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A. Overall Summary

Emphasis during the past year has been on the second and fourth areas of research outlined in the Specific Aims of the 3-year contract proposal submitted August 1, 1980. These are studies of the effects of potential promoting agents on radiation transformation, and of transformation by internal radionuclides emitting high LET radiation. We have also carried out a detailed investigation of the dosimetry of our alpha radiation source; the results confirmed our previous preliminary dosimetric measurements, and now allow us to publish the alpha transformation data described in detail in the previous Progress Report. Preliminary studies on the mechanisms of radiation transformation have been initiated as described in the previous proposal.

Studies on promotion have focused on the effects of 1) the endogenous steroid hormone 17- β -estradiol; 2) the non-steroidal anti-inflammatory agent indomethacin; 3) the endogenous growth factor called Epidermal Growth Factor (EGF); and 4) Melittin, a stimulator of prostaglandins synthesis. In separate preliminary studies, we observed that certain steroidal anti-inflammatory agents induced transformation by themselves. Indomethacin also induced transformation by itself, but clearly did not act as a promoter of x-ray transformation. EGF, on the other hand, enhanced transformation induced by x-rays similarly to the phorbol ester tumor promoter TPA. The time dependence of the EGF effect is currently under investigation. Melittin was inactivate as a carcinogen or promoter

The investigation of transformation by ^{125}I (which emits densely ionizing Auger electrons) incorporated into cellular DNA as $^{125}\text{IUdR}$ indicates that this radionuclide is much more effective per disintegration than ^3H incorporated into DNA as ^3H -thymidine. When normalized for its enhanced cytotoxicity, $^{125}\text{IUdR}$ is still twice as effective as ^3H -TdR in inducing transformation. This result is qualitatively similar to the comparison between x-rays and alpha radiation reported earlier. It also suggests that DNA damage is important in the initial alteration leading to transformation in irradiated cells.

B. Detailed Report

This section includes those areas in the last 3-year proposal in which significant results were obtained during the 11-month period since the last Progress Report (July 1, 1980 - May 31, 1981).

1. Effects of Promoting Agents on Radiation Transformation

In these experiments, we have examined several classes of agents for possible promoting effects on x-ray transformation in mouse $10\text{T}\frac{1}{2}$ cells. The endogenous steroid hormone 17- β -estradiol, Epidermal Growth Factor (EGF), the bee venom toxin melittin-- a stimulator of prostaglandin synthesis, and the non-steroidal anti-inflammatory agent indomethacin. Anti-inflammatory agents have been reported to inhibit promotion in vivo. In all cases, the effects of these agents were compared with those of the known phorbol ester tumor promoter TPA, and with the protease inhibitor antipain-- a potent suppressor of x-ray transformation. In most experiments, the cells were incubated with non-toxic or minimally toxic concentrations of these agents for the entire 6-7 week post-irradiation expression period. The results of some of these experiments are shown in Tables 1 and 2 on the following pages.

TABLE 1. EFFECT OF POTENTIAL PROMOTING AGENTS ON X-RAY TRANSFORMATION

Treatment ^a		Total No. of Dishes	Total No. of Cells	No. Transformed Foci Observed	Average No. Foci per Dish
1	2				
-	-	120	28,640	1	0.01
-	EGF (50 ng/ml)	28	9,575	0	0
-	Mitomycin-C (10^{-9} M)	17	6,075	1	0.06
-	Indomethacin (0.1µg/ml)	33	11,645	37	1.12
-	17-β-estradiol (10^{-6} M)	82	24,600	21	0.26
100 rads	-	105	34,335	21	0.20
100 rads	EGF	29	11,010	26	0.90
100 rads	Mellitin (2µg/ml)	18	6,210	0	0
100 rads	TPA (0.1µg/ml)	32	12,400	36	1.12
100 rads	Mitomycin-C (10^{-9} M)	30	10,530	4	0.13
100 rads	Indomethacin (0.1µg/ml)	11	5,885	2	0.18
600 rads	Antipain (50µg/ml)	26	9,625	1	0.04

^aSecond treatment begun 24 hrs after seeding (immediately after irradiation) and maintained for full 6-7 week expression period. Data pooled from several experiments.

TABLE 2. EFFECT OF 17- β -ESTRADIOL ON X-RAY TRANSFORMATION

Treatment ^a		Total No. of Dishes	No. Transformed Foci Observed	Average No. Foci per Dish
1	2			
-	-	56	0	0
-	17- β -estradiol (10^{-5} M)	72	14	0.19
400 rads	-	102	26	0.25
400 rads	17- β -estradiol (10^{-5} M)	70	31	0.44
400 rads	17- β -estradiol (10^{-5} M) antipain (600 μ g/ml)	20	2	0.10
-	17- β -estradiol (10^{-5} M) antipain (600 μ g/ml)	20	1	0.05

^aSecond treatment begun 24 hours after seeding (immediately after irradiation) and maintained for 5 days. Data pooled from several experiments. Estradiol and antipain given concurrently.

