The cellular effect of 5-bromodeoxyuridine on the mammalian embryo

by JOHN BANNIGAN\textsuperscript{1} AND JAN LANGMAN\textsuperscript{2}

From the Department of Anatomy, University of Virginia

SUMMARY

It is well known that 5-bromodeoxyuridine (BUdR) when injected into pregnant animals may cause exencephaly, cleft palate, and limb abnormalities. Similarly, it is well established that the drug when added to a culture medium may prevent differentiation of embryonic cell systems without affecting cell division or cell viability. The goal of our experiments was to examine whether the congenital malformations resulting from BUdR treatment were due to lack of differentiation of certain cell lines or were due to other mechanisms. The effects of BUdR on proliferating and differentiating cells in the 12-day mouse embryo were therefore examined and special attention was given to the proliferating cells of the rhombic lip which give rise to the Purkinje cells.

When the embryos were treated with BUdR the mitotic index of the neuroepithelium of the rhombic lip doubled in value 3 h after treatment and remained high until 24 h later. By using the colchicine index it was calculated that the mitotic duration in the BUdR-treated embryos lasted at least 2 h and that in the control embryos less than 1 h. When the cell generation time in the BUdR treated animals was calculated the length of the S-phase was increased by about 50\%. It was thus concluded that BUdR caused an increase in the duration of the S-phase and mitosis, together making the cell cycle 5 h longer than normal.

Eighteen hours after treatment many neuroepithelial cells became degenerative. By radio-autography it was demonstrated that the degenerating cells were in their second DNA-synthetic phase following BUdR injection and that cells which incorporated BUdR and were differentiating into neurons were not affected. By injecting [\textsuperscript{3}H]BUdR it was found that many cells which incorporated the analogue were able to leave the proliferative population after their first cell division. They migrated to the periphery where they developed into apparently normal Purkinje cells. The additive effects of cell death and retardation of the cell cycle caused a 15\% deficit of Purkinje cells in the postnatal cerebellum but the BUdR did not interfere with their differentiation. Thus, contrary to the BUdR effect on cultures of embryonic cells, \textit{in vivo} the drug causes cell death and a delay in the cell cycle time. Our experiments therefore seem to indicate that the congenital malformations caused by BUdR in the mammalian embryo are caused by cell death and growth retardation rather than by interference with the process of differentiation.

INTRODUCTION

The pyrimidine analogue 5-bromodeoxyuridine (BUdR) is incorporated as bromouracil (BU) in place of thymine in replicating DNA (Skalko & Packard, 1974). The effects of this substitution have been studied in a variety of differen-

\textsuperscript{1} Author's address: Department of Anatomy, University College, Dublin 2, Ireland.

\textsuperscript{2} Author's address: Department of Anatomy, University of Virginia, School of Medicine, Charlottesville, Virginia 22908, U.S.A.
tiating cell systems in culture (Coleman, Coleman, Kankel & Werner, 1970; Bischoff, 1971; Wilt & Anderson, 1972; Wishnow, Feist & Glowolla, 1974), and in all cases have been found to block terminal differentiation without causing disturbances in cell division or basic cell function (Wilt & Anderson, 1972). In the most thoroughly studied system cultured myoblasts were found to divide in the presence of BUdR but not to fuse to form myotubes (Stockdale, Okazaki, Nameroff & Holtzer, 1964). Holtzer, Weintraub, Mayne & Mochan (1972) stated that the production of contractile proteins needed for myofibrils was suppressed by BUdR but not the production of proteins needed for the cell surface or the mitotic apparatus. Apparently, BUdR affects only those special cell products characteristic of the differentiated state.

In vivo experiments with BUdR to test the influence of the drug on the developing embryo caused many different types of abnormalities. Neural tube defects, dwarfism and polydactyly followed treatment early during development (DiPaolo, 1964; Ruffolo & Ferm, 1965). Cleft palate and limb abnormalities have been observed following treatment later in development (Murphy, 1965; Skalko, Packard, Schwendimann & Raggio, 1971). Hence, in vitro experiments indicate that BUdR can block the terminal differentiation of cells, whereas in vivo experiments have shown that the drug can cause various malformations.

