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Biochemical Studies of Cytokinetic Changes during Tumor Growth[1]

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SUMMARY

We have measured the cytokinetic changes which characterize the growth of a hyperdiploid Ehrlich ascites tumor and have investigated some possible underlying biochemical mechanisms. In this tumor, the cell cycle increases from 9.5 hr on the 2nd day of growth to 39 hr on the 14th day and the proportion of cells in S (thymidine index) declines from 80 to 40% during the same interval of growth. Protein synthesis (measured by uptake of leucine-^{14}C during a 20-min pulse) declines at a rate similar to the decline of the thymidine index, but there are no changes in the specific activity of six enzymes: aryl sulfatase, β-glucuronidase, thymidine kinase, glucose-6-phosphate dehydrogenase, alkaline phosphatase, and glutathione reductase. Transplantation of 10^7 cells from plateau-phase tumors (14 to 16 days) to new hosts causes the rate of protein synthesis to increase immediately. The thymidine index, on the other hand, remains constant for 5 to 6 hr after transplantation and then doubles within the next 4 hr. The rise is prevented (or delayed) when the animals breathe 6% oxygen. Hypoxia also decreases the thymidine index of 2-day tumors, but it does not alter the length of the cell cycle. The results suggest that the progressive accumulation of cells in G_1 during tumor growth may reflect the relative inefficiency of anaerobic metabolism in producing the energy (*e.g.*, ATP) necessary for movement into S phase.

INTRODUCTION

It is well known that development of a tumor rarely follows a constant course. Although the details differ from 1 tumor type to another, the phenomenon of growth deceleration with increasing tumor mass has been ascribed to changes in the length of the proliferative cell cycle, in the growth fraction, and/or in the rate of cell loss. An understanding of these cytokinetic variables is of more than academic interest. Mammalian cells differ in their sensitivity to X-radiation (59) and certain chemotherapeutic drugs (*e.g.*, Ref. 13) according to their position in the cell cycle, and it is reasonable to expect that optimal tumor control doses and fractionation regimes will change if cells are redistributed between sensitive and resistant stages. Moreover, if cells can be recruited from a nonproliferating (G_0) compartment by reduction of tumor mass (16, 43, 45), the proliferating compartment could be considerably

larger after irradiation or chemotherapy than would be predicted from cell survival curves alone.

In this paper we examine some of the biochemical factors that may contribute to the cytokinetic changes which characterize the growth of a hyperdiploid Ehrlich ascites tumor *in vivo*. Although the rate of protein synthesis declines during growth of this tumor, there is no evidence for qualitative defects which might cause the cytokinetic changes. Our results support the view that accumulation of cells in G_1 during tumor growth reflects the progressively larger proportion of anoxic cells in the population.

MATERIALS AND METHODS

Tumor

Hyperdiploid Ehrlich ascites tumor cells (10^7) were inoculated i.p. into female Swiss-Webster mice (20 to 25 g). The growth characteristics of this tumor are described elsewhere (30). Unwashed cells were collected aseptically, suspended in their own ascitic fluid, and diluted with 0.9% NaCl solution as required for inoculation. When tumor enzyme activity was to be assayed on the 1st day of growth, the prospective recipient mice were exposed to 1000 R X-radiation immediately before tumor inoculation to reduce the proportion of small lymphocytes to the level (3 to 10%) found in established tumors (30). Cells were enumerated with a Model B Coulter counter.

Imposition of Hypoxia

In experiments designed to test the effects of hypoxia on tumor growth, mice bearing the Ehrlich ascites tumor were placed in a Lucite chamber which was flushed with a mixture of 6 or 9% oxygen in nitrogen. The oxygen content of the effluent gas was monitored at frequent intervals with a Beckman oxygen analyzer. Food and water were available *ad libitum*.

Measurement of Cell Cycle Parameters

The distribution of cells within the cell cycle was measured by the labeled mitosis method. TdR-^{3}H (0.36 Ci/mmole, 12 μCi/mouse) in 0.15 ml or 0.30 ml of 0.9% NaCl solution was injected i.p. and 2 or 3 mice were sacrificed at appropriate intervals. The thymidine index (proportion of cells in S) was determined by sacrificing animals 20 min after injection of TdR-^{3}H. For the "continuous labeling" experiment, TdR-^{3}H was injected i.p. every 4, 6, or 8 hr. In all cases, the cells were

washed, resuspended in ascitic fluid, and smeared. After fixation of the slides in absolute methanol, autoradiographs were prepared with Kodak NTB-2 emulsion, exposed for 3 weeks, and stained with Giemsa.

Measurement of Protein Synthesis

Mice bearing ascites tumors were given a single i.p. injection of leucine-^{14}C (0.5 μCi, 160 mCi/mmole) 20 min prior to sacrifice. The cells were washed with 0.9% NaCl solution and resuspended in 0.9% NaCl solution containing heparin. For determination of leucine-^{14}C incorporation, 1 volume of cell suspension was added to 2 volumes of ice-cold 10% TCA[3] and then centrifuged for 20 min at 900 X g. The precipitate was dissolved in 0.5 N NaOH, and 50-μl aliquots were dried on Whatman No. 3MM paper discs (2.3-cm diameter). The discs were then carried through cold 10% TCA, hot (90°) 5% TCA, 50% ethanol, 100% ethanol, and acetone (50), dried, placed into vials containing a liquid scintillator, and assayed in a Packard Tri-Carb scintillation spectrometer.

For determination of unincorporated intracellular leucine-^{14}C, a 2nd portion of the washed cell suspension was centrifuged at 600 X g and the cell pellet was extracted with 95% ethanol. After centrifugation, 0.5-ml aliquots of the supernatant were pipetted into scintillation vials for assay.

