Environmental Mutagenesis 5:695–703 (1983)

The Mutagenic Activity of 5-Bromo-2′-deoxyuridine (BrdU) In Vivo in Rats

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BrdU tablets were implanted subcutaneously in rats, and BrdU concentrations were determined in the serum. Within 5 hr peak concentrations of 10 μg BrdU/ml blood were reached. The influence of BrdU in vivo on cell cycling, DNA synthesis, spontaneous sister chromatid exchange (SCE) frequencies, and gene-mutation frequencies (6-TG^r) was determined in freshly isolated cells from a subcutaneous granulation tissue. The most significant effect of BrdU in vivo was a doubling of the spontaneous 6-TG^r frequency.

In reconstruction experiments in vitro the mutagenic activity of BrdU applied in concentrations found in vivo was 2.5–6-fold higher.

With the use of agar-coated tablets, BrdU concentrations in the blood were reduced by half, and no peak concentration was found. The differential staining of chromatids was still sufficient. Since the mutagenic effect of BrdU in vitro was found to be strongly concentration dependent, the use of agar-coated tablets is recommended in experiments in which the compound is used to demonstrate SCE in vivo.

Key words: SCE in vivo/in vitro, granuloma pouch assay, BrdU mutagenicity, BrdU level in vivo, agar-coated tablets

INTRODUCTION

5-Bromo-2′-deoxyuridine (BrdU) incorporation into DNA is a widely used procedure for the demonstration of sister chromatid exchanges (SCE) in metaphases of replicating cell populations in vivo and in vitro. In vitro, BrdU is cytotoxic and genotoxic [San Sebastian et al, 1980]. The nucleoside analogue of thymidine causes gene mutations in vitro either by base pair substitutions or, more likely, by indirect pathways such as perturbation of the intracellular nucleotide pool [Kaufman and Davidson, 1978]. In vivo BrdU exerts cytostatic [Wilmer and Soares, 1980], clasto-

Received February 22, 1983; revised and accepted June 17, 1983.

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genic, and SCE-inducing [Tice et al, 1976; Kram et al, 1979; Wilmer and Soares, 1980] activities. No information is available about its ability to induce gene mutations in vivo.

With the granuloma pouch assay (GPA), gene mutations [Maier et al, 1978, 1980] and SCE [Maier et al, 1982] can be determined simultaneously in an extrahepatic tissue from adult rats. In this study, the animal model was used to investigate the effect of BrdU on cell cycling, SCE frequencies, and gene mutations in concentrations obtained with the BrdU tablet implantation method. BrdU levels were determined and compared in the blood of rats during a 24-hr period after implantation of agar-coated [King et al, 1982] and uncoated tablets [Allen et al, 1978].

MATERIALS AND METHODS

Animals

Random bred albino male Sprague-Dawley rats (SIV 50, Ivanovas, Kisslegg, FRG) weighing 230–260 g (54 ± 4 days old) kept under standard conditions were used (12 hr light-dark cycle; diet, Nr. 890 from NAFAG, Gossau, Switzerland; water, ad libitum).

Growth of granulation tissue was initiated on the inside of a subcutaneous air pocket by the injection of 25 ml germ-free air into the loose connective tissue between the shoulder blades of the animals [Maier, 1983].

Chemicals and Tissue Culture Material

The sources were as follows: 5-bromo-2′-deoxyuridine (BrdU), colchicine, E. Merck (Darmstadt, FRG); Fluo-Chrom-Hoe 33258, Riedel de Haen AG (Seelze-Hannover, FRG); Dulbecco's modified Eagle medium (DMEM), fetal calf serum (FCS), trypsin solution (0.25%), GIBCO (Basel, Switzerland); gentamycin solution, Fakola (Basel, Switzerland); Collagenase I (145 u/mg), Worthington Biochemical Corp (Freehold, NJ); Dispase II, 2.4 U/ml, Boehringer Mannheim AG (Cham, Switzerland); 100-mm tissue culture dishes, Corning (New York, NY); multiplate-4 wells, Lux (Newbury Park, CA); [methyl-^{3}H] thymidine (46 Ci/mmol), Radiochemical Centre Amersham (England, UK).

