Proliferation Kinetics of Recruited Cells in a Mouse Mammary Carcinoma

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ABSTRACT

Solid tumors contain populations of proliferating (P) and quiescent (Q) cells. Shifting between these populations occurs continuously and cells are recruited from quiescence to proliferate (Q→P) as a result of exogenously applied or endogenous cell depleting stimuli. Direct measurements of the proliferation kinetics of these Q→P cells in solid tumors are difficult to make because of the much larger percentage of P-cells. In order to specifically analyze the kinetics of the Q→P cells, double thymidine analogue labeling was used. This was accomplished by first labeling in vivo all of the P-cells in MCaK tumors using continuous exposure to chlorodeoxyuridine (CldUrd) administered by a minibipump over 21 h. About 75% of the aneuploid cells are P-cells based on CldUrd labeling. At different times after the pumps were removed, the tumors were pulse-labeled with iododeoxyuridine (IdUrd) and harvested 6 h later. A 3-color flow cytometry assay was used to simultaneously and independently analyze CldUrd and IdUrd incorporation, as well as DNA content. The Q→P cells were identified as having only been labeled with IdUrd. The length of their S-phase was calculated from the movement of the Q→P cells during the 6 h after IdUrd labeling. The results showed the mean S-phase for the recruited cells to be slightly, but significantly, longer than the length of S-phase for the total cells (11 h versus 9 h, respectively). Thus, the recruited cells appear to have slightly slower kinetics than the proliferating cells in the absence of a perturbing stimulus such as radiotherapy or chemotherapy.

INTRODUCTION

Solid tumors undergo cellular repopulation after being partially depleted of cells by cytotoxic treatments such as radiotherapy or chemotherapy (1–7). There is evidence, in both experimental and human tumors, that tumor cell repopulation after a depleting stimulus may even be accelerated as compared to that expected from the cell proliferation rate in unperturbed tumors (8–11). Moreover, accelerated repopulation can occur during ongoing fractionated radiotherapy, and is probably an important mechanism for treatment failure in a significant number of patients (11). It is unclear whether accelerated repopulation is due to a shortening of the cell cycle time of the repopulating cells, a decrease in cell loss, or other mechanisms.

Tumors are composed of actively proliferating (P) and nonproliferating (Q) cells, the latter of which may retain the capability of reverting into a proliferative state or may lose reproductive ability permanently. After a depleting stimulus that is preferentially toxic to the proliferating cells, such as the resultant mitotic death after X-irradiation, the more resistant quiescent cells will constitute a much larger percentage of the tumor. Under some conditions, these cells may become the primary source of repopulating cells. The cells recruited from quiescence, therefore, may contribute substantially, and may even dominate, the repopulation process. Recruitment of quiescent cells has been documented to occur following radiotherapy, chemotherapy, or other cell depleting stimuli (1–7, 12, 13). Even in unperturbed exponential growth, recruitment may occur secondary to internal stimuli involving changes in nutrition and oxygenation at the cellular level. However, direct measurements of the proliferation kinetics of recruited cells (Q→P) are difficult to make because these cells constitute a small percentage of the viable tumor cell population. Thus, Q→P cells must be distinguished from the other subpopulations before accurate kinetic estimates can be made.

Maurer-Schultz et al. (12, 13) were able to detect and quantify the number of Q→P cells after double labeling with [14C]thymidine and [3H]thymidine using autoradiography. Recently we described a 3-color flow cytometric analysis technique that not only allows for the simultaneous and independent quantification of 2 different incorporated thymidine analogues (CldUrd and IdUrd), but also the DNA content of the cells (14). This simultaneous quantification of CldUrd, IdUrd, and DNA allows for direct measurements of the cell kinetics of the Q→P cells. We present data here using unperturbed solid tumors that show that by first continuously labeling the P-cells with one analogue, the kinetics of the Q→P cells may be assessed by analyzing the second analogue as a pulse-label.

