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KINETIC AND BIOCHEMICAL STUDIES ON TUMOR GROWTH

Comprehensive Progress Report

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SUMMARY

The growth kinetics of four lines of the C3H mammary tumor have been studied in detail by standard autoradiographic procedures in combination with volumetric growth curve analysis. Thus, such parameters as volumetric doubling time, mean cell generation time, growth fraction, and cell loss have been measured. Two of these lines (Slow and S102F) are currently being used for studying perturbed kinetics and the respective values for the above parameters are: Slow; 21.0 days, 34 hours, 0.20, 9% per days and S102F; 2.5 days, 17 hours, 0.60, 27% per day.

A direct method (^{125}I -IUdR Method) for measuring cell loss has also been developed. This method consists of injecting the mice with ^{125}I -IUdR and then measuring the loss of ^{125}I -activity from the tumor. This method has been used in tumors from three of the lines and the results correlate with cell loss as defined by the standard procedures.

The antigenic status of these tumors has been studied as one possible factor underlying the different growth kinetics. The mouse's immunological system was either suppressed (thymectomy and whole-body x-irradiation) or stimulated (previous exposure to tumor cells) and the percent takes, latent period, and growth rates measured. There was no evidence for a strong antigenic factor in any of these tumors.

Hydroxyurea is being used as a "tool" for studying the perturbed cellular kinetics of the duodenum and the Slow and S102F tumors. The methods used are autoradiography, volumetric growth curve analysis, and measurements of the rates of DNA synthesis. Single doses (3 mg/gm body wt.) of hydroxyurea kill the S-phase cells, and produce a temporary G_1/S block, some reduction in the cell-cycle times, and an increase in the growth fraction. Preliminary results from the two-dose experiments suggest that the initial recovery kinetics are similar to the single-dose data, and the perturbed kinetics following a single dose can be used to predict the time when the second dose of hydroxyurea will be most effective.

INTRODUCTION

This work was originally initiated with two major objectives in mind. First, a better understanding of the biology per se of the C3H mouse mammary tumor (specifically, a detailed understanding of the kinetic events at the cellular and subcellular levels which contribute to volumetric growth) and second, the use of this kinetic data in the design of new experimental protocols for the radio- and chemotherapy of these tumors. Such information should lead to logical protocols which will significantly increase the therapeutic gain factor in this animal-tumor system, and hopefully, the insights acquired may have important implications for clinical oncology.

This comprehensive report will be divided along the lines of the major projects which have been undertaken. In each case, the background will be reviewed, the published work briefly discussed, and the work in progress summarized.

I. The ^{125}I -IUdR (5-iodo-2'-deoxyuridine) Method for Measuring Tumor Cell Loss (See enclosed reprints #1-5)

The fact that cells are lost from solid tumors has been recognized for many years by clinical oncologists and pathologists (i.e. distant metastases as well as pyknotic and karyorrhectic cells observed on histopathology slides); however, only recently has cell loss been the subject of extensive quantitative analysis (4,14,28,34). Now, it is well recognized that this parameter must be determined in a comprehensive analysis of unperturbed tumor growth and also in x-ray perturbed growth (5,17). In all these reports, cell loss was estimated by combining autoradiographic and volumetric growth-curve analyses. This method allows one to calculate the potential growth rate, and the actual growth rate and the difference is termed the loss factor. This method is indirect and very time-consuming; thus, the ^{125}I -IUdR technique was devised as a direct way for measuring cell loss. The assumptions are: 1) non-toxic amounts of ^{125}I -IUdR can be incorporated into cellular DNA, 2) the label is lost from the tumor only when the cellular DNA is lost and 3) the ^{125}I -IUdR is not reutilized as extensively as ^3H -TdR (6,20).

