Studies on the Mechanism of the Stimulatory Effect of Lethally Irradiated Cells on Tumor Inocula[1] (34636)

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The inclusion of lethally irradiated tumor cells, or "nuclei" prepared from them, with viable cells in transplant inocula increases the number of tumors which develop from small viable cell numbers, shortens the mean life span of the host mice, and decreases the time from grafting until tumors first appear (the tumor latency) (1–4). This enhancement of tumor growth is eliminated, however, when the integrity of the "nuclear" or lethally irradiated cell membranes is disrupted by sonication (4). We have tentatively proposed that the stimulatory effect of both "nuclei" and radiation-killed cells is dependent on their being intact at the time of inoculation and retained in close proximity to the viable cells. In this relationship, macromolecules or breakdown products slowly released by them could be efficiently utilized by the admixed living tumor cells as a source of nutrients for survival and growth during the critical period prior to the establishment of an adequate vascular supply. Evidence is presented below on the effect of sonication of prelabeled lethally irradiated tumor cells and "nuclei" on the persistence of radioactive label (a) at the inoculation site, (b) in tumors developing from concurrently injected viable tumor cells. Methionine-^{75}Se (met-^{75}Se) and 5-iodo-2′-deoxyuridine-^{125}I (IUdR) were used as protein (5, 6) and DNA (7) labels, respectively.

Methods. The C3H/Wr mammary adenocarcinoma strain MTG-B (8) was used throughout these studies. Young adult male C3H/HeJ mice obtained from Jackson Laboratories were inoculated in the medial aspect of both hind legs with suspensions of tumor cells, 20% by volume, prepared in Eagle's basal medium (BME) as previously described (3, 9). Microscopic examination of such tumor suspensions reveals many intact cells and cell clumps, mixed with cellular debris. The met-^{75}Se(Sethotope, Squibb), with a specific activity of 67.3 Ci/mmole was administered ip to some of the tumor-bearing animals in doses of 6 μCi/0.2 ml/injection at 8-hr intervals on the third through ninth days following tumor transplantation. On the tenth day, for the preparation of lethally-irradiated tumor cells, the tumor-bearing mice were irradiated with a total of 14,000 R at a rate of 1800 R/min in a $4\pi^{137}$Cs irradiator. Tumors were removed, cleaned of extraneous and necrotic tissue and a 15% suspension was prepared as above. An aliquot was taken and a "nuclear" fraction was prepared after homogenization with 50 strokes in a Teflon–glass Potter-Elvehjem homogenizer. The homogenate was centrifuged at 600*g*; the resultant "nuclear" fraction was resuspended and washed three times with BME. Half of both the "nuclear" fraction and the remaining irradiated cell suspension were sonicated with a total of ten 5-sec bursts at 95% maximum power on a Bronwill Biosonik II sonicator. No intact cells or nuclei were found on scanning samples of the resultant sonicated preparations with a phase microscope. Final suspensions containing either 4 or 9% by volume equivalent of centrifugally packed, lethally-irradiated "dead cells" (DC), sonicated DC (SDC), "nuclei" (N), and sonicated "nuclei" (SN) were prepared

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for injection. Groups of young adult C3H/Wr male mice bred in these laboratories were injected subcutaneously with 0.1 ml of either the respective sonicated or unsonicated suspensions in the medial aspect of opposite thighs of the same animal. The use of contralateral thighs in the same animal served as an internal control for any radioactivity that might be recycled to or from distant tissues. Aliquots of each labeled preparation were taken immediately before and after inoculation to determine the total radioactivity injected. The animals were sacrificed at intervals following inoculation and each hind leg was severed at the hip and used for measurement of radioactivity at the inoculation site as indicated below and in Fig. 1.

In a second experiment, a suspension of unlabeled "live cells" (LC)[4] in BME was prepared from tumors of unirradiated unlabeled mice. Final suspensions containing 9% LC were admixed with either 9% equivalent of labeled DC, SDC, N, or SN. Suspensions containing the respective intact and sonicated preparations were injected into contralateral thighs of recipient C3H/Wr mice as described above. In addition, other recipient mice received an inoculation of 9% LC only in the thigh of the right hind leg (RHL) and either 9% DC or SDC in the contralateral left thigh (LHL). The animals were sacrificed on day 6, and the tumors were removed, weighed, and their radioactivity was determined. The hind legs were also removed, as above, to determine residual radioactivity.

