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A Restriction Point for Control of Normal Animal Cell Proliferation

(growth control/cell survival/cancer)

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ABSTRACT This paper provides evidence that normal animal cells possess a unique regulatory mechanism to shift them between proliferative and quiescent states. Cells cease to increase in number under a diversity of suboptimal nutritional conditions, whereas a uniformity of metabolic changes follows these nutritional shifts. Evidence is given here that cells are put into the same quiescent state by each of these diverse blocks to proliferation and that cells escape at the same point in G_1 of the cell cycle when nutrition is restored. The name restriction point is proposed for the specific time in the cell cycle at which this critical release event occurs.

The restriction point control is proposed to permit normal cells to retain viability by a shift to a minimal metabolism upon differentiation *in vivo* and *in vitro* when conditions are suboptimal for growth. Malignant cells are proposed to have lost their restriction point control. Hence, under very adverse conditions, as in the presence of antitumor agents, they stop randomly in their division cycle and die.

Normal animal cells *in vivo* have alternative modes of existence: either proliferative or quiescent. They survive well in either state. "Normal" cells in culture can be shifted from one state to the other by alterations of the growth medium. Several suboptimal nutritional conditions can bring about quiescence: high cell density (1), nutrient (2) or serum insufficiency (3, 4), or high cAMP (5). These cells return to the proliferative state when supplied with complete medium.

Is there a unique quiescent state? The existence of a quiescent state, often referred to as G_0, has been questioned by those who propose that nonproliferating cells are simply stopped somewhere in G_1. We do know that quiescent cells are between M and S, since, when they are supplied with complete medium, they synthesize DNA before they divide. Entry into the quiescent state under a number of conditions leads to a set of metabolic consequences which has been called the pleiotypic response (6).

The purpose of this paper is to provide evidence that cells that have reached quiescence by a variety of means are indeed in the same state. The experiments determine the kinetics of cells' reentry from quiescence into the S phase under a variety of conditions. The results are consistent with the existence of a single switching point, the restriction point (or R-point) in G_1, that regulates the reentry of a cell into a new round of the cell cycle. When cells are stopped by nonphysiological agents such as hydroxyurea or colchicine, they do not stop at the R-point.

We propose that normal animal cells have evolved this ability to shift between proliferative and quiescent states as a mechanism for survival under conditions that lead to differentiation *in vivo*, or under nutritional deprivation to utilize this switching mechanism to achieve quiescence. A fundamental difference between normal and malignant cells may be that the malignant cells have lost their R-point control. As the consequences of this loss, although malignant cells' growth is less restricted, they would have reduced survival ability under adverse conditions. A preliminary report of this work has been presented elsewhere (7).

MATERIALS AND METHODS

Syrian hamster BHK21/C13, J1(PYBHK), and Nil 8 cells were from the Imperial Cancer Research Fund stocks (8). New cultures were started from frozen stocks at monthly intervals. The cells were grown in Dulbecco's modification of Eagle's medium with 10% calf serum, used as complete medium in all experiments, at 37° under 10% CO_2 pressure. Subcultures were carried in NUNC 150-mm petri dishes. Experiments were performed in Linbro FB-16-24-TC trays. Approximately 3×10^3 cells in 1 ml of medium were placed into each well of a Linbro tray and were allowed to grow into a loose network during 2–3 days. The cells remained attached to the bottoms of the wells throughout the experiments. Media were changed by aspiration and rinsing with phosphate-buffered saline (pH 7.2). After the cells had been allowed to incorporate [^{3}H]thymidine (Amersham) added to 0.05 μM and 0.1 μCi/ml, the wells were rinsed with 2 ml of phosphate-buffered saline and then with 1 ml of 5% trichloroacetic acid. The cells were dissolved in 0.8 ml of 2% Na_2CO_3 in 0.1 M NaOH. A 0.6-ml portion of this solution was added to 7 ml of Triton–toluene scintillator to which 0.2 ml of 50% trichloroacetic acid was added to neutralize the alkali. The samples were counted for ^{3}H in a Nuclear Chicago Scintillation Spectrometer. Autoradiography was done with Kodak AR-10 stripping film.

RESULTS

The cell cycle has been divided into four major phases (9) (G_1, S, G_2, and M) but the sequence of essential events within G_1, S, and G_2 is just beginning to be investigated. A cell cannot be positioned within G_1 because there are virtually no biochemical events established that can be used as landmarks.

