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Delayed appearance of ectodermal cell death as a mechanism of polydactyly induction

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SUMMARY

The temporal program of cell death in the apical ectodermal ridge and mesoderm of rat embryo hindlimbs was documented using supravital staining with Nile blue sulfate. Dye uptake indicative of cell death began postaxially at about 290 h of development and was followed in a few hours by preaxial staining, which became more extensive and intense up to 313 h.

Two agents which cause preaxial polydactyly, cytosine arabinoside and 5-fluorodeoxyuridine, postponed the onset of preaxial ectodermal cell death while at the same time having the expected cytotoxic effect on limb-bud mesoderm. In addition, a zone of deep preaxial mesodermal necrosis, thought to control the size of digit 1 in normal embryos, was usually absent in cytosine-arabinoside-treated embryos.

The results suggest that the prolonged survival of ectodermal cells effected an increased inductive activity on the underlying mesoderm, leading to the formation of excess digital tissue. The data further suggest that the rate at which mesodermal cells were killed affected the subsequent delay of ectodermal cell death.

INTRODUCTION

Cytosine arabinoside (ara-C), an inhibitor of DNA synthesis which is lethal to various types of proliferating cells, has previously been shown to induce changes in digital number in the hindlimbs of rat embryos exposed during development. Administration on the morning of the 12th gestational day (288 h of development) results in fetuses with ectrodactylous rear limbs (Ritter, Scott & Wilson, 1971). If ara-C is administered 12 h earlier (276 h of development), many fetuses possess polydactylous rear limbs (Scott, Ritter & Wilson, 1975). Since inhibition of DNA synthesis and cell death produced by ara-C undoubtedly lead to at least a transient reduction in cell number within the hind-limb bud, it was difficult to formulate a theory explaining the induction of malformation with excess tissue, i.e. polydactyly.

Scott *et al.* (1975) showed that the normal pattern of physiological cell death

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in the limb-bud ectoderm was delayed in embryos destined to be polydactylous. Milaire (1965, 1971) had earlier described this same phenomenon in embryos of the mouse strain, dominant hemimelia, in which heterozygotes often have a triphalangous hallux or frank preaxial polydactylism. According to the Saunders–Zwilling model of limb development (Zwilling, 1961), an inductive relationship exists between apical ectoderm and mesoderm. The present study along with the earlier studies of Milaire (1965, 1971) suggested that a delay in ectodermal cell death would allow a prolonged inductive influence of ectoderm on mesoderm at a critical time, and could thus be considered as a possible mechanism of polydactyly production.

Milaire (1976) has also shown that two centers of preaxial mesodermal cell death exist which presumably act to reduce the size of the first digit. Alterations in these centers of physiological necrosis have been demonstrated in cases of preaxial digital excess (Milaire, 1971, 1976; Rooze, 1977).

The purposes of this study were therefore to examine the pattern of physiological ecto- and mesodermal cell death following ara-C treatment and to document the polydactyly-inducing activity of another antiproliferative agent, 5-fluorodeoxyuridine (FUdR), as well as to observe its effects on the inception of ectodermal cell death.

METHODS

For studies on cytosine arabinoside, pregnancy in rats of Royalhart stock, derived from a Wistar strain, was dated by counting time 0 as 9.00 a.m. of the morning on which sperm were found in the vaginal smear of females caged overnight with males of the same strain. At 9.00 p.m. of day 11 (est. 276 h post fertilization) 100 mg/kg of ara-C (supplied by The Upjohn Co., Kalamazoo, Mi.) in aqueous solution was administered *i.p.* Embryos were removed by partial hysterectomy at two intervals on day 12 of pregnancy. Some embryos in each litter were allowed to proceed to day 20 to verify the accuracy of the *i.p.* injection by the presence of polydactylous hindlimbs. Immediately after removal of the uterus the embryos were dissected free of their membranes and placed in a 1:20000 mixture of Nile blue A in Locke's solution for 20–30 min at 37 °C. The mechanism by which this agent preferentially stains non-viable cells has been discussed by Pexieder (1975). Embryos were then placed in Locke's solution and refrigerated until photographed (within 24 h). Times for examination were 288, 291, 294, 297, 300 and 303 h of pregnancy. To determine whether Nile blue sulfate staining could be equated with actual cell death, histological sections stained with H and E were examined microscopically in occasional specimens. Mesodermal cell death was searched for on serial tangential sections of hindlimb bud, and by Nile blue staining on whole 13- and 14-day-old embryos.