The evaluation of the ^{125}I -IUdR method for measuring cell loss from C3H mouse mammary tumors has been completed (8) and can be summarized as follows: 1) IUdR is reutilized but at a much lower rate than thymidine (7,18). 2) The ^{125}I -IUdR method cannot be used in situ because of the high, variable radioactivity in the skin overlying the tumor and in the acid-soluble fraction of the tumor, but 3) it can be utilized in vivo if one removes the tumor and extracts the DNA before counting the ^{125}I -activity.

This method will not give absolute rates of cell loss because of the reutilization. However, the 1971 report showed that this method does agree quite well with the indirect method of combined autoradiography and volumetric growth-curve analysis (8).

Since that time, Mendelsohn and Dethlefsen (28) have published a detailed kinetic analysis on three lines of the C3H mouse mammary tumor, and this same analysis is now completed on the fourth line.* This report shows that loss may occur either from the non-proliferating fraction only (Fast and S102F lines) or from both the proliferating and non-proliferating fractions (Slow line). In the first case there would be considerable delay after the injection of ^{125}I -IUdR before the loss of label occurred from the tumors but in the latter case, one would see loss of radioactivity quite quickly. The specific times, of course, depend upon the kinetics of the particular tumor.

We have used the ^{125}I -IUdR method for measuring cell loss in tumors from these three lines (Dethlefsen and Snively, in preparation). Since the analysis is not complete definite conclusions cannot be made, but qualitatively, the two methods appear to agree. That is, in the S102F line, there is a plateau (no loss of label) for about 140 hours after ^{125}I -IUdR injection and then a rapid loss of radioactivity ($T_{1/2} = 27.0 \pm 5.7\text{hrs}$). In the S102S line, the plateau lasts for 120 hours and then the loss rate is slower ($T_{1/2} = 42.1 \pm 6.4\text{hrs}$). In the Slow line, the loss begins quite quickly (no apparent plateau) and has a $T_{1/2}$ of 84.0 ± 10.0 hours. These data from the Slow line show a shorter half-life (84.0 ± 10.0 vs 121.7 ± 23.2 hrs.) than the previously published results using ^{131}I -IUdR (7). The discrepancy has not been resolved.

In summary, the ^{125}I -IUdR method can be used as a direct measure of cell loss from unperturbed tumors. Thus, it is another useful tool for studies on the growth kinetics of solid tumors. It probably can also be used in studies on perturbed tumors, but the reutilization problem must be evaluated.

IUdR Toxicity: (See preprint #6)

During the course of these experiments, we had observed evidence for

* These lines were developed at the University of Pennsylvania and brought here to the University of Utah. The two original ones were derived from separate spontaneous mammary tumors on different animals and one was very fast growing (Fast) while the other is slow growing (Slow). Unfortunately, the Fast line has been lost.

The next two lines were both derived from the same spontaneous tumor, and in this case, fast growing (S102F) and slow growing (S102S) sublines were vigorously selected. The texture and histological features of these tumors are also very different. The Slow line is very hard, with areas of gross necrosis and other areas of highly organized ducts. The S102 sublines are very soft, almost fluid, with blood filled sinuses and highly repetitious, ductular structures in which the cell linings are only a few cells thick. (This particular work was supported by an NCI grant to Dr. Mendelsohn as well as by this AEC contract.)

IUdR toxicity in the crypts of the duodenum. First, there was a suggestion of cell killing (8) and later unusually low cellular labeling indices were observed in the duodenal crypts (unpublished). This toxicity was observed at doses of 75 μ Ci (0.01-0.02 μ mole) ³H-IUdR per mouse. The problem has been evaluated as discussed in preprint #6. In summary, low doses of IUdR can cause subtle changes in the cellular kinetics; thus, to avoid problems the dose, at least for C3H mice, should be less than 0.01 μ mole per 25 gm. mouse.

II. Immunobiology of the C3H Mouse Mammary Tumors.

Even though the growth kinetics of the tumors from these lines are well understood in a quantitative sense, one does not know the mechanism(s) responsible for the inherently different growth rates. One mechanism which could explain some of this difference is a varying immunological response elicited by the tumors of the different lines.

