The effect of 5-bromodeoxyuridine on cell division and differentiation of preimplantation mouse embryos

By D. R. POLLARD, M. M. BARAN AND R. BACHVAROVA

From the Department of Anatomy, Cornell University Medical College

SUMMARY

Mouse embryos exposed to concentrations of 5-bromodeoxyuridine (BUdR) ranging from 0.01 to 1.0 μg/ml in vitro for two days from the 8-cell stage exhibit a concentration-dependent decrease in the frequency of normal blastocysts and a decrease in average cell number per embryo. A 20-h exposure was adequate to achieve the full BUdR response. Both effects were eliminated in the presence of excess thymidine.

Autoradiographs demonstrated that BUdR[3H] was incorporated into DNA during the first and second day of culture. Thus, BUdR appears to act through incorporation into DNA; and, in this system, cell division is at least as sensitive to BUdR as is differentiation.

INTRODUCTION

The expression of differentiated characteristics can be suppressed in a variety of developing or differentiated cell types (for example, myoblasts, chondrocyte precursors, or chondrocytes) by exposure to appropriate concentrations of the thymidine analogue, 5-bromodeoxyuridine (BUdR). However, other cellular functions such as cell division, viability, and maintenance of enzymes required for general metabolism remain essentially unaffected (for reviews see Holtzer, Weintraub, Mayne & Mochan, 1972; Rutter, Pictet & Morris, 1973; Levitt & Dorfman, 1974). The evidence accumulated from several different systems strongly suggests that this action of BUdR depends upon its incorporation into DNA. Thus, the presence of BUdR in DNA appears to inhibit preferentially the expression of a certain class of genes.

It was of interest to test whether the early mouse embryo would demonstrate the same response to BUdR; namely, the preferential sensitivity to BUdR of differentiated characteristics. Therefore, we studied the effect of BUdR on cell division and on differentiation in mouse embryos cultured in vitro from the 8-cell stage to the blastocyst stage. Some experiments also were performed on embryos exposed to BUdR from the 2-cell stage.

1 Author's present address: Bureau of Surveillance Services, Laboratory Centre for Disease Control, Department of National Health and Welfare, Ottawa, Ontario, K1A 0L2, Canada.

2 Authors' address: Department of Anatomy, Cornell University Medical College, 1300 York Avenue, New York, New York 10021, U.S.A.
MATERIALS AND METHODS

5-Bromodeoxyuridine (B UdR), B UdR[3H], thymidine and deoxycytidine were purchased from Schwartz/Mann, DNase 1 from Worthington Co., and RNase A from Sigma Co.

Eight-cell embryos were recovered from the oviducts of female ICR mice on day 2 of pregnancy (day 0 is the day of finding a vaginal plug), and placed in culture between 16.00 and 17.00 h. The embryos were cultured in Whitten's medium (Hoppe & Pitts, 1973) at 37 °C under 5 % CO₂ in the balanced air. Observations were made each day at approximately 13.00 h. Morphological data from experiments in which more than 70 % of the control embryos developed to the blastocyst stage by day 2 of culture were combined for presentation.

Two-cell embryos were recovered from the oviducts of female ICR mice in the middle of day 1 of pregnancy, and were cultured in tubes in Whitten's medium at 37 °C in the presence of 5 % CO₂, 5 % O₂ and 90 % N₂. Whitten's medium and the B UdR, thymidine and deoxycytidine solutions were made fresh weekly.

Cell counts on embryos were performed according to Tarkowski (1966). Since the control values did not vary significantly from one experiment to another, several experiments were grouped for presentation of the data.

To prepare autoradiographs of embryos exposed to [6-3H]B UdR, the embryos were fixed in Bouin's fixative, embedded in agar in small groups according to the method of Weitlauf & Greenwald (1971), extracted with cold 5 % trichloroacetic acid, and embedded in paraffin. Sections (5–7 μm) from the same sequence were mounted on three separated slides. The slides were hydrated and one slide of each set of three exposed to DNase (0.2 mg/ml in 1 mM Tris, pH 7.4, 3 mM MgCl₂) for 1 h at 37 °C, one slide to RNase (1 mg/ml in water) for 1 h at 37 °C, and one slide to water at room temperature. The slides treated with RNase served as controls to determine the percent of grains removed non-specifically at increased temperature. The slides were then dipped in Kodak NTB-2 emulsion and exposed for 10–20 days.