Total cellular protein was measured by the method of Lowry et al. (47).

Measurement of Enzyme Activities

Ascites tumor cells were washed and then disrupted and analyzed as follows.

β-Glucuronidase. Cells were frozen and thawed 10 times, then incubated for 1 hr at 37° with phenolphthalein glucuronide (pH 4.5) as described by Kerr and Levvy (39). The reaction was stopped by addition of glycine buffer, pH 10.7 (26).

Glutathione Reductase. Cells were homogenized in 0.05% deoxycholate, and the enzyme was assayed by following the oxidation of NADPH at 340 mμ in the presence of oxidized glutathione (49).

Aryl Sulfatase. Cells were frozen and thawed 10 times, then incubated with 6 mM p-nitrocatechol sulfate at 37° for 1 hr. The assay was performed at pH 5.7 as described by Roy (56). Aryl sulfatase activity was maximal at this pH, indicating that the B enzyme is the major one present in ascites cells.

G6PD. Cells were extracted in cold 0.01% saponin, centrifuged, and assayed at 30° by following the reduction of NADP at 340 mμ in the presence of glucose-6-phosphate. A commercial reagent mix (Calbiochem, Los Angeles, Calif.) was used.

Thymidine Kinase. Cells were disrupted by brief sonic extraction, and phosphorylation of TdR-^{3}H was measured by the method of Bianchi et al. (9). Thymidine phosphates were absorbed on DEAE-cellulose discs for counting (14).

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[3] The abbreviations used are: TdR-^{3}H, tritiated thymidine; TCA, trichloroacetic acid; G6PD, glucose-6-phosphate dehydrogenase; DEAE, diethylaminoethyl.

Alkaline Phosphatase. Cells were frozen and thawed 10 times, then incubated with β-glycerophosphate at pH 9.9 for 15 min (37°). The incubation mixture contained 4.4 ml of 0.023 M sodium β-glycerophosphate, 0.1 ml of 0.05 M MgCl$_2$, and 0.5 ml of homogenate. The reaction was stopped by adding 1 ml of 30% TCA, and P$_i$ was measured by a standard technique [Fiske and SubbaRow (23)].

Administration of Actinomycin D

Actinomycin D (Dactinomycin, Merck, Sharp and Dohme, Rahway, N. J.) was dissolved in sterile 0.9% NaCl solution and injected i.p. every hr or every 2 hr after inoculation of 10^7 tumor cells. The dose was 1 μg/injection; controls were given injections of 0.9% NaCl solution.

RESULTS

Cytokinetic Changes during Tumor Growth. The cell cycle of our hyperdiploid ascites tumor lengthens from a median of 9.6 hr on the 1st day of growth to about 39 hr on the 14th day (Chart 1). On the 1st day, the median length of S (50% rise in the curve to the 50% fall) is 8.5 hr and minimum G$_2$ + prophase (earliest appearance of labeled metaphases) is just over 1 hr; there is no detectable G$_1$. By Day 14, S has increased to a median of 21 hr and G$_2$ + prophase has increased to a median of 7.5 hr. Since the cell cycle time is now 39 hr, the 14-day tumor has a G$_1$ of about 10 hr. The failure of the 14-day labeled mitosis curve to reach 100% is probably due to cells which move very slowly through G$_2$ and reach mitosis as long as 20 hr after injection of TdR-^{3}H (see below). S and G$_2$ lengthen proportionally in this tumor (both increase by a factor of about 2.5) between the 1st and 14th day, in agreement with the observation of Lala and Patt (44) in a hypotetraploid ascites tumor.

Ascites tumors become extremely hypoxic during growth [85% of the cells in a 7-day tumor are anoxic (22) as revealed by radiobiological studies] ɔut the data in Chart 1 show that hypoxia does not cause the lengthening of the cell cycle.

Frequent injection of TdR-^{3}H labels 95% of the cells in the 7-day tumor by 24 hr (Chart 2). In the 14-day tumor, 95% labeling is achieved only after 72 hr; 99% of the cells are labeled by 94 hr. Note that the time required for complete labeling of the 14-day tumor greatly exceeds the median length of G$_2$ + M + G$_1$, suggesting a broad distribution of times in 1 or more of these phases. It is highly unlikely that there is much variation in the M phase, but the population does contain some cells with extremely long G$_2$ periods, as evidenced by the failure of the labeled mitosis curve to reach 100% (Chart 1). G$_2$ may also contain a small G$_0$ subpopulation (20). The proportion of TdR-^{3}H-labeled mitotic figures in "continuously labeled " 14-day tumors does not exceed 90% until about 48 hr, although the median G$_2$ is 7.5 hr. Since an additional 24 hr elapse before 95% of the population is labeled (at 72 hr), G$_1$ must also contain some slowly moving cells. Some Ehrlich ascites tumors contain a nonproliferating (G$_0$) compartment between mitosis and S (43–45), but the present data do not permit us to distinguish a combined G$_1$
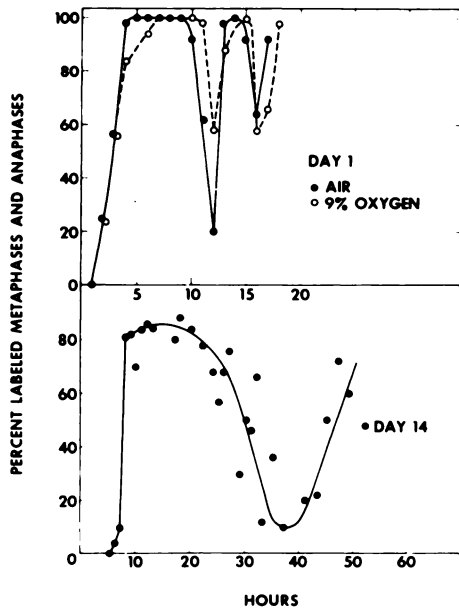
Chart 1. Labeled mitosis curves for hyperdiploid Ehrlich ascites tumor growing in mice breathing air or 9% oxygen. TdR-^{3}H was injected i.p. 1 day or 14 days after inoculation of 10^7 tumor cells. Two or 3 animals were sacrificed at intervals for determination of labeled anaphases and telophases.