For studies of polydactyly induced by FUdR (supplied by Hoffmann–La Roche, Nutley, N.J.) the same strain of rats was used. The lights were on from 10 a.m. to 10 p.m. rather than 5 a.m. to 5 p.m. as in the earlier study. At 8.30 a.m.

females in estrus, indicated by lordosis in response to perineal manipulation, were placed with males of the same strain for about 1 h. Sperm in the vaginal lavage later in the day provided evidence of successful mating. Since the light cycle of these animals is 5 h later, time 0, the supposed time of fertilization is also presumed to be 5 h later (2 p.m.). In any event, elapsed times after time 0 in the two experiments were equivalent. At 4 h intervals between 275 and 291 h of pregnancy females were injected intravenously with 50 mg/kg of an aqueous solution of 5-FUdR. Pregnancy was terminated on day 20, and implantation sites counted *in situ*. Living fetuses were removed and examined for external malformations.

Additional females were treated at 283 h with 50 mg/kg of FUdR followed by partial hysterectomy and immersion of the embryos in Nile Blue A at 298, 304, 307, 310, and 313 h of pregnancy.

RESULTS

Delay of ectodermal cell death by ara-C

Figure 1 displays the pattern of cell death in the ectoderm of the hindlimb in ara-C treated embryos compared to the appropriate controls. In control embryos cell death first became visible at 288 h. At this time dye uptake always occurred in the postaxial ectoderm, but preaxial ectoderm exhibited inconsistent uptake. At 291 h (Fig. 1) the picture was much the same. By 294 h all but a few embryos in one litter exhibited preaxial dye uptake. All control embryos examined at 297 h revealed extensive preaxial dye uptake, covering approximately two-thirds of the preaxial surface, while the postaxial uptake remained circumscribed in the middle third of this surface. At 300 and 303 h the pattern did not change much except that uptake seemed slightly more extensive and intense preaxially.

None of the ara-C treated embryos revealed preaxial ectodermal dye uptake at 288 or 291 h, although slight staining of postaxial ectoderm was evident in a few embryos. At these times drug-induced mesodermal cell death was evident in slight to moderate amounts scattered throughout the limb. At 294 and 297 h all embryos revealed dye uptake in the postaxial ectoderm but preaxial ectoderm was still free of dead cells. Mesodermal cell death due to ara-C was moderate to severe and scattered throughout the limb. At 300 h uptake of dye began in the preaxial ectoderm of about one-half of the embryos, 6–12 h later than in control embryos. Postaxial ectodermal cell death was still obvious and circumscribed as in control embryos, and drug-induced cell death was still visible in moderate amounts in the mesoderm. At 303 h nearly all the embryos had dye uptake in the preaxial ectoderm.

To verify that Nile blue staining indicates cell degeneration, and conversely that lack of staining indicates an absence or paucity of cell death, histological sections of hindlimb-buds of treated and control embryos were prepared. Fig. 2 illustrates the situation at 300 h of development. In control embryos (A) cellular

