Duration of Cell Cycle and Its Phases Measured in Synchronized Cells of Squamous Cell Carcinoma of Rat Trachea

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ABSTRACT

Synchronization of tumor cells can facilitate measurement of cycle time and permit identification of subpopulations which deviate from the average in either total cycle time or duration of specific phases. We utilized exposure to high concentrations of thymidine to arrest cycling cells in tumor fragments in culture. Pieces of six squamous cell carcinomas, induced in heterotopically transplanted rat tracheas by exposure to benzo(a)pyrene, were placed in culture and subjected to two sequential thymidine blocks. The labeling indices in cultures studied at 2 and 8 hr after release from the second block were equal to the growth fractions. By 16 hr after release from the block, no DNA synthesis was observed in any culture. In three tumors for which cycle and cycle phase duration was measured, mitosis occurred synchronously 12 hr after release from the thymidine block, and a second period of DNA synthesis began 16 hr later. The independent effects of the organ culture manipulations were monitored by measuring growth fractions, and those of circadian rhythms were measured by autoradiographic assessment of DNA synthesis over a period of hours after removal of the dThd block to determine whether the labeling index equaled the growth fraction and whether all cells which synthesize DNA start at the same time shortly after release from the block. The independent effects of the organ culture manipulations were monitored by measuring growth fractions, and those of circadian rhythms were measured by comparing mitotic activity and labeling after release at different hours. After the degree of synchrony was determined, the cell cycle and individual phase durations were measured for possible subpopulation deviations and comparisons with previously determined phase durations in normal regenerative tracheal epithelium.

INTRODUCTION

Many morphological and biochemical characteristics of normal cells vary with position in the cell cycle. Differences in types and rates of synthesis of structural proteins, RNA, DNA, and enzymes as well as variations in the transport of nutrients during the generative cycle have been reviewed by Baserga (1–3). It has also been shown that changes in cell surface structure and in the quality and quantity of membrane molecules occur throughout the cell cycle in normal cells (16). Differences in some of these characteristics have been proposed as markers for distinguishing neoplastic cells from the normal population (1, 3, 7, 9, 11, 18, 21). Since the markers may themselves vary with the cell cycle, to evaluate these possibilities one must know in which phase of the generative cycle the cells that are being studied reside. This correlation may be achieved by simultaneously determining the phase of the cycle while examining other features. Alternatively, mitotic activity can be synchronized so that cell cycle phases can be predicted. This approach is preferable because concurrent assessment of cell cycle phase which might impede measures of cell function or structure is then no longer required.

We describe a method for synchronizing mitotic activity in tumor cells and present data concerning duration of the cell cycle and its constituent phases.

MATERIALS AND METHODS

Experimental Design

Fragments of tumor were placed in culture and treated with dThd (2) to arrest cells moving through the cell cycle (8, 24). The success of the synchronization method was tested by autoradiographic assessment of DNA synthesis over a period of hours after removal of the dThd block to determine whether the labeling index equaled the growth fraction and whether all cells which synthesize DNA start at the same time shortly after release from the block. The independent effects of the organ culture manipulations were monitored by measuring growth fractions, and those of circadian rhythms were measured by comparing mitotic activity and labeling after release at different hours. After the degree of synchrony was determined, the cell cycle and individual phase durations were measured for possible subpopulation deviations and comparisons with previously determined phase durations in normal regenerative tracheal epithelium (10).

Source of Tumor Tissues

The study used 6 squamous cell carcinomas induced in heterotopic tracheal grafts treated with benzo(a)pyrene (14). Female Fischer rats (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) weighing 100 to 150 g were used as tissue donors and recipients for the graft procedure. Donor tracheas were placed in s.c. pouches on the backs of hosts. Two weeks later, agar suspensions containing 2.5 mg of benzo(a)pyrene were placed in the lumens of the grafts. Neoplasms which grew to measure 15 to 20 mm in greatest dimension arose in the grafts from 3 to 6 months after onset of exposure to benzo(a)pyrene. The tumors exhibited central necrosis with a viable 3- to 5-mm rim composed of micronodules with intervening vascular connective tissue.