and G_0 population from a pure G_1 with a broad distribution of G_1 times. We will return to this point, but for the present we shall refer to all cells that have divided but have not yet entered S as being in G_1.

The proportion of tumor cells in S declines at a rate of about 3.1%/day (Chart 3). This rate is almost identical to that reported for other Ehrlich ascites tumors with quite different growth rates (*e.g.*, 16, 43). The rate at which the thymidine index of the hyperdiploid tumor declines is not affected by exposing the mice to pure oxygen at 2 atmospheres absolute for 2 hr daily, but it is affected by hypoxia (see below).

Protein Synthesis during Tumor Growth. Since there is considerable evidence that synthesis of specific proteins is necessary for progression of cells from G_1 into S (3, 54), we examined the possibility that the decrease in thymidine index might be due to quantitative or qualitative deficiencies in protein synthesis. Mice bearing tumors of different ages were given injections of leucine-^{14}C and sacrificed 20 min later. The radioactivity of both the protein and nonprotein (amino acid) fractions was measured, and the amount of isotope in the protein fraction was expressed as a fraction of the total cellular radioactivity. Total intracellular radioactivity decreases about 10-fold between the 1st and 14th day of growth. Since the volume of ascites fluid increases approximately 10-fold during this interval, the decrease in intracellular radioactivity represents dilution of the injected isotope. To a 1st approximation, therefore, the rate of leucine transport must remain constant during tumor growth.

Incorporation of leucine-^{14}C into cellular protein declines at a rate of about 2.5%/day (taking the 1-day value as 100%) (Chart 3). The similarity between the slopes of the leucine and thymidine curves might indicate that a progressive decrease in

the rate of protein synthesis caused the accumulation of cells in G_1. However, the relationship could be fortuitous. In the latter case, decreased protein synthesis might simply reflect failure of G_1 cells to move into S, since G_1 cells are reported to synthesize protein more slowly than S cells (2). It is also possible that the 2 parameters might respond independently to some common environmental deficiency. Although injection of amino acids into ascites tumors causes reassociation of polysomes (32), preliminary experiments in this laboratory show no significant elevation of leucine-^{14}C incorporation in animals so treated.

Enzyme Activity during Growth. Since the synthesis (or activity) of certain enzymes increases at discrete times during the mammalian cell cycle (53), we examined the possibility that the quantitative decrease in protein synthesis during tumor growth might be accompanied by qualitative changes in enzyme content. The data indicate, however, that there is no significant change in the specific activity of glutathione reductase, G6PD, aryl sulfatase, alkaline phosphatase, or thymidine kinase during growth of this tumor (Table 1). We cannot confirm the elevated glutathione reductase which Malmgren and Sylven (49) reported for early tumors, probably because these workers did not irradiate their mice or otherwise decrease the number of host peritoneal leukocytes in the 1st few days of growth. Mouse leukocytes contain considerably more glutathione reductase than ascites tumor cells (49) and the presence of even small numbers of host cells can lead to a spurious increase in apparent tumor enzyme content during early growth.

G6PD was assayed because in certain normal tissues this enzyme is responsive to nutritional stress (*e.g.*, Ref. 28). However, the G6PD content of ascites tumor cells does not change during growth (Table 1). The intracellular level of aryl sulfatase, a typical lysosomal enzyme selected because such enzymes have been implicated in tumor growth (37), also remains constant between early and late growth. Similarly, alkaline phosphatase does not change greatly, although in HeLa cells the level of this enzyme does vary around the cell cycle (52).
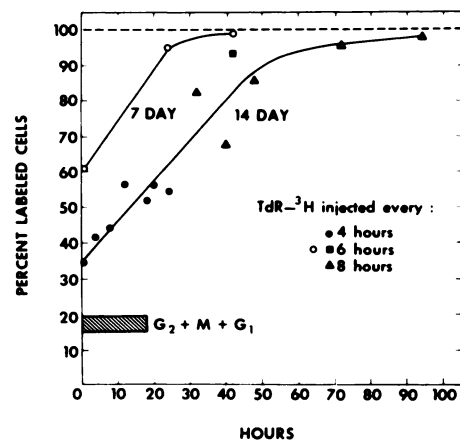

Chart 2. Proportion of cells labeled after repeated injections of TdR-^{3}H into 7-day or 14-day tumors. The mean length of G_2 + M + G_1 for the 14-day tumor, calculated from Chart 1, is shown by the *hatched area*. Each *point* is the mean of 2 or 3 mice and represents 2000 to 3000 cells scored.