This colony of C3H mice has been brother-sister mated for over 15 years, and two of the tumor lines were derived from the same spontaneous tumor. Thus, at first glance, a marked immunological response does not appear to be a likely cause for the different growth rates. However, C3H mouse mammary tumors are antigenic and can exhibit tumor-specific antigens (16,29,39). Antigenic differences have also been observed among tumors arising spontaneously in the same host (38).

Moreover, De Cosse and Gelfant (3) have reported that non-cycling tumor cells can be stimulated to proliferate by the administration of an immunosuppressant, and Janik and Steel (21) studied the cell population kinetics of three transplantable tumors following the perturbation of the immunological status of the host and reported a retardation in tumor growth which appeared to be associated with a reduction in the proportion of actively proliferating cells. Also, Choquet, Chavaudre and Malaise (2) have shown that allogenic inhibition of L1210 leukemia cells (grown as ascites) causes an increase in the doubling time and duration of the cell cycle. Thus, it becomes important to know the antigenic status of the mouse mammary tumors. This is necessary for understanding the tumor biology *per se*, but the information becomes even more significant when one uses this animal-tumor system in studies involving radiation therapy and chemotherapy. A different antigenic status could alter the response, and therefore, the conclusions significantly.

This problem has been studied both by immunological suppression and attempted stimulation of the immune system. In the suppression studies, four week old mice were thymectomized and whole-body irradiated (550-650 rads, 250 Kv x-ray). Then 24 hours later, tumors from one of the lines were transplanted subcutaneously into these mice and an equal number of conventional mice. If the tumors are strongly antigenic, then one would see a higher percent of takes, a shorter latent period and faster growth rate when transplanted into suppressed animals. A check on the degree of suppression consisted of the transplantation of a foreign tumor (BALB/C) into conventional and suppressed C3H mice, and also BALB/c skin grafts onto the

C3H mice.

The second part of this study dealt with an attempt to elicit an immune response to the tumors in conventional C3H mice. Tumor fragments were transplanted subcutaneously in the right flank and allowed to grow. The latent periods and growth rates were measured and then when the tumors reached a volume of 300-400mm³ they were surgically removed. Ten to fourteen days later fragments from the same line were transplanted into the left flanks and growth again observed. A strong antigenic response would be noted by a lower percent of takes, a longer latent period, and lower growth rates in the second transplants.

In both cases, the results were negative. We observed no significant changes in the percent takes, latent periods, or growth rates in any of the experimental situations. Thus, the conclusion is that none of these line tumors is strongly antigenic when transplanted into C3H mice.

III. Differentiation of C3H Mouse Mammary Tumor Cells.

Another mechanism which might partially explain the different growth rates is the extent of cellular differentiation in tumors (35). In the 1971 renewal, I proposed to study this concept by evaluating whether tumor cells could be induced to synthesize milk proteins in organ cultures. Specifically, normal mouse mammary tissue (controls) and tumor tissue would be grown in organ culture in standard medium and in the presence or absence of insulin, corticosterone, and prolactin (22,24,26). These three hormones will stimulate the normal mammary gland to synthesize milk proteins, and I intended to determine to what extent, if any, this applies to tumor cells from the various lines.

Dr. R. Mayne, at the University of Pennsylvania, instructed me in the necessary organ culture techniques. It quickly became apparent, however, that this approach was not adequate. Specifically, the heterogeneity of cells and the small number of cells available did not allow adequate resolution or repeatability when determining the incorporation of radioactive precursors into the various macromolecules. Thus, I employed the single cell suspension and monolayer technique as described by McGrath (27). This procedure appeared to be adequate and more promising but I was still faced with developing the system for the cells from the three mammary tumor lines. Because of this delay, I asked for and received a small grant from the University of Utah and this support was used to develop the method. The pilot studies now suggest that this system will be adequate, and this work will be discussed in detail in the renewal proposal.