To examine whether the congenital malformations caused by treatment with BUdR were due to a lack of cellular differentiation, Webster, Shimada & Langman (1974) gave BUdR to pregnant mice on day 15 and studied the cellular effects on the neuroepithelium of the telencephalon at various time intervals after injection. A gradual increase in the mitotic index of the neuroepithelium was noted beginning at 2 h after treatment, reaching a maximum at 12 h and declining towards normal values by 24 h. No cell death was seen until 24 h after treatment and by 48 h the degenerating particles had been cleared. Since non-radioactive BUdR was used in these experiments, it was impossible to determine which cells had incorporated BUdR and it could not be determined whether any cells had been blocked in their terminal differentiation. Furthermore, it was not possible to determine whether cells blocked in their differentiation were dying or whether the dying cells were those which had re-entered the DNA-synthetic phase.

To examine in more detail the cellular effects of BUdR on the mammalian embryo in vivo, tritiated BUdR was injected in 12-day pregnant mice and the embryos sacrificed at various time intervals. Particular attention was given to the effect of the drug on mitotic activity, the cell cycle of proliferating populations and the fate of differentiating cells. The differentiating Purkinje cells were selected as a model for this study.
MATERIALS AND METHODS

Young DUB/ICR mice (Flow Laboratories, Dublin, Virginia) were maintained on a 12 h light-dark cycle and allowed free access to food and water. They were mated for 4 h during the light phase and the finding of a vaginal plug marked day 1 of pregnancy. BUDR (Sigma Chemical Company) was administered on day 12. Since in the mouse Purkinje cells are formed in the rhombic lip on days 11, 12 and 13, injection of the drug on day 12 of development should affect a significant number of Purkinje cells whose parent cells were in the DNA-synthetic phase at the time of treatment (Andreoli, Rodier & Langman, 1973).

To examine the cellular effects of BUDR the drug was given i.p. at a dose of 500 mg/kg dissolved in 0.6 ml normal saline. The fetuses were recovered at hourly intervals up to 18 h after injection and subsequently at 12 or 24 h intervals until day 19 of pregnancy. Postnatal animals were recovered at birth and at days 1, 5, 9, 16 and 25. Control animals treated with 0.6 ml normal saline were sacrificed at the same times as the experimental animals. The tissues were fixed in modified Karnovsky fixative (Karnovsky, 1965) for 24 h. Following post-fixation for 1 h in osmium tetroxide and dehydration in a graded ethanol series, the material was embedded in araldite and sectioned at 1 μm on a Sorvall Porter-Blum MT2 ultramicrotome. Sagittal sections were made through the rhombencephalic and mesencephalic regions and stained with toluidine blue.

To study the effects of BUDR on the cell generation time the mice were treated as above and immediately after were injected with 12 μCi/g tritiated thymidine (specific activity 20 Ci/mM, New England Nuclear). The embryos were recovered at hourly intervals up to 18 h after treatment. Sections (1 μm) were made and placed on slides covered with egg albumin, dipped in Ilford L4 emulsion and exposed for 4 weeks. After development they were stained with azure II. The different phases of the cell cycle were calculated by plotting the percentage of labelled metaphases against the time after treatment with BUDR.

The duration of mitosis was studied by using the ‘Mitotic index’ method described by Leblond & Walker (1956) and Messier & Leblond (1960). BUDR was given to mice on day 12 of pregnancy as in previous experiments and followed by colchicine 0.5 mg/kg subcutaneously 4 h later. The animals were sacrificed 1, 2 and 3 h after colchicine injection, the embryos sectioned and stained and the number of arrested metaphases counted.

To identify and count the number of cells incorporating BUDR and to trace their course during the following days 500 mg/kg BUDR followed immediately by 15 μCi/g tritiated BUDR (sp. act. 23.7 Ci/mM; New England Nuclear) was injected on day 12 of gestation. Sections were prepared for autoradiography as above except that Kodak NTB3 emulsion was used instead of Ilford L4. The sections were exposed for 5–12 weeks. To examine whether the dying and degenerating cells seen by Webster et al. (1974) were cells blocked in their
Fig. 1. Section through the rhombic lip of a 12-day mouse embryo 6 h after injection with saline. Note the neuroepithelial (NE) or matrix layer and the occasional neuroblast at the periphery adjacent to the mesenchyme. M, mitotic zone; S, DNA-synthetic zone; arrows indicate neuroblasts.

