.010	10	" (sonicated)	15.2 ± 4.1	40

* Percentage by volume of centrifugally packed tumor suspension.
† Concentration approximately equal to 3.2% packed whole cells or equivalent percentages of nuclei and cytoplasm.

of takes but to a lesser extent (Table I, Fig. 1). Again, the changes observed were more pronounced when fewer viable cells were contained in the inocula. At the 0.032% level of viable cells, latency was decreased by 3 days, concomitant to a 3-fold increase in tumor takes, whereas, the addition of the "cytoplasmic" fraction of 0.01% viable cells decreased latency by 4 days and increased the number of takes 6-fold.

Sonication of supra-lethally irradiated tumor cells or "nuclei" essentially eliminated their ability to stimulate tumor growth (Table II).

Discussion. The stimulation of tumor growth induced by supra-lethally irradiated cells, "nuclei," or "cytoplasm," has been evaluated on the basis of latency and percentage of tumor takes. Both parameters are dramatically affected by addition of supra-lethally irradiated tumor cells to the tumor inocula. We have interpreted the parameter, latency, as a measure of tumor growth rate; *i.e.,* the faster the growth rate, the shorter the latency(3). The percentage of tumor takes can be thought of as an indication of cell survival; *i.e.,* if supra-lethally irradiated cells promoted a greater survival of the viable tumor cells in the inocula, more tumors would establish, and develop. Revesz(2) has shown that the stimulation of tumor growth rate and percentage of tumor takes is contingent upon the presence of metabolizing cells in the stimulating material. Those cells which produce the effect, although they are not able to proliferate, still carry on active metabolic processes. In contrast, cells not metabolizing, such as those killed by heating or prolonged incubation, are not effective in promoting tumor growth. A possible explanation may be that the radiation killed cells are donating substances which are critical in the establishment and growth of the tumors. The effect appears to be most critical in a small population of viable cells, becoming more pronounced as the percentage of viable cells is decreased.

Separation of supra-lethally irradiated tumor cells into "nuclear" and "cytoplasmic" fractions enabled us to evaluate the respective contributions of each to tumor enhancement. Our data suggest that the primary source of stimulation is in the nucleus in that the stimulatory effect of the "nuclear" fraction was similar to the action of intact radiation killed cells. The "cytoplasmic" fraction also reduced the latency and increased the number of tumor takes. However, it was not as effective as either whole cells or the "nuclear" fraction. Along these lines, the role of the nucleus and the cytoplasm in radiation recov-

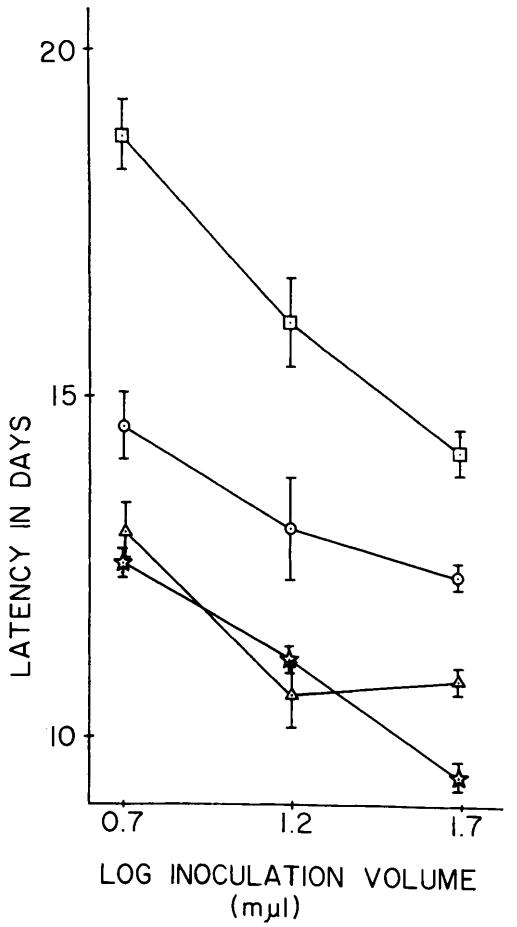

FIG. 1. Tumor latency following inoculation of 3 concentrations of viable cells (squares), of viable cells plus supra-lethally irradiated cells (stars), and of viable cells plus a nuclear fraction (triangles) and a cytoplasmic fraction (circles) prepared from supra-lethally irradiated cells. Supra-lethally irradiated cells given at a final concentration of 31.6%, and fractions at 31.6%-equivalent. Vertical lines indicate standard errors. Number of tumors as shown in Table I.

ery has been discussed by Horikawa, Sugahara, and Doida(6). Recovery in L-cells after x-irradiation was enhanced by addition of subcellular fractions to the culture medium. Both nuclear and cytoplasmic fractions enhanced recovery, increasing the formation of colonies. However, the fractions were not as effective as whole cell homogenates. Although the viable cells in our study were not irradiated, the situation is analagous in that both systems were under stress.