Four experiments for autoradiography were performed on different days, using 30–50 embryos in the treated groups. The grains over each nuclear cross-section observed on the upper surface of the specimen were counted and the data were standardized to 20 days of exposure.

RESULTS

Morphology of mouse embryos exposed to B UdR from the 8-cell or 2-cell stage

Differentiation in the preimplantation embryo is characterized mainly by the appearance at the blastocyst stage of a single layer of flattened trophoblast cells surrounding the blastocoel cavity. Thus, B UdR effects on differentiation were determined by observations of embryo morphology.
Eight-cell embryos were cultured continuously in various concentrations of BUdR. The results obtained on day 2 of culture, when the highest frequency of blastocysts was observed in all cultures, are presented in Fig. 1. Development of 8-cell embryos was arrested upon exposure to 50 µg/ml of BUdR. At lower doses (0.01–15 µg/ml), abnormal blastocysts were obtained which were characterized by irregular shape, one or more small blastocoel-like cavities, ill-defined inner cell mass cells, and, occasionally, partial necrosis by day 3 or 4 of culture. These characteristics were more pronounced in the abnormal blastocysts than in normal early blastocysts.

The frequency of abnormal blastocysts reached a maximum at 0.1–1.0 µg/ml of BUdR; while the frequency of normal blastocysts increased steadily with decreasing concentrations of BUdR. At 0.01–0.1 µg/ml of BUdR, embryos reached the morula stage during the first day of culture with the same frequency as the control sample. In order to determine whether the abnormal blastocysts observed on day 2 might pass through a normal blastocyst stage first, embryos were monitored every 6 h from day 1 to day 2 of culture. It was observed that the abnormal blastocysts developed directly from normal-looking morulae. Thus, exposure to intermediate concentrations of BUdR impairs differentiation in the early embryo.

The effect of excess thymidine on the inhibition of development by BUdR was investigated. As shown in Table 1, thymidine alone in the range of 1.0–
50-0 μg/ml had no effect on the frequency of blastocysts observed on day 2 of culture. However, a significant decrease in the frequency of blastocysts on day 3 was observed, suggesting that continued exposure to thymidine had an effect on the maintenance of the blastocysts and resulted in a collapse of the blastocoel wall. As is also shown in Table 1, thymidine completely reversed the morphological effects of 0-1 μg/ml of BUdR: on day 2, normal blastocysts were obtained at the same frequency as in the controls, and on day 3 at a frequency closely similar to that observed in thymidine alone, and distinctly higher than that in 0-1 μg/ml of BUdR alone.

Table 1. Effect of thymidine on BUdR inhibition of blastocyst formation by 8-cell embryos

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 2 in culture</th>
<th>Day 3 in culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of embryos</td>
<td>Normal blastocyst (%)</td>
</tr>
<tr>
<td>Control</td>
<td>131</td>
<td>82</td>
</tr>
<tr>
<td>1-0 μg/ml thymidine</td>
<td>109</td>
<td>70</td>
</tr>
<tr>
<td>10-0 μg/ml thymidine</td>
<td>84</td>
<td>76</td>
</tr>
<tr>
<td>50 μg/ml thymidine</td>
<td>151</td>
<td>78</td>
</tr>
<tr>
<td>Control</td>
<td>134*</td>
<td>84*</td>
</tr>
<tr>
<td>0-1 μg/ml BUdR</td>
<td>146*</td>
<td>31*</td>
</tr>
<tr>
<td>Control</td>
<td>50</td>
<td>80</td>
</tr>
<tr>
<td>0-1 μg/ml BUdR + 1-0 μg/ml thymidine</td>
<td>54</td>
<td>80</td>
</tr>
<tr>
<td>0-1 μg/ml BUdR + 5-0 μg/ml thymidine</td>
<td>50</td>
<td>90</td>
</tr>
</tbody>
</table>

* Data of Fig. 1.

In contrast to thymidine, deoxycytidine had only a marginal effect in reversing the inhibition of blastocyst formation (Table 2). The effect is less pronounced when compared with the development of embryos cultured in 0-1 μg/ml of BUdR as shown in Tables 1 and 4.

The effect of continuous exposure of 2-cell embryos to 0-01 μg/ml of BUdR is shown in Table 3. The 2-cell embryos are more sensitive to this concentration of BUdR than the 8-cell embryos. The frequency of blastocysts was only 30 % and of these blastocysts 67 % were abnormal.