Fig. 2. Similar section as in Fig. 1 of a 12-day mouse embryo treated with BUdR 6 h before sacrifice. Note the increased number of mitotic figures compared with Fig. 1.

differentiation or were cells which had started again with DNA synthesis, two pregnant mice were given 500 mg/kg BUdR on day 12 of gestation and 12 μCi/g tritiated thymidine 15 h later. These animals were sacrificed 3 h after injection of the tritiated thymidine, and the embryos fixed, sectioned and prepared for autoradiography.

To study the effects of BUdR treatment on the pattern of dendritic branching of the Purkinje cell a number of brains were removed from 25-day-old treated and control animals and prepared for Golgi–Cox staining according to the method described by Ramon-Moliner (1970). This material was mounted in celloidin and sectioned at 40–60 μm on a sledge microtome.
Table 1. Mitotic indices of neuroepithelial cells after treatment with BUdR or saline

<table>
<thead>
<tr>
<th>Hours after treatment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>6</th>
<th>10</th>
<th>12</th>
<th>13</th>
<th>15</th>
<th>18</th>
<th>24</th>
<th>48</th>
</tr>
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<tbody>
<tr>
<td>Mitotic index</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>after BUdR</td>
<td>2.9</td>
<td>4.2</td>
<td>5.3</td>
<td>5.8</td>
<td>5.7</td>
<td>5.4</td>
<td>5.2</td>
<td>5.2</td>
<td>4.9</td>
<td>2.8</td>
<td>1.6</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>0.3</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.4</td>
<td>0.4</td>
<td>0.7</td>
<td>0.3</td>
<td>0.6</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Mitotic index</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>after saline</td>
<td></td>
<td></td>
<td></td>
<td>3.3</td>
<td></td>
<td></td>
<td>3.3</td>
<td>2.8</td>
<td>2.9</td>
<td>2.3</td>
<td>1.9</td>
</tr>
<tr>
<td>S.E.M.</td>
<td></td>
<td></td>
<td></td>
<td>0.2</td>
<td></td>
<td></td>
<td>0.05</td>
<td>0.3</td>
<td>0.2</td>
<td>0.03</td>
<td>0.3</td>
</tr>
</tbody>
</table>

The number of embryos from which counts were derived ranged between 6 and 16 in the BUdR-treated group and between 5 and 9 in the control group.

RESULTS

When 12-day-old embryos were treated with BUdR, the first visible effect of the drug was an increase in the number of mitotic neuroepithelial cells along the lumen of the neural tube (Figs. 1 and 2). This increase started 2 h after treatment and was almost twice that of the controls at 3 h (Table 1). The mitotic index remained at this high level until 15 h after treatment and then declined gradually towards normal values, which were reached by 24 h (Table 1). No distinct morphological abnormalities could be detected in the mitotic figures, although they were strikingly clear (Figs. 1 and 2). It was concluded that BUdR either caused difficulty in the separation of the chromosomes during the mitotic process, or greatly accelerated the DNA-synthetic phase of the neuroepithelial population, thus explaining the higher than normal number of mitotic figures at the lumen.

When the cell cycle curves in controls and experimental animals were analyzed (Fig. 3), it became evident that the cell generation time of the neuroepithelial cells in normal 12-day mouse embryos was 10 h, and in BUdR-treated embryos nearly 15 h. Hence, the drug caused a lengthening of the total cell generation time of nearly 5 h. By taking the duration of the DNA-synthetic phase (S-phase) as the interval between 50% labelling on the first ascending limb and 50% labelling on the first descending limb, the S-phase was found lengthened by about 3.5 h and the duration of $G_2+M+G_1$ phases by 1.5 h. It may thus be concluded that the increased mitotic index is not caused by an acceleration of the S-phase. On the contrary BUdR apparently caused a delay in the DNA-synthetic process, which in turn would cause a decrease in the mitotic index, instead of an increase.