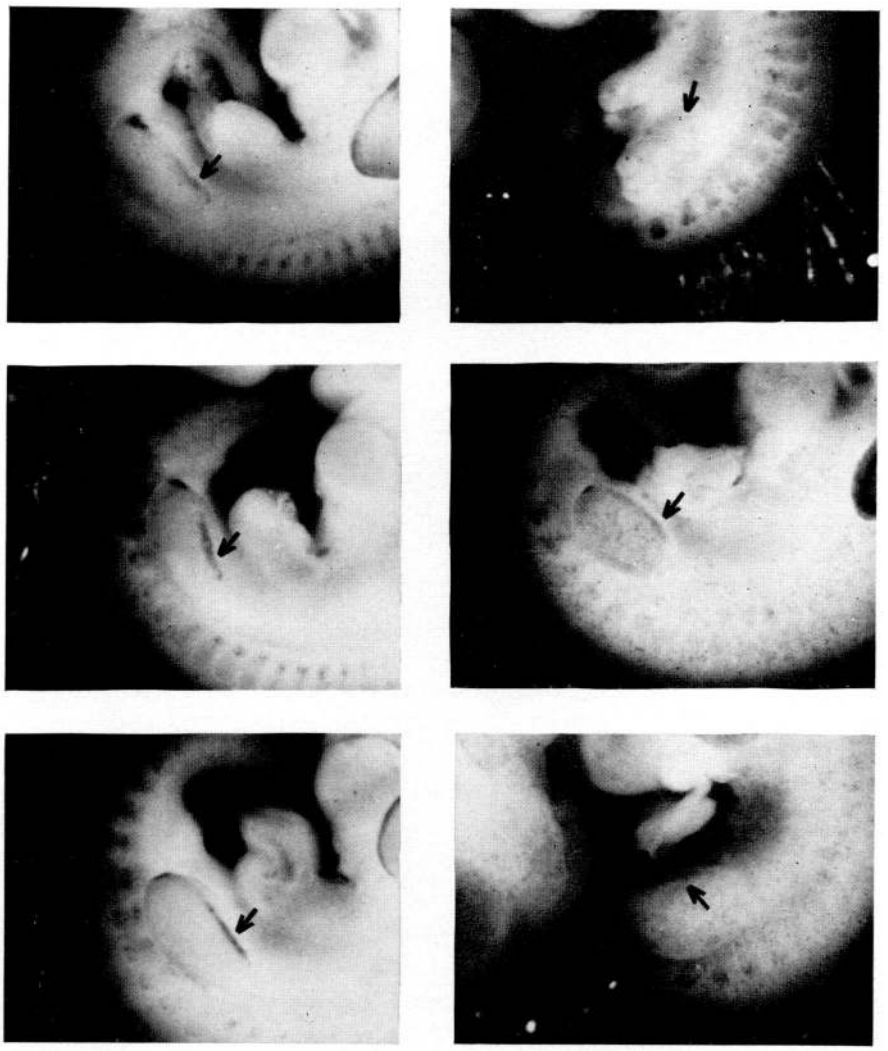

Fig. 1. Uptake of Nile blue, indicating dead cells in hindlimb buds of rat embryos. Control embryos on left, ara-C treated embryos on right. Top, 291 h; middle, 297 h; bottom, 303 h. Arrows indicate preaxial surface.

debris indicates a substantial number of degenerating cells in the thickened 'active' ectoderm, while the ectoderm from the treated embryo (B), although likewise showing a thickened active appearance, is devoid of signs of cell death.

Delay of mesodermal cell death by ara-C

Figures 3 and 4 demonstrate the appearance of a preaxial mesodermal necrotic zone which has been designated as *foyer primaire preaxial* (*fpp*) by Milaire (1976). In the present study this zone appears early on day 13 (312–315 h) in control