Cell number of embryos exposed to BUdR from the 8-cell stage

The effect of BUdR on the increase in cell number per embryo during three days in culture from the 8-cell stage was also tested. As shown in Fig. 2B, little effect of BUdR was observed on the doubling of cell number which occurs in controls on the first day of culture. However, during the second day of culture, the control embryos, on the average, increased their number by 42 cells; while
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embryos exposed to 0.01 μg/ml or 0.1 μg/ml of BUdR increased by only 25 cells. During the third day in culture, while control embryos continued to increase in cell number rapidly, there was only a slight increase in cell number in 0.01 μg/ml of BUdR and a net decrease in cell number in 0.1 μg/ml of BUdR.

For the above data, the results of cell counts for both normal and abnormal blastocysts from the BUdR-treated cultures were combined. Within the 0.1 μg/ml BUdR-treated cultures, the average number of cells per embryo on day 2 of culture for normal blastocysts alone was 49.4 ± 2.5 and for abnormal blastocysts 33.3 ± 2.5.

Table 2. Effect of deoxycytidine on BUdR inhibition of blastocyst formation and of cell number of 8-cell embryos cultured for two days

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Series 1</th>
<th></th>
<th>Series 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of</td>
<td>Normal</td>
<td>No. of</td>
<td>Mean</td>
</tr>
<tr>
<td></td>
<td>embryos</td>
<td>blastocyst (%)</td>
<td>embryos</td>
<td>cell no.</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>—</td>
<td>11</td>
<td>62.4 ± 4.3</td>
</tr>
<tr>
<td>0.5 μg/ml deoxycytidine</td>
<td>54</td>
<td>78</td>
<td>25</td>
<td>68.9 ± 3.2</td>
</tr>
<tr>
<td>0.1 μg/ml BUdR</td>
<td>99</td>
<td>25</td>
<td>23</td>
<td>37.0 ± 2.8</td>
</tr>
<tr>
<td>0.1 μg/ml BUdR + 0.5 μg/ml deoxycytidine</td>
<td>113</td>
<td>49</td>
<td>13</td>
<td>36.1 ± 4.3</td>
</tr>
</tbody>
</table>

Table 3. Morphology of embryos exposed to BUdR for four days from the 2-cell stage

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of embryos</th>
<th>8-cell (%)</th>
<th>Morula (%)</th>
<th>Blastocyst (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>Control</td>
<td>68</td>
<td>4</td>
<td>12</td>
<td>72</td>
</tr>
<tr>
<td>0.01 μg/ml BUdR</td>
<td>36</td>
<td>6</td>
<td>14</td>
<td>11</td>
</tr>
</tbody>
</table>

The effect of BUdR on embryo cell number in the presence of excess thymidine was then examined. As shown in Fig. 2A, thymidine alone exerted a marked effect at all tested concentrations greater than 0.1 μg/ml. These results confirm the observations of Snow (1973) that normal-appearing blastocysts with greatly reduced cell numbers can be obtained by exposure of early mouse embryos to thymidine at concentrations above 0.5 μg/ml. Nevertheless, clear reversal of the BUdR effect on cell number was obtained when embryos were cultured in 0.1 μg/ml of BUdR plus 1.0 μg/ml thymidine (Fig. 2B, line e). The resulting curve is similar to that obtained in 1.0 μg/ml of thymidine alone (Fig. 2A, line c), and significantly different from that in 0.1 μg/ml of BUdR. In another set of experiments, reversal of the effect of 0.01 μg/ml of BUdR by 0.1 μg/ml of
thymidine was attempted. However, in this series less inhibition of the normal increase in cell number by 0-01 μg/ml of BUdR was obtained; while the inhibition by 0-1 μg/ml of BUdR remained similar to that obtained previously. Thus, at 0-01 μg/ml the results were too variable to obtain significant reversal by excess thymidine.

Thymidine was specific in overcoming the inhibitory effects of BUdR on cell number since deoxycytidine had no such activity (Table 2).

Fig. 2. A, Effect of thymidine on cell number ± S.E.M. of 8-cell embryos cultured for three days. Concentrations of thymidine (μg/ml): a, 0; b, 0-1; c, 1-0; d, 10; e, 50.

B, Effect of BUdR or BUdR plus thymidine on cell number ± S.E.M. of 8-cell embryos cultured for three days. Concentrations of BUdR (μg/ml): a, 0; b, 0-01; c, 0-1; d, 1-0. Line e, 0-1 μg/ml of BUdR plus 1-0 μg/ml of thymidine. Points are staggered for purposes of illustration only.

