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5-IODO-2'-DEOXYURIDINE INHIBITION OF *DICTYOSTELIUM DISCOIDEUM* DIFFERENTIATION AND CYCLIC AMP PHOSPHODIESTERASE ACTIVITY

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Abstract—5-Iodo-2'-deoxyuridine (IdUrd), at a concentration of 0.25 mM, had little or no effect on the growth of *Dictyostelium discoideum* for at least four doubling times. However, when these cells were allowed to differentiate, the number of fruiting bodies obtained was only about half of that obtained from cells grown in normal medium, and a great majority of them were about ¼ to ⅛ the size of normal fruiting bodies. The inhibition of differentiation could be reversed if the cells were washed free of IdUrd and grown in normal medium for two generations before differentiation. If the IdUrd-grown cells were mixed with only 15 per cent as many normal cells just prior to differentiation, there was no inhibition of differentiation. Since it is known that, as a thymidine analogue, IdUrd can be incorporated into DNA, the induction patterns of several enzymes during growth and differentiation were compared in normal and IdUrd-treated cells. There was a continuous increase in the treated relative to untreated cells in the activities of the enzymes *N*-acetyl-glucosaminidase, α-mannosidase, β-glucosidase and acid and alkaline phosphatases during growth. There was also an increase in activity relative to control cells for all of these enzymes except alkaline phosphatase during the differentiation of cells previously grown in medium containing IdUrd. The reverse, however, was found for 3',5'-cyclic adenosine monophosphate (cAMP) phosphodiesterase. There was a decrease caused by IdUrd in both the extracellular and cellular phosphodiesterase activity during development. In contrast, during growth, although IdUrd treatment also reduced the extracellular phosphodiesterase activity, the cellular phosphodiesterase activity remained unchanged from control.

The cellular slime mold, *Dictyostelium discoideum*, is an excellent system for studying cellular differentiation. When nutrition is removed, the amoeboid cells cease further replication and begin to adhere to each other in chains and streams in response to the pulses of an attractant produced by a small percentage of the cells at the centre of the developing multicellular aggregate which then enters into a developmental cycle [1]. Thus, the phase of differentiation is completely separated from the phase of cellular growth. Only two major tissue types are differentiated, spores and stalk cells, constituting the fruiting body.

Since 1963, several investigators have reported a fascinating property of IdUrd and 5-bromo-2'-deoxyuridine (BrdUrd). Cells that are scheduled to differentiate are blocked in this process by BrdUrd or IdUrd and cells which have differentiated apparently lose their differentiated properties. Both of these effects on differentiated functions occur at analogue concentrations which have little or no effect on cell replication.

Recently Felenbok *et al.* [2] have demonstrated that BrdUrd, at concentrations that had little effect on the vegetative growth, blocked differentiation of *Dictyostelium discoideum*. The present paper shows that the same is true for IdUrd and presents evidence that activity of some developmentally regulated enzymes is also affected.

MATERIALS AND METHODS

Enzymes and reagents. Culture medium for growing strain AX-2 was HL5, prepared according to the method of Firtel and Bonner [3]. Filter papers (5.5 cm) no. 3 and no. 50 were purchased from Whatman. The saturation buffer used for differentiation was prepared according to the method of Newell *et al.* [4] and contained 40 mM phosphate buffer, pH 6.4, with 1.5 mg/ml of KCl and 0.5 mg/ml of $MgCl_2 \cdot 6\,H_2O$ but with no streptomycin sulfate added.

p-Nitrophenyl phosphate, *p*-nitrophenyl-α-D-mannoside, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide and *p*-nitrophenyl-β-D-glucoside were purchased from Sigma Chemical Co. Alkaline phosphatase and cAMP were obtained from Boehringer Mannheim. [^{3}H]-cAMP and Aquasol were purchased from New England Nuclear Corp.

Cell culture. Dictyostelium discoideum strain AX-2 was grown in axenic medium HL5 in ½ gallon size round bottles (Wheaton) with slow rotation (0.5 rev/min) in the dark at room temperature (22–23°). The doubling time was between 8 and 12 hr.