Culture and Synchronization of Tumor Fragments

With the rats lightly anesthetized with ether, fragments of viable tumor tissue were removed from the well-vascularized periphery of the cancers and minced. These pieces were immediately immersed in serum-free McCoy's modified Medium 5A with streptomycin and penicillin where they remained for 1 hr. The cells composing the tumor pieces were then synchronized by the use of a double dThd block which arrests cells at the G1-S interface (8, 24). The McCoy's modified

1 Supported by Contract NO 1 CP 33361, National Cancer Institute.
2 The abbreviation used is: dThd, thymidine.

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Medium 5A was replaced with the same medium containing 2 mm dThd. After 30 hr, fresh McCoy’s modified Medium 5A was substituted for the dThd-containing medium to release cells which had collected at the interface of G1 and S. Six hr later, the medium containing 2 mm dThd was again substituted for the McCoy’s medium without dThd, and the tissue was incubated for 30 hr before return to medium without dThd. The second block serves to increase the degree of synchrony.

Effects of Culture and Circadian Rhythms on Labeling

Labeling indices were measured without dThd block at 6 a.m., 12 noon, 6 p.m., and 12 midnight on the first day to test for circadian rhythm. Labeling indices were repeated on the second and third days to determine whether the size of the normal in situ proliferative population changed due to culture conditions. The labeling was performed by placing the tissue for 30 min in fresh McCoy’s medium to which [3H]dThd (specific activity, 20 Ci/mmol) was added to make a final concentration of 1 μCi/ml of medium. The tissue was then cold chased with two 10-min washes in McCoy’s medium containing excess dThd (1.2 μg/ml) in order to displace any unincorporated [3H]dThd. Labeling indices of arrested organ cultures were also measured at 2, 8, and 16 hr after release from the second dThd block to determine if synchrony was achieved. Unsynchro- nized tumor tissue was pulse labeled at each medium change in the above protocol to determine if the medium changes in themselves had an effect on the labeling indices.

Growth fractions were measured on the first, second, and third days of culture by replacing the medium with fresh McCoy’s medium containing [3H]dThd (0.5 μCi/ml; specific activity, 20 Ci/mmol). The tissue was incubated in this medium for 24 hr. After this interval, the tissue was cold chased with two 10-min washes of medium containing excess dThd (1.2 μg/ml).

One additional tumor-bearing animal was pulse labeled in vivo with an i.p. injection of [3H]dThd at a concentration of 1 μCi/g body weight to compare in vivo versus in vitro labeling indices. The animal was sacrificed 1 hr after pulse labeling, and tumor fragments were prepared for autoradiography.

Autoradiographic Procedure

After labeling, the tissue was immediately immersed in fixa- tive containing 3% glutaraldehyde with 0.2 M sodium cacodylate buffered at pH 7.3. After overnight fixation, the tissue was transferred to 1% unbuffered osmium tetroxide. One hr later, the tissue was dehydrated in graded steps of ethanol and embedded in Epon 812. One-μm-thick plastic sections were heat fixed to acid-cleaned glass slides and dipped in 1:1 dilution of NTB2 photographic emulsion. The slides were kept under cool dry conditions in light-tight boxes for 4 weeks, developed in full-strength Kodak developer D19, and fixed with full-strength Kodak Rapid Fix. After drying, the slides were stained with methylene blue and azure II.

An 80-μm-wide peripheral rim of each tumor fragment section was scored for percentage of cells labeled. A minimum of 2000 cells was counted for each tumor at each time studied. Only nucleated cells were counted, and cells showing pyknosis or karyolysis were excluded. Cells were scored as positive for [3H]dThd incorporation if 3 or more silver grains were present directly over the nucleus. This threshold value was greater than background which was never more than 2 grains per cell. However, in the area used for scoring, there were no cells with grain counts in the range of 2 to 10 grains. For several sections in which absolute grain counts were done, the counts were unimodal and fell within 1 S.D. from the mean.

All slides were read by a single observer in a double-blind protocol, and 5% of the slides were selected at random for repeat scoring by that observer and by another observer as a measure of reproducibility and absence of bias.