Irreversibility of BUdR effects

To determine whether the effects of BUdR were reversible upon removal of BUdR, embryos were exposed to BUdR for 20 h from the 8-cell to the morula stage and then transferred to fresh medium. As shown in Table 4, embryos which had been pulse-treated with BUdR developed in a manner indistinguishable from those treated continuously with BUdR, both with respect to embryo morphology and cell number. Addition of thymidine to the fresh
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medium had no significant effect. These results demonstrate that exposure to BUdR for approximately one cell cycle is adequate to obtain the full BUdR effect.

Incorporation of BUdR\(^{[3H]}\) into DNA of developing embryos

The incorporation of BUdR\(^{[3H]}\) by embryos treated continuously from the 8-cell stage was measured by autoradiography. Embryos exposed to 0·1 \(\mu g/ml\) of BUdR\(^{[3H]}\) (1·3 mCi/\(\mu m\)) were fixed on day 1 of culture at the morula stage or on day 2 as normal or abnormal blastocysts. Essentially all nuclei observed

Table 4. Effect of a 20-h pulse of BUdR on morphology and cell number of 8-cell embryos cultured for two days

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of embryos</th>
<th>Normal blastocyst (%)</th>
<th>Abnormal blastocyst (%)</th>
<th>No. of embryos</th>
<th>Mean cell no.</th>
<th>S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 h in 0·1 (\mu g/ml) of BUdR followed by 24 h in:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No additions</td>
<td>70</td>
<td>44</td>
<td>36</td>
<td>20</td>
<td>41·0</td>
<td>2·2</td>
</tr>
<tr>
<td>0·1 (\mu g/ml) BUdR</td>
<td>57</td>
<td>39</td>
<td>42</td>
<td>12</td>
<td>40·4</td>
<td>2·6</td>
</tr>
<tr>
<td>0·1 (\mu g/ml) thymidine</td>
<td>74</td>
<td>35</td>
<td>43</td>
<td>17</td>
<td>44·2</td>
<td>1·5</td>
</tr>
</tbody>
</table>

Table 5. Autoradiographic study of the incorporation of BUdR\(^{[3H]}\) (0·1 \(\mu g/ml\)) by 8-cell embryos cultured for one or two days

<table>
<thead>
<tr>
<th>Fixed on</th>
<th>No. of nuclear cross-sections counted</th>
<th>Mean grains per nuclear cross-section</th>
<th>S.E.M.</th>
<th>Grains removed in RNase (%)</th>
<th>Grains removed in DNase (%)</th>
<th>Grains per nuclear cross-section in DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>197</td>
<td>55</td>
<td>2</td>
<td>13</td>
<td>78</td>
<td>36</td>
</tr>
<tr>
<td>Day 2</td>
<td>82</td>
<td>118</td>
<td>6</td>
<td>5</td>
<td>59</td>
<td>64</td>
</tr>
</tbody>
</table>

were labelled, and the number of grains per nuclear cross-section specifically removed by DNase calculated. As shown in Table 5, significant incorporation took place during the first 20 h of exposure. In fact, the relative grain counts for day 1 and day 2 indicate that approximately 0·7 times as much labelled precursor was incorporated by one cell during one cell cycle in the first 20 h as during one cell cycle in the next 24 h (total radioactivity per nucleus in day-2 embryos should be 1·2 times that of day 1 embryos given the observed increases in cell number, if the specific activity of the thymidine-BUdR\(^{[3H]}\) pool remained constant throughout the experiment). Similar results were obtained in experiments using BUdR at the same concentration but ten times lower specific activity.
The results presented here demonstrate that 8-cell mouse embryos are more sensitive to BUdR than systems studied previously in which at least 10-fold higher concentrations have been used to obtain consistent inhibitory effects on differentiation. Our observations on the BUdR-sensitivity as measured by development to the blastocyst stage of 2-cell and 8-cell embryos are in accord with those of Garner (1974).

Our main aim was to define the relative sensitivity to BUdR of differentiation and of cell division in the mouse embryo system. In this case differentiation refers to the activities of developing trophoblast cells which contribute to the formation of the blastocyst, such as the transfer of materials from the external medium to the blastocoel and the formation of tight junctions (Nadijcka & Hillman, 1974). Our study of developing 8-cell embryos in several concentrations of BUdR has enabled us to observe partial effects of BUdR on both morphological development and on the increase in cell number. In embryos treated continuously from the 8-cell stage, the lowest concentration of BUdR (0.01 μg/ml) which had a significant effect on the frequency of appearance of normal blastocysts also significantly decreased the cell number. However, normal blastocysts with decreased cell number were observed. At 0.1 μg/ml, under the various conditions used, effects on morphology and on cell number occurred in parallel. At higher concentrations, initiation of the blastocoel was eliminated only when cell number was reduced to 20 cells or less. These results suggest that cell division is at least as sensitive to BUdR as differentiation.