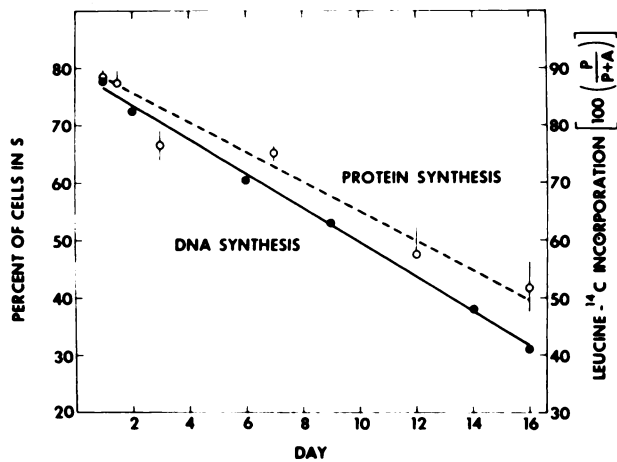John W. Harris, Frank Meyskens, and Harvey M. Patt

Chart 3. Proportion of cells synthesizing DNA (thymidine index) and rate of protein synthesis during growth of hyperdiploid Ehrlich ascites tumor. Protein synthesis (leucine-^{14}C incorporation), is expressed as cpm in the acid-insoluble fraction (P) over cpm in acid-soluble plus acid-insoluble fractions ($P + A$). Each point is the mean ($\pm$ S.E.) of 3 to 13 mice for protein synthesis and 2 mice for DNA synthesis.

Thymidine kinase activity often decreases as cells enter the plateau phase of growth (40, 66). In many systems, thymidine kinase and the enzymes required for DNA synthesis are synthesized de novo at or immediately prior to the initiation of DNA synthesis (3, 66). However, cellular thymidine kinase activity does not change during growth of the Ehrlich ascites tumor (Table 1).

β-Glucuronidase does appear to decrease during tumor growth (Table 2). However, this decrease seems to be due to the presence, in early tumors, of some host cells which contain large amounts of the enzyme since (a) the specific activity of β-glucuronidase on Day 1 is lower when the inoculum is increased 20-fold, presumably because of a decrease in the relative contribution of host cells to the measured activity (Table 2), and (b) admixture of tumor cells and mouse peritoneal cells in vitro or in vivo causes a large and immediate increase in mean β-glucuronidase activity. The artifactual increase of β-glucuronidase in early tumors occurs although the recipient mice are previously exposed to whole-body X-radiation. As reported earlier, X-irradiation of the host greatly reduces the proportion of small lymphocytes in early tumors (30). These results suggest that the contaminant cells are probably macrophages, since these cells contain large amounts of β-glucuronidase and are not removed from the population by X-irradiation.

We conclude that the specific activities of the 6 enzymes studied remain constant during growth of the Ehrlich ascites tumor. The same conclusion has been reached, for other tumors, with respect to glutaminase (41) and nicotinamide adenine dinucleotidease (27). On the other hand, uridine phosphorylase and uridine kinase increase between the 9th and 12th day of ascites tumor growth (55).

Thymidine Index in Transplanted Tumors. When 10^7 Ehrlich ascites tumor cells are transplanted, an increasing number of cells progress into S, and the thymidine index rises (Chart 4). With cells from 3-day and 7-day tumors, the in-

crease usually (although not invariably) begins immediately and proceeds for 8 to 10 hr (Chart 4). When 14-day cells are transplanted, there is a lag of 5 to 6 hr before the index rises; in only 2 of 16 experiments with 14-day cells was the lag completely absent.

Protein Synthesis in Transplanted Tumors. Incorporation of leucine-^{14}C increases dramatically within 20 min after transplantation; it then increases gradually during the next 10 hr (Chart 5). The same result is obtained when labeled leucine is injected into the donor tumor and a portion of undiluted tumor cell suspension is transplanted to fresh mice 5 min later. Since there is no significant difference, at any posttransplantation time studied, between cells transplanted from 7-day, 12-day, and 16-day tumors, the data in Chart 5 are expressed as the weighted mean of all values obtained for each time. The rate of protein synthesis does not increase sharply between 6 and 10 hr, although the proportion of cells in S doubles during this interval (compare Charts 4 and 5). This observation eliminates the possibility that the decrease of protein synthesis during growth passively reflects the decreasing proportion of S cells.

Leucine was chosen for this study because of its rapid utilization and small pool size in ascites tumor cells (36), but in the

Table 1

Enzyme activities during growth of Ehrlich ascites tumor (inoculum, 10^7 cells)

Day of growth	Protein[a]	Glutathione reductase[b]	G6PD[c]	Aryl sulfatase[d]	Thymidine kinase[e]	Alkaline phosphatase[f]
1[g]	17.4	1.24	6.47		1.74	
2		1.23	8.40	1.27		
7	18.5	1.74	8.25		1.12	9.5
12	20.8	1.99	5.03			
14			7.15	1.33	1.44	12.5

[a] mg/10^8 cells.
[b] ΔA_{340} of 0.001/min/10^5 cells (22°).
[c] ΔA_{340} of 0.001/min/10^9 cells (30°).
[d] μg 4-nitrocatechol liberated/min/10^8 cells (37°).
[e] mμmoles thymidine phosphorylated/min/10^8 cells (37°).
[f] μg phosphorus liberated/15 min/10^7 cells (37°).
[g] Mice received 1000 R whole-body X-radiation before inoculation.

