Incorporation of 5-bromo-2′-deoxyuridine into mesenchymal limb-bud cells destined to die: relationship to polydactyly induction in rats

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SUMMARY

The thymidine analogue, 5-bromo-2′-deoxyuridine (BUdR), given at the proper dose and time to pregnant rats produces preaxial hindlimb polydactyly in a high proportion of near term foetuses. The lack of physiological cell death in an area of preaxial mesenchyme known as the foyer primaire preaxial (fpp) is thought to be important in the pathogenesis of this defect. This study addresses the question of whether BUdR’s well-known antidifferentiative effects, which are due in some way to drug incorporation into DNA, are operative in this in vivo system. The dose and temporal response of BUdR for the induction of preaxial polydactyly inversely parallels the frequency of embryonic hindlimbs with an fpp. Incorporation of BUdR into degenerative fragments within the fpp of these treated limbs is demonstrated with indirect immunofluorescence using an antibody to bromouridine. Hindlimbs exposed to a threshold dose of BUdR at the optimal time for producing polydactyly have incorporated the drug into degenerative fragments within the fpp. This suggests that a higher, teratogenic dose of BUdR might likewise be incorporated. The resulting higher level of incorporation presumably alters the normal course of terminal differentiation for these cells originally destined to die.

Teratogenic doses of BUdR injected at later than the optimal time are also incorporated into dead cell fragments within the fpp, suggesting that presumptive dead cells have additional rounds of DNA synthesis which are BUdR-insensitive. Approximately 12 h prior to overt death presumptive fpp cells no longer incorporate the drug.

Results reported support the hypothesis that incorporation of teratogenic levels of BUdR prevent cell death in the fpp. The extra cells are thought to contribute directly or indirectly to the added digit. Contrary to other views, it is suggested that BUdR-induced teratogenesis can be a result of the drug’s antidifferentiative effects on specific, ‘sensitive’, populations of cells.

INTRODUCTION

The thymidine analog 5-bromo-2′-deoxyuridine (BUdR) is well known for its ability to block differentiation in cell and organ culture (reviews by Wilt & Anderson, 1972; Rutter, Picket & Morris, 1973; Goz, 1977). It will do this at concentrations which exert little effect on the rates of total RNA, DNA, or

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proteinsynthesis, or the general viability of cells. Effects of BUdR on developing embryonic tissues are not always reversible, whereas inhibition of differentiative functions in cultured tumour cells is generally completely reversible (Goz, 1977). In all cases, however, the drug is readily incorporated in place of thymidine during DNA replication and, indeed, the presence of BUdR during a single period of DNA synthesis is usually required for the specific inhibition. Although many details need to be resolved, it is for the most part agreed that incorporation of BUdR into nuclear DNA is required to affect a specific cell function (Goz, 1977).

Administration of BUdR to pregnant rodents results in defects in a variety of organ systems including palate and facial structures (Murphy, 1965; Shah & MacKay, 1978; Skalko, Packard, Schwendiman & Raggio, 1971), neural tube (Ruffolo & Ferm, 1965; Webster, Shimada & Langman, 1974) and limbs. Preaxial hindlimb polydactyly is a common limb defect encountered in all rodent species studied (mice, DiPaolo, 1964; Skalko et al. 1971; hamsters, Ruffolo & Ferm, 1965; rats, Scott, 1981). The pathogenesis of BUdR-induced polydactyly in rats was previously examined and found to differ from other drugs which cause the same defect in that drug-induced mesenchymal cell death and delayed appearance of physiological cell death in the apical ectodermal ridge were not found (Scott, 1981). The common feature BUdR shares with other polydactyly situations so far examined, both drug-induced (Scott, Ritter & Wilson, 1977, 1980; Klein, Scott & Wilson, 1981) and genetic (Milaire, 1976; Rooze, 1977; Knudsen & Kochhar, 1981), is the absence of a zone of physiological cell death in the preaxial mesenchyme. This zone, termed the foyer primaire pre-axial (fpp) by Milaire (1976), is found early in limb development in mesenchyme approximately 10–20 cell diameters in from the preaxial extent of the AER (see Scott, 1981; fig. 2). The fpp is thought to be important in controlling the size and number of preaxial digits in the three species of rodents studied so far (Milaire, 1976).