Cell Cycle and Phase Durations

Cell cycle and phase durations were measured by the techniques described by Gordon and Lane (10).

Determination of Duration of DNA Synthesis (T_s). Two hr after release from the synchronization procedure, the tissue was exposed to [3H]dThd at a concentration of 5 μCi/ml McCoy’s medium (specific activity, 60 μCi/mmol) for 30 min followed by three 10-min cold chases with McCoy’s medium containing excess dThd (1.2 μg/ml). The tissue was transferred to McCoy’s medium, and, at hourly intervals between 6 and 12 hr after the [3H]dThd pulse, groups of fragments were labeled for 30 min with medium containing [14C]dThd at a concentration of 1 μCi/ml medium (specific activity, 42 mCi/mmol). The fragments were cold chased in excess dThd, immediately fixed in 3% glutaraldehyde with 0.2 M sodium cacodylate buffered at pH 7.3, and prepared for double-label autoradiography. Data obtained from slides of 3 tumors raised on separate animals were statistically compared to one another for each time studied by standard error of the mean. The initial label was given 2 hr after release because preliminary studies on a similar tumor in which replicate fragments were pulse labeled at 0.5-hr intervals from 20 min to 4 hr after removal from dThd excess showed maximal labeling at 2 hr thereafter, and there was less than 0.5% labeling at 1.5 hr. As an independent test of the dThd effect on cycle progression, unsynchronized fragments from one tumor were also pulse labeled with [3H]dThd and then pulse labeled with [14C]dThd from 6 to 12 hr later. The disappearance of double-labeled cells was at 9 hr. Thus, flux through S of unsynchronized cells equaled progress through S by synchronized cells. Another set of unsynchronized specimens was pulse labeled with [3H]dThd and then subjected to a pulse of [14C]dThd at hourly intervals from 12 to 32 hr later. The earliest appearance of double label is a measure of G1 + G2 + M, and the last appearance of double-labeled cells is a measure of G1 + G2 + M + 2S. These values were used as controls for the effects of dThd on cycling.

Technique for Determining Duration of the Cell Cycle (T_c). Two hr after release from synchronization, the fragments taken from 3 separate tumor-bearing animals were pulse labeled with [3H]dThd at a concentration of 5 μCi/ml in McCoy’s medium for 30 min, cold chased with excessive dThd, and then placed in nonradioactive McCoy’s medium. Beginning 14 hr after the [3H]dThd exposure at 1-hr intervals through 40 hr, groups of fragments were pulse labeled with [14C]dThd for 30 min, cold chased with excess dThd, and immediately fixed with glutaraldehyde. One-μm sections of Epon-embedded tissue from alternate hours were prepared for double-label autoradiography to determine the shape of the curve. Specimens from the remaining hours which fell within the periods of DNA synthesis were studied.
Double-label autoradiograms were prepared by the technique described by Gordon and Lane (10). A first layer of Kodak NTB2 photographic emulsion was applied by dip technique to the 1-μm sections at 1:3 dilution. This layer was exposed for 9 days, developed, and counted for the number of labeled cells by phase microscopy. A second layer of Kodak NTB2 at 1:1 dilution was applied by dip technique over the first layer labeled cells by phase microscopy. A second layer of Kodak NTB2 at 1:1 dilution was applied by dip technique over the first layer.

Technique for Determining Duration of G2 (Ts). Two hr after release from synchronization, fragments were exposed to [3H]dThd at a concentration of 5 μCi/ml McCoy’s medium for 30 min. The fragments were cold chased with excess dThd and then placed in normal McCoy’s medium. Groups of fragments were fixed with glutaraldehyde at hourly intervals between 10 and 14 hr after the [3H]dThd pulse label. They were embedded in Epon 812, sectioned, and prepared for single-label autoradiography. A minimum of 100 mitotic figures in at least 10 fragments per time point per tumor were scored for the percentage of mitotic cells exhibiting silver grains over their chromosomes.

Calculation of the Duration of G2 (Ts) and Approximation of (Ts). Ts was calculated by subtracting the Ts + Ts2 + Ts3 from the TC. Ts was approximated and supported by measurements made from other rat tissues (4).