For differentiation, the medium was removed by centrifugation. The cells were washed twice with cold sterile water, then resuspended in water at a concentration such that in 0.5 ml of suspension 1 to 4×10^7 cells could be plated out as needed for the experiment. The cells were plated onto a Whatman no. 50 filter paper (5.5 cm) supported by three Whatman no. 3 filters underneath in a Petri dish. All the

filter papers were pre-washed with distilled water several times, then autoclaved in the Petri dish and dried. Just prior to applying the cells, the filter pads were first saturated with 4 ml of the saturation buffer, and excess moisture was removed by suction; 0.5 ml of an appropriate cell suspension was then spread over the filter pad, and after removing excess fluid by suction, the cells were allowed to develop in a sealed moist chamber in the dark at room temperature (22–23°).

The cells at any particular differentiated state could be scraped off the top filter paper for experimentation at various intervals after the initiation of starvation. For assay of the contents of the extracellular fluid, the three supporting filter papers underneath were pressed, and the fluids were collected.

By allowing the cells to differentiate on agar plates, the morphological changes during development could be followed more closely, especially during the early stages. For this purpose, the saturation buffer used to wet the filter papers was diluted 4-fold and 2% (w/v) Bacto-Agar (Difco Laboratories) was added. The cells were plated the same way as described for the filter pads. Complete development usually took 24–27 hr.

Enzyme assays. Enzyme activities were measured as previously described—*N*-acetyl-glucosaminidase by the method of Loomis [5], β-glucosidase by the method of Coston and Loomis [6], α-mannosidase by the method of Loomis [7], and alkaline phosphatase and acid phosphatase by the method of Gezlius and Wright [8]. Cells in the exponential phase of growth were harvested by centrifugation after about two doublings in normal medium or medium containing IdUrd, washed twice with cold water and resuspended in water at a concentration of 1–3 $\times 10^7$ cells/ml. The cells were stored frozen. When ready for assay, the cells were thawed out, and sonicated with a short burst of probe energy of about 50 W of a Sonifier Cell Disruptor (Heat Systems-Ultrasonics Inc., Plainview, NY) until the cells were broken (usually about 5–10 sec). The broken cells were then centrifuged at 3000 rev/min in an International centrifuge with a no. 269 rotor and the supernatant fluids were assayed for enzyme activities within the hour. One unit of enzyme activity is defined as the amount producing 1 nmole nitrophenol/min.

To assay the cAMP phosphodiesterase activity, the sonicated cell suspensions were not centrifuged; instead, the suspensions were dialyzed against 0.01 M Tris–HCl buffer, pH 7.5, exhaustively before being assayed. Growth medium and extracellular fluid collected during development were also dialyzed before being assayed for cAMP phosphodiesterase activity.

The phosphodiesterase activity was measured by a modification of the method of Henderson [9]. Activity was assayed at 30°, in a reaction mixture of total volume 0.5 ml and consisting of 50 mM Tris–HCl (pH 7.5), 12.5 $MgCl_2$, 52 μM [^{3}H]-cAMP (5.8 μCi/μmole) and 7 μg alkaline phosphatase (0.4 units/μg). The assay was initiated by the addition of [^{3}H]-cAMP. A 0.1-ml, zero time, sample was immediately withdrawn and added to 1.4 ml of an aqueous slurry of Bio–Rad AG 1–X2 (Cl^- form, 200–400 mesh; 6 g resin/20 ml of water). After thorough mixing, the suspension was centrifuged, and 0.5 ml of the supernatant fluid counted in 5 ml Aquasol. After 20 min of incubation, three more 0.1-ml samples were withdrawn from the reaction mixture and treated as the zero time sample. One unit of enzyme activity was defined as the amount that hydrolyzed 1 nmole cAMP/hr.

RESULTS

Effect of IdUrd on cell growth and differentiation. Figure 1 shows that 0.25 mM IdUrd had a negligible effect on the growth of vegetative amoeba for about four doublings, after which there was a modest inhibition. With higher concentrations (0.5 and 1.0 mM) the growth rate becomes reduced by about a third at two doublings and more severely thereafter.