These and other observations led to the speculation that incorporation of BUdR into prospective fpp cells in some way prevents their death and thereby provides additional mesenchyme to the developing hindlimb which is subsequently converted into an extra preaxial digit (Scott, 1981). In contrast, Bannigan & Langman (1979) working with the embryonic neuroepithelium suggested that congenital malformations caused by administration of BUdR to the pregnant rodent are not the result of interference with the processes of differentiation, but instead are caused by drug-induced cell death and disturbances of the cell cycle.

This matter of whether incorporation of BUdR affects the differentiation of cells in vivo is of considerable importance to delineating the drug's chemical and cellular mechanism of action. The first critical point to the hypothesis explaining BUdR-induced polydactyly presented above requires the demonstration that the drug is incorporated into cells normally destined to die in the
BUdR in cells destined to die

fpp. Since these cells do not die in hindlimbs exposed to a polydactyly inducing dose of the drug, we have used dosing regimes that do not alter the appearance of the fpp. An antibody to bromouridine was then used to probe histological sections of treated limbs for incorporation of the drug into dead fragments within the fpp.

**MATERIALS AND METHODS**

Rats of the Royalhart stock, derived from a Wistar strain, were maintained on a 12 h light-dark cycle and allowed free access to food and water in hanging metal cages. Pregnancy was dated by counting time 0 as 09.00 h of the morning on which sperm were found in vaginal smears of nulliparous females caged overnight with males of the same strain. BUdR (Sigma) was suspended in 0.3% carboxymethyl-cellulose (in 0.9% NaCl) by grinding in a teflon-glass homogenizer immediately prior to use. Females were injected intraperitoneally with 100, 250, 500 or 750 mg/kg at 4 h intervals between 288 and 300 h of pregnancy. Foetuses removed by Caesarean section on day 20 of gestation were fixed in 95% ethanol preparatory to double staining of cartilage and bone by the method of Watson (1977).

Additional females were treated but their embryos were removed at 324 h (21.00 h, day 13) of development, an optimal time for seeing the fpp in control hindlimb buds. Immediately after removal the embryos were dissected free of their membranes and placed in a 0.005% solution of Nile Blue A in Tyrode’s saline for 20 min at 37 °C. Embryos were then placed in Tyrode’s and refrigerated until the pattern of dye uptake could be recorded (within 12 h). Selected embryos possessing a clear fpp were fixed in Carnoy’s for 8–12 h and processed for indirect immunofluorescence as described below.

The procedure for production of anti-nucleoside antibodies was exactly that of Gratzner, Leif, Ingram & Castro (1975). The IgG-containing fraction from pooled rabbit immune sera was further separated by affinity chromatography. The solution was passed through a column of thymine riboside linked to Sepharose and then one of bromouridine-Sepharose. The columns were eluted separately with acid to obtain solutions of anti-thymine (anti-T) and anti-bromouridine (anti-BU) antibodies (details in Gratzner et al. 1975).

Fixed embryos were processed automatically and embedded in Paraplast. Three to five serial sections through the dorsal–ventral plane were cut at 3–4 μm and dried onto gelatin-coated slides. Every other slide was stained with Feulgen (Lillie, 1954) in order to accurately locate the fpp.

Selected slides were deparaffinized in xylene and hydrated through a graded ethanol series to distilled water. They were then immersed in 1.0 N HCl for 10 min at room temperature, followed by three washes of distilled water. Slides were treated in 95% formamide, 4.9% saline citrate (0.15 M-citric acid, 0.15 M-NaCl, pH 7.0), 0.1% formaldehyde (v:v) for 1 h at 65 °C in order to denature the nuclear DNA (Dev et al. 1972). Following three brief washes in phosphate-
buffered saline (PBS) at room temperature, sections were ringed with wax and coated with normal goat serum diluted 1:40 in PBS for 20 min in a humid chamber. The slides were rinsed in three 200 ml beakers of PBS for 5 min, rinsed and coated with the desired antibody diluted to 0.4 mg/ml in PBS which contained BSA (1 mg/ml). After incubating at room temperature in the humid chamber for 30 min, slides were rinsed in PBS three times for 5 min each. Fluorescein-labelled goat anti-rabbit IgG (Miles/Yeda; diluted 1:20 in PBS) was centrifuged 20000 g for 20 min prior to application for 30 min at room temperature. The slides were once again rinsed in PBS as above and then stained with toluidine blue (0.025% in PBS) for 20 sec. Following a brief wash in PBS the sections were coverslipped in Difco mounting medium and sealed with nail polish.