Table 2

β-Glucuronidase activity during growth of Ehrlich ascites tumors

Tumor (inoculum)	Day of growth	Phenolphthalein formed (μg/min/10^8 cells)
Hyperdiploid (1×10^7)	1[a]	4.56 ± 0.36 (3 pools of 14)
	7	2.56 ± 0.18 (10)
	14	1.49 ± 0.12 (8)
Hypotetraploid (1×10^6)	1	9.44 (pool of 6)
	4	3.98 ± 0.18 (3)
	7	3.53 ± 0.35 (3)
Hypotetraploid (20×10^6)	1	3.85 ± 0.32 (3)

[a] Mice received 1000 R whole-body X-radiation prior to inoculation.
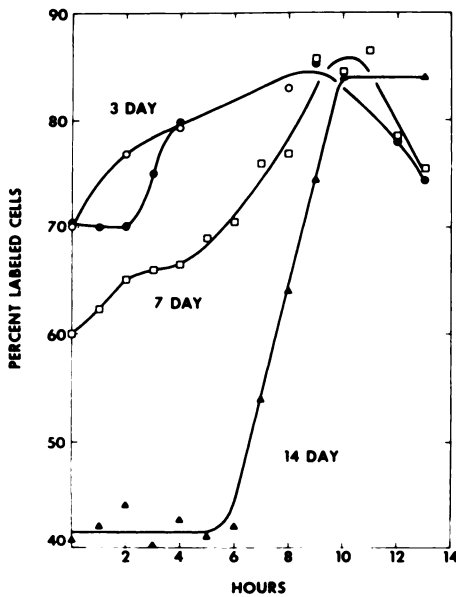
Chart 4. Thymidine index after transplantation of 10^7 hyperdiploid Ehrlich ascites tumor cells into female Swiss-Webster mice. Cells were obtained from 3-day, 7-day, or 14-day tumors. Each *set of symbols* represents a single experiment, in which 2 or 3 mice were given injections of TdR-^{3}H at each interval, then sacrificed 20 min later. The cells were pooled, resuspended in ascitic fluid, and smeared.

absence of actual measurements of the pool it is possible that the results may be influenced by pool size changes. If the leucine pool decreases during growth, but leucine transport does not, the apparent rate of protein synthesis in old tumors will be greater than the actual rate. On the other hand, if the pool increases during growth, the apparent rate will be less than the true rate. However, since the "0-time" rates of leucine incorporation are similar for cells transplanted from tumors of several ages (Chart 5), it seems unlikely that changes in pool size influenced the results presented in Chart 3.

The gradual increase in leucine-^{14}C incorporation during the first 10 hr after transplantation (Chart 5) probably represents increased synthesis, although the possibility of a gradual decrease in the pool size cannot be ignored. Total cellular radioactivity (protein plus amino acid) remains constant during this time, suggesting that the ability of the cells to transport leucine does not change.

Relationship between Cell Concentration and Protein Synthesis *in Vivo*. Dilute suspensions of ascites cells synthesize protein *in vitro* more rapidly than dense suspensions (12). If such a relationship exists *in vivo*, it is obviously of no significance for ascites tumor growth after the 1st few days, since the cell concentration remains constant at about 2×10^8 cells/ml. However, it is possible that there is some relationship between protein synthesis and cell concentration in the early hours of growth. This possibility was examined.

First, we repeated the experiments of Bladé *et al.* (12). Ascites tumor cells (in ascitic fluid diluted with 0.9% NaCl solution) and medium (containing leucine-^{14}C) were warmed separately, then mixed and incubated at 37° for 15 min. The suspensions were equilibrated with air during incubation by the action of small jets impinging upon the surface of the

dishes (depth of fluid, 2 mm). The results confirm those of Bladé *et al.* (12) in that protein synthesis increases with decreasing cell density. At 35×10^6 cells/ml, incorporation amounts to 200 cpm/10^6 cells, compared to 1800 cmp/10^6 cells at 0.5×10^6 cells/ml; the shape of the curve connecting these 2 extremes is that described by the French workers (12).

We then examined the cell concentration *in vivo* during the first 24 hr after transplantation of 1×10^7 or 8×10^7 tumor cells into mice that had been exposed to 1000 R whole-body X-radiation 24 hr before. The concentration of cells in the ascites lagged for a few hr, then rose gradually (Chart 6). There are distinct differences in cell concentration during the first 6 hr, a longer "lag period" being noted for cells transplanted from older tumors. When 8×10^7 cells are inoculated, the cell concentration rises more gradually, but the lag is still evident. When recipient mice are not irradiated, the cell concentration rises more slowly than shown in Chart 6 and the lag period extends to about 15 hr (F. Meyskens, unpublished data). This suggests that the lag period may be due, at least in part, to immunological reactions of the host.

Comparison of Charts 5 and 6 shows quite clearly that there is no correlation between cell concentration and the rate of protein synthesis during the first 10 hr after transplantation. Therefore, we conclude that the *in vitro* relationship described by Bladé *et al.* and confirmed by us has no obvious relevance to tumor growth *in vivo*.

RNA Synthesis after Transplantation. Total cellular RNA declines during ascites tumor growth at a rate (about 3.5%/day) similar to that observed in the present experiments for protein synthesis and the thymidine index (46). Diminished RNA synthesis might explain the failure of postmitotic tumor cells to initiate DNA synthesis, since RNA synthesis is necessary for progression of cells from G_1 into S. To test this possibility, 1 μg of actinomycin D was injected every hr or
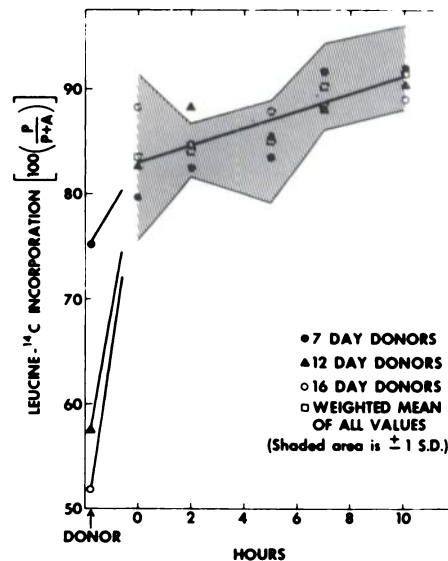