In a third experiment, young adult $BC3F_1$/Wr mice were grafted with the MTG-B tumor as above, and some were treated with ^{125}IUdR, specific activity 2.5 Ci/mmole, 0.15 μCi/g of body weight/injection at 6-hr intervals on days 1 through 6 following transplantation. On day 7, tumors were removed from ^{125}IUdR and placebo-treated mice and suspensions were prepared. An aliquot of ^{125}IUdR-treated tumor suspension was irradiated *in vitro* with 18,000 R delivered at 1800 R/min in the 4π ^{137}Cs irradiator. Half the irradiated suspension was then sonicated as described above. Final suspensions containing either 10 or 1% unlabeled LC admixed with either 32% ^{125}IUdR-labeled DC or 32% ^{125}IUdR SDC were prepared. Aliquots were taken to determine total radioactivity before injection into $BC3F_1$ mice. The animals were sacrificed 7 to 9 days after injection and the tumors were removed, weighed, and the total radioactivity was determined.

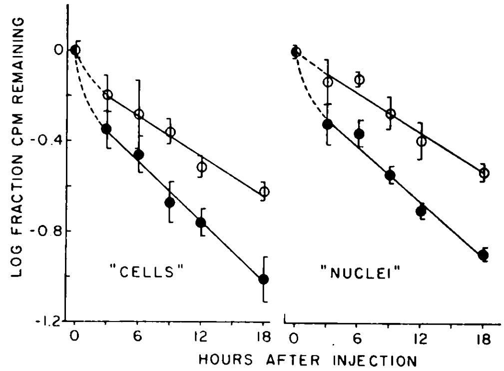

FIG. 1. Decrease in ^{75}Se activity from site of injection of lethally irradiated labeled "whole cell" suspension (○, left), sonicated lethally irradiated "whole cell" suspension (●, left), "nuclear" preparation (○, right), or sonicated "nuclear" preparation (●, right). Vertical bars are standard deviations. Statistical parameters in Table I.

All radioactivity measurements were performed with an iodide crystal well scintillation counter with low-background shielding and a single channel pulse-height analyzer. Radioactivity measurements were made on legs or whole individual tumor samples containing ^{75}Se. Tumor samples containing ^{125}I were pooled for determination of radioactivity as indicated in Tables III and IV. All results were appropriately corrected for radioactive decay. The maximum standard deviation of the counting procedure (for low activity samples) was ± 13% of the net sample counts per minute, and the majority of samples were counted with a standard deviation of 5% or less.

Results. The rate of disappearance of radioactivity from the site of injection of

[4] LC and "live cells" denote centrifugally packed material obtained from a suspension prepared as previously described (3, 9) from unirradiated tumors. It does *not* imply that all such material is comprised of viable tumor cells.

TABLE I. Statistical Parameters for Slopes Indicated in Fig. 1.

Suspension	Slope	95% Limits	Correlation coefficient
"Whole cells"	—0.030	—0.024——0.035	—0.863
Son. "whole cells"	—0.045	—0.040——0.050	—0.942
"Nuclei"	—0.029	—0.024——0.034	—0.892
Son. "nuclei"	—0.041	—0.037——0.045	—0.959

TABLE II. Radioactivity in Tumors from Inocula Containing 9% LC Plus 9%-Equivalent of Either ^{75}Se-labeled DC, SDC^{75}Se, N^{75}Se, or SN^{75}Se.

Suspension	Day of autopsy	Tumor wt		Tumor radioactivity (cpm)	
		No.	(mg ± SD)	Total	(per mg)
LC + DC^{75}Se	6	8	72 ± 25 (0.05>p>0.02)	121.0 ± 31.3 (0.01>p>0.001)	1.9 ± .9 (0.02>p>0.01)
+ SDC^{75}Se	6	8	37 ± 34	48.8 ± 42.1	0.7 ± 0.4
+ N^{75}Se	6	9	52 ± 22 (0.3>p>0.2)	43.3 ± 18.5 (0.001>p)	0.9 ± 0.4 (0.05>p>0.02)
+ SN^{75}Se	6	9	36 ± 26	13.9 ± 7.9	0.5 ± 0.3

TABLE III. Radioactivity in Tumors from Inocula Containing LC Plus ^{125}IUdR-Labeled DC or SDC.