The effects of potential tumor promoters on x-ray transformation are shown in Table 1. Indomethacin and 17- β -estradiol both induced a fairly high yield of transformants by themselves. In cultures treated with x-rays and indomethacin, however, the yield was reduced over that expected. EGF did not transform by itself, but consistently enhanced the yield of transformants induced by either x-rays (Table 1) or ultraviolet light exposure (data not shown). The enhancement was similar in magnitude to that induced by TPA in the same experiments; it was not significantly inhibited by indomethacin (data not shown). Melittin, on the other hand, appeared to suppress both x-ray and UV-induced transformation. Also included as a control in these experiments was the protease inhibitor antipain. Antipain induced no transformation by itself, but markedly suppressed transformation induced by x-rays (Table 1). As previously reported, it also completely inhibited the promotion of x-ray transformation by TPA. Indomethacin also inhibited the TPA enhancement of x-ray transformation (data not shown).

As is also shown in Table 1, no promoting effect was observed when x-irradiated cells were incubated during the entire expression period in the presence of low concentrations of mitomycin-C. Some investigators have found that TPA induces a low level of sister chromatid exchanges (SCE) in mammalian cells, and it has been hypothesized that this phenomenon may reflect mitotic recombinational activity which leads to promotion by facilitating the segregation of specific recessive mutations in daughter cell populations. Mitomycin-C is a potent inducer of SCE; at the concentration employed it enhanced the spontaneous SCE frequency by a factor of about 1.5, a similar enhancement to that produced in 10T $\frac{1}{2}$ cells by TPA.

The interactions between x-irradiation and the endogenous steroid hormone 17- β -estradiol are shown in Table 2. This hormone induced transformation by itself, and increased x-ray transformation in an additive fashion. Thus, these two agents may act as carcinogens, but estradiol does not appear to act as a true promoter of x-ray transformation. As can be seen in Table 2, the protease inhibitor antipain also suppressed transformation induced by estradiol.

Perhaps the most important finding to arise so far from these experiments, in terms of hazards of low level radiation exposure, is the observation that EGF can act as a potent enhancer of x-ray transformation. EGF was not carcinogenic by itself (Table 1). To our knowledge, these results provide the first evidence that a purified polypeptide growth factor can enhance malignant transformation in vitro. On a molar basis, EGF is about 10 times more potent than the highly active plant-derived tumor promoter TPA. The concentration range in which it was effective in these experiments (10^{-9} - 10^{-10} M) is within physiologic levels. Thus, the origin of human tumors may result from a complex interaction between environmental factors including radiation and endogenous growth factors.

2. Transformation by Internally Emitting Radionuclides

This research has focused on the effects of ^{125}I incorporated into the DNA of cells as $^{125}\text{IUdR}$, and tritium incorporated into DNA as ^3H -thymidine ($^3\text{H-TdR}$). Decay of ^{125}I is associated with the release of a shower of very low energy Auger electrons. There are thus two special characteristics of this radiation in terms of its biologic effect: Its LET is high; and its

effects are highly localized with the DNA of the cell. Haseltine and coworkers (unpublished) have recently shown that the decay of ^{125}I UdR in DNA destroys both of the opposing bases, but that its effects are limited to a few bases either side of the site of incorporation. Thus, damage is not only localized to the DNA, but highly localized with the DNA with a high probability of inducing double strand breaks.

We have studied transformation by ^{125}I UdR and ^3H -TdR in mouse BALB/3T3 cells. The cells were incubated with the radiolabeled precursor for a single S phase of synchronous growth following their release from density inhibition as described in the proposal. Results have been expressed on a per disintegration basis by actual measurements of the incorporated radioactivity in each experiment, and parallel autoradiographic controls to assess the fraction of labeled cells.

Survival studies have indicated that both radionuclides yield an exponential decline in survival with increasing dose (amount of incorporated radioactivity), with no shoulder on the survival curve. ^{125}I UdR is approximately seven times more cytotoxic to 3T3 cells per disintegration than is ^3H -TdR. Results on the induction of transformation by the two radionuclides are shown in the Figure below.

As can be seen, for transformation ^{125}I UdR is about fifteen times more effective than ^3H -TdR per disintegration. If these results are normalized for the differential cytotoxic effects of the two isotopes, ^{125}I remains twice as effective as ^3H for a given level of survival. A 2-3 fold greater amount of energy is absorbed in the nucleus per disintegration following ^{125}I decay as compared with ^3H decay. If we assume that nuclear irradiation is important for the cytotoxic and transforming effects of radiation, this factor should be accounted for. Under these conditions, however, ^{125}I UdR remains 5-7 fold more effective in inducing transformation than ^3H -TdR.