Chart 5. Protein synthesis (leucine-^{14}C incorporation) after transplantation of 10^7 hyperdiploid Ehrlich ascites tumor cells. Recipient mice were X-irradiated with 1000 R before use. The results are expressed as in Chart 3. Each *point* is the mean of 3 to 9 measurements; each measurement represents cells pooled from 2 or 3 mice.
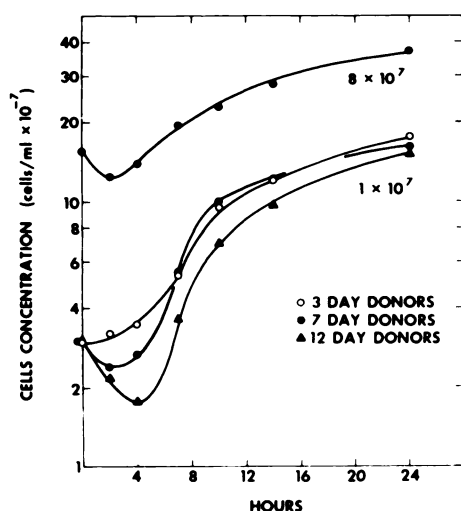
Chart 6. Cell concentration in ascitic fluid after transplantation of 10^7 ascites tumor cells. All mice were X-irradiated with 1000 R 24 hr prior to inoculation of tumor cells to reduce contamination by small host leukocytes to <10%. Cells inoculated for these experiments were not washed; ascitic fluid was diluted with 0.9% NaCl solution as appropriate. For the lower curves (1 X 10^7 cells inoculated), the inoculum was 0.3 ml; for the upper curve (8 X 10^7 cells inoculated), it was 0.5 ml. Each *point* is the mean of at least 3 mice.

every 2 hr after transplantation of 10^7 cells and the thymidine index was measured at appropriate intervals. Hourly injection of actinomycin D inhibits the incorporation of uridine-^{3}H into RNA by 52% after 4 hr.

Actinomycin D does not affect the initial rise of the thymidine index 5 to 8 hr after transplantation (Chart 7). However, the thymidine index begins to level off in actinomycin-treated tumors after 8 hr, while continuing to rise in the 0.9% NaCl solution-injected controls. Since ascites cells in the last 2 to 3 hr of G_1 have already synthesized the RNA necessary for progression into S (4), these results indicate that the cells which initially enter S come from late G_1. The plateauing of the thymidine index after the 8th hr probably reflects interference with cells which originate earlier in the cell cycle.

Transplantation of Irradiated Cells. Further evidence regarding the origin of the cells which enter S after transplantation was sought by transplanting cells from mice bearing 14-day tumors just after the latter had been exposed to 1250 R whole-body X-radiation (300 kVp, 20 ma, dose rate 100 R/min). Since X-radiation blocks ascites cells in G_2 for 8 hr under these conditions (30), any cells which move into S before 8 hr must originate in M or G_1. As shown in Chart 7, the thymidine index of the irradiated tumor decreases after about 2 hr as cells move out of S without replacement, rises at the normal rate for 2 hr, and then decreases rapidly. Considering the low starting point for the rise and the complete absence of cell progression from G_2 through M during the first 8 hr, the cells which initiate DNA synthesis 5 to 7 hr after transplantation must come from G_1. However, the cells which normally move into S 8 to 10 hr after transplantation may originate in G_2 (see Ref. 67), since the thymidine index in the irradiated tumor does not rise after 7 hr.

Effect of Hypoxia on the Thymidine Index. During growth,

ascites tumors progress from complete oxygenation (immediately after transplantation) to a state where 85 to 90% of the cells are anoxic at any given time (22). Although hypoxia does not affect the length of the cell cycle (Chart 1), the possibility remains that it may play a role in the decline of the thymidine index.

Female Swiss-Webster mice were inoculated with 10^7 tumor cells and, 24 hr later, placed in a chamber which was continually flushed with a mixture of 9% oxygen in nitrogen. After 22 hr in 9% oxygen, each mouse received a single i.p. injection of TdR-^{3}H and was returned to the chamber for 20 min before sacrifice. The thymidine index after 22 hr of hypoxia was 59.3 ± 4.9% (approximately the index characteristic of 7-day tumors) compared to 72.5 ± 1.6% for air-breathing controls.

We then examined the effect of hypoxia on the thymidine index of newly transplanted tumors. Cells from 14-day tumors were transplanted and the mice immediately were placed in a chamber flushed with 6% oxygen. At intervals, 2 mice were removed from the chamber, given injections of TdR-^{3}H, and returned to the chamber for 20 min prior to sacrifice. Under these conditions, the thymidine index increases only slightly (Chart 8). However, when the animals are returned to air after 6 hr of hypoxia, the thymidine index rises at the normal rate after a brief lag. This requirement for oxidative metabolism is not merely a "preparatory" one: if hypoxia is imposed after the mice have breathed air for the first 5 hr following transplantation, the continued rise of the thymidine index is prevented although, as in the experiment shown in Chart 8, some additional cells have already initiated DNA synthesis prior to the imposition of hypoxia.

