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KINETIC AND BIOCHEMICAL STUDIES ON TUMOR GROWTH

Comprehensive Progress Report

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ABSTRACT

The objectives for this contract are to develop a comprehensive understanding of the growth kinetics of the unperturbed C3H mammary tumor and then use these data in the design of logical therapeutic protocols.

The growth kinetics of three lines of the C3H mammary tumor have been studied by standard autoradiographic procedures in combination with volumetric growth curve analysis. Thus, such parameters as volumetric doubling times, mean cell generation times, growth fraction and cell loss have been measured. A new 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 the tumors from the three lines and the loss of ^{125}I -activity correlates 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.

The perturbing effects produced by single doses of hydroxyurea have been studied in the duodenum and S102F tumors. Again the methods used have been standard autoradiographic and volumetric growth curve analysis along with measurements on the rates of DNA synthesis. Single doses of hydroxyurea kill the S-phase cells, produce some degree of cell synchrony, some reduction in the cell-cycle times, and an increase in the growth fraction. Two-dose experiments are now in progress.

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. Hopefully, such information will lead to logical protocols which will significantly increase the therapeutic gain factor in this animal-tumor system, and 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

The fact that cells are lost from solid tumors has been recognized for many years by clinical oncologists and pathologists (i.e., distant metastases and pyknotic and karyorrhectic cells on histopath slides); however, only recently has it been the subject of extensive quantitative analysis. (Steel, 1968; Frindel, Malaise and Tubiana, 1968; Denkamp, 1970 and Mendelsohn & Dethlefsen, 1972.) 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 (Hermens and Barendsen, 1969; Denkamp, 1972). In all these reports, cell loss was estimated by combining autoradiographic and volumetric growth-curve analyses. This allows one to calculate the potential growth rate and the actual growth rate then 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 being: 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. (See Hughes *et al*, 1964; and Dethlefsen, 1969).

The evaluation of the ^{125}I -IUdR method for measuring cell loss from C3H mouse mammary tumors has been completed (Dethlefsen, 1971) and can be summarized thusly: 1) IUdR is reutilized but at a much lower amount than thymidine (Hofer, Prenskey & Hughes, 1969; Dethlefsen, 1970). 2) ^{125}I -IUdR 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 did agree quite well with the indirect method of combined autoradiography and volumetric growth-curve analysis.

Since that time, Mendelsohn and Dethlefsen (1972) have completed a detailed kinetic analyses on three lines of the C3H mouse mammary tumor, and this same analysis is now being completed on the fourth line. * This report shows that loss may occur through the non-proliferating fraction only (Fast and S102F lines) or from both the proliferating and non-proliferating fractions (Slow line). In the first case,

* These lines were developed at the University of Pennsylvania and brought here to the University of Utah. The two original ones were each 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 intentionally selected. The texture and histological features of these tumor cells 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.

there would be considerable delay 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, depending on the individual kinetics of the particular tumor.

We are now in the process of using the ^{125}I -IUdR method to verify these quantitative estimates of cell loss. Data collection is not complete so definitive quantitative results cannot be given, but qualitatively, the two methods appear to agree. That is, in the S102F line tumors, there is a delay of at least two days before a significant loss of radioactivity occurs while in the Slow line, the loss of radioactivity begins quickly (within several hours). These data from the Slow line agree with the previously published results using ^{131}I -IUdR (Dethlefsen, 1970).

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

II. IUdR Toxicity

During the course of these experiments, we have observed evidence for IUdR-produced toxicity in the crypts of the duodenum. First, there was a suggestion of cell-killing (Dethlefsen, 1971) and later we noted unusually low cellular labeling indices in the duodenal crypts. (unpublished) This was at doses of 75 μCi (0.01 to 0.02 μM) ^3H -IUdR per mouse. These data are in contrast to previously established data from tumor DNA which showed that incorporation was proportional to dose up to at least 10 μM IUdR (labeled with 10 μCi ^{125}I) per mouse (Dethlefsen, 1969). Subsequent autoradiographic studies were done with 25 μCi (0.002 μM) ^3H -IUdR per mouse with no detectable toxic effect, but it was obviously crucial to establish non-toxic tracer levels for ^3H -IUdR. Three variables needed to be evaluated: 1) The ethanol concentration per injection [Stock ^3H -IUdR (Schwarz/Mann) is shipped in 50% ethanol]. 2) The chemical dose per mouse, and 3) The radioactivity injected per mouse.