The exposure of early sea urchin embryos to BUdR gives results which in some respects are similar to ours: a decrease in the rate of cell division and arrest as blastulae with abnormal blastocoels (Gontcharoff & Mazia, 1967; Tencer & Brachet, 1973).

High concentrations of BUdR cause some inhibition of the normal increase in cell number in most systems, but a lower concentration can usually be found which inhibits cell differentiation with essentially no effect on cell number (Holtzer et al. 1972; Levitt & Dorfman, 1974). However, Ingram et al. (1974), using chick erythropoietic cells at the concentration required for complete inhibition of haemoglobin synthesis, observed a decrease in the normal cell number, apparently due to cell death. In HeLa cells a significant increase in the generation time and a decrease in cell viability are seen in concentrations of BUdR from 2.5 to 10 μg/ml (Kajiwara & Mueller, 1964). In our system it is most likely that the effect of BUdR over the first two days in culture is mainly to slow the cell cycle, followed by some cell death on day 3.

Our evidence suggests that the mechanism of BUdR depression of the increase in cell number is different from that of thymidine, since BUdR acts at lower concentrations and the BUdR effect is reversed by thymidine. Thymidine presumably inhibits enzymatic activities required for DNA synthesis. The effect
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of low concentrations of BUdR on morphology is not simply a secondary effect of the decrease from the normal cell number since thymidine decreases cell number to the same extent with no effect on the size or form of the blastocoel.

The inability of BUdR-treated embryos to differentiate fully to the blastocyst stage could be a consequence of altered membrane properties. Changes in cellular adhesiveness to substrates or to other cells are commonly observed in BUdR-treated cells (for example, Abbott & Holtzer, 1968). Presumably normal intercellular contacts are required for formation of a normal blastocyst.

In early mouse embryos, as in other systems, the evidence is consistent with a mechanism of action of BUdR which requires its incorporation into DNA. The following points support this view. First, the presence of excess thymidine completely reverses the effects of BUdR on morphology and on cell number. Golbus & Epstein (1974) also demonstrated that thymidine reverses the BUdR effect on morphology of early mouse embryos; and, in addition, that thymidine reversal acts at some intracellular site.

Secondly, 8-cell embryos express the full BUdR effect during the second day of culture, even when exposed to BUdR only during the first day of culture. Golbus & Epstein (1974) found that when morulae are exposed to 30 µg/ml of BUdR for more than 2 h the effects cannot be reversed, and showed that BUdR leaves the cells rapidly upon exposure to fresh medium. Unfortunately, in this system it is not possible to pulse with BUdR during S phase only since it has been shown that in 8-cell embryos S phase is relatively long and asynchronous (Barlow, Owen & Graham, 1972). However, it should be noted that lack of reversibility of the effects of a BUdR pulse has been observed in several systems early in their developmental pathway, such as potential chondrocytes derived from chick somites (Abbott, Mayne & Holtzer, 1972) or limb-buds (Levitt & Dorfman, 1972), or developing neural retina (Morris, 1973).

Thirdly, our autoradiographic studies show that BUdR is incorporated into nuclear DNA during the first and the second day of culture of 8-cell embryos. It is apparently the BUdR incorporated during the first day which is responsible for the subsequent effects on development.

Thus, in the early embryo as in other systems, the effects of BUdR may be interpreted as due to faulty transcription or regulation of transcription of BUdR-substituted DNA.

Our main conclusion is that BUdR has different effects on the preimplantation embryo from those observed on differentiating tissues from post-gastrula stages, although it appears to act by a common mechanism in both cases. In the early mouse embryo no differential BUdR-sensitivity of differentiated functions is observed. One possible interpretation of these results is that BUdR in the early embryo inhibits a variety of enzymes required for general metabolism and that the effect on differentiation is a secondary result of this inhibition. Another interpretation is that both cell division and differentiation in early mouse embryos depends on a transcription process which has some common step.
sensitive to BUdR. In later stages the BUdR-sensitive step in the transcription of 'essential' genes may be by-passed or modified to become more BUdR resistant.

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REFERENCES


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