We suggest several tentative conclusions from the results to date.

(1) The higher LET radiation emitted by ^{125}I is not only more efficient in killing cells, but is relatively even more efficient in transforming cells than is low LET radiation. The increased effectiveness of ^{125}I is qualitatively similar to that previously observed for ^{238}Pu alpha irradiation in exponentially growing 3T3 cells.

(2) The site of the initiating event in radiation induced transformation is within the nucleus of the cell, and, more specifically, involves DNA damage, as the low energy electrons emitted by ^{125}I are largely absorbed within the DNA molecule. Furthermore, highly localized areas of radiation within DNA such as is induced by ^{125}I decay appear more carcinogenic than the more homogenous distribution of radiation which results from ^3H decay.

(3) The type of molecular DNA damage important in transformation may involve gross structural changes such as double strand breaks rather than simple base damage. ^{125}I and alpha radiation produce double strand breaks with a higher frequency than do ^3H beta radiation or x-rays.

3. Dosimetry of Alpha Radiation Source

We previously carried out preliminary dosimetric studies on the alpha radiation source used throughout the previous grant period with a silicone surface barrier detector. The results indicated the dose-rate to the cells was 24 rads/min. Because of the possible errors inherent in this method, the dose-rate from the ^{238}Pu source was again measured in a separate series of experiments which utilized a different technique. We employed an air-filled ionization chamber of parallel plate geometry having an entrance window of aluminized Mylar whose thickness was $1.8 \times 10^{-3} \text{ g cm}^{-2}$. The sensitive volume of this chamber was defined by the 0.318 cm gap between the plates and by the 1.00 cm diameter of the collecting electrode connected to the electrometer. The grounded guard electrode surrounding the collector extended to a diameter of 7.6 cm. The electrometer used was a Keithley Model 616 digital electrometer. Bias voltage was applied from a 300 volt battery, and no polarity effect has been observed.

The construction of the source, the "honeycomb" collimating grid and its clamping assembly are such that the alpha particles had to travel through at least $0.75 \times 10^{-3} \text{ g cm}^{-2}$ of air before reaching the entrance foil of the chamber. The air path was increased in steps to measure the ionization versus depth curve. The air path was adjusted for the thickness and stopping power of the window, and for half the thickness of the air gap between electrodes. The positions of the entrance and exit faces of a typical cell attached to the bottom of the culture dish were calculated. The measurements made with the ionization chamber at minimum air gap correspond in depth to the center of the typical cell during the cell irradiations.

The traverse uniformity of the dose-rate was investigated by moving the ionization chamber to several points along two diameters of the source assembly. Spatial resolution is coarse, but the data show a gradual fall-off from the central intensity amounting to 15% at 2.5 cm radius. Conversion of the ionization signal to dose-rate was based on the dimensions of the sensitive volume and published values of W, the energy deposited per ion pair formed. The mean alpha energy at the point of interest was estimated to be 3 MeV from range-energy curves. The dose delivered to the cells can then be calculated using the formula:

$$D = \frac{10^{**5} Q W S_m}{V}$$

where D is the dose (rads), Q is the charge collected from the ionization chamber (coulombs), W is the energy per ion pair (eV), ρ is the density of the gas (gm/cm^{**3}), V is the sensitive volume of the chamber (cm^{**3}), and S_m is the ratio of the mass-stopping power of the target material to that of the gas. We thus obtained a central dose-rate of 24.2 rads tissue/minute at the center of the irradiated cell.

As a check, the same ionization chamber was exposed to a uniform beam of 155 MeV protons whose calibration is well known. By use of estimates of the ratios of W for 3 MeV alphas and 155 MeV protons, the sensitivity of the chamber was recalculated giving a central dose-rate of 24.4 rads tissue/minute. Such good agreement is fortuitous. Taking account of the estimated uncertainties of all factors involved we believe the doses stated to accurate to $\pm 10\%$.

C. Publications

1. Fisher, P.B., Mufson, R.A., Weinstein, I.B. and Little, J.B. Epidermal growth factor, like tumor promoters, enhances viral and radiation-induced cell transformation. *Carcinogenesis* 2: 183-187, 1981.
2. Kennedy, A.R. and Little, J.B. Effects of 17B-estradiol on radiation transformation in vitro; inhibition of effects by protease inhibitors. *Carcinogenesis* 2: 67-69, 1981.
3. Little, J.B. Influence of non-carcinogenic secondary factors on radiation carcinogenesis. *Radiation Res.* 87: 1981 (in press).