Our purpose in this paper is to compare the relative positions between M and S ("within G_1") of a number of different quiescent cell populations, in order to ascertain whether or not they are at the same point. In the simplest approach, complete medium was added to quiescent cells and the transit time to the beginning of DNA synthesis was determined. For reasons to be summarized below, the results of these experiments were not fully adequate to locate the cells' positions.

Abbreviation: R-point, restriction point.

We then developed methods to compare directly the positions into which different blocking conditions put a cell population. A cell population was shifted from one blocking condition to another, and the kinetics of DNA synthesis were then examined. Using these methods, we were able to support our hypothesis of a unique restriction point.

In the simplest experiments, the cells in an exponentially growing culture were stopped. Complete medium was then added and the kinetics of incorporation of [^{3}H]thymidine were measured. After starvation for isoleucine, glutamine, or serum, about an 8-hr interval elapsed between restoration by returning the cells to complete medium and the onset of thymidine incorporation above background level (Fig. 1). A similar 8-hr interval was observed after prestarvation for phosphate, or when the cAMP level was increased by exposure of the cells to 0.05 mM papaverine, or 1.0 mM dibutyryl cAMP plus 2.0 mM theophylline, or 30 μg/ml of prostaglandin E1 + 1 mM theophylline (10) for 46 hr. The length of this transit time was not seriously affected by leaving the cells under the various inhibitory conditions for times up to 3 days. In each experiment, the quiescent cells required the same length of time to recommence DNA synthesis. These results are consistent with each of the cell populations being blocked at the same point.

There are, however, several limitations to these experiments. First, the measurement of transit time is not precise enough to detect a difference of an hour or two that might exist between recovery of different quiescent cell populations. Secondly, the well-known variability in the length of G_1 (9) is still present in these experiments; in autoradiographic experiments, different cells begin thymidine incorporation at different times. Thus, measurement of the time of initiation of DNA synthesis by a cell population depends upon the behavior of an early initiating subclass of the population. We can only conclude, therefore, that this subclass is at the same point in different quiescent cultures. Assessment of initiation times for the population as a whole from these data involves many complications, both conceptual and experimental. Consequently, we turned to another kind of experiment.

A basic test of whether quiescent cells inhibited by different means are in the same state is to impose one block and then shift the cells to another kind of block and determine whether or not they are able to proceed to DNA synthesis. If the block applied second stops cells at an earlier time in G_1 than does

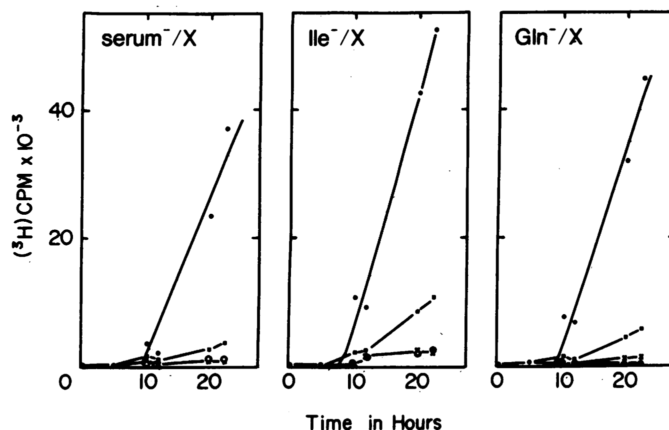

FIG. 1. Thymidine incorporation by BHK cells after transfer of quiescent cells into new media. Growing cells were starved in medium with (*left*) 0.25% calf serum + 0.1 mM hypoxanthine + 0.1 mM ornithine, (*middle*) 4% serum minus isoleucine, and (*right*) 4% serum minus glutamine. After 64 hr (for the first three points) or 52 hr (final three points) the medium was replaced with either complete medium (●) or with each of the deficient media (serum⁻, ■; Ile⁻, ×; Gln⁻, ○), all plus [^{3}H]thymidine. Samples were taken at the times shown to measure ^{3}H incorporation. Values on *ordinate* have been multiplied by 10^{-3}.

the block applied first, the cells should make DNA. But if the block applied second acts later or at the same point as the one applied earlier, the cells should not progress on to DNA synthesis (except to the extent that the blocking effect is delayed and some cells will slip by).