MATERIALS AND METHODS

Tumor Model. The mouse mammary carcinoma MCaK is a spontaneously arising, serially passed tumor that was used in the 7th to 10th isotransplant generations. Early isotransplant generations were kept in liquid nitrogen. The tumors were grown s.c. in the flanks of syngeneic C3Hf/Kam specific pathogen-free mice as described previously (14–17). An inoculum of 5 × 103 cells was used and the labeling was performed when the tumors had a mean geometric diameter of 8–9 mm.

Tumor Cell Labeling. Continuous labeling with CldUrd, or BrdCyt, was accomplished by implanting Alzet minibipumps (pump 2001; Alza Corp., Palo Alto, CA), containing approximately 0.25 ml of 0.3 M of thymidine analogue, s.c. in the flank opposite the tumor. Both CldUrd and BrdCyt were dissolved in calcium- and magnesium-free phosphate-buffered saline. Pulse-labeling with IdUrd was accomplished by injecting host animals with 60 mg/kg body weight of 3H thymidine using autoradiography. Recently we described a simultaneous quantification of 2 different incorporated thymidine analogues (CldUrd and IdUrd), but also the DNA content of the cells (14). This simultaneous quantification of CldUrd, IdUrd, and DNA allows for direct measurements of the cell kinetics of the Q→P cells. We present data here using unperturbed solid tumors that show that by first continuously labeling the P-cells with one analogue, the kinetics of the Q→P cells may be assessed by analyzing the second analogue as a pulse-label.

Received 6/25/93; accepted 11/17/93.

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Funding was provided by the Radiological Society of North America Research and Development Fellowship (A. P.); the American Society of Therapeutic Radiology and Oncology Fellowship (A. P.); National Cancer Institute Grants CA16672, CA06294, and CA11430; and the Katherine Unsworth Annuity Trust

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1 The abbreviations used are: P-cell, proliferating cell; Q-cell, quiescent cell; Q→P, initially quiescent cells that were recruited to proliferate; P→Q, initially proliferating cells that became quiescent; Q→D, initially quiescent cells that died and disintegrated; P→D, initially proliferating cells that died and disintegrated; BrdUrd, bromodeoxyuridine; BrdCyt, bromodeoxyuridine; CldUrd, chlorodeoxyuridine; IdUrd, idodeoxyuridine; LI-P, P-cell labeling index; LI-Q, Q-cell labeling index; TC, cell cycle time; Tqc, potential doubling time; Td, length of S-phase; PBS, phosphate-buffered saline containing 0.5% Tween 20 (Sigma) and 0.5% bovine serum albumin (Miles Laboratories, Kankakee, IL); Tc, cell cycle time; Tq, potential doubling time; Tq, length of S-phase; PBPTB, phosphate-buffered saline containing 0.5% Tween 20 (Sigma) and 0.5% bovine serum albumin (Miles Laboratories, Kankakee, IL); Tc, cell cycle time; Tq, potential doubling time; TC, relative movement method; TC, relative movement method.

Isolation of Nuclei. As described previously (14–17), a small piece of tumor tissue (usually 0.05–0.1 g) was removed from the ethanol solution, finely minced, and suspended in 5 ml of 0.04% pepsin (EM Science, Cherry Hill, NJ) in 0.1 M HCl. The tissue was digested for 1 h at 37°C, filtered through 35-μm nylon mesh, spun down, and incubated in 1.5 ml of 2 M HCl for 20 min. Three ml of 0.1 M sodium borate buffer were then added, and the nuclei centrifuged and resuspended in 7 ml of PBPTB. The nuclei were counted, and 2.5 × 106 nuclei were aliquotted per tube for staining.

Staining of Nuclei. The nuclei were spun down and 0.2 ml of Br3 antibody (catalogue no. MD5300; Caltag, South San Francisco, CA) diluted 1:500 in Tween 20 (Sigma) and 0.5% bovine serum albumin (Miles Laboratories, Kankakee, IL); Tc, cell cycle time; Tq, potential doubling time; TC, relative movement method.
were incubated overnight at 4°C before they were analyzed.