To determine the specificity of fluorescent staining, the following control experiments were performed: (a) pre-immune rabbit sera (diluted 1:20) was used as the primary immunoreagent, (b) the fluorescein-labelled goat-anti-rabbit IgG fraction was used without prior exposure of the sections to anti-BU, (c) sections of hindlimbs from embryos not exposed to BUdR were processed as described above using anti-BU as the primary immunoreagent and (d) the affinity purified anti-BU was incubated for 1 h at room temperature with an aliquot of Sepharose–bromouridine before applying it to tissue sections.

Slides were examined with a Zeiss ICM 405 using a 100 W mercury burner. Fluorescence was excited by epi-illumination using the following sequence of filters (all Zeiss); BG 12 (red suppressor), PB 450-490 (exciter), FT 510 (dichromic mirror), LP 520 (barrier filter). Photographs were taken with Kodak Tri-X film using 2–4 sec exposures.

**RESULTS**

Day-20 foetuses stained for cartilage and bone revealed a range of polydactylyous hindlimbs (Fig. 1). The extra preaxial digit was either diphalangous or triphalangous. Extra tarsal and/or metatarsal elements usually associated with

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**Table 1. Percent of total rat hindlimbs affected with polydactyly in BUdR**

<table>
<thead>
<tr>
<th>Time of administration (h of pregnancy)</th>
<th>Dose (mg/kg)</th>
<th>100</th>
<th>250</th>
<th>500</th>
<th>750</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>51.7 (346)</td>
<td>74.7 (450)</td>
<td>61.2 (268)</td>
<td></td>
</tr>
<tr>
<td>288</td>
<td>0.9 (224)*</td>
<td>51.7 (346)</td>
<td>74.7 (450)</td>
<td>61.2 (268)</td>
<td></td>
</tr>
<tr>
<td>292</td>
<td>0 (52)</td>
<td>49.4 (158)</td>
<td>45.3 (128)</td>
<td>57.0 (228)</td>
<td></td>
</tr>
<tr>
<td>296</td>
<td>—</td>
<td>28.2 (238)</td>
<td>21.9 (196)</td>
<td>11.4 (228)</td>
<td></td>
</tr>
<tr>
<td>300</td>
<td>0 (60)</td>
<td>0 (56)</td>
<td>2.3 (86)</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

* Parentheses indicates total number of hindlimbs examined. Bold numbers include previously published results (Scott, 1981).
Fig. 1. Ventral views of day-20 rat hindlimbs stained for cartilage and bone. 
(a) Untreated right hindlimb, (b, c, d) polydactylous hindlimbs from litters treated 
with 500 mg/kg BUdR at 09.00 h, day 12 (288 h). (b) Preaxial diphalangous extra 
digit on a right limb with fused extra metatarsal and partially fused extra tarsal. 
(c) Preaxial triphalangous extra digit of left hindlimb showing fused extra metatarsal 
and two separate extra tarsal elements. (d) Two extra preaxial digits of a right hind- 
limb. The lateral diphalangous extra digit has separate metatarsal and tarsal 
elements while the adjacent extra triphalangous digit has a fused extra metatarsal. 
Note ossification is only present in metatarsals of digits 2, 3, 4 and 5 as indicated by 
central dark bands.

the digit were either fused with the normal elements or separate. All types of 
polydactyly have been summed and expressed as the percent of total hindlimbs 
examined.

Table 1 summarizes the dose and temporal response relationships after 
BUdR administration during the first half of day 12 of gestation with regard 
to the induction of polydactyly. As reported earlier (Scott, 1981), the period
Table 2. *Percent of total rat hindlimbs with the fpp at 324 h of development*

<table>
<thead>
<tr>
<th>Time of administration (h of pregnancy)</th>
<th>100</th>
<th>250</th>
<th>500</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>288</td>
<td>89.6 (134)†</td>
<td>1.9 (54)</td>
<td>0 (66)</td>
</tr>
<tr>
<td>292</td>
<td>—</td>
<td>11 (82)</td>
<td>4.2 (48)</td>
</tr>
<tr>
<td>296</td>
<td>—</td>
<td>72 (100)</td>
<td>51.7 (120)</td>
</tr>
<tr>
<td>300</td>
<td>90.9 (22)</td>
<td>90.6 (32)</td>
<td>84 (50)</td>
</tr>
</tbody>
</table>