BrdU Application In Vivo

BrdU tablets were implanted subcutaneously (sc) in the lumbar region of ether anesthetized rats 48–64 hr after formation of the air pouch. The 1-cm skin incision was closed with 3 surgical clips. Agar-coated (5% agar, 2 mm coat, dried aseptically) and uncoated BrdU tablets, weighing 250 mg with a diameter of 7 mm and a thickness of 3 mm, were used. With the uncoated tablet, macroscopically visible fragments were found 24 hr after implantation at implantation sites. This indicates that the tablet size chosen contains more BrdU than the tissue can absorb maximally within 24 hr.

Competition With Thymidine Incorporation In Vivo

The intracellular BrdU concentration can be estimated by its influence on ^{3}H-thymidine incorporation into DNA of granuloma cells. In intervals of 2 hr beginning either 22 or 16 hr prior to dissection of the tissue, 3 × 10 μCi in 1 ml 0.9% NaCl was injected into the pouch. Dissociated cells were cultured in vitro on glass coverslips for 4 hr, fixed, and then exposed to Kodak AR 10 stripping films for 3 wk. After

development, fixation, and staining, the morphology of cells, the number of labeled cells, and their degree of labeling were evaluated. The stripping technique resulted in an extremely low background of grains.

BrdU Concentrations in Blood

BrdU was determined in samples of 100-μl blood, withdrawn from the tail vein. The tablet was implanted in the morning and samples taken according to the schedule indicated in Figure 1. The blood was immediately added to an equal volume of 3.65% Na-Citrat in 0.9% NaCl. Cells and plasma proteins were precipitated with 30 μl trichloroacetic acid (TCA) and centrifuged. Twenty microliters of the supernatant were subjected to analytical reversed-phase high pressure liquid chromatography (HPLC) in a 30-cm μ Bondapak C_{18} column 300 × 14 mm. Elution occurred with ammonium phosphate buffer (pH 4.0, 1% methanol) and a flow rate of 1.6 ml/min at room temperature. The absorbance was determined at 280 nm.

The BrdU concentration was calculated from the area under the peaks. Calibration was achieved with blood samples to which known amounts of BrdU were added and treated as described above.

Cell Recovery

Animals were killed by cervical dislocation 24 hr after tablet implantation. The granulation tissue was dissected aseptically and dissociated into single cells by incubation twice for 45 min at 37°C in 10 ml Dulbecco's PBS containing collagenase (600 u) and Dispase (8U).

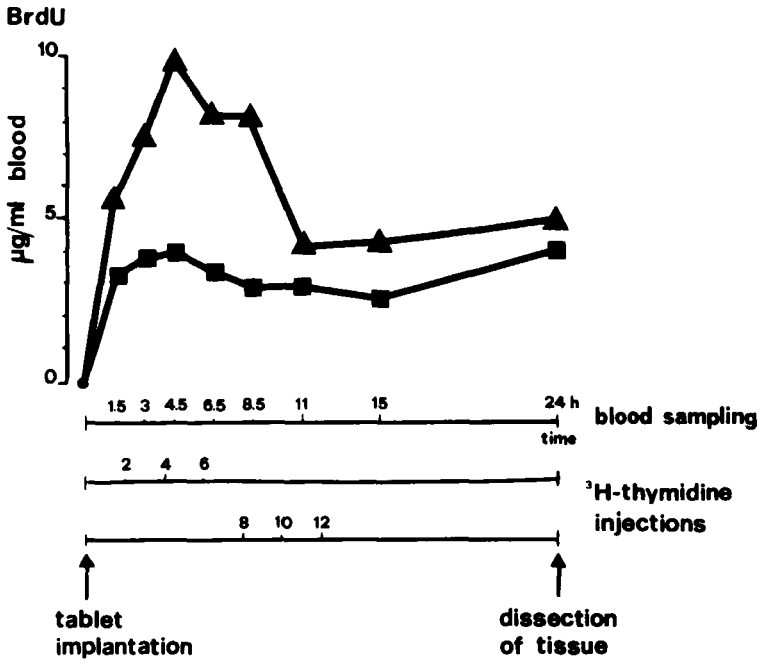

Fig. 1. BrdU blood levels as a function of time after implantation of agar coated (■) or uncoated (▲) BrdU tablets. Interaction with the intracellular nucleotide pool was determined by the application of ^{3}H-thymidine at intervals indicated.