When control and IdUrd-treated cells were allowed to differentiate on moist filter pads, the cells grown in normal medium form fruiting bodies within 27 hr while those grown in medium containing 0.25 mM IdUrd showed marked reduction both in the number of fruiting bodies formed and in their sizes. Differentiation of cells grown beyond four doublings in 0.5 mM and 1.0 mM IdUrd was almost completely blocked. Almost no fruiting bodies of any size were formed. This inhibition of development could be reversed if the cells were washed free of IdUrd and allowed to grow in normal medium for two doublings just prior to differentiation.

When the cells were allowed to develop on agar plates, more detailed changes in morphology could be seen. After doubling once in 0.25 mM IdUrd, very little streaming of the cells toward aggregating centers was seen (after 9–10 hr) compared to the normal cells. Instead mostly clumps of cells were

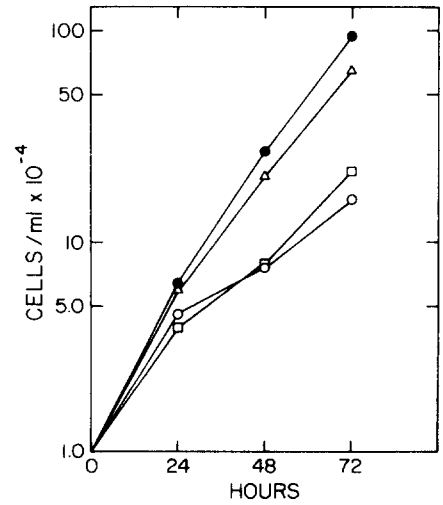

Fig. 1. Effect of IdUrd on the growth of *Dictyostelium discoideum* in axenic medium. Cells were grown in HL5 medium containing no IdUrd (●—●); 0.25 mM IdUrd (△—△); 0.5 mM IdUrd (□—□); and 1 mM IdUrd (○—○). The initial cell concentrations ranged from 1.1 to 1.7$\times 10^4$ cells/ml, and for the purpose of comparison, they were normalized to an initial value of 1.0$\times 10^4$ cells/ml.

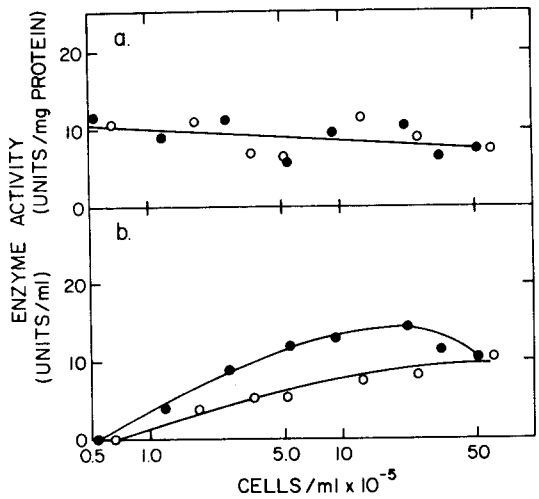

Fig. 2. Effect of IdUrd on cAMP phosphodiesterase activity during growth. Cells were grown in either HL5 medium containing no IdUrd (●—●) or 0.25 mM IdUrd (○—○). At various intervals during the exponential growth, portions were removed and the cells separated from the medium by centrifugation. Both (a) the cell fractions and (b) the medium were assayed for enzyme activity, as described in Materials and Methods.

observed. The fruiting bodies that did form from cells grown in medium containing 0.25 mM IdUrd were often formed a few hours later than in the case with normal cells. When the cells doubled only once in IdUrd medium, the fruiting bodies produced were

