EFFECTS OF 5-BROMO-2'-DEOXYURIDINE (BUdR) ON IN VITRO COLONY FORMATION IN CULTURES OF MOUSE BOND MARROW CELLS

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1. Introduction

Colony stimulating factors (CSF) appear to be glycoproteins [1] and have been prepared from many sources including serum and urine of both normal and leukemic individuals [2]. The biological activity of CSF is generally measured by its ability to promote the formation of granulocyte and/or mononuclear cell colonies in cultures of bone marrow cells suspended in either soft agar [3,4] or methyl cellulose [5]. CSF has been shown to stimulate the in vitro incorporation of labeled precursors into DNA [6–8], but colony formation cannot be explained simply in terms of a clonal proliferative model [8–11]. Moreover, morphological observations and histochemical evidence have suggested that CSF promotes the in vitro differentiation and/or selection of granulocyte and mononuclear cell precursors in both normal and leukemic marrow cell populations [12–14].

Low concentrations of the thymidine analog BUdR inhibit the differentiation of specific cell types and prevent normal development in embryos [15–17]. It was of interest, therefore, to investigate what effect low concentrations of BUdR might have upon CSF-dependent colony formation. In this study, evidence is presented that CSF-dependent in vitro colony formation in cultures of mouse bone marrow cells is significantly inhibited by as little as 10^{-7} M BUdR, and that BUdR appears to exert its inhibitory effect at a relatively early stage of colony formation.

2. Materials and methods

All isotopes were purchased from New England Nuclear Corp.: thymidine-^{3}H-methyl, specific activity 6.7 Ci/mmole; deoxycytidine-5-^{3}H, specific activity 30 Ci/mmole; 5-bromodeoxyuridine-6-^{3}H, specific activity 25.2 Ci/mmole. Methylcellulose (4000 centipose) was obtained from Fisher Scientific. Fetal calf sera were the product of Flow Laboratories and North American Biologicals. Sera were kept frozen at −20°C in small batches to avoid repeated freezing and thawing. All other reagents were of the highest purity commercially available.

2.1. *Preparation of CSF*

CSF was obtained from the urine of leukemic patients admitted to Emory University Hospital. Twenty-four hour urines were collected in iced containers to which liquid phenol (0.1%) had been added, and were then frozen. Urines from several patients were pooled and the protein from 6 liters was precipitated at 4°C by the addition of one volume of acetone. The precipitate was dissolved in 200 ml of H_2O and then dialyzed against 5 liters of cold, distilled water for 72 hr (5 changes). The dialyzed material was centrifuged to remove any insoluble material and the resulting supernatant was passed through a column of Sephadex G-75 (5.5 × 55 cm, 4°C) which had been equilibrated in distilled water. The resulting active fractions were pooled, lyophilized and stored at −20°C

2.2. *Culture systems for CSF assay*

Both male and female C57 B1/6J mice (2–3 months) were sacrificed by cervical dislocation at 8–10 a.m. Marrow was expressed from each femur with 2 ml of medium (100 parts McCoy's medium 5A (modified): 30 parts fetal calf serum). Generally, marrow was pooled from at least one male and one female

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mouse (1 femur each) for each experiment. Nucleated cells were counted using a Coulter counter.

2.2.1. *Colony forming activity*

Colony formation activity of CSF was assayed in agar containing medium using a double layer technique [18]. Feeder layers were prepared as follows: Double strength medium at 37°C was mixed with an equal volume of 1% agar at 40°C. Two ml of this mixture were added to 35 mm Petri dishes (Falcon Plastics) containing an aliquot of the CSF preparation to be assayed (0.3 ml or less). The contents of the dish were mixed, allowed to gel, and stored at 37°C under a humid atmosphere of 7.5% CO_2 in air until inoculated with a marrow cell layer prepared in the following way: marrow cells were added to medium containing 0.3% agar (37°C) to give a concentration of 2.5×10^4 cells per 0.7 ml. This volume was pipetted onto each feeder layer and allowed to gel. The inoculated culture dishes were incubated for 7 days at 37°C in moist 7.5% CO_2 in air at which time colonies were counted as described previously [19]. All cultures were done in duplicate, and dose response curves were constructed using a minimum of 3 CSF concentrations. The lines drawn through the data points were calculated by the method of least squares. Similar experiments were carried out in cultures containing 0.8% methylcellulose.