RESULTS

Cultured fragments of squamous cell carcinoma retained their histological organization and cellular differentiation when cultured. There were no avascular or necrotic fragments. Labeling indices of unsynchronized tissues were the same throughout the 3-day study and did not demonstrate any appreciable circadian rhythm (Table 1). Comparison of in vivo and in vitro labeling indices for one of the tumors revealed no differences. There was also very little difference in labeling index among the 6 tumors studied. Growth fractions were measured to determine whether there was any change in the size of the proliferative population during the study. These 24-hr pool labels showed no significant differences during the 3-day period (Table 1). There was no variation of labeling indices associated with changes of culture medium. Grain counts in the areas studied in specimens which had been labeled in vitro were 10 grains or more per nucleus with background less than 2 grains.

In cell populations not dividing synchronously, the labeling index is usually a fraction of the proliferative population, and, if the cells are completely asynchronous, the percentage of cycling cells which are labeled during a short exposure to [3H]dThd is an accurate reflection of the relationship between the duration of S and the duration of the cell cycle (19). After synchronization, pulse label of the cells in the S phase should produce a labeling index equal to the growth fraction. As the cells leave the S phase, the labeling index should decline rapidly to zero.

Two hr after the release from the second dThd block (Table 2), the labeling index in synchronized tumor fragments equaled the measured growth fraction of unsynchronized tumor fragments on their third day of culture. Eight hr after release, the labeling indices of all the tumors were still equal to the growth fractions of tumor fragments on their third day of culture (Table 2). By 16 hr after release, the labeling index declined to zero (Table 2). These data show that the double dThd block was suitable for synchronization of the proliferative population of tumor cells.

Application of the double label method permits direct measurement of the duration of S and determination of the degree of synchrony which was achieved by the double dThd arrest. The specimens subjected to a pulse label of [3H]dThd 2 hr after release from the dThd arrest and to a pulse label of [14C]dThd at hourly intervals thereafter (Chart 1) show a sharp decline in double-labeled cells between 8 and 9 hr after introduction to the [3H]dThd. This indicates that the duration of S is 8 to 9 hr in a well-synchronized population of tumor cells. The unsynchronized tumor cells in fragments not exposed to the dThd block exhibited double-labeled cells until the second pulse was given at 9 hr. This is evidence that the dThd block did not increase the length of the S phase.

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Table 1
Daily labeling indices and growth fractions of culture tumor fragments
Tumor fragments from 3 carcinomas were pulse labeled in vitro with [3H]dThd (5 μCi/ml medium) for 30 min at 3 different times of day to determine if tumor cell proliferation exhibits a circadian rhythm. Pulse labels were done on 2 successive days to determine whether circadian rhythms appeared after a period in culture. Additional tissue samples were pool labeled with [3H]dThd (0.5 μCi/ml medium) for 24 hr on Days 1, 2, and 3 to determine if the size of the proliferative population changes over the duration of culture. The specimens, pulse labeled, fixed, embedded, and sectioned, were prepared for autoradiography and represent the labeling index. Pool-labeled specimens prepared similarly represent growth fractions.