Sonication of either supra-lethally irradiated cells or "nuclei" obtained from such cells prevented the stimulation of tumor growth regularly observed when non-sonicated aliquots of the above are added to viable tumor cell inocula. Since sonication disrupts the cellular and nuclear membranes, the enhancement appears to be dependent upon an intact membrane. The response of viable tumor cells to an unsonicated "cytoplasm" is enigmatic unless one considers that cytoplasm contains membrane bound structures. The stimulatory effect of added radiation killed cells, "nuclei," and "cytoplasm" may be dependent upon their retention in proximity to the viable cells. Products slowly released by them could be efficiently utilized by the living cells. In contrast, following sonication, cells products which may be needed by tumor cells for survival and growth during the critical period before establishment of a vascular supply may be dispersed too rapidly for efficient utilization. The observations of Revesz (2) and Wallace(7) that, for maximum effect, the lethally irradiated cells need be admixed with the viable cells at the time of tumor implantation are in agreement with the above hypothesis.

Summary. Separation of supra-lethally irradiated tumor cells has been carried out to determine their effectiveness in reducing tumor latency and enhancing tumor takes. These experiments indicate that the most pronounced stimulation comes from the centrifugal fraction containing the "nuclei" from supra-lethally irradiated cells. The degree of stimulation by the "nuclear" fraction closely parallels the stimulation by whole radiation killed cells. The "cytoplasmic" fraction was also capable of augmentation of tumor survival and growth, however, less than either the "nuclear" fraction or whole radiation-killed cells. Disruption of the membranes of whole cells or "nuclei" by sonication eliminated the tumor growth stimulation. A tentative mechanism is proposed for the action of radiation killed cells, "nuclei," and "cytoplasm" in enhancing growth and survival of transplanted isologous tumors.

1. Revesz, L., Nature, 1956, v178, 1391.
2. ———, J. Nat. Cancer Inst., 1958, v20, 1157.

3. Clifton, K. H., Draper, N. R., Int. J. Radiat. Biol., 1963, v7, 515.
4. Yatvin, M. B., Toda, J. K., Clifton, K. H., Third International Congress of Radiation Research, Cortina, Italy, June 26-July 7, 1966.
5. Clifton, K. H., Briggs, R. C., Stone, H. B., J. Nat. Cancer Inst., 1966, v36, 965.
6. Horikawa, M., Sugahara, T., Doida, Y., Exp. Cell Res., 1964, v34, 198.
7. Wallace, A. C., Cancer Res., 1965, v25, 355.

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Potentiation of Diethylstilbestrol Induced Aortic Ruptures of Turkeys with Thiouracil.* (32060)

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A high incidence of aortic ruptures can be induced in turkeys by feeding(1) diethylstilbestrol(DES). Hyperlipemia, hypercholesterolemia, and hypotension are accompanying features of the syndrome. It has been reported that thiouracil counteracted the protection afforded by estrogens in cholesterol induced coronary atherosclerosis in cockerels(2). Because of this report, the experiments in the present paper were conducted in an attempt to modify, by use of thiouracil, the incidence of DES induced aortic ruptures of turkeys.

Materials and methods. Broad-Breasted Bronze and Broad-Breasted White male turkeys were raised by conventional methods and fed a 20% protein commercial-chick-starter mash until 4 weeks of age. At this time they were changed to a 19% protein diet containing 0.5% NaCl(3). They were changed again at 6 weeks of age to a basal diet containing 23% protein, 6% animal fat, 3% NaCl(3), and various supplements.

Three separate trials were conducted. In each trial the turkeys were randomized into 7 groups of 8 birds each at 6 weeks of age. The following supplements were added to the basal diet and were fed to 3 groups of poults in each trial: (1) 16 g DES†/100 lb of feed; and (2) 16 g DES/100 lb of feed plus 0.1% thiouracil. One group of turkeys was fed unsupplemented basal diet and served as controls. Since 3 trials were conducted, a total of 9 groups were fed each supplemented diet, and 3 groups the unsupplemented diet. In addition, 1 group of turkeys in the third trial was fed basal diet supplemented with 0.1% thiouracil.

Indirect systolic blood pressure measurements were made on 4 poults per group in each trial at 10 weeks of age(4). Total plasma lipid(5) and cholesterol(6) determinations were recorded on 4 poults per group in each trial at 11 weeks of age. The experiment was terminated when the turkeys were 11½ weeks of age, at which time body weights were recorded.

Small pieces of thyroid glands were fixed in 10% neutral formalin at the conclusion of the trials. Sections of this material were stained with hematoxylin-eosin stain for histologic examination. Small pieces of abdominal aortas from turkeys that died from aortic rupture and from poults that were sacrificed at termination of the experiments were similarly fixed and stained prior to histologic examination. Frozen sections of abdominal aortas were also prepared and stained with oil red O-luxol fast blue stain.

Results. As there were no significant interactions between trials and treatments, the results of the 3 trials were combined. Thirty-seven percent of the poults fed DES died of aortic rhexis; however, when DES was fed with thiouracil, mortality was significantly increased to 69%. Rhexis occurred only in the abdominal aorta, and was a consequence of

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† Stilbosol, Eli Lilly Co., Greenfield, Ind., contained 20 g/lb in a carrier of polyethylene glycol 200.