Suspension	Day of autopsy	Tumors		
		No.	Mean wt (mg ± SD)	(cpm/tumor)
10% LC, 32% DC^{125}I	7	30	108 ± 50	9.7[a]
" " 32% SDC^{125}I	7	29	83 ± 40	<1[a]
1% LC, 32% DC^{125}I	8	30	83 ± 51	12.4 ± 2.0[b]
" " 32% SDC^{125}I	9	19	89 ± 43	<1[a]

[a] All tumors were pooled for determination of radioactivity.

[b] Tumors were pooled in four groups of 4 to 13 tumors each. Indicated standard deviation was calculated from the four average (cpm/tumor) values so obtained ($n = 4$).

lethally irradiated ^{75}Se-methionine-labeled DC or N is shown in Fig. 1. The slopes of the disappearance curves of DC and of the N fraction prepared from them were very similar. Sonication of either the DC or N preparations caused a more rapid disappearance of the label from the site of inoculation (Fig. 1). The change in slope due to sonication was similar in both preparations (Table I).

When unirradiated, unlabeled tumor cells were inoculated with either whole lethally irradiated met-^{75}Se-labeled DC or N prepared from them, more radioactivity was recovered in the resultant tumors 6 days after transplantation than from tumors arising from inocula that contained either labeled SDC or SN preparations (Table II). This was true regardless of whether comparisons are made on the basis of radioactivity per whole tumor or per milligram of tumor weight. Furthermore, the mean weights of tumors which developed from preparations containing unsonicated labeled DC were twice as great as those from preparations containing SDC (Table II).

Substitution of either sonicated or unsoni-

TABLE IV. Radioactivity Recovered After Injection of LC in One Leg and DC^{75}Se or SDC^{75}Se in the Other.

Suspension	Leg	Day of autopsy	Tumors No.	Tumors Wt (mg)	Tumor (cpm) Total	Tumor (cpm) (per mg)	Leg cpm RHL[a]	Leg cpm LHL
9% LC	RHL	6	8	83 ± 31[b]	10.1 ± 5.3	0.12 ± 0.03	102.4 ± 18.2	
							↖ ($p<0.001$) ↘	
+ 9% DC^{75}Se	LHL		—					247.1 ± 66.8
				↑ ($p>0.1$) ↓	↑ ($p>0.3$) ↓	↑ ($p<0.01$) ↓	↑ ($p<0.02$) ↓	↑ ($p<0.01$) ↓
9% LC	RHL	6	8	108 ± 28	8.0 ± 2.4	0.08 ± 0.02	79.0 ± 12.0	
							↖ ($p<0.001$) ↘	
+ 9% SDC^{75}Se	LHL		—					164.9 ± 30.9

[a] The cpm remaining after tumor removal.
[b] All values ± standard deviation where indicated.

cated nuclei obtained from equivalent amounts of lethally-irradiated ^{75}Se-labeled cells gave results comparable to that observed for whole cells. However, as might be expected as a result of the smaller quantities of both tissue and total radioactivity involved, when nuclei were employed, both the size and radioactivity of tumors arising from the unsonicated preparations were reduced (Table II).

When unlabeled tumor cells were inoculated with lethally irradiated ^{125}IUdR-labeled cells, or irradiated sonicated labeled cells, more radioactivity was recovered in the tumors which developed from preparations containing unsonicated DC (Table II). Furthermore, the decreased retention of total tumor ^{125}I radioactivity resulting from sonication of the DC preparation was even more striking than when the ^{75}Se label was used. Although the mean weight of tumors developing from inocula containing 10% LC and unsonicated DC was greater than that of tumors from 10% LC and SDC, the difference was not significant.

When only LC were placed in the thigh of one hind leg and ^{75}Se-labeled DC or SDC in the contralateral thigh, the radioactivity per milligram of tissue was higher in tumors of animals receiving the unsonicated cells. In addition, the total radioactivity contained in the tumor and both hind legs was greater in animals inoculated with the unsonicated labeled cells (Table IV).