The results of experiments of this sort are quite clear. Some examples are shown in Fig. 1. Only a small thymidine incorporation was noted relative to the controls, and this can be attributed to the slow effect of low serum as the second blocking condition.

Table 1 summarizes many double-block experiments. The pairs of blocking conditions were applied in both sequences. Values of about 20% or less indicate that the second block was effective in preventing reinitiation of DNA synthesis. Values around 60% would be expected if all of the cells passed through one round of DNA synthesis and then stopped, because in the control the cells have reinitiated a second round of DNA synthesis. We conclude that all physiological blocking

TABLE 1. *Thymidine incorporation after shifts from one blocking condition to another*

	Second condition*							
First condition	−Ile	−Gln	Low serum	Dibutyryl cAMP + theophylline	Papaverine	PGE1 + theophylline	Hydroxyurea	Colchicine
Minus Ile	10	12	13	37	18	32	9	46
Minus Gln	13	4	10	30	25	30	3	73
Low serum	7	4	8	22	14	10	—	—
Dibutyryl cAMP + theophylline	7	6	7	6	3	34	—	—
Papaverine	16	16	22	22	16	36	—	—
Hydroxyurea	—	78	85	85	60	88	—	—
Colchicine	26	27	22	47	23	50	—	—

Growing BHK cells were placed under the first condition for approximately two days and then transferred to the second condition, and also into complete medium as a control. Concentrations are as given in Fig. 3. [^{3}H]Thymidine was added and the incorporation of radioactivity was measured after 1 day, except that when hydroxyurea was present initially, incorporation was for 9 hr.

* Results are presented as % of [^{3}H]thymidine incorporation by controls.

PGE1, prostaglandin E1.

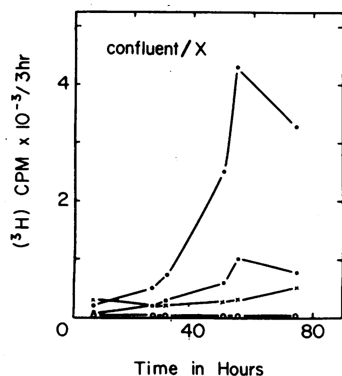

FIG. 2. Rate of thymidine incorporation after confluent quiescent Nil 8 cells were replated in various media. The pattern of the experiment was the same as that for Fig. 1, except that confluent Nil 8 cells were scraped from petri dishes and replated below confluence in the fresh media. Thymidine incorporation during a 3-hr interval was determined at each sampling time.

conditions are effective as second blocks, and hence all must act at the same point.

This kind of experiment has been extended to the inhibition of growth at high cell density (density-dependent inhibition) (1). Nil 8 cells were used in these experiments because they are more strongly contact-inhibited than are BHK cells.

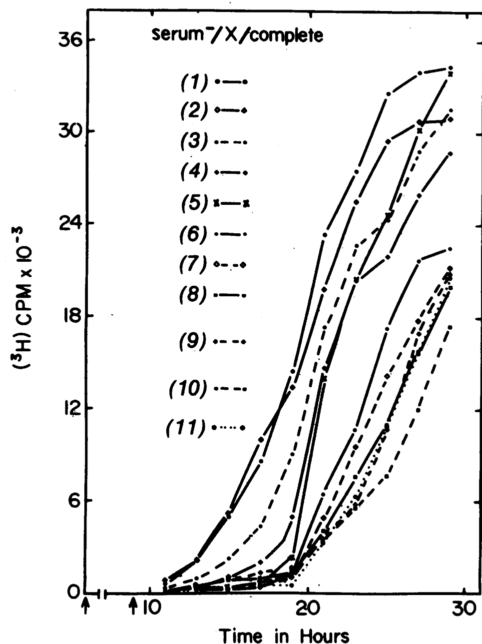

FIG. 3. Thymidine incorporation by BHK cells in complete medium after two sequential blocking conditions. Growing BHK cells were starved 60 hr in medium containing 0.5% serum plus hypoxanthine and ornithine. Then they were transferred for 9 hr into (*1*) complete medium + 4% serum +25 μM adenosine; (*2*) same + 1 mM hydroxyurea; (*3*) same + 10 μg/ml of colchicine; (*4*) same + 0.05 mM papaverine; (*5*) same minus isoleucine; (*6*) same minus glutamine; (*7*) same + 4 μg/ml of cytochalasin B; (*8*) same with 0.25% serum + hypoxanthine + ornithine; (*9*) same + 1 mM dibutyryl cAMP + 2 mM theophylline; (*10*) same + 30 μg/ml of prostaglandin E1 + 1 mM theophylline; (*11*) left in old low-serum medium. To all cells was then added complete medium with 10% serum + adenosine + [^{3}H]thymidine. Samples were assayed for thymidine incorporation at intervals.

