Proc. Natl. Acad. Sci. USA
Vol. 74, No. 5, pp. 2041–2044, May 1977
Cell Biology

In vivo analysis of cellular replication

(rat bone marrow/BrdUrd-differential staining/ metaphase chromosomes)

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Communicated by Roscoe O. Brady, March 2, 1977

ABSTRACT **The number of previous cell replications that a metaphase cell has undergone in the presence of BrdUrd can be determined by the differential fluorescent patterns of metaphase chromosomes stained with Hoechst dye 33258. To examine if this technique could be applied to analyzing cell cycle kinetics *in vivo*, we infused Wistar rats with BrdUrd for 7.5–33 hr at concentrations of the nucleotide analog that did not inhibit cellular replication. Examination of the frequency of one, two, and three or more replication cycle cells as a function of BrdUrd infusion time indicates that cell replication times for rat bone marrow cells are relatively homogeneous. Analysis of this data with a computer simulation model produced a mean cell cycle duration of 9.2 hr, which is compatible with the fastest times obtained with radioisotope studies. These results support the potential of nonradioisotope analysis of cell replication *in vivo*.**

Previous attempts at examination of cellular replication *in vivo* have involved radioactive nucleotide labeling of cells coupled with autoradiographic analysis of tissues after labeling (1–4). However, this approach is limited by the reutilization of radioisotope nucleotides, potential inhibition of cell replication by incorporated radioisotope, and by the restricted resolution and long processing times for autoradiography. The recent advent of the bromodeoxyuridine–differential staining techniques permits the identification of replicating cells without the necessity of autoradiography (5–7). It has the additional advantage of permitting rapid and unequivocal identification of cells which have undergone one, two, and three or more replication cycles in the presence of this nucleotide analog (see Fig. 1). Metaphase cells that have replicated once in the presence of BrdUrd have chromatids with equal replacement of thymidine by its analog and, therefore, fluoresce with equal intensity when stained with Hoechst 33258 (Fig. 1*A*). After two replication cycles in the presence of BrdUrd, the chromatid that is bifilarly substituted with BrdUrd will fluoresce less intensely (because of BrdUrd quenching of the fluorescent excitation and emission of the DNA/Hoechst 33258 complex) than its unifilarly substituted sister chromatid (Fig. 1*B*). Finally, metaphase cells that have replicated three or more times (3+) in the presence of BrdUrd will contain chromosomes which have both chromatids with DNA bifilarly substituted with BrdUrd and, therefore, will show equally dull fluorescence (Fig. 1*C*).

Based on this ability to identify the replicative history of metaphase cells, we have developed an analytical system, designated as BISACK (bromodeoxyuridine incorporation system for the analysis of cellular kinetics), for examining the proliferative kinetics of cellular populations (8). The validity of BISACK requires the demonstration that cell replication kinetics can be examined under noninhibitory concentrations of BrdUrd. This has been achieved *in vitro* where BISACK has been utilized to analyze the cellular kinetics of phytohemagglutinin-stimulated human peripheral lymphocytes (8).

BrdUrd labeling of metaphase chromosomes *in vivo* was first accomplished in chick embryos by injection *in ovo* (9), and has subsequently been achieved in plants (10) and fish (11). Differential staining of mammalian metaphase chromosomes has been obtained in mice by multiple intraperitoneal injections (12, 13) or multiple subcutaneous infusions (14). In contrast to these latter techniques for BrdUrd administration *in vivo*, we have developed containment devices for both mice and rats which permit the constant intravenous infusion of controlled doses of BrdUrd over extended time periods (15). It was therefore decided to examine if intravenous infusion of low doses of BrdUrd would permit analysis of cell proliferation kinetics *in vivo* at noninhibitory concentrations of BrdUrd.

MATERIALS AND METHODS

Animals. Twelve-month-old random-bred Wistar female virgin rats were obtained from the colony of the Gerontology Research Center, Baltimore, MD. These rats weighed from 300 to 375 g and were fed ad lib. a National Institutes of Health open-formula 07 diet.

BrdUrd Inhibition of Cellular Replication. BrdUrd in phosphate-buffered saline at concentrations ranging from 1.0 to 123.0 mg/kg per hr was administered by tail vein infusion for 24 consecutive hours commencing at 1:30 p.m. During this infusion the rats were restrained in specially designed, modified Bollman cages (15). Two hours prior to sacrifice, 2 ml of Colcemid (10 μg/ml, Gibco) was administered intravenously. Bone marrow cells were removed from the rat femurs, washed with phosphate-buffered saline, and centrifuged at 200 $\times$ *g* for 10 min. The pelleted cells were suspended in 0.06 M KCl for 30 min and then recentrifuged for 10 min at 200 $\times$ *g*. The cells were then fixed in methanol/acetic acid (3/1), placed onto slides, and stained with Hoechst 33258 as previously described (15). One hundred consecutive metaphase cells were analyzed and classified as having undergone either one, two, or three or more replication cycles based on the differential fluorescent patterns of their chromosomes.

