EFFECT OF 5-BROMODEOXYURIDINE ON GROWTH, ENCYSTMENT, AND EXCYSTMENT OF *ACANTHAMOEBA CASTELLANII*

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INTRODUCTION

Differentiation of several cell types is inhibited by 5-bromodeoxyuridine (BUdR) (1–3, 9, 12). Since BUdR can be incorporated into DNA in place of thymidine (TdR) (12), the inhibition of differentiation may be due to the presence of BUdR in the DNA of the cell.

In *Acanthamoeba castellanii*, DNA synthesis continues through overt differentiation of the trophozoite into the dormant cyst.[1] Since cell division is terminated before encystment is induced, continuation of DNA synthesis suggested that the DNA synthesized at this time might be important for normal differentiation. Consequently, we undertook experiments to determine whether BUdR would interfere with differentiation of *A. castellanii*. The present report deals with preliminary observations on the effects of BUdR on growth, encystment, and excystment.

Culture Conditions

A. castellanii (Neff strain) was grown axenically in optimal growth medium (OGM) in aerated suspension culture as described by Neff et al. (5). The modifications introduced for inoculation and sampling of the cultures were described by Stevens and O'Dell (10). Cell counts were made on appropriately diluted samples of the cultures using a Coulter Counter model B (Coulter Electronics Inc., Fine Particle Group, Hialeah, Fla.). The kinetics of growth and spontaneous encystment in the optimal growth (OG) cultures have been reported previously (11). All manipulations for growth, encystment, and excystment were performed under sterile conditions.

[1] Roti Roti, L. W., and A. R. Stevens. 1974. DNA synthesis during growth and encystment of *Acanthamoeba castellanii*. Submitted for publication.

Induction of Encystment

Using cells from exponentially growing cultures at a density of 1–2 $\times$ 10^6 cells/ml, encystment was induced by using the medium replacement method (constant pH 8.9) described by Neff et al. (5). Cell density in the encystment medium (EM) was between 2 and 2.5 $\times$ 10^5 cells/ml. Encystment synchrony was evaluated morphologically by phase-contrast microscopy (5). More than 95% of the cells formed mature cysts within 24 h after induction under these conditions (5, 11).

Induction of Excystment

Approximately 5 days after induction of encystment, samples were removed from the encysted culture, and the cysts were collected by centrifugation at 800 $\times$ *g* for 4 min. The cysts were washed, resuspended in OGM, and inoculated into aerated suspension culture at a final density of 4–6 $\times$ 10^4 cysts/ml. Conditions for growth in the excysting culture were as described above. Excystment was monitored morphologically using the staining method described by Mattar and Byers (4).

Inhibitor Studies

Solutions of BUdR (Sigma Chemical Co., St. Louis, Mo.) and 5-fluorodeoxyuridine (FUdR) (Calbiochem, San Diego, Calif.) at 100 times the final concentration were prepared in 0.14 M NaCl and sterilized by filtration before addition to cultures. All cultures containing BUdR-treated cells were shielded from visible light by covering the culture flask with aluminum foil.

RESULTS

The effect of BUdR on growth of *A. castellanii* was evaluated after addition to exponentially growing suspension cultures (Fig. 1). Initially, the normal population doubling time of 7 h was maintained for a period which depended upon the concentration of BUdR. After six generations with 3.3×10^{-5} M BUdR, or three to four generations with 3.3×10^{-4} M, the doubling time increased to 13 h. Before the culture reached stationary phase, the cells treated with 3.3×10^{-4} M BUdR were subcultured into fresh medium, also containing BUdR (3.3×10^{-4} M). Exponential growth in the presence of BUdR was maintained in this way through three subcultures for a total of 29 cell generations with a doubling time of 10–13 h. The culture was then allowed to enter stationary phase without further subculture. Normal stationary phase density was reached in all BUdR-containing cultures.

To determine whether BUdR had an effect on differentiation of *A. castellanii* into dormant cysts, spontaneous encystment was evaluated after the cultures containing BUdR (3.3×10^{-4} M) reached stationary phase. Even after the cells had been maintained in BUdR for up to 29 generations, the time required for encystment induction and the degree of encystment were approximately the same in BUdR-containing as in control stationary phase cultures. Also, BUdR had no effect on experimentally induced encystment (see Materials and Methods). Cells that had been maintained in exponential growth for 7–20 generations in the presence of 0.25–3.3×10^{-4} M BUdR encysted within the normal 24-h period after transfer to EM. Further, experimentally induced encystment of cells not previously exposed to BUdR was not affected by addition of 0.25×10^{-4} M BUdR at the time cells were transferred to EM.