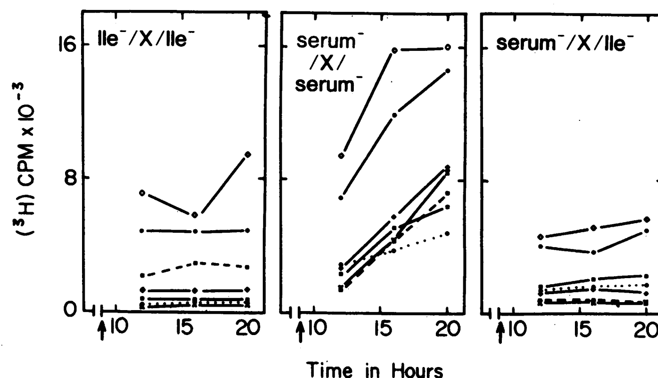

FIG. 4. Thymidine incorporation by BHK cells in deficient media after two sequential blocking conditions. The experiment was the same as for Fig. 3, except that the initial starvation for 50 hr was either in medium minus isoleucine + 4% serum or with 0.5% serum + hypoxanthine + ornithine. After the middle period of 9 hr in various media, the cells were supplied with medium + [^{3}H]thymidine and deficient in either isoleucine or serum. Incorporation of thymidine was then determined. *Symbols*, see *legend* for Fig. 3.

When contact-inhibited Nil 8 cells were replated at low cell density in a complete medium, thymidine incorporation was initiated after about 20 hr (Fig. 2). When these cells were similarly replated in media lacking isoleucine or glutamine, or with only 0.25% serum, the cells did not resume incorporation. Thus, the latter blocking conditions arrest cells at the same point or later than does contact inhibition.

It could be argued that the quiescent cells were in such poor condition that any inhibitory or suboptimal environment would prevent their return into the proliferative cycle. But when quiescent cells were transferred into complete medium containing 5 μg/ml of colchicine or 1 mM hydroxyurea (for 9 hr), both toxic agents, the cells then rapidly initiated DNA synthesis (see Fig. 3).

Several more critical tests for the existence of a unique R-point in G_1 have been applied. The experiments depend upon three sequential treatments, during the last of which thymidine uptake is measured. The first method is to apply a blocking condition so that cells become quiescent, then to apply a second blocking condition for a period of usually 9 hr, in order to allow any possible escape from the first block, and finally to put cells into complete medium and measure thymidine uptake. This procedure is similar to some experiments by Froehlich and Rachmeler (11) with cAMP. Any escape during the middle period should allow DNA to be made earlier than in control cultures in which cells are left in the first blocking medium through the 9-hr intervening period. Fig. 3 shows that none of the physiological blocking conditions allowed any appreciable escape towards DNA synthesis; all extrapolated to 9 hr after complete medium was supplied. In contrast, intervening exposures to media containing hydroxyurea or colchicine did allow escape, as would be predicted from the experiments described before.

Another three-step experiment is shown in Fig. 4. In this sequence, the first blocking condition was either serum or isoleucine. Then a variety of second blocking conditions were applied for 9 hr. Finally, the cells were returned to low serum or isoleucine and ability to incorporate thymidine was measured. If the middle period had allowed escape of cells from the initial block, then DNA synthesis would have occurred

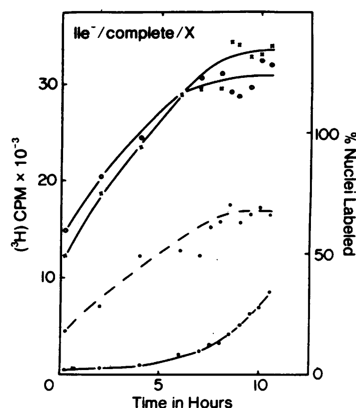

FIG. 5. Recovery from isoleucine starvation by BHK cells in complete medium. Growing cells were starved for 45 hr in isoleucine-deficient medium + 10% serum (a minimal period). For the *top two curves*, cells then (zero time) were placed into complete medium + 10% serum + adenosine for the intervals shown on the *abscissa*. After this recovery period, they were put into medium with 4% serum + [^{3}H]thymidine and lacking either isoleucine or glutamine, until 27.5 hr, when they were assayed for thymidine incorporated. The *middle curve* shows the percent labeled mitoses, determined by autoradiography as the cells recovered in complete medium + 1 μCi/ml of [^{3}H]thymidine. The *bottom curve* shows total thymidine incorporation in complete medium by the recovering cells.