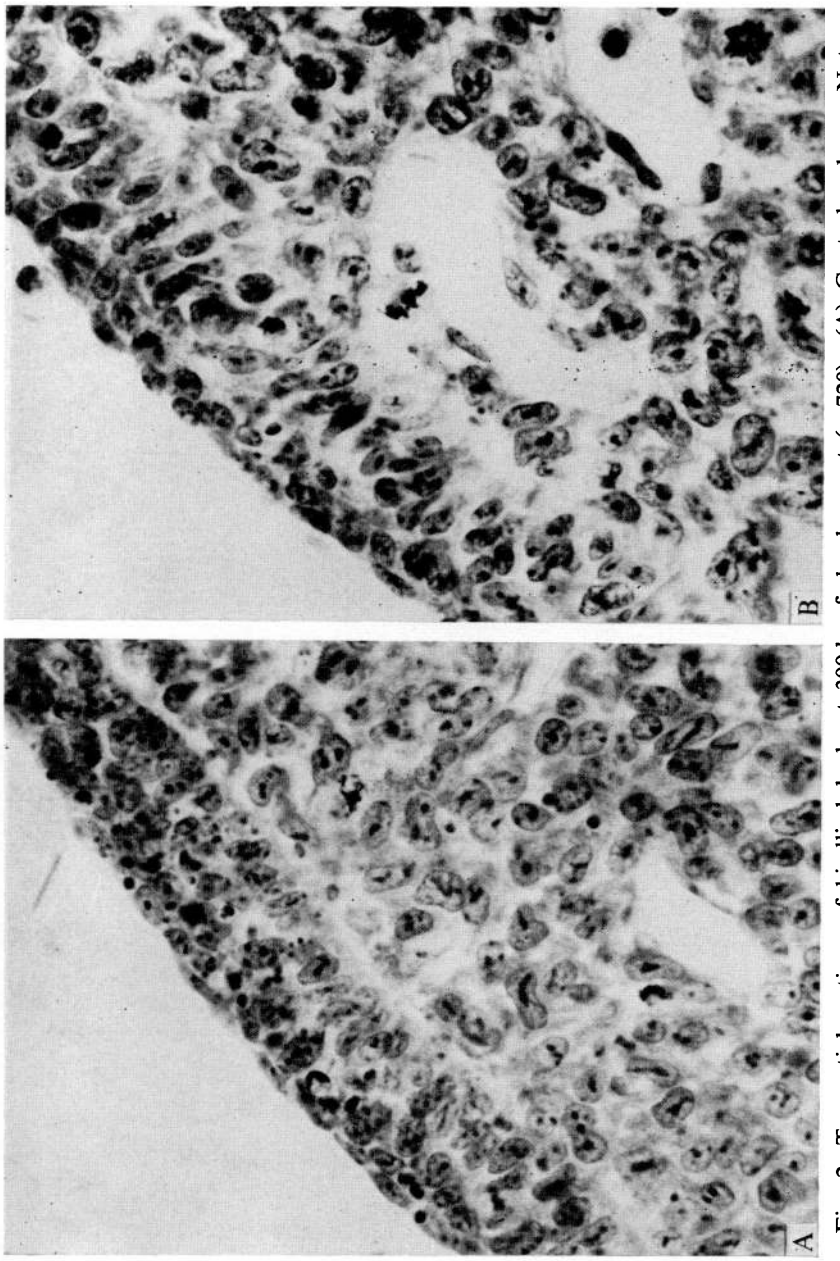

Fig. 2. Tangential sections of hindlimb-buds at 300 h of development (×720). (A) Control embryo. Note large number of degenerating egg cells marked by darkly staining spherules in ectoderm. (B) Embryo treated with ara-C at 276 h of development. Note absence of degenerating cells in ectoderm but retention of thickened, 'active' appearance.

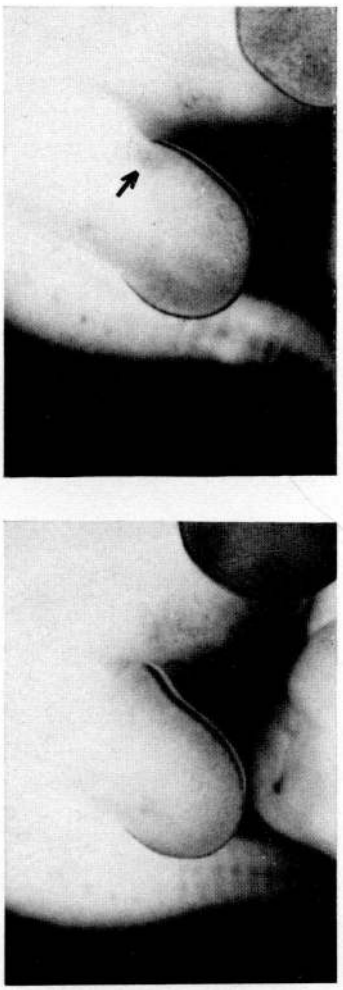

Fig. 3. Uptake of Nile blue in deep preaxial mesoderm. Control embryo on top, ara-C treated embryo on bottom. 324 h. Arrow indicates the foyer primaire preaxial in the control embryo. No such area is present in treated embryo. Diffuse marginal coloration is non-specific dye adsorption.

embryos and is well established by 318 h of development. It continues to be a prominent feature of hindlimb development throughout the 13th and 14th days but is absent early on the 15th day of development (360 h). The fpp usually lies below the marginal venous sinus and at the termination of the most proximal extension of the AER.

On the other hand, examination of 97 treated embryos throughout this period (312–360 h) revealed only two with a deep preaxial necrotic zone, at 324 h of development. The necrotic zone lay at the base of the extra digit.

Two zones of marginal mesodermal necrosis termed *foyer marginal I* (fMI)