DISCUSSION

Our hyperdiploid tumor contains some cells which remain in G_1 much longer than the median length of G_1, and it is appropriate to consider the possibility that such cells may be in a nonproliferative (G_0) compartment resembling that of liver cells. There is evidence for such a compartment in at least 1 ascites tumor line, a rapidly growing hypotetraploid (43—45). Although all the cells in this same tumor line can be labeled within 24 hr (for the 7-day tumor) by frequent injection of TdR-^{3}H (J. Harris, unpublished data), this fact alone does not disprove the existence of a G_0 compartment. Unlabeled G_0 cells would be diluted out as labeled postmitotic cells flow into the compartment, and, if there is appreciable loss of unlabeled G_0 cells, complete labeling could be attained within a relatively short time. Although our labeling data (Chart 2) provide a reasonably good fit to the "G_0 model" proposed by Brown (15), analysis of similar data has recently led Tannock (62) to conclude that the growth fraction probably remains close to 1 throughout most of ascites tumor growth.

On kinetic grounds, we cannot completely exclude the possibility that our tumor may contain G_0 cells analogous to those observed in solid tumors (62), since the position of each cell within the tumor is constantly changing and this may permit cells to move between G_1 and G_0, depending upon their position in the tumor mass at any given time. However, our biochemical studies provide no support for the view that
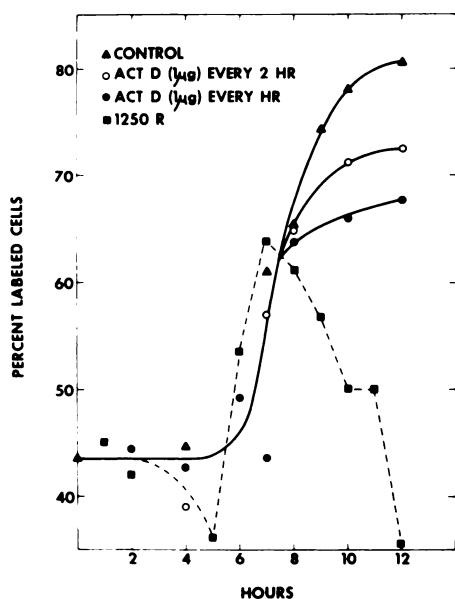
Chart 7. Effect of actinomycin D or 1250 R X-radiation on the thymidine index of transplanted Ehrlich ascites tumor cells. The inoculum was 10^7 cells; each *point* was obtained by pooling cells from 2 or 3 mice 20 min after injection of TdR-^{3}H. Controls in this experiment were given hourly injections of 0.9% NaCl solution.

the ascites cells in G_1 are similar to cells classically described as G_0 (*e.g.*, liver and salivary gland). Actinomycin D prevents the onset of DNA synthesis in stimulated G_0 systems (3), but does not prevent the rise in thymidine index after transplantation of ascites tumor cells (Chart 7). Moreover, synthesis of thymidine kinase is said to be characteristic of recycling systems [*e.g.*, hepatectomized liver (3)], but the enzyme remains constant during ascites growth (Table 1) and therefore presumably does not increase in the hours following transplantation. We conclude that cells in ascites tumors (and by implication, in solid tumors) stop in G_1 and do not have the biochemical characteristics generally ascribed to true G_0 cells.

What can be said regarding the biochemical mechanisms responsible for the cytokinetic changes during tumor growth? Specifically, does deficient protein synthesis play a role in the accumulation of cells in G_1? Although protein synthesis often declines as mammalian cells enter the plateau phase of growth in depleted medium (16, 18), the plateau state can be maintained without quantitative changes in protein synthesis if fresh medium is supplied continuously (42) or frequently (29), possibly due to the presence of adequate amino acid levels (32, 38). Therefore, diminished protein synthesis is not an obligatory concomitant of the plateau phase of growth. Although we cannot exclude the possibility that some specific protein required for initiation of DNA synthesis is synthesized after transplantation, we can conclude that such synthesis, if it occurs, is not sufficient to initiate the movement of cells into S, since this can be prevented by hypoxia (Chart 8). [This argument is based, of course, on the assumption that cells synthesize the same proteins aerobically as they do anaerobically in the presence of glucose; there is some evidence that this may not be true (31, 34), although anaerobic ascites cells synthesize protein at the same *rate* as fully oxygenated cells

when glucose is present (10).] Imposition of hypoxia does not affect the incorporation of leucine-^{14}C in early tumors (J. Harris, unpublished data).

In view of the demonstration that the thymidine index is responsive to changes in oxygen tension (Chart 8), the decrease in the index during growth could be caused by the progressively larger proportion of the cells which become anoxic as the tumor mass enlarges. Ascites cells cannot divide under anaerobic conditions, even in complete medium (11), and radiobiological studies show that 85 to 90% of the cells in a 7-day tumor are completely anoxic (22). The probability that a given cell will move from G_1 into S could, therefore, be a function of the frequency with which it becomes oxygenated, *i.e.*, moves within 100 to 150 μ of a blood vessel (22). Obviously, this frequency will diminish as tumor volume increases. When the inoculum is small or when tumor volume is reduced by paracentesis (16), the probability of cell/blood vessel "contact" increases and additional cells move into S. If the inoculum is large, reoxygenation is incomplete and the thymidine index rises only slightly (30). Hypoxia probably delays movement of cells from G_1 into S, rather than preventing it absolutely, since the thymidine index does rise somewhat in hypoxic animals (Chart 8).