when the initial conditions were again restored. As can be seen, much less DNA was made, except when the middle period was one in which hydroxyurea was applied. The middle panel again exhibits the incomplete blocking effect of low serum medium.

A final variation was to interpose a variable interval in complete medium between two blocking conditions that may be the same or different. Serum-starved cells are capable of DNA synthesis if they are given an intervening period of several hours in complete medium, and then returned to serum-free medium (3, 4). We asked whether the interval in complete medium that allows escape from the initial block simultaneously allows escape from another blocking condition. Have cells in complete medium moved at the same time to a position beyond both blocks? In the experiment shown (Fig. 5), cells were first made quiescent by isoleucine deprivation, then provided with complete medium for the intervals shown, and finally media were added that contained [^{3}H]-thymidine but lacked either isoleucine or glutamine. Essentially, the ability of cells to go through one round of DNA synthesis was determined by measuring incorporation until 27.5 hr. This experiment was successful in showing that when cells escape from their isoleucine requirement for DNA replication, they simultaneously escape from their glutamine requirement. The kinetics of escape (*top two curves*) is paralleled by autoradiographic measurements (*middle curve*).

The sum total of these five kinds of experiments allows us to conclude that the same quiescent state exists for the various physiological blocking conditions: in each condition the cells are blocked at the same R-point between M and S.

Experiments with colchicine and hydroxyurea were designed to find out whether the sequential blocking method would identify other positions in the cycle at which cells are blocked. Colchicine and hydroxyurea are nonphysiological, toxic agents that stop cells in M and in S, respectively. In our

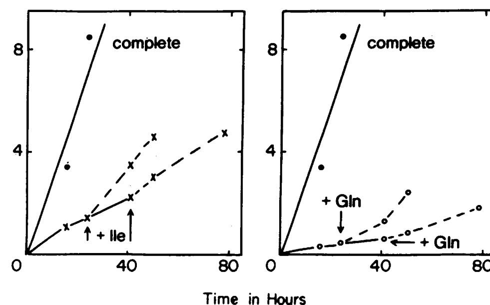

FIG. 6. Ability of J1 (PYBHK) cells to incorporate thymidine during and after starvation for isoleucine or glutamine. Growing cells were placed into media lacking Ile or Gln and then into complete medium + [^{3}H]thymidine at 24 and 41 hr. Assays were made for incorporated thymidine at intervals. Control in complete medium (●).

experiments, many cells are killed in 24 hr and do not incorporate thymidine. Treatments such as isoleucine starvation were found to proceed through DNA synthesis in complete medium containing colchicine, showing that the double-block method distinguishes this inhibitor from the more physiological ones used above. Experiments with hydroxyurea were more complex since hydroxyurea inhibits DNA synthesis and so prevents assay by [^{3}H]thymidine incorporation. In these experiments, cells were transferred from a physiologically induced quiescent state into complete medium containing 1 mM hydroxyurea for 9 hr. Then they were transferred into complete or deficient media without hydroxyurea, and thymidine incorporation was measured (Figs. 3 and 4). Thymidine uptake began immediately, showing that the cells had escaped from the G_1 block and had moved to the G_1–S interphase in the presence of hydroxyurea. The same experiments using colchicine showed a partial escape. The reciprocal experiments were hard to interpret because of extensive cell death (Table 1). Cells transferred from low hydroxyurea into nutrient-deprived or high cAMP conditions incorporated thymidine rapidly; those first exposed to low colchicine showed only moderate incorporation relative to controls in complete medium. Results are consistent with relative positions of the block points.

Can similar experiments be done with transformed cells? Attempts were made to put BHK cells transformed by simian virus 40 into the quiescent state by isoleucine and by glutamine starvation (Fig. 6). After a period of 40 hr of starvation, these cells did not recover their ability to incorporate thymidine when complete medium was provided. This result suggests that there is a fundamentally different response of transformed cells to nutritional insufficiency as compared to normal cells.