To determine the duration of mitosis independent from the cell cycle curve, 12-day pregnant mice were treated with colchicine and the number of arrested metaphases counted at 1, 2 and 3 h after treatment. Table 2 shows the values of the colchicine index as found in control and BUdR-treated embryos, with
Fig. 3. Cell generation curves of the neuroepithelial cells of 12-day control and BUdR-treated mouse embryos. Note the differences in the duration of the S-phase and the total generation time.

Table 2. Mitotic indices and mitotic durations of neuroepithelium in BUdR- and saline-treated embryos after injection of colchicine

<table>
<thead>
<tr>
<th>Hours after colchicine*</th>
<th>Colchicine index</th>
<th>Mitotic duration (hours)†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BUdR</td>
<td>Saline</td>
</tr>
<tr>
<td>1</td>
<td>3·3 ± 0·8</td>
<td>4·1 ± 0·8</td>
</tr>
<tr>
<td>2</td>
<td>5·4 ± 1·3</td>
<td>6·6 ± 1·7</td>
</tr>
<tr>
<td>3</td>
<td>6·6 ± 0·7</td>
<td>8·9 ± 0·5</td>
</tr>
</tbody>
</table>

* Colchicine was injected 4 h after treatment with BUdR or saline.
† Mitotic duration was calculated from the formula $T_m = \frac{I_m}{I_c} \times T_c$, where $T_m =$ duration of mitosis, $I_m =$ mitotic index in material not treated with colchicine, $I_c =$ mitotic index of material treated with colchicine and $T_c =$ time in hours after colchicine injection (Hooper, 1961).

the corresponding values for the durations of mitosis. It is seen from these calculations that the average duration of mitosis in the saline-treated embryos was 0·95 h and in the BUdR-treated ones more than 2 h. Therefore, between 2 and 18 h after treatment with BUdR, the average duration of mitosis was at least twice as long as in the controls. Furthermore, the increase in $G_2 + M + G_1$ found in the cell generation curve (Fig. 3) in all probability was due to an increased duration of mitosis and not to an interference with either the $G_1$- or $G_2$-phase.

When the embryos were treated with tritiated BUdR and sacrificed 12 h later,
most of the labelled cells were found in the DNA-synthetic zone. Since none of the labelled cells had migrated beyond the DNA-synthetic zone, it was impossible to determine whether any of them were differentiating Purkinje cells, or had returned to the proliferative pool to start DNA synthesis for the second time after treatment.

At 18 h after treatment (that is during the second cell cycle after treatment with BUdR) many cells with dark staining nuclei were seen in the neuroepithelial zone throughout the developing CNS (Fig. 4), but damage appeared most severe in the roof of the mesencephalon. Degenerating cells were rarely seen in the neuroblast layer. Some large cells, evidently phagocytic in function, were also seen in the DNA-synthetic zone (Fig. 4).

To examine whether the dying cells were neuroblasts blocked in their differentiation or were cells which had re-entered DNA synthesis for the second time,
Fig. 6. Cerebellum on day 19 of gestation following injection of $[^3]$H]BUdR on day 12. Note the large pale cells (arrows) considered to be immature Purkinje cells. Some of these cells are labelled. EGL, external granular layer.

Fig. 7. Cerebellar cortex on postnatal day 9. About 15% of Purkinje cells are labelled (arrows) following treatment with $[^3]$H]BUdR on day 12 of gestation. The labelled cells are indistinguishable from unlabelled Purkinje cells and from Purkinje cells in control animals. P, pial surface; PCL, the layer of Purkinje cells.
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Table 3. Average number of Purkinje cells seen in sagittal sections of cerebellar vermis on postnatal day 16

<table>
<thead>
<tr>
<th>Means* of counts of Purkinje cells in sagittal sections of vermis of single animals</th>
<th>Overall mean and s.d.</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUdR</td>
<td>201</td>
</tr>
<tr>
<td>Saline</td>
<td>234</td>
</tr>
</tbody>
</table>

* Each figure represents the mean of counts of at least 24 serial sections from a single animal. Two litters are represented in each treatment group.