The effect of hypoxia on the progress of cells around the cell cycle provides a logical explanation for the failure of transplanted solid tumors to grow in hypoxic mice (1). Similar cytokinetic responses can be observed in hypoxic normal tissues (51). Mice are particularly useful for study of the cytokinetic effects of hypoxia, since the oxygen dissociation curve for mouse blood is less steep than for other species, including man (57), and it is therefore relatively easy to decrease tissue and tumor pO_2. Increasing the oxygen concentration of the inspired gas above that of air, on the other hand, or exposing
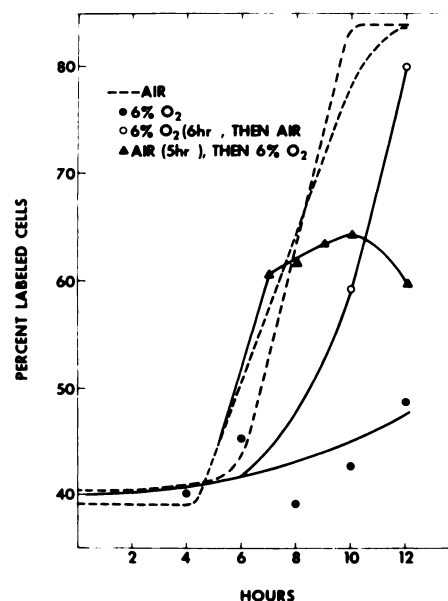

Chart 8. Effect of hypoxia on the thymidine index in transplanted Ehrlich ascites tumor. Each *point* was obtained by pooling cells from 2 or 3 mice. Hypoxic animals (breathing 6% oxygen in nitrogen) were removed from the chamber for injection of TdR-^{3}H, returned for the duration of the pulse (20 min) and then sacrificed.

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the animals to hyperbaric oxygen, does not affect either tumor growth (19, 35, 60, 61), or the thymidine index (J. Harris, unpublished data) because blood is already saturated with oxygen when the animals breath air. Moreover, the large size of the tumor mass and the rapid utilization of oxygen by the cells (22) preclude any significant elevation of tumor pO_2 even when the host is exposed to hyperbaric oxygen. Measurements of oxygen tension in ascites tumors of increasing size provide direct evidence on this point (21).

Our results do not explain the lengthening of the cell cycle time. Since this phenomenon is not observed in solid tumors (62), it seems likely that elongation of the cell cycle reflects some condition unique to ascites growth (*e.g.*, decreasing pH). Decreasing pH causes reversible elongation of the generation time of cultured mammalian cells (48), and it may be relevant that the pH of ascites fluid decreases to 6.5 by the 14th day of growth.

In summary, we do not yet know the precise mechanisms which account for the accumulation of cells in G_1, although we can construct a working hypothesis which is consistent with the available data: as the tumor grows, its volume increases, and the frequency with which individual cells become oxygenated decreases. As a consequence, the energy required for growth (presumably ATP) must be derived from glycolysis for increasing lengths of time. Since anaerobic ATP production is far less efficient than aerobic ATP production, each cell requires longer to produce a given amount of ATP. If more energy is required to initiate DNA synthesis than to sustain it, cells will accumulate in G_1 until they accumulate sufficient ATP to move into S and progress through the cycle at the normal rate. Starvation, and alternate means of retarding energy production by the tumor cells, also causes cells to stop cycling—in this case, in G_2 (5).

Decreased efficiency of ATP production under anoxic conditions could also explain the similarity between the rates at which the thymidine index, the rate of protein synthesis, and the total RNA content decline, since there is a close proportionality, in other systems, between ATP content and the rates of protein synthesis (6) and RNA synthesis (33). Oxygen stimulates protein synthesis (34, 58) and this is often accompanied by synthesis of ATP (58). Isolated nuclei, for example, synthesize protein 3 times faster under oxidative conditions than in the absence of respiration (68). It may be relevant that a cell-free protein-synthesizing system prepared from Ehrlich ascites tumors of various ages shows no difference in the rate of protein synthesis (phenylalanine incorporation) in the presence of exogenous ATP (24).

Although this explanation is reasonable, it is not entirely satisfactory and we regard it as nothing more than a working hypothesis. It does not explain, for example, the 5- to 6-hr lag period which precedes the rise of the thymidine index in transplanted cells (Chart 4). This lag suggests that cells must synthesize something or overcome some immunological barrier before they can move into S. Moreover, preliminary results indicate that dinitrophenol does not prevent the rise of the thymidine index which follows transplantation. Although some workers believe that there are "energy barriers" in the cell cycle (64, 65), this concept has been disputed by others (25). Clearly, we require further experimental evidence before

we can reach firm conclusions as to the mechanism(s) responsible for the effects of hypoxia.

The results presented in this paper may have practical implications apropos of 2 questions. Does redistribution of cells within the cycle during tumor growth affect the radio-curability of the tumor? Does loss of cells after radiotherapy or surgery affect the size of the proliferating pool in the surviving population? A partial answer may be made to the 1st question: P388 lymphocytic leukemia tumors harvested from mice on the 2nd and 7th day of growth are equally sensitive to radiation *in vitro* (7), although the proportion of cells which are in S falls from 68 to 15% during this interval (J. Harris, unpublished data). This apparent lack of correlation between radiosensitivity and the proportion of cells in S is most surprising, and further studies are necessary before its importance can be assessed. As to the 2nd question, it is clear that if cells in solid tumors respond to oxygen like those in ascites tumor [and there is evidence that they do (61)], reoxygenation of the tumor during fractionated radiotherapy may cause non-proliferating cells to enter the proliferative cell cycles. Since about 20% of the cells in most solid tumors are anoxic and since reoxygenation frequently does occur after X-radiation (8, 17, 63), the contribution of such redistribution to tumor growth could be highly significant.

ACKNOWLEDGMENTS

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Biochemical Studies of Cytokinetic Changes during Tumor Growth

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