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Time</th>
<th>Labeling index</th>
<th>Day</th>
<th>Growth fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12 noon</td>
<td>0.217</td>
<td>1</td>
<td>0.590</td>
</tr>
<tr>
<td>1</td>
<td>10 p.m.</td>
<td>0.210</td>
<td>1</td>
<td>0.591</td>
</tr>
<tr>
<td>1</td>
<td>12 noon</td>
<td>0.203</td>
<td>1</td>
<td>0.598</td>
</tr>
<tr>
<td>1</td>
<td>6 a.m.</td>
<td>0.190</td>
<td>1</td>
<td>0.608</td>
</tr>
<tr>
<td>1</td>
<td>12 noon</td>
<td>0.206</td>
<td>2</td>
<td>0.597</td>
</tr>
<tr>
<td>1</td>
<td>12 noon</td>
<td>0.211</td>
<td>3</td>
<td>0.597</td>
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<tr>
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<td>0.202</td>
<td>1</td>
<td>0.586</td>
</tr>
<tr>
<td>2</td>
<td>6 a.m.</td>
<td>0.196</td>
<td>1</td>
<td>0.586</td>
</tr>
<tr>
<td>2</td>
<td>12 midnight</td>
<td>0.214</td>
<td>1</td>
<td>0.586</td>
</tr>
<tr>
<td>2</td>
<td>6 a.m.</td>
<td>0.216</td>
<td>1</td>
<td>0.586</td>
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<tr>
<td>2</td>
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<td>2</td>
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<td>0.586</td>
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<tr>
<td>2</td>
<td>12 noon</td>
<td>0.205</td>
<td>1</td>
<td>0.586</td>
</tr>
<tr>
<td>2</td>
<td>3 p.m.</td>
<td>0.213</td>
<td>1</td>
<td>0.586</td>
</tr>
<tr>
<td>2</td>
<td>12 midnight</td>
<td>0.193</td>
<td>1</td>
<td>0.586</td>
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<tr>
<td>2</td>
<td>6 a.m.</td>
<td>0.198</td>
<td>1</td>
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<td>2</td>
<td>12 noon</td>
<td>0.206</td>
<td>2</td>
<td>0.586</td>
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<tr>
<td>2</td>
<td>12 noon</td>
<td>0.203</td>
<td>3</td>
<td>0.595</td>
</tr>
</tbody>
</table>

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Table 2
Labeling indices for determining effectiveness of double dThd block on synchronization of tumor cells
Tumor fragments were cultured in medium containing 2 mM dThd for 30-hr periods, returned to normal medium for 6-hr intervals, and then exposed for 30 hr more to 2 mM dThd. To measure the extent of cell cycle synchronizing to tumor, samples were pulse labeled with [3H]dThd (5 μCi/ml medium) at 2, 8, and 16 hr after release from the second dThd block.

<table>
<thead>
<tr>
<th>Tumor</th>
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</thead>
<tbody>
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</tr>
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<td>5</td>
<td>0.589</td>
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<tr>
<td>6</td>
<td>0.603</td>
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</table>

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Labeling the synchronized cells as they enter the S phase and then observing them at a later time autoradiographically as labeled mitosis occurs (Table 3) permit accurate measurement of the interval from the onset of S to mitosis. This period which includes $T_s$, $T_A$, and $\frac{1}{2} T_M$ was determined to be 12 hr. Since $T_s$ and $\frac{1}{2} T_M$ are 8.5 to 9.5 hr, $T_A$, is, by subtraction, 2.5 to 3.5 hr.

The duration of the cell cycle ($T_C$) was measured by labeling the synchronized population of cells with $[^3H]dTdd$ as they entered S and then, at hourly intervals after the wave of mitosis, pulse labeling the fragments with $[^{14}C]dTdd$ to determine the onset of the second phase of S for cells which had participated in the first S phase (Chart 2). The 28-hr interval between these points was considered to be the $T_C$.

$T_A$, was indirectly calculated to be 14.5 to 16.5 by subtracting $T_s$, $T_A$, and the approximation of $T_M$ from $T_C$. Measures of $S$ and overall cycle time of unsynchronized cells gave similar values for the random sample of cells which were pulse labeled.

### DISCUSSION

The ability to culture tumor fragments the in vivo structure and function of which are maintained (13, 20, 26) may prove helpful in the study of some characteristics likely to be affected by uncontrolled extraneous variables. One such variable is duration of the individual phases of the cell cycle. In most studies of biochemical function or structural features, it is not possible to simultaneously recognize the cycle phase. If the cells were pulse labeled with radioactive $dTdd$, one can ascertain when some of the cells were in the S phase of the cycle. However, the phase cannot be determined by many cells which were not labeled during the pulse. A way in which one can be certain of the phase of all cells is to synchronize their entry into a particular phase of the cell cycle.