The problem was evaluated thusly: Tumor fragments (S102F line) were transplanted into both flanks of young mice, then when the volume reached about 1000 mm^3 , the mice were injected with ^3H -IUdR and killed one hour later. The proximal duodenum and entire tumor were removed, the DNA was extracted, and radioactivity measured. In one experiment, all mice received 10 μCi (about 0.001 μM) of ^3H -IUdR in 0.2 ml of ethanol but the ethanol concentration was either 2.5, 6, or 18%. In another experiment, all mice received 25 μCi ^3H -IUdR in 0.2 ml of 12% ethanol but the chemical dose per mouse was either 0.002, 0.02, 0.2, or 2.0 μM . The third experiment consisted of two groups in which the mice received either 25 μCi (0.002 μM) or 75 μCi (0.006 μM) of ^3H -IUdR. All injections were intraperitoneal, and there were at least 6 mice with 10 tumors per group. The weight of the mice ranged between 27 and 34 grams.

The results are as follows: 1) There is a 23% reduction in ^3H -IUdR incorporation into the duodenal DNA and a 20% reduction into tumor DNA when the ethanol concentration is increased from 2.5% to 18%. 2) There is also inhibition of incorporation (μM IUdR/ μg DNA) when one increases the amount of IUdR injected per mouse. If one takes the 0.002 μM group as a base-line, then there is no effect in the 0.02 μM group but in the 0.2 μM group there is a 26% inhibition in the duodenum and 38% in the tumor. These figures for the 2.0 μM group are 81 and 68% respectively. 3) There is no inhibition of incorporation if one injects 75 μCi ^3H -IUdR per mouse but keeps the IUdR dose at 0.006 μM each.

In summary, some inhibition of incorporation is observed in both tissues when the ethanol is 18%, but the more significant inhibition occurs when the IUdR dose per mouse exceeds 0.02 μM .

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

Even since the initial work on the current concept of the cell cycle by Howard and Pelc (1951) and the mammalian cell cloning and single cell survival techniques by Puck and Marcus (1956), 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 the fact that most of the laboratory procedures are too cumbersome and time-consuming to be used in the clinic.

We became involved with the HU because we felt that this drug could be used to study the basic mechanisms involving cell-cycle perturbations in an animal-tumor system and then this data could be used to design logical protocols for fractionated chemotherapy and combined chemo- and radiotherapy. HU was chosen because it had been studied extensively in vitro (Sinclair, 1965, 1967; Kim, Gelbard and Perez, 1967; and Bacchetti and Whitmore, 1969) 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 (Mauro & Madoc-Jones, 1969; Madoc-Jones & Mauro, 1970; Gillette, Withers & Tannock, 1970; and Rajewsky, 1970).

Studies on the cell-cycle effects in the mouse duodenum and mammary tumor (S102F line) have been published (Dethlefsen and Riley, 1973, I, II). A single large (3mg/gm body wt.) dose was given intraperitoneally, and 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 the mean cell-cycle time, an increase in the proliferating fraction, and an elongation of the crypts. The HU also produces some synchronization of the 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.

Ongoing work in this area includes studies on the clinical aspects as well as cellular effects from two doses of HU (each dose being 3 mg/gm body wt.) The so-called "clinical aspects" are concerned with evidence for host toxicity and include such parameters as loss of body mass, diarrhea (severity and duration), rough hair coat, and perturbation in the tumor growth curves. There are three groups of mice in each study. One group receives the second dose of HU 9 hours after the first, in another group, the second dose is given 12 hours after the first, and in the third group the second dose is given 24 hours later. This timing correlates with the HU-produced synchronization in the duodenum, i.e. 9 hours, first wave of DNA synthesis; 12 hours; minimal number of cells synthesizing DNA; and 24 hours, cellular recovery from HU perturbation essentially complete.