Flow-Cytometric Analysis. The staining was quantified on an EPICS 751 flow cytometer (Coulter Electronics, Hialeah, FL) equipped with narrow-beam excitation optics (5 μm) and a quartz flow cell as described previously (14–17). A 5-W argon-ion laser set at 488 nm and 200 mW was used. A minimum number of 20,000 nuclei from each specimen was analyzed and the data collected in list mode.

Calculation of TN Using the Relative Movement Method. The quantities fD (fraction of labeled undivided cells), fM (fraction of labeled divided cells), and RM (relative movement of the labeled undivided cells) were obtained from the aneuploid DNA versus IdUrd histograms (18). The pulse-labeling-to-harvest times (t) were longer than the length of G2M, and shorter than TN and, therefore, the following equations were used as described elsewhere (15–17, 19):

\[ \nu = \ln[(1 + fD(t))(1 - fD(t))/2] \]  
\[ RM(t) = (F(t) - FG)/FGM - FG \]

where RM(t) is the calculated back extrapolate of RM at time t = 0; F(t) is the mean DNA fluorescence channel of the aneuploid-labeled undivided cells; FG is the mean DNA fluorescence channel of the aneuploid G1 nuclei; and FGM is the mean DNA fluorescence channel of the aneuploid G2M nuclei.

\[ TN = \nu/(2\times TN(RM) \times RM(t) - RM(0)) \]

In addition, for the total aneuploid cell population, the TN may also be obtained from the above parameters as follows:

\[ TN = \ln(2) \times TN(RM)/\nu \]

Calculation of TN Using the % S-Depletion Method. TN was also calculated using the percentage of aneuploid cells depleted from S-phase over the time interval (t) between the IdUrd pulse-labeling and tumor harvest (20). These measurements were made on the labeled total cells (IdUrd positive), the P-cells (IdUrd positive, IdUrd negative), and the Q→P and P→Q cells (IdUrd negative, IdUrd positive). It should be emphasized that the rate of exit of the labeled cells from S-phase is being measured and that depletion from S-phase represents the relative changes over time. Moreover, cell death and disintegration could occur directly from the quiescent (Q→D) or proliferating (P→D) compartments.

The first step in the measurement of the proliferation kinetics of the recruited cells is to continuously label all of the P-cells such that the Q-cells are identified. In order to accomplish this, the continuous labeling theoretically should last for at least one TN minus TS. However, due to the variability in TN, labeling times longer than TN-TS may be needed (12, 13). Another consideration is that when the continuous labeling is too long, then a significant percentage of the cells undergoing the transition from proliferating to quiescent (P→Q) could be IdUrd-labeled. Experiments were performed to delineate the optimal continuous-labeling time.

Fig. 1A shows the results of the continuous labeling of MCaK tumors with IdUrd for different periods of time. The estimated TN and TC from prior studies (15, 21) are approximately 10 and 28 h, respectively. The data show an initial steep slope in terms of the percentage of aneuploid nuclei incorporating IdUrd during the first 18 h. This correlates with the progressive labeling of the P-cells. After approximately 18 h, the slope becomes more shallow, indicating that labeling of the P-cells is essentially complete at approximately 18 h. This 18-h P-cell labeling time is the same as the estimated TC-TS time of 17–18 h. Based on these data and allowing for variability in TC, a continuous labeling time of 21 h was chosen.