2.2.2. *DNA precursor incorporating activity*

CSF dependent incorporation of [^{3}H]TdR or [^{3}H]CdR was measured in liquid cultures or cultures containing methylcellulose (0.8%) as follows: Cells were added to single strength medium to give a concentration of 10^5 or 10^6 cells per ml, and 1 ml aliquots were pipetted into 13×100 mm culture tubes containing the CSF to be assayed (0.1 ml or less). The contents of the tubes were mixed and allowed to incubate at 37°C in an atmosphere of moist 7.5% CO_2 in air. At 48 hr, each tube received the indicated amount of [^{3}H]TdR or [^{3}H]CdR (specific activity 0.02 Ci/mmole). After a 24 hr labeling period, 5 ml of the following mixture were added to each tube: cold isotonic saline buffered with 10 mM sodium phosphate, pH 7.4 (PBS) containing 0.5 mg of unlabeled TdR or CdR and approximately 10^5 fresh mouse bone marrow cells per ml (unnecessary when original inoculum was 10^6 cells/ml). The tubes were centrifuged for 25 min (all centrifugations were carried out at 4°C and 2500 *g*),

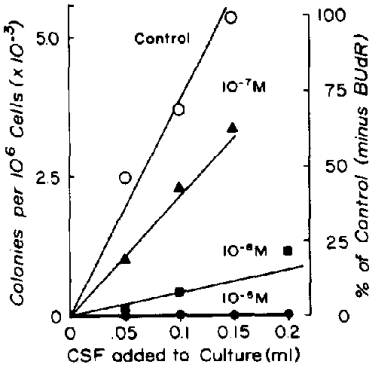

Fig. 1. Colony-CSF dose response curves in cultures containing different concentrations of BUdR. o—o Control, no BUdR; ▲—▲, 10^{-7} M; ■—■, 10^{-6} M; ●—●, 10^{-5} M.

the supernatants were discarded, and the tubes were allowed to drain. After carefully wiping the lip of each tube with a Kimwipe, 0.4 ml of PBS was added. The cell pellet was dispersed using a Vortex mixer and 5 ml of cold 0.2 M perchloric acid (PCA) were added. After centrifuging for 25 min the supernatant was discarded. The pellet was then washed 3 times each with 5 ml of 0.2 M PCA: centrifugation was for 10 min. Following the final wash, the tubes were allowed to drain, 1 ml of 0.2 M PCA was added, and hydrolysis was carried out in a boiling water bath for 20 min. On cooling, 0.9 ml was counted in a Triton–toluene scintillation cocktail using a Beckman liquid scintillation spectrometer. Cultures were routinely done in triplicate and dose response curves were obtained using a minimum of 3 CSF concentrations. Regression lines were drawn by the method of least squares.

2.3. *Isolation and centrifugation of DNA*

DNA was isolated by Sarkosyl–pronase treatment [20]. CsCl gradient solutions were prepared by adding CsCl to citrate–phosphate buffer (0.0625 M Na_2HPO_4, 0.015 M Na_3 citrate, pH 7.2) containing 20–30 μg DNA to achieve an average starting density of 1.70 g/cm^3. Samples were overlayed with mineral oil and gradients were formed by centrifuging in a Spinco SW 50.1 rotor for 48 hr at 35 000 rpm and 20°C using a Beckman L2-65B ultracentrifuge. Gradients were fractionated by piercing the bottom of the tubes and dripping fractions directly into counting vials. Samples were prepared for counting as described by Taylor et al. [20].

Table 1
Effect of time of addition of BUdR (10^{-6} M) on colony formation.

Time	Colony formation*		% of No. drug control
	No BUdR	10^{-6} M BUdR	
0	5964 ± 590	173 ± 138	3
24	5721 ± 629	154 ± 123	3
48	5712 ± 415	1100 ± 704	23
72	3714 ± 111	3462 ± 380	93

* Colony formation is reported as the slope of the dose response curve ± S.E. in units of colonies per 10^6 cells plated per ml CSF. Experiments were carried out using dual chamber tissue culture slides (Lab-Tek); one side served as the no. drug control and the other as the drug treated culture. Standard errors were large for BUdR treated cultures at 0, 24 and 48 hr owing to the small number of colonies.

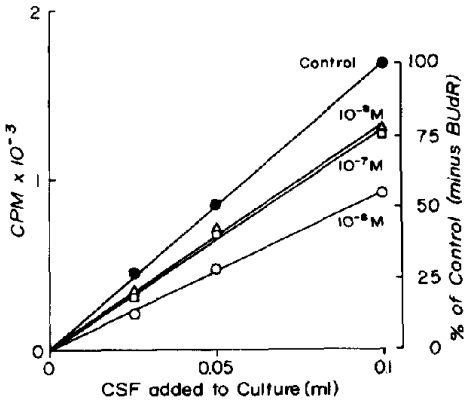

Fig. 2. Effect of different concentrations of BUdR on CSF-dependent incorporation of [^{3}H]CdR. •—•, Control, no BUdR; △—△, 10^{-8} M; □—□, 10^{-7} M; ○—○, 10^{-6} M. Cultures (10^6 cells) were labeled with 0.2 μCi/ml of isotope for 24 hr. The no. CSF cultures containing 0, 10^{-6} M, 10^{-7} M and 10^{-8} M BUdR incorporated 725, 502, 713 and 701 cpm, respectively.

3. Results and discussion

The effect of different concentrations of BUdR on colony-CSF dose response curves was determined in soft agar, marrow cell cultures. These data are presented in fig. 1 and show that 10^{-5} M, 10^{-6} M and 10^{-7} M BUdR inhibited colony formation 100%, 84% and 42% respectively.