IV. Work Done While on Scholarly Leave (Reprint #11)

I had the opportunity to take a scholarly leave (July 1, 1970-June 30, 1971) during the course of this contract and spent the time with Dr. G.M. Tomkins, Dept. of Biochemistry and Biophysics, University of California Medical Center, San Francisco. This time was intended to broaden my experience in the molecular biology of mammalian cells and in the current laboratory techniques of biochemistry and molecular biology. This year was extremely productive in this regard and is best summarized in the

in the publications which have or will emanate from this work. (See publication list.)

V. Hydroxyurea (HU) Effects on Duodenal Crypt Cell and Tumor Cell Kinetics

A. Single and Double-dose Effects (See reprints and preprints #'s 7-10)

Ever since the initial work on the current concept of the cell cycle by Howard and Pelc (19) and the mammalian cell cloning and single cell survival techniques by Puck and Marcus (30), it has been the hope of tumor biologists that formal cellular kinetic analysis of normal and malignant tissue would lead to logical improvements in protocols for radiation therapy, as well as chemotherapy. Unfortunately, direct impact on the clinic has been minimal. The reasons are multiple and complex, but basically they center around two facts: 1) There still is not enough information on the complex interactions between therapeutic agents and tissues even in animal models and 2) Most of the laboratory procedures for collecting these data are too cumbersome and time-consuming to be used in the clinic.

We became involved with HU because we felt that this drug could be used to study the basic mechanisms involving cell-cycle perturbations in an animal-tumor system. Then these data could be used to design logical protocols for fractionated chemotherapy and combined chemo- and radiotherapy. HU was chosen because its effects on cells had been studied extensively in vitro (1,23,32,33) and the major effects which are of interest to us (i.e., specific killing of cells synthesizing DNA, a block preventing G₁ cells from entering the S-phase, and partial synchronization upon recovery from the drug), were also reported for in vivo studies (15,25,31).

Studies on the cell-cycle effects in the mouse duodenum and mammary tumor (S102F line) have been published (11,12). A single large (3 mg/gm body wt.) dose was given intraperitoneally. This dose of HU kills the S-phase cells and produces a G₁ block of 4-5 hours in both tissues. The duodenal crypt cells are partially synchronized upon the return of DNA synthesis, with the first cohort of cells synthesizing DNA at 8-9 hours post-HU. Crypt recovery is manifested by a decrease in mean cell-cycle time, an increase in the proliferating fraction, and an elongation of the crypts. HU also produces some synchronization of the S102F tumor cells, but this appears minimal and desynchronization occurs quickly. There is also a 2-3 day plateau in the tumor growth curve before the growth rate returns to the pre-HU rate. The tumor cell-cycle time is not reduced significantly but the growth fraction is increased.

These data along with other published studies on HU effects have been used to develop models for optimizing a 2-dose regime of HU and HU followed by x-irradiation (discussed in preprint #9). These models are currently being evaluated in the laboratory, and the two-dose studies on duodenum and the S102F tumor line are nearing completion (See preliminary draft, #10). In general, the results predicted by the model are confirmed.

Single and double-dose HU studies are also being conducted on tumors from the Slow line. In the single-dose experiments, 80 mice, bearing tumors in both flanks, were injected with HU (3 mg/gm body wt.). Then at selected times 3 or 4 mice were injected with ^3H -TdR (20 μCi ea.) and killed one hour later. Tumor DNA was extracted, the amount measured by U.V. absorption and the ^3H -activity determined. In the double-dose experiments, 70 tumor-bearing mice received two doses of HU (each 3 mg/gm body wt.) 12 hours apart. At selected intervals, after the last HU dose, ^3H -TdR was injected into the mice and the tumors were handled as above. The data show that the DNA content (μg DNA/mg tumor) does not vary as a function of time post-HU. Also, the radioactivity in the acid-soluble fraction does not vary significantly as a function of time between HU and ^3H -TdR injections. ^3H -TdR incorporation (dpm/ μg DNA) is completely inhibited in both sets for 4-5 hours; then in the single-dose set, it increases to about 55% of control at 8 hours post-HU and stays in this range until 36 hours. Incorporation then increases to 138% at 48 hours before dropping back to 100% at 72 hours post-HU. In the double-dose set, the incorporation also starts increasing after 5 hours but in contrast to the single dose results, it reaches control levels at 20 hours post-HU and 194% of control at 24 hours. This level is maintained until 48 hours post-HU. These data along with previously published data show that the duration of the HU-induced G_1/S block is independent of the cell cycle time. In contrast to the S102F data, there is no evidence for partial cell synchrony in the Slow line, but there is killing of the S-phase cells, and early recruitment from the non-proliferating fraction is a significant factor in recovery.