In order to confirm the completeness of P-cell labeling after continuous exposure to IdUrd for 21 h, the curve in Fig. 1 was fitted using a nonlinear least squares algorithm (22) to fit a model to the data that allowed for variation in the transit times through each phase of the cell cycle. It was assumed that the phase transit times were described by a shifted exponential such that there is a minimal residence time in each phase followed by an exponentially decaying distribution leading to variability in the transit times. These data yielded estimates of TC consistent with 28 h ± 2 h (± SD) so that the coefficient of variation was derived from the following:

\[ LI = e^{b(t + 1)} \]

For the calculation of LI-Q, the distribution of IdUrd-labeled aneuploid Q-cells (Q→P cells) was used to determine the numbers of labeled-undivided and labeled-divided cells; the total number of aneuploid Q-cells (IdUrd negative) was used in the denominator to calculate fM and fD. For the calculation of LI-P, the total number of aneuploid P-cells (IdUrd positive) was used in the denominator to calculate fM and fD; the distribution of the IdUrd-labeled aneuploid P-cells was used to determine the numbers of labeled-undivided and labeled-divided cells. For the calculation of total cell labeling index, the total number of aneuploid cells was used in the denominator to calculate fM and fD; the distribution of all IdUrd positive aneuploid cells was used to determine the numbers of labeled-undivided and labeled-divided cells.

RESULTS

The term P-cells will be used in the context of this paper to mean cells that were proliferating at any time during the IdUrd continuous labeling. Q-cells are defined as cells that remained quiescent during the entire time of IdUrd continuous labeling and during the IdUrd pulse-labeling. Q→P cells represent those that were initially quiescent (IdUrd negative), became proliferating after termination of IdUrd continuous labeling, and were in S-phase at the time of IdUrd pulse-labeling (IdUrd positive). Thus, the designation Q→P represents cells recruited from quiescence to proliferate. P→Q cells represent those that were initially proliferating (IdUrd positive) and became quiescent (IdUrd negative). However, since a proliferating cell in G1, or G2M at the time of the IdUrd pulse-label would appear to be quiescent, the absolute numbers of Q→P and P→Q cells cannot be accurately measured, and the value of these measurements is in examining the relative changes over time. Moreover, cell death and disintegration could occur directly from the quiescent (Q→D) or proliferating (P→D) compartments.

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of the transit time distribution is at most 20%. Using the estimated kinetic parameters, the fraction of unlabeled P-cells at 21 h was estimated to be less than 1% of the total population of P-cells. The growth fraction from the data presented in Fig. 1 and prior studies (21) is 70–75%.

Since chloride has a smaller van der Waals radius than iodide or bromide, and is closer to that of fluoride, we were concerned that Cl4Urd might alter the cell kinetics by inhibiting thymidylate synthetase (23, 24). For this reason the kinetics of continuous labeling with Cl4Urd were compared to those of BrdCyt (Fig. 1B). BrdCyt is deaminated in vivo to BrdUrd and, therefore, it is the incorporation of the transit time distribution is at most 20%. Using the estimated kinetic parameters, the fraction of unlabeled P-cells at 21 h was estimated to be less than 1% of the total population of P-cells. The growth fraction from the data presented in Fig. 1 and prior studies (21) is 70–75%.

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The experiments to measure the kinetics of the recruited aneuploid tumor cells were structured as follows: (a) Cl4Urd administered as a continuous label over 21 h; (b) recruitment period of 6–36 h; (c) IdUrd pulse label; and (d) tumor harvest approximately 6 h later. By administering the labels in this manner, the P- and Q-cells are first identified by Cl4Urd-labeling (Fig. 2A), and the Q→P cells are identified by IdUrd-labeling in the absence of Cl4Urd labeling. The movement of the pulse-labeled Q→P cells over the 6 h prior to tumor harvest is used to estimate T_s (15, 17). Fig. 2 illustrates the movement of the Q→P cells, the IdUrd-labeled P-cells, and all of the IdUrd-labeled cells in the 2-parameter DNA/IdUrd histograms. Two examples of the fitting of the IdUrd-labeled Q→P cells for the calculation of T_s for the recruited cells using the %S-depletion method are shown in Fig. 3. Of note is that there is considerable overlap between S and G2M, and as a consequence the percentages in these compartments are subject to error. This overlap region was fitted automatically using computer modeling, without operator bias.