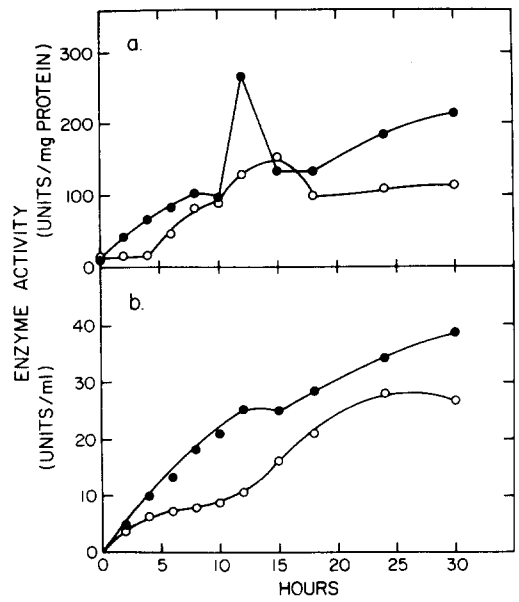

Fig. 3. Effect of IdUrd on cAMP phosphodiesterase activity during development of *Dictyostelium discoideum*. Cells were grown in HL5 medium containing either no IdUrd (●—●) or 0.25 mM IdUrd (○—○) for two doublings. Cells were then allowed to differentiate on filter pads (4×10^7 cells/pad). At various times during development, cells were scraped off the top filters and the extracellular fluid was expressed from the supporting pads as described in Materials and Methods. Both the (a) cellular fraction and the (b) extracellular fluid were assayed for enzyme activities, as described in Materials and Methods.

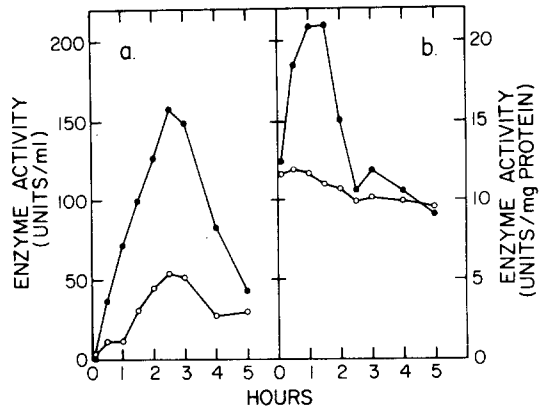

Fig. 4. Effect of IdUrd on cAMP phosphodiesterase activity during the shaking of *Dictyostelium discoideum* in phosphate buffer. Cells were grown in HL5 medium containing either no IdUrd (●—●) or 0.25 mM IdUrd (○—○) for two doublings. Cells were then harvested, washed twice with cold water and resuspended in 10 mM phosphate buffer, pH 6.4, containing 4.75 mM KCl and 0.5 mM $MgCl_2$, at a concentration of 4 10^7 cells/ml. The suspensions were then shaken on a rotary shaker at 230 rev/min at 22–23°. At various times, aliquots of cell suspension were removed. The fluid was separated from the cells by centrifugation and the cells were resuspended in water. cAMP phosphodiesterase activity of (a) the extracellular fluid and (b) the cellular fractions were assayed, as described in Materials and Methods.

about ½ to ¼ the normal size and many cells were still seen in a heap at the base of the stalk, whereas in the case of a normal fruiting body no cells were seen at the stalk base. When the cells had doubled two to four times in IdUrd medium, the fruiting bodies formed were about ⅛ the normal size and with many cells still in a heap at the stalk base. However, there was a greater number of micro slug-like structures that never developed any further. When cells that had doubled more than four times in IdUrd medium were plated out, no fruiting bodies of any size were seen. Instead the plate contained slug-like structures of various sizes, some very slender and long with many cells at their bases.

In terms of quantity, the amount of fruiting bodies obtained from cells doubled twice in medium containing 0.25 mM IdUrd was only about half of that obtained from cells grown in normal medium, and of that half, only about 5 per cent approximated normal size. However, when increasing amounts of normal cells were mixed with cells grown in IdUrd, more and more normal size fruiting bodies began to appear. At a ratio of normal cells to IdUrd-grown cells of 1:7, the recovery was about 90 per cent.

When cells grown in normal medium were plated onto filter pads saturated with buffer containing 0.25 mM IdUrd, no inhibition of differentiation was observed. Thus, IdUrd inhibits differentiation only when present during growth.