12-day pregnant animals were injected with tritiated thymidine 15 h after BUdR treatment and sacrificed 3 h later. When the radioautographs of the embryos were studied, almost all degenerating cells and phagocytosed particles were labelled, indicating that the dying cells were in their second S-phase or had just finished their second replication phase after BUdR treatment.

Twenty-four hours after treatment with tritiated BUdR a number of labelled cells had migrated beyond the neuroepithelial zone into the neuroblast layer and were probably differentiating into neurons. Most of the labelled cells, however, had remained in the proliferative population to synthesize DNA for the next cell cycle. At 48 h after treatment with tritiated BUdR the picture was essentially the same, but a few degenerating cells could still be seen (Fig. 5). Seventy-two hours after treatment the neuroepithelium appeared normal. The phagocytes and degenerating particles had disappeared, and labelled cells were seen in the neuroblast zone as well as in the DNA synthetic zone. From these experiments it may be concluded that few, if any, of the differentiating neuroblasts had died and that 72 h after treatment the tissue had recovered from the cell damage.

When fetuses were recovered on the nineteenth day of development, their weight was approximately 20 % less than that of the controls. In sagittal sections through the cerebellum, however, no obvious differences could be detected between the treated and control animals. At this stage of development the cerebellum has four well-marked fissures and an external granular layer of three to four cell layers in width. Immediately beneath it were seen some large, pale nucleated cells, considered to be Purkinje cells (Fig. 6). A number of them were labelled following treatment with tritiated BUdR on day 12 of development, indicating that a substantial number of Purkinje cells had incorporated the radioactive BUdR. Most of the grains were concentrated at the periphery of the nucleus and around the nucleolus, that is, in regions where the chromatin is concentrated, thus supporting the notion that the BU is bound to the DNA. No morphological differences were noted between labelled and unlabelled maturing Purkinje cells, indicating that BUdR had failed to block their differentiation.
The number of Purkinje cells in the cerebellum on postnatal day 16 was estimated in the following way. Counts were made of the total number of Purkinje cells in serial 1 µm sagittal sections of the entire vermis in treated and control embryos. The averages of these counts are presented in Table 3 where it is seen that treatment with BUdR caused a 16% reduction in the Purkinje cell population. In similar animals treated with tritiated BUdR 15% of the Purkinje cell population was labelled (Fig. 7). In addition examination of the Golgi–Cox material failed to reveal any differences in Purkinje cell morphology or the pattern and extent of dendritic branching between treated and controls. These observations indicate that the number of Purkinje cells was reduced, but that the ones which had incorporated BUdR had differentiated according to their normal pattern.

DISCUSSION

Bromouracil is identical to thymine except for the presence of a bromine atom at the 5-position normally occupied by a methyl group. In addition, the bromine and methyl group have the same van der Waals’ radius (Skalko & Packard, 1974). Because of these similarities BUdR is probably handled by the same enzyme systems as thymine and can be incorporated into DNA without great difficulty. The bromine atom, however, is considerably heavier than the methyl group and more electronegative (Freese, 1959), while in addition Lin, Lin & Riggs (1976) have found that BU-DNA binds more tightly to histones than normal DNA. These differences in physical properties are probably sufficient to interfere with the uncoiling of the DNA strands during replication and with separation of the chromosomes during cell division, thus accounting for the prolonged S- and M-phases found in our experiments.