The 2 methods used to calculate T_s are compared in Table 1. The statistical comparisons shown were made using analysis of variance, and nearly identical results were obtained using Mann-Whitney non-parametric analysis (data not shown). The results show that similar estimates of T_s for the recruited T_s were obtained using the %S-depletion and relative movement methods. Both estimates were approximately 11 h. The length of the recruitment period, between the Cl4Urd continuous label and the IdUrd pulse label, did not affect the T_s values. The T_s of the total, and the proliferating T_s populations were found to be significantly shorter than the recruited T_s. The T_s of the total calculated using the %S-depletion and the RM methods were similar. The only differences between the T_s-Dep and T_s-RM estimates were seen in the proliferating cell population. The T_s-Dep-P estimates were slightly shorter than the corresponding T_s-RM-P estimates. The reason for this difference is unclear.

The data were also analyzed to examine the shifts in the P- and Q-cell populations during the recruitment period. At the end of the 21-h labeling period, approximately 76% of the cells were Cl4Urd-labeled (Fig. 1). Table 2 shows that 6–12 h and 30–36 h after the labeling period, 81 and 86% of the cells are Cl4Urd-labeled, respectively. These data indicate that the proportion of labeled P-cells increased as a result of further proliferation, although reutilization of label could also contribute.

The percentages of the IdUrd-labeled and unlabeled Q- and P-cells are also shown in Table 2. The percentage of the labeled P-cells (Cl4Urd positive and IdUrd positive) decreased over time, while that of the unlabeled P-cells increased. It should be emphasized that many of the unlabeled P-cells were in G1 or G2M at the time of the IdUrd pulse-label. Assuming unperturbed exponential growth, a relative increase over time of the percentages of P-cells that are not labeled with IdUrd indicates an increase in the proportion of P-cells exiting the proliferating compartment. These data suggest that the transitions from P→Q and/or P→D occurred to a greater degree than the transition from P→2P (production of 2 proliferating daughter cells). Conversely, the percentage of IdUrd-labeled Q-cells (Q→P) increased while the percentage of Q-cells not labeled with IdUrd decreased. The depletion of unlabeled Q-cells is caused by the Q→P transition, death and disintegration of Q cells (Q→D), and the inability using the assay described to quantify the Q→P transition (Q-cells by definition are Cl4Urd negative). In addition to continued recruitment, a shortening of the G1 or G2M transit times of the Q→P cells recruited earlier would also result in an increase in the percentage of labeled Q-cells.
Another parameter that also reflects these population shifts is the labeling index. Labeling index is corrected for cell division (Eqs. A and H) while the percentages of labeled cells described above are not. Thus, LI-Q is not affected by a change in cell cycle traverse time (assuming that relative phase durations remain unchanged), but is affected by continued recruitment. The results of the labeling indices for the P, Q, and total cells are shown in Table 3. The data show that as the recruitment period is increased, the LI-Q increases. These results reflect the increase in the percentage of labeled Q-cells described above. The LI-P decreased slightly, but significantly, over time. These results reflect the decrease in the percentage of labeled P-cells described above.

Table 3 also displays the estimates of labeling index and T\textsubscript{pop} for the total population. These values are similar to those reported previously using one or 2 pulse-labels (15, 16), indicating that the continuous label with CldUrd did not significantly alter the kinetics of the tumors.

DISCUSSION

The recruitment of noncycling, quiescent, cells during chemotherapy (1–4) or radiotherapy (5–7) has been documented primarily by indirect monitoring of changes in labeling index, or other parameters that correlate with proliferation (i.e., mitotic index, Ki-67 staining). In these settings, the kinetics of the much larger percentage of P-cells are not separated from the Q→P cells. Thus, an increase in labeling index could represent: (a) semisynchrony, as is seen after radiotherapy or chemotherapy (25, 26); (b) shortening of the P-cell G\textsubscript{1} or G\textsubscript{2} phase durations; (c) a lengthening of the P-cell S phase duration; (d) increased Q-cell loss; (e) decreased P-cell loss; (f) decreased P→Q transition; or (g) increased Q→P transition. The only accurate approach to defining the kinetics of recruitment is to distinguish the initially quiescent cell population.