* fpp present in 86.5% of untreated control embryos at 324 h (Scott, 1981).
† Parentheses indicates total number of hindlimbs examined after staining with Nile Blue A. Bold numbers include previously published results (Scott, 1981).

between 288 and 292 h represents the time in development of highest sensitivity to polydactyly induction with BUdR. Administration of the analogue 4 or 8 h later results in a notably lowered frequency in each dosage group. The lowest dose examined (100 mg/kg) represents a threshold dose, while the next higher dose (250 mg/kg) gives an intermediate response. The 500 mg/kg dose appears to give the upper limit of effect, since increasing the dose to 750 mg/kg gave approximately the same percentage of polydactylous hindlimbs. Reductional defects, such as ectrodactyly and brachydactyly, began to appear in the highest dosage groups (data not shown).

The first macroscopic change in early hindlimbs exposed to BUdR is the absence of physiological cell death in the preaxial region termed the fpp. Table 2 illustrates the effect of BUdR exposure on the presence of the fpp in rat hindlimbs which were supravitally stained with Nile Blue at 324 h of development (21.00 h, day 13). With reference to Table 1 it is seen that with each dose as the frequency of polydactyly goes down with later times, the frequency of hindlimbs with an fpp goes up. There is, however, a prevalence of limbs missing the fpp which do not develop polydactyly (Table 1). For example, at 288 h a dose of 250 mg/kg produces 52% polydactyly but 98% of the hindlimbs examined lack an fpp at 21.00 h, day 13. This disconcerting fact is also apparent in control hindlimbs where only 86% show an fpp (Scott, 1981). It is possible that a reduced fpp is not detectable by our observational method. Treated or control hindlimbs possessing a clear fpp at 21.00 h, day 13 were used for subsequent indirect immunofluorescence.

Sections of untreated hindlimbs were coated with antibody to thymine (anti-T) in order to examine whether nuclear debris within the fpp was available for antibody binding. Figure 2 demonstrates that, indeed, dead cell fragments are fluorescent as well as all whole cell nuclei when anti-T is used as the primary immunoreagent. Cross-reactivity of anti-BU toward thymidine was examined on sections of hindlimbs not exposed to BUdR (Fig. 3). No fluorescence was observed in this or any of the other immunocytochemical controls (not shown).
Fig. 2. Histological section through the fpp of a control (untreated) hindlimb at 21.00 h, day 13 coated with anti-T. Fluorescent pattern at top, bright-field image at bottom. Many dead cell fragments are fluorescent (arrows). Some apparent dead cell fragments are not fluorescent (circled). Scale bar = 12 µm.
Fig. 3. Histological section through the fpp of control (untreated) hindlimb at 21.00 h, day 13 coated with anti-BU. Lack of any specific fluorescence is seen in the top photo. Arrows in the bright-field photo point to examples of dead cell fragments. Scale bar = 12 µm.
$BuD$R in cells destined to die

Fig. 4. Histological section through the hindlimb of an embryo exposed to 500 mg/kg BuD$R$ at 09.00 h, day 12 (288 h) and coated with anti-Bu at 21.00 h, day 13. Some nuclei in both ectoderm and mesenchyme are seen to be fluorescent (top). Bright-field image at bottom. Scale bar = 12 $\mu$m.
Fig. 5. Histological section through the fpp of hindlimb exposed to 100 mg/kg BUdR at 09.00 h, day 12 (288 h) and coated with anti-BU at 21.00 h, day 13. Most dead cell fragments are fluorescent (arrows in lower photo). A few apparent dead cell fragments are not fluorescent (circled in lower photo). Scale bar = 12 μm.
Fig. 6. Histological section through the fpp of hindlimb exposed to 500 mg/kg BUdR at 21.00 h, day 12 (300 h) and stained with anti-BU at 21.00 h, day 13 (324 h). Many dead cell fragments are seen to be fluorescent (arrows in lower photo). Some fragments are not fluorescent (circled in lower photo). Scale bar = 12 μm.
Fig. 7. Histological section through the fpp of a hindlimb exposed to 250 mg/kg BUDR at 09.00 h, day 13 (312 h) and coated with anti-BU at 21.00 h, day 13. Most dead cell fragments are not fluorescent (circles). A very few apparent dead cell fragments are fluorescent (arrows). Scale bar = 12 μm.
The fluorescent staining pattern when anti-BU is used on sections of limbs exposed to BUdR is quite distinct from the pattern produced with anti-T. Figure 4 demonstrates the fluorescent pattern obtained with anti-BU on a section from a hindlimb exposed to 500 mg/kg BUdR at 288 h (09.00 h, day 12) and processed 36 h later. Many, but not all, nuclei are fluorescent in both the ectoderm and mesenchyme. The area normally occupied by the fpp is devoid of cell death and fluorescent nuclei were not localized to this or any other discrete area of the hindlimb.