Discussion. In an earlier study we demonstrated that for either lethally irradiated cells or nuclei to enhance tumor growth they must be intact, since sonication, which disrupts membranes and/or denatures macromolecules, abolishes tumor growth enhancement. Similarly, Revesz (2) showed that heat denaturation of cells eliminates their action. From this information we postulated that enhancement of tumor growth by radiation-killed cells or their "nuclei" is, at least in part, a nutritionally mediated phenomenon (4). The rationale for this hypothesis is that enhancement occurs as a result of the slow release of nutritional elements from the irradiated cells or "nuclei" at the site of transplantation with the subsequent utilization of these nutrients by the viable cells during the critical period prior to the establishent of a vascular supply to the tumor. The current studies, using radioactively labeled tumor cells, provide data consistent with this hypothesis.

The correlation between the effect of sonication on the rate of clearing of radioactivity after the injection of either intact or sonicated ^{75}Se-labeled DC or N from contralateral thighs, on the retention of radioactivity in tumors developing from admixed living cells, and on the relative ability of such preparations to enhance tumor growth (4) strongly suggests that retention of DC and N at the injection site is an important aspect of their action. Furthermore, it should be noted that although the disappearance slopes were similar during the first 18 hr after injection, the radioactivity of DC was less efficiently retained than that of N. This may result from the fact that many cells are damaged prior to sonication by our cytoseive procedure for preparing tumor cell suspensions. Thus much of the radioactivity inoculated as "intact" DC would not have been enveloped by a membrane, whereas the procedure for isolation of N would remove most of the cytoplasmic tags and damaged nuclei, and a proportionately greater percentage of the radioactivity would be present in membrane-bound units. This further stresses the importance of the intact cell or nuclear membrane in both retention of macromolecules and tumor growth enhancement. Furthermore, the radioactivity retained in the disappearance studies of labeled DC, SDC, N, and SN correlates well with the amount of label that is ultimately found in the tumor.

Similarly, sonication of DC which had been labeled with ^{125}IUdR also reduced the persistence of label in tumors arising from inoculations of LC admixed with the respective labeled DC preparations. ^{125}IUdR is incorporated into DNA in place of thymidine. That portion of the labeled DNA precursor which is not incorporated is rapidly deiodinated and catabolized (7). Although it should be noted that the mean ^{125}I radioactivity retained in tumors 7 or 8 days after inoculation of mixed LC and labeled DC suspensions was only 1.5–2.0% of the radioactivity injected, analyses in this and other experiments (to be reported) indicate that most of the retained radioactive label is in the DNA hydrolysates of the acid-insoluble fraction.

This retention of radioactivity of protein and DNA labels could be due to incorporation of whole or of fragmented proteins and DNA, to engulfment of intact labeled DC and N by the growing tumor, or to recycling of radioactivity from nontumor cells coincidentally labeled in the host. Recycling of materials from coincidentally labeled cells is apparently not a major factor in stimulation of tumor growth in that both Revesz (2) and Wallace (10) found that for maximum effect, the lethally irradiated cells need be admixed with the viable cells at the time of inoculation. However, recycling of DC radioactivity to sites distant from the injection occurs, and the DC condition (sonicated or not) appears to govern the overall distribution of radioactivity. The animals injected in one thigh with ^{75}Se-labeled DC and in the other thigh with unlabeled LC had significantly more total radioactivity present in both hind legs in addition to more in the tumor on a per milligram basis than when sonicated material was used. Radioactivity introduced into the mouse in the macromolecules of lethally irradiated intact cells is therefore more efficiently retained and, probably, utilized by the animal as a whole.

Thus, whether by means of cell nutrition in the classical sense or in some other way, such as engulfment, retention of radioactivity from macromolecular labels of lethally irradiated cells does occur concomitantly with enhancement of tumor growth.

Summary. Addition of lethally irradiated cells or "nuclei" prepared from them to tumor inocula increased the frequency of tumor takes and decreased latency and host survival. In previous studies, sonication of either cells or "nuclei" abolished this effect. It was postulated that the stimulatory effect is due to the transfer of nutrients from the membrane-bound structures in the lethally irradiated preparations to living tumor cells prior to the establishment of a vascular supply. In the current studies it was found that sonication of ^{75}Se-methionine-labeled lethally irradiated cells or "nuclei" resulted in a more rapid disappearance of radioactivity from the site of injection and reduced the uptake of the label into simultaneously injected viable tumor cells. Similarly, tumors developing from inocula containing sonicated ^{125}IUdR-labeled irradiated cells retained

less radioactivity than tumors which developed from inocula containing unsonicated lethally irradiated cells.

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