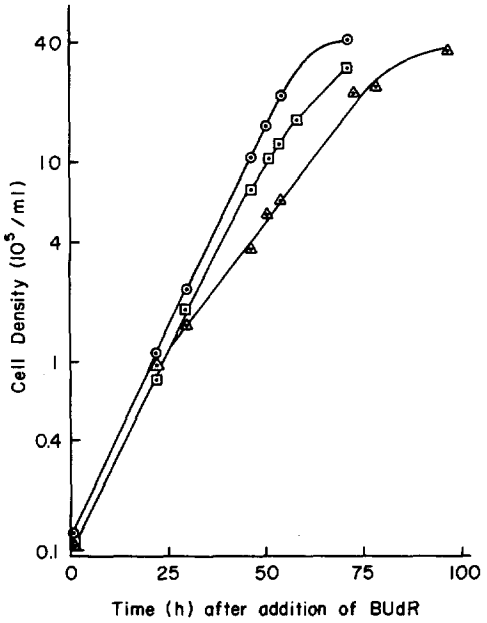

FIGURE 1 Effect of BUdR on growth. BUdR, 3.3×10^{-5} M (—□—) or 3.3×10^{-4} M (—△—), was added to exponentially growing cultures. An equivalent amount of 0.14 M NaCl was added to the control culture (—○—). The results shown for 3.3×10^{-5} M are from a single experiment, and those for 3.3×10^{-4} M are typical of four similar experiments.

The failure of BUdR to affect encystment of *A. castellanii* could have been due to a limited incorporation of the analogue into DNA. If endogenous synthesis of TdR were inhibited by addition of FUdR to the cultures along with BUdR, the degree of substitution might be increased relative to that in cultures treated only with BUdR. When BUdR and FUdR were added simultaneously to exponentially growing cultures of *A. castellanii*, cell growth continued longer than with FUdR alone (Fig. 2). After addition of FUdR and BUdR together, growth continued for two generations at the normal rate and for one

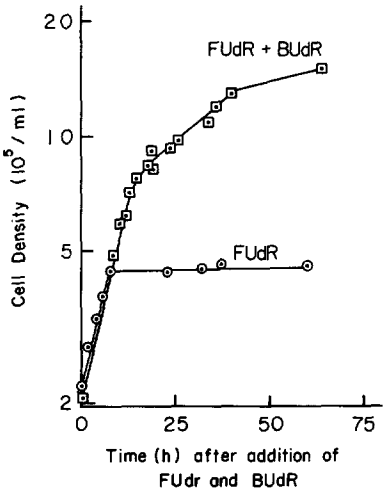

FIGURE 2 Effect of BUdR on inhibition of growth by FUdR. FUdR (3×10^{-6} M) alone (—○—) or concomitantly with BUdR (3.3×10^{-4} M) (—□—) was added to exponentially growing cells. Results shown are a composite from two experiments.

more generation at a reduced rate, whereas with FUdR alone growth was terminated after only one generation. This alteration of the FUdR effect by BUdR might have been due to a reduction in the amount of FUdR that entered the cell, especially since BUdR was added at 100 times the FUdR concentration. To test this possibility, BUdR was added 7 h after FUdR, when growth (Fig. 2) and DNA synthesis[1] had already been terminated. Under these conditions, addition of BUdR still led to a second doubling of the population, indicating that BUdR did indeed substitute for TdR in FUdR-treated cells. As in cultures treated with only BUdR, spontaneous encystment was essentially unaffected in cultures containing both FUdR and BUdR. After growth termination by simultaneous addition of FUdR and BUdR, the time-course of encystment was quite similar to that in untreated stationary phase cultures, or in cultures where FUdR and TdR had been added simultaneously. In cultures where the FUdR effect was circumvented by addition of BUdR after 2–7 h, the time-course of encystment was closely comparable to that in corresponding cultures containing FUdR and TdR.

Although BUdR had no effect on the formation of morphologically identifiable mature cysts, the possibility remained that cysts formed from BUdR-treated cells might be physiologically defective. Therefore, excystment of the cysts formed by BUdR-treated cells was evaluated. The numbers of cysts in the various morphologically defined stages of excystment (4) were scored at intervals after transfer of cysts to OGM. "Activation" is defined as the change in staining properties of the cysts that is indicative of the preliminary stages of excystment of *A. castellanii* into viable trophozoites (4). The results are shown in Fig. 3 for control cells and for cells that had been grown seven generations in the presence of 3.3×10^{-4} M BUdR before encystment induction. In the control cultures, about 70% of the cysts were "activated" within the time period of observation. On the other hand, only about 25% of the cysts formed by BUdR-treated cells were activated and the remaining cysts stayed dormant for the duration of the experiment. Eventual emergence of the activated forms as evaluated by the increase in the number of empty cyst walls, however, did not appear to be impaired by BUdR. In both control and BUdR-treated cultures roughly half of the activated cysts emerged within the time of the experiment (Fig. 3).