When a dose of 100 mg/kg is given at 288 h (09.00 h, day 12) the majority of hindlimbs possess the fpp (Table 2). Anti-BU localization in one of these hindlimbs is seen in Fig. 5. The intensity and quantity of fluorescence per nuclei is reduced compared to the 500 mg/kg treatment. The majority of dead cell fragments are seen to be fluorescent. The stain used in the brightfield image is not specific to nuclear material so we are unsure whether non-fluorescent fragments represent nuclear or cytoplasmic debris. Positive fluorescence in pycnotic fragments is a consistent feature in a number of identically treated hindlimbs from different litters.

We next examined limbs exposed to BUdR at later than the optimal time to produce polydactyly. Figure 6 shows a section containing the fpp from a hindlimb exposed to 500 mg/kg BUdR at 300 h (21.00 h, day 12). Many necrotic fragments are stained with anti-BU, showing that presumptive fpp cells have incorporated some BUdR prior to their death. Hindlimbs exposed to 250 or 500 mg/kg BUdR at 296 h (17.00 h, day 12) also display fluorescence in a relatively large proportion of observable dead fragments (not shown). To examine when the embryo can be treated with BUdR and the fpp not stain with anti-BU, litters were treated with 250 or 500 mg/kg at 312 h (09.00 h, day 13). In both cases hindlimbs were stained at 21.00 h, day 13 with Nile Blue and almost all had an fpp. Indirect immunofluorescence revealed very few fluorescent dead cells in the fpp of either treatment group when they were stained with anti-BU (Fig. 7).

**DISCUSSION**

The thymidine analogue, BUdR, has been called an ‘ideal’ teratogen because of its ability to specifically alter development without significantly affecting normal metabolic or proliferative processes (Rutter, Picket, Githens & Gordon, 1975). This statement is supported by the majority of studies using in vitro systems, but the few in vivo systems examined in detail have fallen somewhat short of this ideal. In mammalian embryos exposed in utero to high doses of BUdR (> 500 mg/kg) cell death is found in organs expected to be malformed (limbs, Packard, Skalko & Menzies, 1974; Scott, 1981; neural tube, Webster, Shimada & Langman, 1974; Bannigan & Langman, 1979). In addition, perturbations of the cell cycle and general growth retardation have been noted. Bannigan & Langman (1979) were unable to show that BUdR interfered with
cellular differentiation in the mouse neuroepithelium. Instead they suggested that a variety of BUdR-induced malformations are caused by cell death and/or slowing of the cell cycle.

Contrary to this view, the treatment of Drosophila melanogaster larvae with BUdR induces a variety of growth lesions in adult flies which are not correlated with cell death in the imaginal discs (Rizki & Rizki, 1977). Data from this system indicates that following incorporation of BUdR into critical sequences of DNA an informational change is transmitted to the progeny which differentiate at metamorphosis as a clone of phenotypically altered cells. Similarly, drug-induced cell death is believed not to be an important feature in the pathogenesis of BUdR-induced polydactyly because it is seen only at high-dosage levels and then only late in limb development when an abnormal contour is already evident. In addition, lower doses which cause no appreciable cell death still produce a significant number of affected limbs (Scott, 1981). Whether a slowing of the cell cycle is integral to production of defects cannot be determined by results presented here or in either of the aforementioned studies.