Cell Replication Analysis by BISACK. Rats were infused as described above with BrdUrd at concentrations of 7.0–8.0 mg/kg per hr for time periods ranging from 7.5 to 33 hr. Colcemid was added 1 hr prior to sacrifice of these animals. Bone marrow metaphase cells were prepared and 100 consecutive metaphase cells were analyzed for each time point as described above. Although cells which have not traversed the S cell cycle phase (zero-replication cycle cells) cannot be distinguished from first-replication cycle cells, the experimental conditions of *in vivo* cell replication analysis (7.5 hr, initial time point) make their appearance unlikely. For purposes of BISACK, cells in the S cell cycle phase at the onset of BrdUrd infusion, which display discontinuous chromatid labeling, are considered to have replicated.

Abbreviation: BISACK, bromodeoxyuridine incorporation system for the analysis of cellular kinetics.

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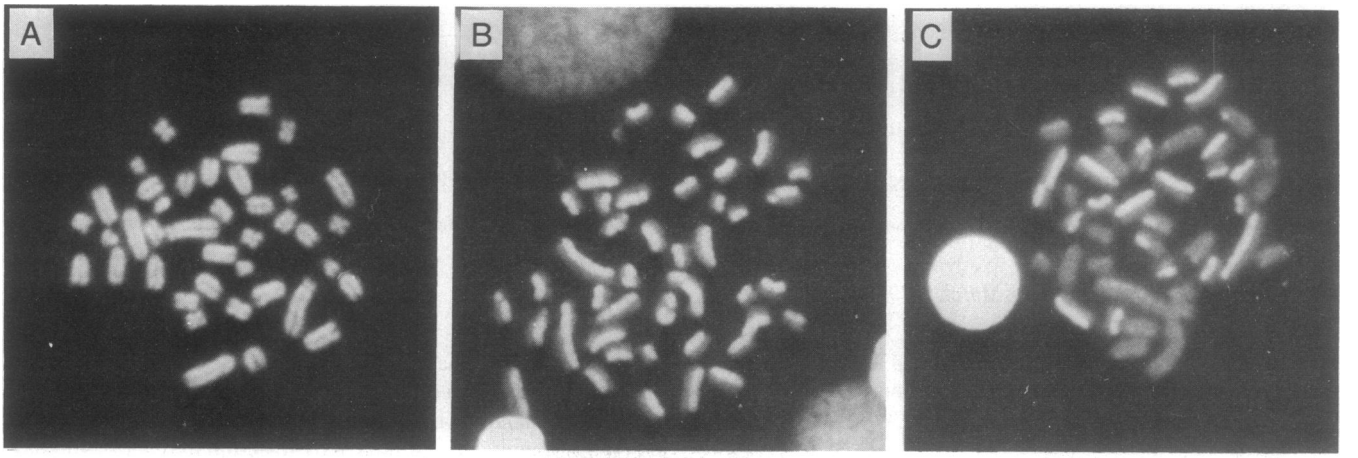

FIG. 1. Photomicrographs of rat bone marrow metaphase cells stained with Hoechst 33258 dye which have undergone one (*A*), two (*B*), and three (*C*) replication cycles in the presence of BrdUrd.

RESULTS

Examination of Inhibition of Cellular Kinetics by BrdUrd. Because BrdUrd can inhibit cellular replication (8), it was imperative to determine the concentration range of BrdUrd that could be infused without inhibiting cell cycle kinetics. We therefore examined the frequency of metaphase cells that had replicated one, two, or three or more times during a 24-hr infusion of BrdUrd as a function of various concentrations of the nucleotide analog. BrdUrd concentrations below 1.9 mg/kg per hr did not permit accurate classification of metaphase cells by replication cycle. At BrdUrd concentrations between 1.9 and 10 mg/kg per hr, the relative proportions of cells in the different replication cycles remain relatively stable (Fig. 2). However, above 10 mg/kg per hr, inhibition of cellular replication was observed as indicated by the decline in the percent of 3+ replication cycle cells and a corresponding increase in one or two replication cycle cells. Similar results were also obtained after a 30-hr BrdUrd infusion; the relative proportions of the first, second, and third or more replication cycle cells remained constant between 4.0 and 10.0 mg/kg per hr. To insure measurement of cellular replication under noninhibitory conditions, we conducted all further experiments at BrdUrd concentrations ranging from 7.0 to 8.0 mg/kg per hr.