Maurer-Schultze et al. (12) used \[^{14}\text{C}\]thymidine and \[^{3}\text{H}\]thymidine double-labeling and found an increase in the number of Q→P cells when a majority of the total population was depleted. The stimulus of...
Table 1 Calculation of $T_S$ for the recruited, proliferating, and total aneuploid tumor populations using the %S-depletion and RM methods

<table>
<thead>
<tr>
<th>Group</th>
<th>$N$</th>
<th>$T_{S}$-Dep-R</th>
<th>$T_{S}$-RM-R</th>
<th>$T_{S}$-Dep-P</th>
<th>$T_{S}$-RM-P</th>
<th>$T_{S}$-Dep-T</th>
<th>$T_{S}$-RM-T</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>17</td>
<td>11.4 ± 0.7c</td>
<td>10.9 ± 0.3</td>
<td>7.9 ± 0.3d</td>
<td>10.0 ± 0.3e</td>
<td>8.9 ± 0.3d</td>
<td>9.5 ± 0.2d</td>
</tr>
<tr>
<td>6–12 h</td>
<td>8</td>
<td>11.2 ± 0.9</td>
<td>11.2 ± 0.5</td>
<td>7.8 ± 0.3d</td>
<td>10.0 ± 0.5e</td>
<td>9.0 ± 0.3d</td>
<td>9.5 ± 0.3d</td>
</tr>
<tr>
<td>30–36 h</td>
<td>9</td>
<td>11.5 ± 1.1</td>
<td>10.7 ± 0.3</td>
<td>8.0 ± 0.4d</td>
<td>10.2 ± 0.2c</td>
<td>8.8 ± 0.5d</td>
<td>9.6 ± 0.2d</td>
</tr>
</tbody>
</table>

* Tumors were grouped by the time after continuous labeling with CldUrd that the IdUrd pulse-label was administered. The tumors were harvested approximately 6 h after the pulse-label was given.

b R, recruited; T, total.

c $P < 0.05$ compared to the matched $T_{S}$-Dep or -RM for the recruited cells using analysis of variance, least significant difference.

d $P < 0.05$ compared to matched deletion group to the left using analysis of variance, least significant difference.

e $P < 0.05$ compared to the matched $T_{S}$-Dep-R or -RM-R for the recruited cells using analysis of variance, least significant difference.

Previously we reported that similar cell kinetic estimates are obtained when CldUrd and IdUrd are used singly or in combination, and that the results are not affected by the order of the labeling (16). Bakker et al. (28) and Aten et al. (29) have also shown that CldUrd and IdUrd may be used together without evidence of altering the cell kinetics. Whereas these studies confirmed that pulse labeling with both CldUrd and IdUrd does not alter the cell kinetics, continuous exposure to a thymidine analogue could lead to added effects. There is evidence that CldUrd increases chromosome exchange (30, 31) and strand breaks (32), as compared to BrdUrd. Although we did not observe any difference in the kinetics of continuous labeling between CldUrd and BrdUrd, continuous labeling with any of the halogenated pyrimidine analogues could potentially alter the cell cycle kinetics of these cells.

Fig. 3. Two examples of the distribution of Q→P nuclei. These are CldUrd-unlabeled, IdUrd-labeled aneuploid nuclei, and the relative movement of the labeled cells is illustrated. The histograms were fitted to determine the percentages of aneuploid G1, S, and G2M phase nuclei for the calculation of %S-depletion. The diploid G2M phase nuclei were also fitted and subtracted. In addition, the $f_{n}$, $f_{m}$, and $RMs$ were determined from the histograms for the calculation of $v$ by Eq. A, and $T_{S}$-RM by Eq. F, as described previously (15–17).
any of the populations studied. The similarity of the \( T_{\text{rec}} \) values reported (Table 3) after continuous CldUrd labeling, as compared to previously published values (15, 16), indicates that the continuous labeling did not perturb cell cycle transit. Moreover, Bakker et al. (26) did not observe any perturbation of the distribution of cells in the cell cycle phases after continuous labeling of \( R_1 \) cells with CldUrd in vitro.