The only cellular effect studied, at this writing, is the rate of DNA synthesis after two doses of HU. Both doses of HU are given, then at various times after the second dose, ³H thymidine is injected and one hour later the mice are killed. Then the DNA is extracted from duodenum and tumor and radioactivity determined. The first group of experiments (2nd HU 9 hours after 1st HU), are complete and in this case, the DNA synthesis in both tissues is completely inhibited for 6 hours after the second HU dose. Then the duodenal DNA radioactivity increases to a peak [8% of control (No HU) levels] at 8 hours, drops to 2% at 12 hours and then gradually increases so as to reach about 15% of control by 20 hours after the second dose of HU. The tumor DNA radioactivity increases until a peak (50% of control) is reached at 14 hours and then drops back to 30% of control by 20 hours after the second HU injection. These duodenal data suggest that the crypt cells surviving two doses of HU behave very similar to the crypt cells surviving a single dose of HU. The data from the 12 and 24 hour groups and the clinical data are too preliminary to warrant discussion at this time.

IV. 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 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 markedly 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 (Weiss, 1969; Morton, Miller and Wood, 1969; and Heppner and Pierce, 1969). Antigenic differences have also been observed among tumors arising spontaneously in the same host (Vaage, Kalinovsky, and Olson, 1969).

Moreover, De Cosse and Gelfant (1968) have reported that non-cycling tumor cells can be stimulated to proliferate by the administration of an immunosuppressant, and Janik and Steel (1972) upon studying the cell population kinetics of three transplantable tumors following the perturbation of the immunological status of the host, reported a retardation in tumor growth and this retardation appeared to be associated with a reduction in the proportion of actively proliferating cells. Also, Choquet, Chavaudre and Malaise (1970) 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 very important to know the antigenic status of these tumors. This is necessary for understanding the tumor biology per se, but the information becomes even more significant when one used this animal-tumor system in studies involving radio- 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 (manuscript in preparation). In the suppression studies, four week old mice were thymectomized and whole-body irradiated (550-650 rads, 250 KvP 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 a 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 dwelt with an attempt to elicit a response 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 300-400 mm³ 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 should 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 are strongly antigenic when transplanted into C3H mice.

V. Differentiation of C3H Mouse Mammary Tumor Cells.

Another mechanism which may explain the different growth rates is the extent of terminal differentiation of tumor cells (Steel, 1969). In 1972, I proposed to evaluate this concept by stimulating the tumor cells to synthesize milk proteins in organ cultures. Specifically, normal mouse mammary tissue (the controls) and tumor tissue would be grown in organ culture in standard medium and in the presence or absence of insulin, corticosterone, and prolactin. (See Juergens et al, 1965; Lockwood et al, 1967, and Mayne and Barry, 1970). 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 the 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 have gone to the single cell suspension and monolayer technique as described by McGrath (1971). This procedure appears to be more adequate and promising but I am 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. This support will be used to develop the method, then when the pilot studies have proven the system to be adequate, I will seek additional support for this problem in differentiation and oncogenesis.

VI. Work Done While on Scholarly Leave

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 publications which have or will emanate from this work (Dethlefsen, Levinson, and Tomkins, 1971; Dethlefsen and Tomkins, 1972; Tomkins et al, 1972; and Dethlefsen, submitted 1973).

VII. Summary

In summary, the growth kinetics of the tumors from the three lines of the C3H mammary tumor are quite adequately understood. The combined autoradiographic and volumetric growth curve analysis and the ^{125}I -IUdR method for measuring cell loss have probably carried the kinetic analysis to a practical limit for in vivo work. At least, the kinetic information is now very adequate, so one can readily use this information in studies on perturbations from various therapeutic agents. The first step in this analysis has been done with studies on cell cycle effects in duodenum and tumor following single doses of HU. As outlined in the renewal proposal, this work will continue with two and more doses of HU and combined HU and x-irradiation.

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TRAINING SUPPORT

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.

FINANCIAL SUPPORT

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