Analysis of Cellular Kinetics. The frequencies of metaphase cells which have undergone one, two, and three or more replication cycles as a function of BrdUrd infusion time are displayed in Fig. 3. Each time point represents data from a single animal. Second generation cycle cells were first seen at 9 hr after the commencement of the BrdUrd infusion, while third generation cycle cells appeared at 18 hr.

To aid analysis of cell cycle kinetics, a computer simulation model was developed which permits the determination of cell cycle times (P. Thorne, personal communication). This model assumes that cells, once they begin to cycle, continue cycling

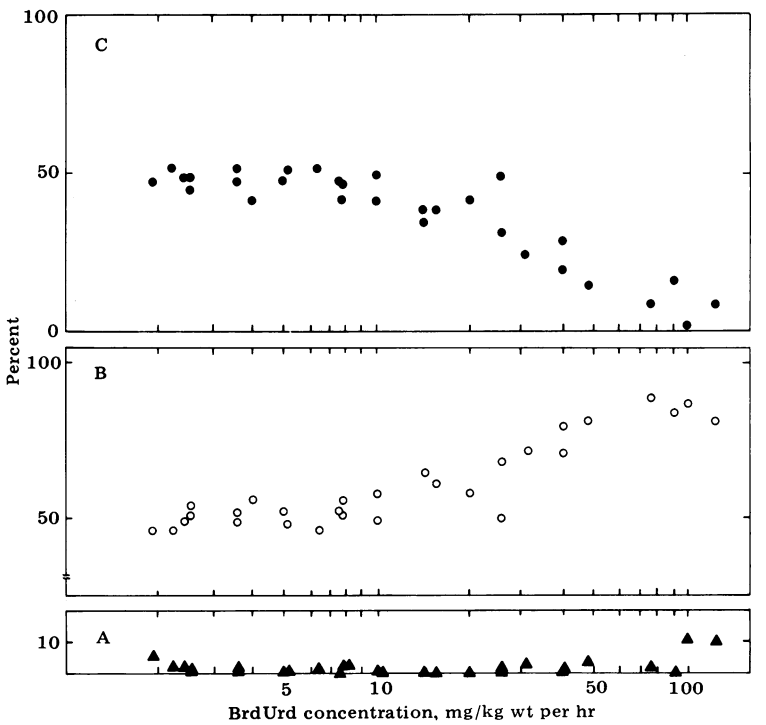

FIG. 2. Percentage of metaphase cells which have replicated once (*A*), twice (*B*), or three or more times (*C*) as a function of infused BrdUrd concentration. Each point represents data from a single animal.

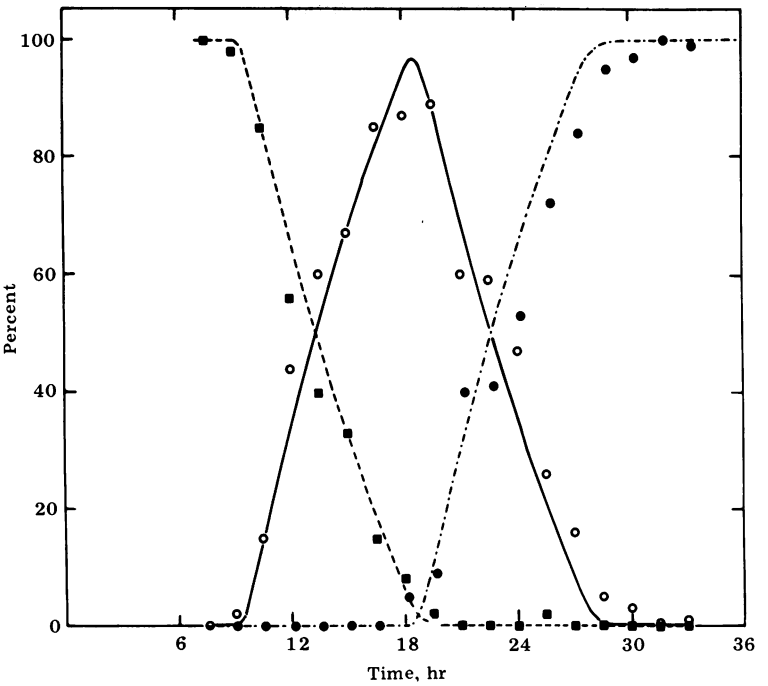

FIG. 3. Percentage of metaphase cells that have replicated once (■), twice (○), and three or more (●) times in the presence of BrdUrd. Each time point represents data from a single animal. Note the appearance of second replication cycle cells at 9 hr and third replication cycle cells at 18 hr. The curves representing first (- - -), second (—), and third or more (· - · -) replication cycle cells were derived from the computer simulation model (P. Thorne, personal communication).

and that cell cycle durations follow a gamma distribution (16). By utilizing data from Fig. 3, this mathematical model produced a mean cell cycle duration of 9.2 hr. This estimated rat bone marrow cell cycle time is compatible with the most rapid times determined by autoradiography (17).