B. HU Induced Cell Synchrony in vivo and Tissue Radiation Response

A review of the literature on HU effects in vivo showed that, in general, HU does cause partial cell synchronization in rapidly proliferating tissues, i.e., duodenal crypts, bone marrow, and some rodent tumors. These data suggest that one might use HU to synchronize the cells and then administer x-irradiation to the tumor at a time which is less detrimental to the normal tissues, i.e., a higher proportion of the cells are in a relatively radioresistant phase.

The model presented in preprint #9 predicts that whole-body x-irradiation at 24 hours after HU (3 mg/gm body wt.) will be more destructive to both the duodenum and bone-marrow than x-irradiation at 20 hours post-HU. The following experiments have been done:

- *1. Gut Death ($\text{LD}_{50/5}$) (The head and proximal thorax are shielded.)
 - a) 13 groups of 8-10 mice each to establish the control $\text{LD}_{50/5}$ (Dose range 800-1700 rads)
 - b) X-irradiation at 24-hours post-HU
 - 3 groups of 10 mice each at 1284 rads and 1 group of 10 mice each at 1530 and 1700 rads
 - c) X-irradiation at 20-hours post-HU
 - 5 groups as in part b

*This work is being done by Dr. Joel Ohlsen (a second-year radiotherapy resident).

These preliminary data suggest a control LD_{50/5} of approximately 1250 rads. The mice irradiated at 20 hours post-HU appear to have the same LD_{50/5} as the control mice while those irradiated at 24 hours post-HU seem to have an appreciably lower LD_{50/5}.

*2. Bone marrow death (LD_{50/30}) (Whole-body irradiation)

a) Control LD_{50/30}

- 1) One group of 10 mice @ 550 rads - 3/10 alive at 30 days
- 2) One group of 10 mice @ 750 rads - 0/10 alive at 30 days

b) Colony-forming Units (C.F.U.'s) [The spleen colony assay technique of Till and McCulloch (37)]

- 1) One group of 20 recipient mice. The donor mice received 300 rads. Approximately 15% of the control C.F.U.'s survived.
- 2) One group of 20 recipient mice. The donor mice received HU and the bone marrow cells were collected one hour later. The HU treatment reduced the C.F.U.'s/femur to 50% of the control value (2.6×10^3).

*This work is being done by Dr. Joel Ohlsen (a second-year radiotherapy resident).

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Dethlefsen, L.A. and Snively, J.: ^{125}I -IuDR and Tumor Cell Loss Revisited: Cell Loss Measured from Three Established Lines of the C3H Mouse Mammary Tumor.

Dethlefsen, L.A. and Tomkins, G.M.: Effects of Cordycepin on Cell Division, Macromolecular Synthesis and Induction of Tyrosine Aminotransferase in Rat Hepatoma Cells in Suspension Culture.

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ABSTRACTS (cont.)

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This Contract has supported, in part, the training of two veterinary students in the Summer Research Program, one third-year undergraduate from Lincoln University, and two minority high school students from Philadelphia. These programs were designed to introduce the students to careers which are available in biochemical research and to give them actual experience in a research environment.

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