The immunocytochemical demonstration that 100 mg/kg BUdR given at the optimal time to produce polydactyly (09.00 h, day 12) is incorporated into degenerative fragments of the fpp (Fig. 5) leads to the conclusion that a higher, teratogenic dose given at the same time must likewise be incorporated into these cells which were destined to die. If one accepts the suggestion that physiological cell death represents a form of terminal differentiation (Basile, Wood & Braun, 1973; Coffino, Bourne & Tomkins, 1975; Skalko, Perrins & Niles, 1977), then this study has made the first correlation between the in vivo incorporation of BUdR and inhibition of differentiation in a discrete population of mammalian cells. Furthermore, the failure of fpp cells to die is closely correlated with the subsequent formation of an extra digit as shown in this study (Table 2) and other polydactylous situations (Scott et al. 1977, 1980; Klein et al. 1981; Milaire, 1976; Rooze, 1977; Knudsen & Kochhar, 1981).

It is assumed that the genetic programme leading to cell death is in some way altered because a critical level of substitution is achieved with the higher doses. Results from this laboratory using tritiated BUdR to follow incorporation into single embryos show that radioactivity in acid-insoluble material is, indeed, dose related (Wise, Scott & Wilson, 1980). Preliminary attempts using histochemical methods to localize either the presumptive fpp cells in normal limbs or 'saved' fpp cells in treated limbs have been unsuccessful. This lack of a cell marker prevents a direct demonstration of the hypothesis that cells prevented from dying have incorporated the drug.

The demonstrated presence of BUdR in the fpp of those limbs exposed to teratogenic doses at later times and the fluorescence in whole cell nuclei at all times examined shows that cells are not affected equally by a teratogenic dose. We must speculate that cells in vivo have a very restricted developmental period during which the incorporation of BUdR will inhibit or alter terminal
BUdR \textit{in cells destined to die}

Differentiation. Thus, presumptive fpp cells exposed at later times are not undergoing a BUdR-sensitive phase of the cell cycle, and likewise fluorescent ‘healthy’ cells were unaffected by its incorporation. Tables 1 and 2 show that there is a relatively limited developmental period when BUdR is effective in producing polydactyly and preventing the appearance of the fpp respectively. It should be emphasized here that preaxial hindlimb polydactyly is the only major structural malformation found in litters treated with 250 and 500 mg/kg at 288 h.

Our results and the suggestion made above lead us to speculate about possible mechanisms for other BUdR-induced defects. BUdR-induced cleft palate might be caused by inhibition of medial edge epithelium to undergo physiological cell death prior to fusion (Hudson & Shapiro, 1973). This could be examined by in vitro techniques (Tyler & Koch, 1977). Alternatively, the cellular mechanism for shelf rotation could be inhibited by incorporation of the drug. Lastly, syndactyly (Skalko \textit{et al.} 1977) would be explained by the inhibition of interdigital cell death.

The incorporation of BUdR at 296 and 300 h, and the lack of incorporation into fpp debris at 312 h (Fig. 7) supports the view that the presumptive fpp goes through a limited number of BUdR-insensitive rounds of DNA synthesis. This phenomenon is similar to the quan tal mitosis theory described for embryonic myoblasts by Holtzer, Weintraub, Mayne & Mochan (1972). Both situations appear to include a BUdR-sensitive phase during which the genome expresses an intrinsic bias toward a certain state of differentiation. After a ‘quantal’ division one or both daughter cells exhibit a progressive decline in mitotic activity. This is what we observe, for at 09.00 h, day-13 cells destined to be the fpp no longer incorporate BUdR (Fig. 7). This timing is very similar to that found in the posterior necrotic zone (PNZ) of the chick wing bud (Pollack & Fallon, 1976) where a decrease in nucleic acid synthesis was detected with autoradiography approximately 16 h prior to any morphological manifestation of cell death in the PNZ.

Our results do not rule out other non-genetic effects of BUdR on cells of the developing rat hindlimb destined to be polydactylous. Our lack of knowledge about the control of physiological cell death episodes in limbs makes any proposed mechanism somewhat speculative. The recent model for control of cell death in the anterior necrotic zone of chick wing buds by long-range signals emanating from the postaxial border (Hinchliffe & Gumpel-Pinot, 1981) might offer an alternative explanation. The changes induced by BUdR might include any or all of the events from production to reception and interpretation of the signal. The developing chick limb may then be the system to further examine these possibilities. In this regard, BUdR is one of two exogenous agents which cause duplication of digital elements when administered \textit{in ovo} (Karnofsky, 1965). Insulin is the other (Romanoff, 1972).
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