Using double thymidine analogue labeling, the \( Q \rightarrow P \) cells were isolated analytically. We chose to initially investigate the kinetics of the \( Q \rightarrow P \) cells in an unperturbed solid tumor model to determine the feasibility and consistency of these measurements. One assumption underlying these experiments is that the transitions into and out of quiescence are occurring constantly in the absence of an external perturbing stimulus. There is evidence for this in the studies using \( [\text{H}] \)thymidine and \( [\text{H}] \)thymidine (12, 13), as well as the data presented herein. Approximately 4% of the total aneuploid population in MCAK tumors in exponential growth were identified as labeled \( Q \rightarrow P \) cells during the recruitment period (Table 2), in the absence of an external stimulus. In addition, approximately 10% of the total aneuploid population were documented to have undergone the \( Q \rightarrow P \) transition during this time. Obviously, internal stimuli such as hypoxia and nutritional deprivation play an active role in the movement between these cell kinetic states.

The finding that the total number of recruited cells is 4% is an underestimate since a pulse-label was used and only recruited cells in S-phase were labeled. However, the fact that only 4% of the cells were identified as \( Q \rightarrow P \) cells raises the question of whether slowly proliferating \( P \) cells were included in these measurements. Such slowly proliferating \( P \) cells with \( T_{\text{rec}}-T_{\text{rec}} \) times of \( >21 \) h would not be labeled with the CldUrd pulse-label. The tumors were harvested approximately 6 h after the pulse-label was given.

<table>
<thead>
<tr>
<th>Group*</th>
<th>( n )</th>
<th>( % ) labeled**</th>
<th>( % ) unlabeled**</th>
<th>( % ) total**</th>
</tr>
</thead>
<tbody>
<tr>
<td>All ( 6-12 ) h</td>
<td>8</td>
<td>92.0 ± 1.3</td>
<td>92.0 ± 1.3</td>
<td>92.0 ± 1.3</td>
</tr>
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</table>

A T<sub>50</sub> value of 20 h was attained, the majority of cells being recruited \( Q \rightarrow P \); then a fall in these parameters over time would be expected. The results consistently show the opposite; the proportion of CldUrd-unlabeled and IdUrd-labeled cells increased over the recruitment period. Thus, over 75% of the cells designated \( Q \rightarrow P \) are recruited cells.

Two methods were used to confirm the accuracy of the \( T_{\text{rec}} \) measurements, and both provided similar estimates. Significant differences between \( T_{\text{rec}} \) and \( T_{\text{rec}} \) were found only for the subtraction of \( P \)-cells and the mechanism for this is unclear. The relationships between the estimates of \( T_{\text{rec}} \) for the \( P \), \( Q \), and total cells were similar for the 2 methods used. The relative movement method described by Begg et al. (18) is only applicable to unperturbed populations; a \( G_2 \) block would distort the estimate of \( T_{\text{rec}} \). Although the %S-depletion method inherently has more variability due to the dependence on computer modeling and the overlap between S-phase and G2-M phase, it is also applicable in the presence of a \( G_2 \) block. Therefore, the %S-depletion method will be more desirable in the presence of an external perturbing stimulus such as X-irradiation.