Sister Chromatid Differentiation

Dissociated cells were distributed in wells of a multiplate-4 wells (24 × 67 mm) test plate containing a clean glass coverslip and 8 ml DMEM with 0.31 μg BrdU/ml (= 1 μM). 10^6 Cells were filled into wells from which SCE were analyzed after 24 hr and 7.5 × 10^5 cells into wells from which SCE were determined after 48 hr.

Metaphases were prepared on coverslips in situ 24, 48, and in some cases 72 hr after in vitro culture. Mitotic arrest was initiated with 1 μg colchicine/ml DMEM, 3 hr prior to hypotonic treatment with 0.75% KCl for 15 min at 37°C. The hypotonic solution was then replaced continuously by fixative (acetic acid:methanol = 1:3). Subsequently coverslips were transferred to freshly prepared fixative for 10 min and air dried. The staining procedure was essentially that of Perry and Wolff [1974]: Coverslips were stained for 20 min in Fluo-Chrom-Hoe 33258 (5 μg/ml in M/15 Sorensen's buffer, pH 6.8), washed in distilled water, and covered with a layer of Sorensen's buffer and a quartz coverslip. The UV-exposure was 30 min under a shortwave UV-lamp (UVS 54, Mineralight®, 6 W) at a distance of 3.6 cm (= 81 kJ/m^2). The staining in Fluo-Chrom-Hoe was not essential for the demonstration of SCE, but it provided a better differentiation of chromatids. Subsequently coverslips with cells were washed in distilled water, incubated for 2 hr at 55°C in 0.6 M NaCl and 0.06 M $Na_3C_6H_5O_7 \cdot 2H_2O$ adjusted with HCl at pH 7.0, and again washed in distilled water. Cells on the coverslips were then stained in 7% Giemsa (Merck) solution (Sorensen buffer, pH 6.8) for 8 min, washed, air dried, transferred for 15 min in xylene, and mounted in Eukitt.

Simultaneously, the primary cloning efficiency (I CE) of dissociated cells from most animals was determined in DMEM. This I CE was used as an indicator for the possible cytotoxic effect of BrdU [Maier et al, 1982]. Cells were tested for 6-thioguanine resistance (6-TG^r) as reported previously [Maier et al, 1980] but with slight modifications (expression time in vivo 24 hr, in vitro 2 × 72 hr, 6-TG concentration in selective medium 15 μM).

RESULTS

BrdU Concentration in Blood and Competition With Thymidine Incorporation

Concentrations found in blood are summarized in Figure 1. With uncoated tablets maximal blood concentrations were twice as high as with coated BrdU tablets within 5 hr. After 10 hr the blood concentrations with uncoated tablets were only 20% higher than with coated tablets. Accordingly, with coated tablets, 145 mg (58%) of the original 250 mg tablet was recovered after 24 hr.

Measured by autoradiography, BrdU concentrations from uncoated tablets reduced the ^3H-thymidine incorporation. Cells showed a decreased grain density over the nucleus and the number of heavily labeled cells (more than 50% of the nucleus area is black) dropped from 49.6% without BrdU to 1.5% when BrdU was applied with uncoated tablets. Compared to untreated cells, 2–8 hr after tablet implantation in 63% and 8–14 hr in 37% of individual cells, the label was reduced to an undetectable level (Table I).

TABLE I. Effect of BrdU Application In Vivo

	Induction of 6-TG^r cells		^{3}H-thymidine incorporation[a] (percent of labeled cells)		
				^{3}H-thymidine pulse after tablet implantation	
Treatment dose	Number of animals	6-TG^r $\times 10^{-6}$ cells	Number of animals	2–8 hr	8–14 hr
Without BrdU	6	5.3 ± 5.1	2	39.5 (100%)	26.9 (100%)
BrdU 250 mg tablet sc (63%)	6	9.9 ± 11.4[b]	2	14.5 (37%)	16.8

[a]Label of cells was determined 4 hr after culture in vitro. Analysis was peformed with coded slides.
[b]Mutation frequencies are obtained from pooled data (number of cells tested, corrected by the cloning efficiency/number of clones found in selective medium). SD are calculated from the means of mutation frequencies obtained from individual animals. Value is statistically different from control, α calculated = 0.027 (Kastenbaum and Bowman [1970]).