Effect of IdUrd on the activity of cAMP phosphodiesterase and other enzymes during growth and differentiation. Our observation of the morphologically visible effects of IdUrd on differentiation and the mixing experiments with normal and IdUrd-treated cells led us to suspect that cAMP and/or its related enzymes, known to be important in *Dictyo-*

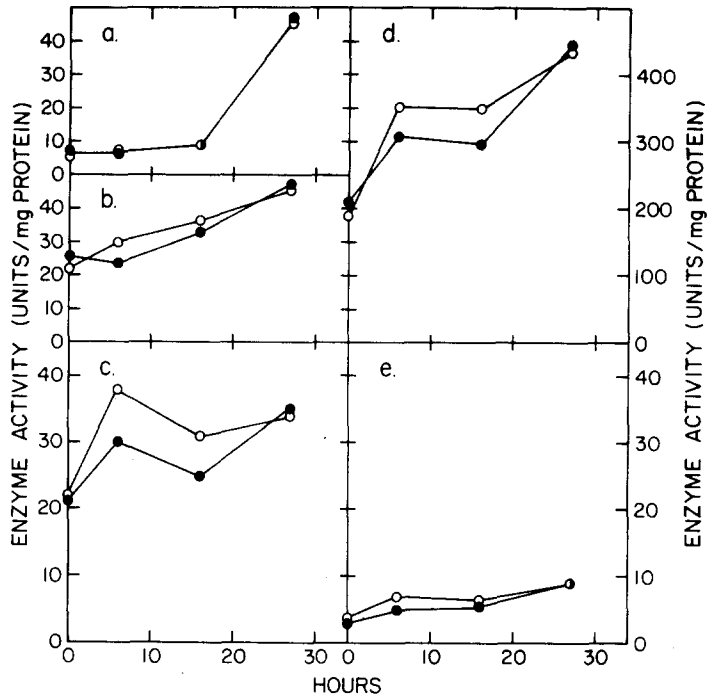

Fig. 5. Effect of IdUrd on developmentally regulated enzymes. Cells were grown in HL5 medium containing no IdUrd (●—●) 0.25 mM IdUrd (○—○) for about two doublings. The cells were then plated onto filter pads (2×10^7 cells/pad) and collected at the indicated times for enzyme assays, as described in Materials and Methods. The enzymes assayed were: (a) alkaline phosphatase, (b) acid phosphatase, (c) α-mannosidase, (d) *N*-acetylglucosaminidase and (e) β-glucosidase.

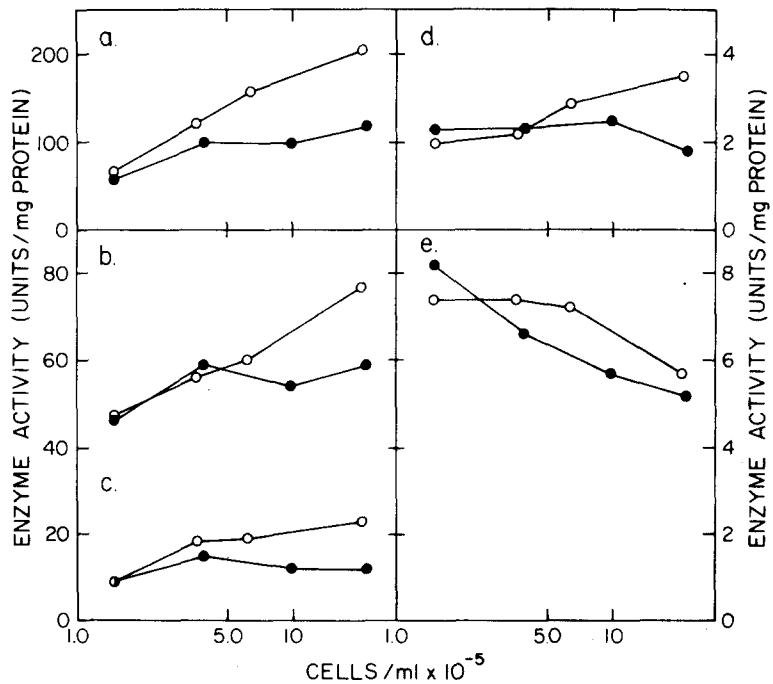

Fig. 6. Effect of IdUrd on enzyme activity during the growth of *Dictyostelium discoideum*. Cells were grown in HL5 medium containing either no IdUrd (●—●) or 0.25 mM IdUrd (○—○). At regular intervals, aliquots of cells were removed and assayed for enzyme activities, as described in Materials and Methods. The enzymes assayed were: (a) *N*-acetylglucosaminidase, (b) acid phosphatase, (c) α-mannosidase, (d) β-glucosidase and (e) alkaline phosphatase.

stelium differentiation, might somehow be involved in the inhibition of differentiation caused by IdUrd. Accordingly, we examined the effect of IdUrd treatment on cAMP phosphodiesterase activity during growth and differentiation.