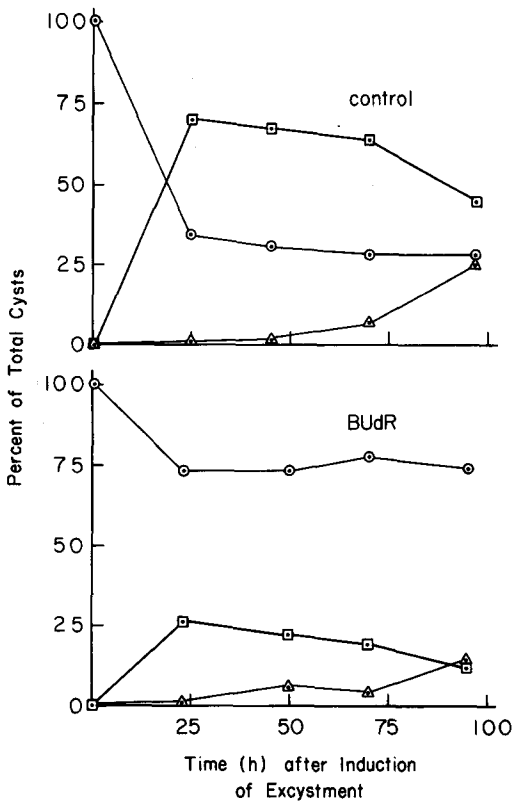

FIGURE 3 Effect of BUdR on excystment. Cells were grown without (control) or with (BUdR) 3.3×10^{-4} M BUdR for seven generations before induction of encystment (in the absence of BUdR). Excystment was induced 5 days later, and the number of dormant cysts (—○—), activated cysts (—□—), and empty cyst walls (—△—) was evaluated at intervals as percent of total cysts. The number of "preemergent" cysts observed at any one time was quite small or zero, and was included in the number of activated forms. The points represent average values from two similar experiments.

DISCUSSION

The effects of BUdR on differentiation of *A. castellanii* were first studied using a concentration of BUdR (3.3×10^{-4} M) which caused lengthening of the population doubling time, but permitted continuation of exponential growth at the new rate (Fig. 1). Encystment, either spontaneous or experimentally induced, occurred normally in the BUdR-treated cells, but when the cysts formed by experimental induction were returned to OGM, their excystment was reduced (Fig. 3). The inhibition of excystment appears analogous to the inhibition of differentiation observed in other eukaryotic cells (1–3, 9, 12), where

differentiation is also impaired at concentrations of BUdR that do not interfere with growth, or retard it only slightly.

The possibility remained that BUdR might inhibit encystment as well as excystment if the degree of substitution of BUdR for TdR could be increased. When FUdR and BUdR (at 100 times the FUdR concentration) were added simultaneously to exponentially growing cultures, growth continued longer than in the presence of FUdR alone (Fig. 2), apparently analogous to circumventing FUdR inhibition by adding TdR. Therefore, the degree of BUdR substitution in these cultures must have been greater than in cultures containing BUdR alone. Furthermore, simultaneous addition of BUdR and FUdR led to termination of growth after three population doublings, whereas when TdR was added to FUdR-containing cultures, normal stationary phase density was reached. Since spontaneous encystment of these cells proceeded normally once cell division was terminated, even a growth-limiting degree of BUdR substitution apparently did not interfere with this form of differentiation in *A. castellanii*. Indeed, the effect of combined treatment with FUdR and BUdR might be interpreted as induction of differentiation, such as occurred in the case of mouse neuroblastoma cells treated with BUdR (8). However, encystment in *A. castellanii* normally occurs when growth is terminated, either in stationary phase cultures or when encystment is induced experimentally. Encystment in the cultures treated with both BUdR and FUdR was, therefore, probably secondary to the cessation of growth.

Both encystment and excystment of *A. castellanii* can be considered as forms of differentiation. Both involve morphological and physiological changes in the cells and require synthesis of RNA and protein (4, 6, 7). Thus, in *A. castellanii* one type of differentiation was inhibited by the presence of BUdR in the cell, while the other seemed to be completely insensitive to it. The reason for the difference in the action of BUdR on two forms of differentiation in the same unicellular organism remains obscure. Further work with this system may provide basic information about the mechanism of action of BUdR, and about the role of DNA in differentiation of *A. castellanii*. Physical-chemical studies of DNA isolated from *A. castellanii* are being undertaken to determine the degree of BUdR substitution in the various fractions of DNA from cells in different states with respect to growth and differentiation.

SUMMARY

When *A. castellanii* was grown in the presence of 3.3×10^{-4} M BUdR, the population doubling time was increased from the normal 7 h to 10–13 h, and was maintained for as many as 29 generations. Nevertheless, the cells retained the capacity for differentiation into dormant cysts, either when it was allowed to occur spontaneously in stationary phase cultures, or when it was induced experimentally by starvation. Excystment, on the other hand, was significantly impaired when cysts formed by experimental induction of BUdR-grown cells were returned to OGM.

When FUdR (3×10^{-6} M) was added concomitantly with BUdR (3.3×10^{-4} M) to exponentially growing cells, cell division continued for three generations instead of only one generation as in cultures treated with FUdR (3×10^{-6} M) alone. Although incorporation of BUdR into DNA must have been much greater under these conditions than in the presence of BUdR alone, spontaneous encystment of the FUdR-BUdR cultures was still unaffected. Thus, it appears that of the two types of differentiation observed in *A. castellanii*, only excystment is sensitive to the presence of BUdR in the cell.

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