Influence on Cell Cycling and SCE Frequency

The influence of BrdU on cell cycling in vivo was estimated by the mitotic indices obtained after the 3 hr colchicine treatment of cells cultured for 24 and 48 hr in vitro. Cells exposed to BrdU in vivo only were compared with those exposed to in vivo and in vitro or in vitro only. The spontaneous SCE frequencies were not affected by the different ways of BrdU exposure (Table II).

Gene Mutations

The induction of 6-TG^r mutants in vivo was determined in three experiments. The thymidine analogue doubled the spontaneous mutation frequency (Table I). In reconstruction experiments in vitro, cells isolated from untreated animals were exposed to concentrations and time intervals comparable to those found in vivo. The two exposure intervals were chosen in order to define the effect of the constant level achieved with uncoated and coated tablets (5 μg/ml, 24 hr) versus the short high-pulse exposure found with uncoated tablets in vivo (10 μg/ml, 4 hr). Induced mutation frequencies in vitro were higher than those obtained in vivo (Table III).

DISCUSSION

Absorption of BrdU

Results from ^{3}H-thymidine incorporation studies with untreated animals (Table I) demonstrate that the majority of cells (39.5–26.9%) recovered in vitro were synthesizing DNA in vivo 50–62 hr after pouch initiation, ie, during the exposure to BrdU. The lower percentage of cells which incorporate ^{3}H-thymidine during the second interval, 16–10 hr prior to dissection is due to the declining proliferative stimulus beginning already 48 hr after pouch initiation. In animals with implanted uncoated BrdU tablets, we observed that BrdU competes efficiently with ^{3}H-thymidine incorporation (Table I). Corresponding to the BrdU concentrations found in the blood (Fig. 1), the number of cells in which ^{3}H-thymidine is substituted to an

TABLE II. Influence of Tissue Dissociation and BrdU Application on Cell Cycling and SCE*

BrdU		24 hr cultured in vitro						48 hr cultured in vitro					
In vivo	In vitro	Mitotic index	M_1 (%)	M_2 (%)	Iso-nonlabeled (%)	M_3 (%)	SCE frequency	Mitotic index	M_1 (%)	M_2 (%)	Iso-nonlabeled (%)	M_3 (%)	SCE frequency
Yes	No	3.7	67.7	28.7	0	3.7	11.4	6.3	70.2[a]	3.4	26.3	—[b]	15.5
Yes	Yes	3.1	74.0	25.4	0	0.5	11.1	6.6	9.3	26.7	0	64.0	13.5
No	Yes	2.8	98.0	2.0	0	0	14.1	6.1	16.5	48.0	0	35.5	14.40

*For each BrdU application schedule, data from eight animals are summarized. All the values represent the interindividual mean of intraindividual means. The numbers for individual animals were defined as follows: Mitotic index = number of metaphases among 1,000 cells. The relationship between M_1:M_2:M_3 was obtained after classification of 100 metaphases according to their corresponding labeling pattern. Isononlabeling refers to the % of second division metaphases with isononlabeled regions in one or more chromosomes. SCE frequencies = 20 M_2 metaphases examined.

[a]Sum of M_1 and M_3.

[b]Not found, not distinguishable from M_1.

TABLE III. Gene Mutation Induction In Vitro*

BrdU dose	Exposure time (hr)	II Cloning efficiency (%)	Number of cells tested/ mutants scored	6-TG^r mutation frequency ($\times 20^{-6}$)	Mean
Control		81.3	976,600/2	2.1	
0.31 μg/ml	24	52.7	632,400/4	6.3	
	24	75.2	902,400/6	6.7	6.5
5.00 μg/ml	24	24.2	266,200/7	26.3	
	24	31.5	378,000/2	5.3	15.8
10.00 μg/ml	4	54.7	601,700/30	49.9	
	4	56.8	681,600/19	27.9	38.9

*Freshly isolated granulation tissue cells were cultured for 48 hr, treated with BrdU, subcloned after 24 hr and again after 72 hr. Subsequently cells were cultured in selective medium (DMEM + 6-TG) and in DMEM similar to cells exposed to BRDU in vivo. Number of cells tested are corrected values according the cloning efficiency in cultures without 6-TG.

undetectable level was higher (63%) 2–8 hr after tablet implantation than after 8–14 hr (37%) (Table I). These data, combined with the observed reduced labeling pattern, demonstrate that BrdU levels of 5–10 μg/ml blood affect the intracellular nucleotide pool drastically. The subsequent decrease of the blood concentration after 10 hr, although the uncoated BrdU tablets were not completely absorbed, may be due to local tissue necrosis, induced by a cytotoxic BrdU concentration around the tablet. The lower blood concentrations obtained with the agar-coated tablet was sufficient for the differential labeling of sister DNA strands.