Many attempts have been made to induce synchrony of various cells in culture and tissue in situ. Methods which use physical agents, such as heat, cold, mechanical trauma, or surgical excision, and chemical agents, such as isoproterenol, actinomycin D, puromycin, bromodeoxyuridine, 1-β-D-arabinofuranosylcytosine, and the stathmokinetic drugs, can also be used to induce synchrony (15). The problem associated with physical agents is that the resultant synchrony is not as complete as with chemical means. However, most of the chemicals used are toxic and have their own independent effects. $dTdd$ is an exception in that it is normally available to and used by the cells, but, when given in excess, reversibly arrests some mammalian cell types at the G1-S interface without any toxic effects (8, 15, 24). While $dTdd$ has been shown to be toxic in some cell systems (4) and to be more toxic for tumor cells than normal cells (25), in our experiments the histology and growth
fraction of tumor fragments treated with dThd were no different than those which had not been so treated.

Labeling of tumor fragments by incubation in medium containing [3H]dThd is known to result in variable amounts of incorporation which is in part attributable to limited penetration (12) and may also be affected by oxygen tension (6). It has been shown that scoring only the outermost 100-μm perimeter of the labeled fragment avoids counting as negative those cycling cells which have not had access to label or with incorporation that is depressed by low oxygen tension. By using high concentrations of [3H]dThd and restricting scoring to the peripheral zones of high labeling, we apparently excluded from our sample cells with supranuclear grain counts near background. The data also reveal that, while it has been shown biochemically that in some systems there is a low level of DNA synthesis throughout the cycle (22) and that even within S phase there are periods of varying amounts of DNA synthesis, such features may not be demonstrated by autoradiographic methods.

The labeling indices under these conditions were similar to those in the control in which the tissue was labeled in vivo, indicating that the level of radioactivity used in vitro did not produce necrosis or inhibit cycling. Differences were also not seen between the fragments subjected to the dThd block and those not synchronized by this technique. This is empirical evidence that, in this tumor cell system, the toxic and cytokinetic effects of high doses of dThd reported for other models under other conditions (4) do not occur.

There has been concern about whether cells treated with excess dThd are arrested at the G1-S interface or are just slowed substantially in their movement through the S phase (15). We conclude that this tumor population was arrested at the G1-S interface because there was a plateau between 2 and 8 hr and a sharp decline of the labeling index by 16 hr after release from synchronization. In addition, the study of T S further substantiates the good degree of synchrony attained by the double dThd arrest. If cells had only been slowed in their movement through S phase as a result of the dThd block, the cells would have continued to be distributed throughout S phase, and, after release, there would be a gradual decline of labeling indices with time rather than a sharp drop observed.

Tumors have been known to vary with regard to cell cycle kinetics from the normal tissue from which they arose (1, 3, 5, 23). The durations of cell cycle phases might themselves be characteristics useful in recognizing tumor cells. Heterogeneity with regard to the generative cycle could be an indication of instability of the tumor population while consistent deviations of subpopulations might be indicative of clonal origins of tumors. Previous studies in which variations have been detected involved the averaging technique of Quastler and Sherman (19) or modifications thereof. This method does not reveal whether the observed differences from normal tissue are limited to a specific cellular subpopulation or are true for the entire tumor. In tumors where variations in cycle time might exist, only gross changes can be observed because small groups of cells have minor effects on the population averages. Synchrony of tumor populations allows analysis of cell cycle kinetics while avoiding the averaging methods required with randomly distributed populations.

We measured the duration of the phases of the cell cycle looking for differences from normal in a specific phase for the entire tumor cell population or for deviant clones. T S of 8 to 9 hr, T D of 2.5 to 3.5 hr, T G1 of 14.5 to 16.5 hr, and T c of 28 hr were determined for the tumor population. The phase durations we have found for the tumor cells are the same as those of the proliferative cells in healing mild wounds of the tracheal epithelium (10). We have therefore concluded that cell cycle kinetics is not one of the characteristics which will distinguish these tumor cells from normal cells. On the other hand, this similarity between cancer cells and normal cells will facilitate study of cycle-specific features which may be useful markers of cancer, since the homogeneity of the tumor cell population increases the predictability that, once synchronized, all cycling cells are in the same phase and comparisons can be made between normal and tumor cells without corrections for differences in cycle time or phase durations in the 2 populations.

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