From the cell cycle curves it is evident that 15 h after the injection of BUdR the neuroepithelial (matrix) cells, which had incorporated the analogue, had passed through mitosis. Subsequently, the daughter cells either stayed in the matrix population to start with their second DNA synthesis or proceeded to differentiate as neuroblasts (Langman, Guerrant & Freeman, 1966). Thus, the primitive labelled neurons seen 24 h after injection with tritiated BUdR must have left the proliferative population after the first cell division, and were apparently able to develop as morphologically normal Purkinje cells as judged by light microscopy and Golgi–Cox staining. A preliminary study with electron microscopy revealed no changes in the Purkinje cell body. However, this study has not yet been extended to the dendritic tree and its synapses. Hence, although our study clearly showed that BUdR affects DNA synthesis, duration of cell division and cell viability, it did not show that the drug interfered with cellular differentiation as described for many in vitro experiments. The most probable explanation for this difference is that in the in vitro experiments the cells were continuously exposed to BUdR allowing almost complete substitution of thymine sites, whereas in vivo the drug is present for only a short time and
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probably does not replace more than a limited number of thymine sites (Packard, Skalko & Menzies, 1974). Indeed Skalko & Packard (1974) and Packard et al. (1974) showed that following intraperitoneal injection of tritiated BUdR maximum incorporation into DNA of the embryo does not occur until 2 h after injection. It is therefore likely that the DNA synthesizing cells incorporate only a small amount of BU. A second and related explanation is provided by the finding of Kasupski & Mukherjee (1977) that the expression of certain genes in synchronized mouse L cells can be prevented by BUdR only during specific periods within the S-phase. Exposure to BUdR in the first hour of S greatly reduced the ability of the cells to produce glucose-6-phosphate dehydrogenase (G6PD) and alcohol dehydrogenase (ADH), but exposure in the third hour of S reduced the production of acid phosphatase, but not that of G6PD or ADH. Thus it appears that genes are replicated in an orderly sequence during the S-phase. In unsynchronized proliferating populations, however, a certain number of cells may incorporate the analogue during critical phases of DNA synthesis affecting genes essential for differentiation, while other cells may incorporate the analogue without affecting essential genes.

The occurrence of cell death in vivo is another difference between the effects of BUdR in vivo and in cell culture. It is likely that this difference has little to do with the different degrees of thymine substitution. When Garner (1974) and Pollard, Baran & Bachvarova (1976) exposed mouse embryos at the stages of two and eight cells to BUdR in culture for up to 3½ days they found no effects on differentiation but on the contrary noticed considerable cell death. Thus it appears that fundamental differences in cell physiology exist between the cells of an intact embryo and cells attached to a substrate in a culture dish. Garner (1974) has pointed out that the cytotoxic effect of BUdR may be due to the ability of any nucleoside, when present in excess, to inhibit the entry of heterologous nucleosides into the nucleotide precursor pool (Steck, Nokata & Bader, 1969). This mechanism probably does not operate in our experiments, since the cells had no difficulty incorporating tritiated thymidine either in the first or second S-phase following BUdR injection.

At postnatal day 16 the number of Purkinje cells was significantly reduced. This is clearly caused by the additive effects of BUdR on cell death and the slowing of the cell cycle for a 24 h period. These two effects possibly are responsible for the congenital malformations resulting from BUdR treatment during early stages of development. Thus, excencephaly in the mouse following treatment on day 9 of development (Ruffolo & Ferm, 1965) is in all likelihood caused by excessive cell death and a subsequent cell deficit during the time of closure of the neural groove. The occurrence of cleft palate can probably be explained by similar mechanisms.

The syndactyly caused by treatment with BUdR (Skalko et al. 1971) might be explained in the following way. Fallon (1972) showed that if normal cell death occurring in the interdigital mesoderm of the chick was prevented with
Janus Green B syndactyly resulted. Assuming that the mesodermal cells which degenerate to free the digits are programmed to die at a specific time, the lengthening of the cell generation time by the BUdR may cause them to die later than normal or not at all, thus causing syndactyly.

The occurrence of polydactyly is even more difficult to explain. Stark & Searls (1973) have shown that the apical ectodermal ridge (AER) exerts an inductive influence on the underlying mesoderm to form the digits and that the inductive capability of the AER lasts for only a restricted period of time. It may be that BUdR, by lengthening the cell cycle in the AER, prolongs the period of its competence as an inducer and thus causes polydactyly.

Hence, our experiments indicate that BUdR does not cause fetal malformations by inhibiting or altering differentiation of embryonic cells, but rather by its growth retarding and cytotoxic effects.

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