Cell Cycling and Spontaneous SCE Frequencies

With the BrdU-labeling technique of sister chromatids, the cell cycle kinetics can be estimated. The relationship between M_1:M_2:M_3 in vitro 24 hr after cell isolation (Table II) indicates that the delay in the cell cycle is caused by the dissociation procedure and not by BrdU exposure in vivo. Cells which incorporated BrdU in vivo only showed isononlabeled chromosomes (Giemsa isostained) later than 24 hr of culture in vitro. That means cells which were in the process of the second DNA S-phase in vivo at the time of tissue excision and dissociation required at least 24 hr in culture in order to terminate their S-phase, passing G_2- and entering M_2-phase. Therefore, the continuous BrdU application, in vivo and in vitro, does not result in a relative increase of M_2 within 24 hr. The 74% of M_1 indicates, however, that cells respond to a mitogenic stimulus most likely induced by protease treatment during the tissue dissociation [Sefton and Rubin, 1970; Hart and Streilein, 1976]. After 48 hr in culture, the number of cells in cycle doubled compared to 24 cultures. Newly stimulated cells decreased drastically (M_1:BrdU in vivo and in vitro). The cell cycle was accelerated compared to 24 hr (M_3:BrdU in vitro only).

Comparing the labeling pattern of cells exposed to BrdU in vitro only, one can estimate that after continuous BrdU exposure not more than half of the M_2 cells recovered after 48 hr in culture are labeled additionally in vitro only.

The spontaneous SCE frequency in vivo (BrdU in vivo only) was equal to in vitro and 3–4 times higher than that found in bone marrow cells [Maier et al, 1982].

This indicates that it is not the dissociation procedure or the culture in vitro that causes the high spontaneous SCE value.

In conclusion, BrdU exposure in vivo and/or in vitro neither reduced the replication capacity of the freshly isolated cells nor enhanced the spontaneous SCE frequency in vivo or in vitro significantly.

Induction of Gene Mutations In Vivo

The most significant effect of BrdU in vivo, when applied with uncoated tablets, was the doubling of the spontaneous mutation frequency at the 6-TG^r locus. This mutagenic activity in vivo should be considered when combined studies for gene mutation and SCE formation are performed.

Reconstruction Experiments

In vitro exposure of cells from untreated animals to BrdU concentrations and time intervals found in blood resulted in a much higher mutagenic activity of BrdU than in vivo (Table III). Clearly the 24-hr exposure is more cytotoxic/cytostatic than the pulse treatment, whereas with the short pulse treatment, 6-TG^r mutants are more efficiently induced. This is indirect evidence that incorporation of BrdU into DNA is not the critical lesion. These findings are in agreement with other cell culture studies. It was found that the mutagenicity of BrdU is not dependent on the substitution degree of DNA, and therefore base mispairing cannot be the critical mutagenic event [Aebersold, 1976; Kaufman and Davidson, 1978]. Accordingly BrdU does not induce Oua^R mutants in vitro [Nakamura and Okada, 1979]. It was postulated that starvation for deoxycytidine (dCyd) [Kaufman and Davidson, 1978] or imbalances in the intracellular deoxyribonucleotide pool in general [Kunz, 1982] are responsible for the induction of mutations. Therefore, we assume that changes at the 6-TG^r locus, as obtained in our experiments, are mainly caused by small deletions. The discrepancy between mutation frequencies obtained in vivo and in vitro suggests that in vivo the intracellular nucleotide pool is more stable or can more efficiently be regulated than in vitro.

Furthermore a short pulse with a high concentration of BrdU disturbs the nucleotide pool more efficiently and therefore causes specific locus mutations more efficiently. For this reason it is preferable to use agar-coated BrdU tablets in experiments in which in vivo induced SCE and gene mutations are recovered simultaneously. The coated tablets prevent initial high and short pulses in the BrdU blood level.

ACKNOWLEDGMENTS

This work was supported by a grant of the Swiss National Science Foundation, contract Nr. 3.819.79.

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