DISCUSSION

Most animal cells *in vivo* exist in a nonproliferating state in which they remain viable and metabolically active. They arose from proliferating cells whose metabolic patterns were switched to quiescence at some time during differentiation. In contrast to this pattern of normal differentiation, cancer cells appear to arise from quiescent cells that have been switched back to active proliferation. If this formulation is correct, it becomes of great importance first to characterize the switching mechanism that regulates cell proliferation at the cellular level, and then to explore its molecular basis.

The experiments described in this paper were designed to find out whether cells switch between quiescence and the proliferative state at one or at several points in the cell cycle. We have concluded from the results presented here that several environmental conditions that are suboptimal for growth act in culture at a unique R-point located in G_1 at the R-point shifting the metabolic pattern of cells between quiescence and growth. The identification of this unique point greatly strengthens the hypothesis that the R-point plays a key role both in normal differentiation and in the origin of malignancy.

Conditions tested in our experiments include deprivation for isoleucine, glutamine, serum, or phosphate, elevated cAMP levels (brought about by three different methods), and inhibition by cytochalasin B, a compound that seems principally to affect cell separation. In each of these experiments, BHK cells were blocked at the same place in the G_1 phase of the cell cycle. Also, density-inhibited Nil 8 cells were found to behave like nutritionally blocked BHK cells, indicating that density-dependent inhibition ("contact inhibition") also stops cells at the R-point. Reinitiation by BHK cells of growth after infection with polyoma virus has a sequence similar to nutritional release from R-point control (1). So does the starting and stopping of WG, a temperature-dependent mutant hamster cell line (12). We conclude that the R-point phenomenon is widespread and has a correspondingly great significance in the pattern of cell behavior.

Evidence from experiments at the cellular level lead to questions regarding the molecular mechanisms underlying the the R-point switch. We need to determine the biochemical sequences that lead from the original physiological blocking condition to the final change that inhibits progress of the cell towards DNA synthesis. Could, for instance, entry or exit of growth-controlling factors through the cell membrane (13) be the primary step, one affected by serum factors? If so, many of the conditions that stop cell proliferation could have the same effect, namely, a change in the internal concentration of the same essential factor or nutrient (14).

Two rather different views can be proposed to account for the shift from proliferation to quiescence: either that poor conditions shift cells from proliferation into a less active metabolic state (6, 14), or alternatively, that all cells go into a quiescent state as part of their normal growth cycle and emerge from this state probabilistically, as proposed by Smith and Martin (15). By their hypothesis, quiescence would be achieved by a greatly decreased probability of escape from this universal phase of the cell cycle. Unfortunately, direct experiments to measure the timing of entry into quiescence are impracticable because the various blocking conditions act slowly (taking effect sometimes after more than one growth cycle) (5), and they affect metabolism at multiple sites. For these reasons, in our experiments we examined the release from quiescence back to the proliferative state.

An R-point more sensitive to environmental conditions than any other point in the cell cycle may have evolved to protect cells; permitting them to shift gears, so to speak, and to move into a lower metabolic activity characteristic of quiescence. Cells unable to shift into quiescence at the R-point would stop at random points in the cycle, or possibly at secondary control points, at which they would not survive. In our experiments, e.g., cells blocked at M with colchicine or at S with hydroxyurea mostly died within a generation.

Growth characteristics of some transformed, malignant cells suggests that they have lost their R-point control. One evidence is that transformed cells do continue to grow at low serum or suboptimal nutrient concentrations at which normal cells are blocked (1). Second, when cells are severely restricted nutritionally, they stop at random points in the cycle and die (Fig. 6; refs. 16–18). *In vivo*, tumor cells need ample nutrition to survive (19). This concept can provide a basis for the well-known lower resistance of tumor cells to antitumor agents, many of which create nutritional deficiencies (e.g., folic acid analogs, purine and pyrimidine analogs, or asparaginase). Reports of lower tumor incidence in rats on restricted diets could be related to the poorer survival of tumor cells when nutrients are insufficient.

We intend to examine various normal and transformed cell lines to learn whether their R-point controls have been lost, how their survival has been affected after various deprivations, and the relation of these parameters to other characteristics of transformed cells such as surface changes and the transport changes that permit better nutrient uptake (20).

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