Figure 2a shows that, during vegetative growth, the cellular activity of cAMP phosphodiesterase in the control cells remained relatively constant, perhaps decreasing slightly with time, and that growth of the cells in 0.25 mM IdUrd caused no observable difference from control cells. By contrast, growth of the cells in the presence of IdUrd did cause a reduction in the extracellular activity of cAMP phosphodiesterase as compared to control cells (Fig. 2b).

Figure 3 demonstrates, however, that a reduction in activity relative to control cells was observed in both the cellular and extracellular enzyme activity when cells previously grown in medium containing IdUrd were allowed to differentiate on filter papers. Riedel *et al.* [10] showed that some of the early events of *Dictyostelium* differentiation, such as elaboration of cAMP phosphodiesterase activity, can occur if the cells are shaken in a buffered salt solution instead of placing them on filter papers. Figure 4a shows that when cells were removed from growth medium and shaken in phosphate buffer containing KCl and $MgCl_2$ there was, as previously reported by Riedel *et al.* [10], a sharp rise in the extracellular cAMP phosphodiesterase activity that reached a peak after 2 hr. Prior growth of cells with 0.25 mM IdUrd considerably reduced this peak of phosphodiesterase activity. In the case of cellular activity (Fig. 4b), the peak activity appeared in control cells about an hour earlier than the peak of extracellular activity. The IdUrd-grown cells showed no peak of cellular phosphodiesterase activity at all.

IdUrd had no effect on the general pattern of the activities during the course of differentiation of the five enzymes, as depicted in Fig. 5. However, the activities of *N*-acetylglucosaminidase and α-mannosidase were somewhat elevated. The increase in activity was less for β-glucosidase and acid phosphatase, and unchanged in the case of alkaline phosphatase.

Figure 6 shows the effect of IdUrd on the same five enzymes during the growth phase. In all cases, to varying degrees, continuous growth in the presence of IdUrd corresponded to an increase in enzyme activity.

DISCUSSION

The data presented in this report show that IdUrd can inhibit the differentiation of *Dictyostelium* with little or no effect on the rate of cell replication. As a first approach to understanding the phenotypic mechanism for IdUrd inhibition of differentiation, we have examined the activities of several enzymes associated with development. Goz and Prusoff [11] had found a differential inhibition of the phenotypic expression of the various genes of phage T_4 with IdUrd-substituted DNA. It was, therefore, interesting to see that IdUrd had a similar effect on induction patterns in *Dictyostelium*. Although the activity of cAMP phosphodiesterase was reduced, the activity of some other enzymes actually increased.

There is now good evidence to indicate that cAMP plays an important role in bringing about aggregation as well as in the control of morphogenetic movement and differentiation throughout the developmental cycle of *Dictyostelium discoideum* [1, 12, 13]. In light of the critical role of cAMP phosphodiesterase in differentiation, it may be possible that the reduction in phosphodiesterase activity, as shown in Figs. 2 and 4, brought about by IdUrd is an important factor in the inhibition of differentiation. However, since differentiation is inhibited, the reduced phosphodiesterase activity could just as well be the result of the blocked differentiation as the cause. Further work is needed to resolve this question.

Another consideration is whether the decrease in cAMP phosphodiesterase activity in cells grown with IdUrd is due to the endogenous inhibitor of cAMP phosphodiesterase reported by Riedel *et al.* [10]. A preliminary check of the cAMP phosphodiesterase inhibitor has indicated that this decrease in diesterase activity was not due to an over abundance of the cAMP phosphodiesterase inhibitor or its premature appearance.

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