Reproducibility of BISACK at Single Time Point. To examine the reproducibility of determinations of the frequencies of metaphase cells in the different replication cycles, we infused eight animals with BrdUrd at 8.0 mg/kg per hr for 24 hr. The results seen in Table 1 indicate that, although some variation exists between animals, the determinations are relatively consistent. The most extreme difference was 18% between animals 3 and 6.

DISCUSSION

Despite the known heterogeneous nature of bone marrow cells, two aspects of these cell cycle measurements suggest relatively homogeneous cell replication times: (*i*) the high frequency of second replication cycle cells (88%) at 18 hr and (*ii*) the relative absence of first replication cycle cells after 18 hr and second replication cycle cells after 30 hr of BrdUrd infusion. Although this does not exclude the presence of slowly replicating cell populations, these cells would have to be present at very low cell concentrations and/or have extremely long cell replication times.

Analysis of cellular proliferation kinetics *in vivo* with BISACK has several advantages over previous autoradiographic techniques: (*i*) cell replication can be easily followed over several generations, (*ii*) label dilution and reutilization does not present a problem, (*iii*) identification of first, second, and third or more replication cycle cells is rapid and unequivocal, and (*iv*) cell replication can be analyzed under conditions which can be shown to not inhibit cell proliferation. For *in vivo* BISACK analysis, constant intravenous infusion of BrdUrd has several advantages over multiple intraperitoneal or subcutaneous injections of the nucleotide analog: (*i*) the experimental animal is traumatized only once (at the onset of infusion), (*ii*) fluctuations of BrdUrd concentration at the tissue level are kept to a minimum, and (*iii*) reproducible serum concentrations of BrdUrd can be obtained.

While BISACK involves the determination of cellular kinetics based on the sampling of individual metaphase cells, some information can be obtained with this technique regarding the relative proportion of cells which have replicated (18). Further insight into cell cycle analysis can be achieved by close examination of differential staining patterns. Cells in the S phase of the cell cycle at the onset of the BrdUrd infusion will exhibit discontinuous staining patterns (which appear as a speckling of the bright fluorescent chromatids). The relative proportion of the chromosomal length and the particular chromosomal locations (late replicating regions) involved in speckling can be utilized to distinguish cells that were in early S phase from those in late S phase at the onset of BrdUrd infusion.

Further research will be focused on examining the replication kinetics of defined cell populations. However, not all tissues and cell types may be amenable to cell cycle analysis by BISACK since BrdUrd (even at low concentrations) may inhibit cell

Table 1. Kinetics of metaphase cells of rat bone marrow after a 24-hr BrdUrd infusion

	% of cells in different replication cycles		
Animal	1	2	3+
1	0	56	44
2	1	55	44
3	0	65	35
4	1	59	40
5	0	58	42
6	0	47	53
7	1	51	48
8	1	60	39

replication in some rapidly differentiating tissues (R. R. Tice, unpublished results).

BISACK is most readily applied to easily dissociable tissues such as bone marrow, spleen, and testes. Since the nuclei of cells that have replicated in the presence of BrdUrd will have diminished fluorescent excitation and emission when stained with Hoechst 33258 [in proportion to the number of DNA replication cycles (19)], this technique may be extendible to other rapidly multiplying cell populations which are not easily dissociable (such as intestinal epithelia and epidermis).

While mathematical models for analysis of cell cycle times need to be more completely developed, the results described above clearly reveal the potential of this nonradioactive approach to examining cell replication *in vivo*.

The same containment apparatus and intravenous infusion technology employed for the analysis of cellular proliferation kinetics can also be readily utilized to examine sister chromatid exchanges (8). The ability to continuously infuse low concentrations of BrdUrd has permitted us to examine spontaneous sister chromatid exchanges *in vivo* (20). This methodology can also be used to analyze the frequency of sister chromatid exchanges induced by mutagenic and/or carcinogenic compounds that require host mediated activation (21). In this case the intravenous route has the advantage of allowing concomitant administration of the agent to be tested with the onset of BrdUrd infusion, permitting highly reproducible results (D. Kram, R. Tice, and E. L. Schneider, in preparation).

We thank P. Thorne, G. Senula, B. Gilman, and P. Gianas for expert technical assistance and Dr. H. Loewe, Farwerke Hoechst, Frankfurt, for donating Hoechst 33258. The suggestions of Drs. A. Carrano and S. Latt are gratefully acknowledged. H.S. is presently a graduate student in the Department of Chemistry, University of Maryland, Baltimore County, Catonsville, MD. R.T. was supported by National Cancer Institute Award 5T32CA09021.

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