The \( T_{\text{rec}} \) values of \( Q \rightarrow P \) cells were unaltered over the 36-h recruitment period examined. However, alterations in the proportions of the CldUrd-labeled and unlabeled \( P \)- and \( Q \)-cell populations were seen. A reduction in the percentage of labeled \( P \)-cells over the recruitment time was offset by an increase in the percentage of unlabeled \( P \)-cells. These results were reflected as a drop in the \( P \)-cell labeling index over time, suggesting a reduction in the proliferative fraction of the \( P \)-cell population. Using the data from Table 3, approximately 15% of \( P \)-cells exit the proliferating compartment over 24 h. Therefore, an estimated 11% of the total aneuploid population exits the proliferating compartment over 24 h. One possible mechanism is the \( P \rightarrow Q \) transition, whereby the numerator of the LI-P is reduced. This mechanism appears to be predominant since the total percentage of \( P \)-cells increased while the percentage of labeled \( P \)-cells decreased over the recruitment period. However, cell death and disintegration (\( P \rightarrow D \))

Table 3: Labeling indices for the \( Q \), \( P \), and total aneuploid populations, and \( T_{\text{rec}} \) for the total aneuploid population at different times after continuous labeling

<table>
<thead>
<tr>
<th>Group*</th>
<th>( n )</th>
<th>( \text{LI}^P )</th>
<th>( \text{LI}^P )</th>
<th>( \text{LI}^T )</th>
<th>( T_{\text{rec}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>All ( 6-12 ) h</td>
<td>8</td>
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</tr>
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</table>

* Tumors were grouped by the delay between the continuous label with CldUrd and the IdUrd pulse-label. The tumors were harvested approximately 6 h after the pulse-label was given.

** IdUrd labeling index of the aneuploid \( Q \)-cells using the number of CldUrd negative nuclei as the denominator in calculating \( f_{\text{Id}}^P \) and \( f_{\text{Id}}^T \).

** IdUrd labeling index of the aneuploid \( P \)-cells using the number of CldUrd positive nuclei as the denominator in calculating \( f_{\text{Id}}^P \) and \( f_{\text{Id}}^T \).

** IdUrd labeling index of all aneuploid cells.

\( T_{\text{rec}} \) of the total aneuploid population.

\( \text{Mean} \pm \text{SE} \).

\( P < 0.05 \) compared to 6–12 h group, Student’s \( t \)-test and Mann-Whitney test.
would also reduce LI-P by reducing the numerator proportionally more than the denominator.

A significant cell loss factor might contribute to these findings. Indeed, tumor volume curves (33) indicate a tumor doubling time of 50 h, and labeling studies a $T_{doubling}$ of 30 h (Table 3) (16, 17), giving a calculated cell loss factor of approximately 60% for the MCaK line. Proliferating cell loss could occur via a number of mechanisms including P→Q→D, P→D (mitotic death, apoptosis), or exfoliation.

Another finding was that LI-Q and %Q-labeled increased over the recruitment period while the %Q-unlabeled decreased. These data are partly explained by the fact that P→Q cells are not included with the Q-cell population because of prior labeling with CluUrd. Thus, as the Q-compartment is depleted by the P→Q and/or P→D transitions, there is no measurable replenishment of the unlabeled Q-cell population because of limitations in the way the measurements are made. This results in increases in LI-Q and %Q-labeled. Using the data in Table 3, 4% of Q-cells, corresponding to 1.1% of the total aneuploid cell population, move from quiescence to become labeled over 24 h. Since only 30% of the proliferating cells are labeled (LI-P), we calculate that 3.5% of the total aneuploid population actually moves from the quiescent compartment to the proliferating compartment over 24 h.

The experiments described show that the kinetics of the Q→P cells can be measured directly and with consistency. The ultimate objective is to determine the proliferation kinetics of the Q→P cells after X-irradiation. Preliminary results indicate that such measurements can be made, at least up to 48–72 h after irradiation with 3.5–7 Gy. We are currently investigating whether accelerated repopulation is caused by an alteration in the kinetics of the Q→P cells or some other mechanism.

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Proliferation Kinetics of Recruited Cells in a Mouse Mammary Carcinoma

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