The following experiment was carried out in an attempt to learn if there was a critical time period in which BUdR was acting to inhibit CSF-dependent colony formation. BUdR (10^{-6} M) was added to marrow cell cultures in methylcellulose after various times of preincubation in the presence of different concentrations of CSF (table 1). These data indicated that the drug had essentially no effect on colony formation when added after 72 hr of preincubation. BUdR (10^{-6} M) inhibited colony formation to approximately the same extent in either agar or methylcellulose containing cultures. Control cultures to which medium (but no drug) was added showed a progressive decrease in colony formation (table 1). At the present time, the reason for this decrease remains obscure, although it is not unreasonable to assume that mixing of the viscous culture medium following addition of a given material may lead to the disruption of incipient colonies. Nevertheless, it has been observed repeatedly that agitation of the cultures after 24 hr has a deleterious effect on colony formation.

The CSF-dependent incorporation of ^{3}H-CdR into marrow cell DNA was investigated in cultures containing methylcellulose and to which different concentrations of BUdR had been added. The data shown in fig. 2 indicate that 10^{-6} M BUdR inhibited incorporation by about 50% whereas 10^{-7} M and 10^{-8} M BUdR each resulted in about a 20% inhibition as compared to the non-drug control. Thus it is evident that a equivalent concentration of BUdR inhibited colony formation and precursor incorporation into DNA to different degrees, its effect on colony formation being greater. These data lend additional support to the idea that non-proliferative as well as proliferative events are involved in the process of CSF-dependent in vitro colony formation [8–11].

To investigate whether BUdR was substituting for TdR under somewhat similar conditions to the experiments previously described, DNA was isolated from CSF-stimulated marrow cell cultures in liquid medium which had been labeled with either [^{3}H]BUdR or [^{3}H]TdR. The DNA was then subjected to equilibrium isopycnic centrifugation in buffered CsCl. The results of such an experiment are shown in fig. 3, and indicate that BUdR does in fact substitute for TdR in the marrow cell DNA. DNA isolated from cultures labeled for 72 hr with 10^{-6} M [^{3}H]BUdR had a buoyant density indistinguishable from that of the unsubstituted DNA. Labeling of the cultures for 24 hr using

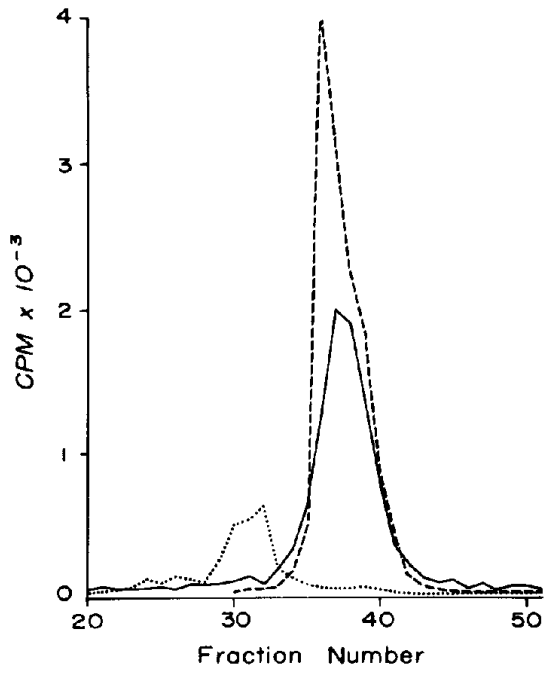

Fig. 3. Cesium chloride-density gradient profiles of DNA isolated from 72 hr CSF-treated cultures (10^6 cells/ml). Cultures were labeled with 0.4 μCi/ml of isotope for (a) 72 hr with [^{3}H]BUdR, specific activity 0.4 μCi/mM (——) or (b) 24 hr with [^{3}H]TdR (----) or [^{3}H]BUdR (·····), specific activity 0.02 Ci/mM following a 48 hr preincubation period.

a concentration of [^{3}H]BUdR of 2×10^{-5} M resulted in sufficient substitution such that the DNA banded at a higher buoyant density. While these results might suggest that a rather limited substitution of the DNA by BUdR may be involved in the inhibition of CSF-dependent colony formation, further data, obtained under strictly identical culture conditions, are needed to establish such a relationship.

4. Conclusions

These studies demonstrated that low concentrations of BUdR were effective in interfering with some aspect of CSF-dependent in vitro colony formation in cultures of mouse bone marrow cells. This interference appeared to be associated with an early event(s), and might be associated with the substitution of BUdR for TdR in the marrow cell DNA. The inhibitory effect of BUdR was more pronounced on colony formation than it was on precursor incorporation into DNA [8–11]. Based on the known effects of BUdR on differentiative events in other biological systems [15–17], we suggest that its effect in our system is of a similar nature. Further progress in understanding the mechanism of action of CSF and its role in proliferative and differentiative events of bone marrow cell development must await the availability of relatively homogeneous fractions of marrow cells and more fully characterized preparations of CSF.

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