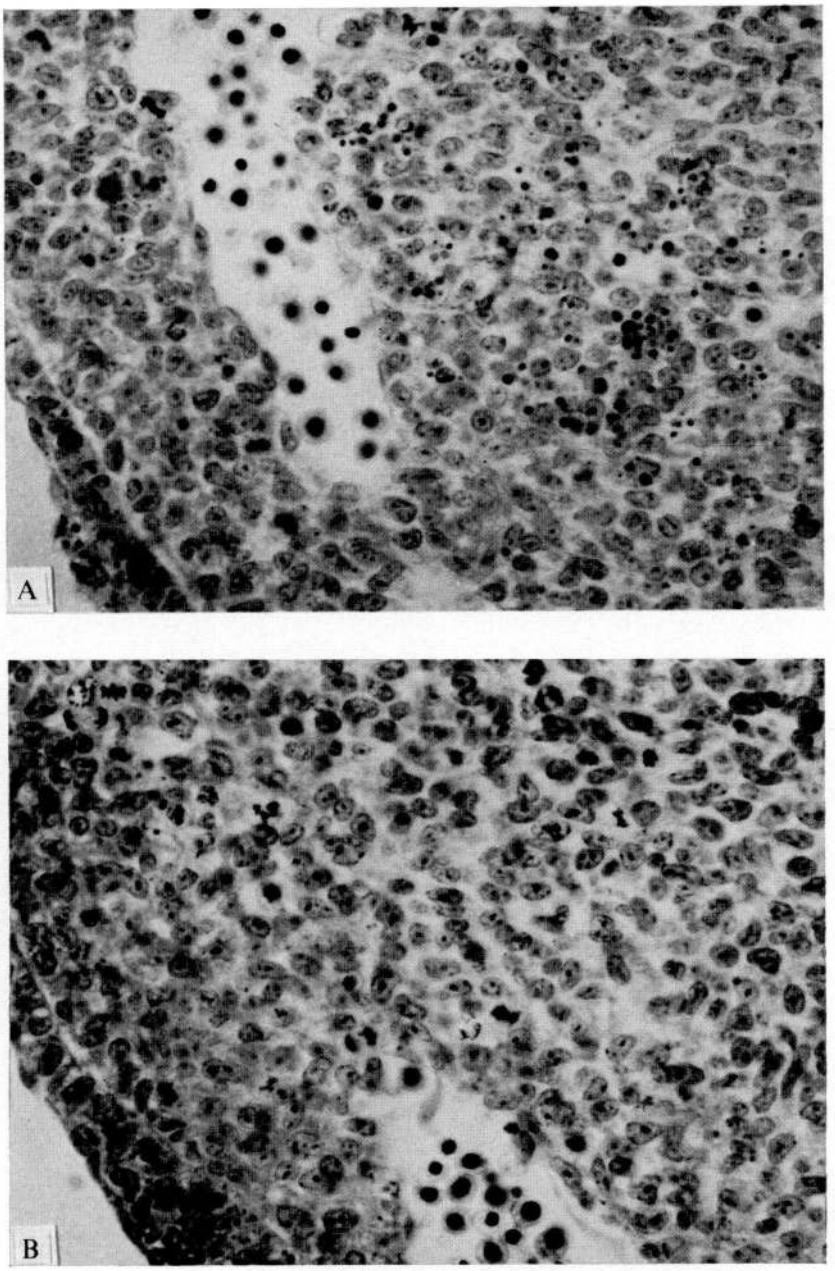

Fig. 4. Tangential section through hindlimb at 324 h of development. (×470.) (A) Control embryo. Note large number of degenerating cells in preaxial mesoderm beneath marginal venous sinus. Also note location at the most proximal extension of preaxial AER. (B) Ara-C treated embryo. Note absence of degenerating cells in the same area.

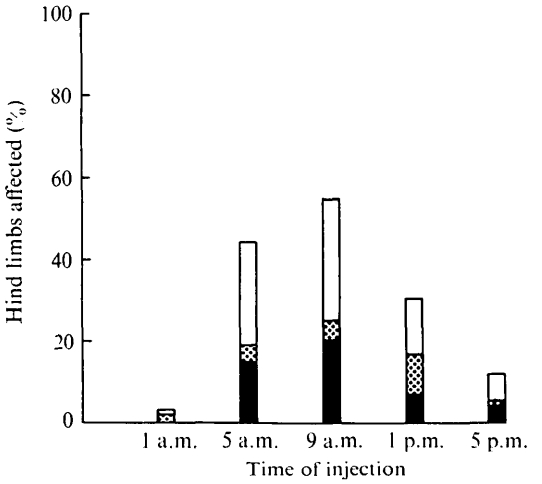

Fig. 5. Incidence of digital excess deformity in hindlimbs following *i.v.* treatment with 50 mg/kg of FUdR on day 11 or 12 (275–291 h) of pregnancy. Clear bar indicates limbs with digital giantism only. Dotted bar indicates limbs with extra digits of normal size. Solid bar indicates limbs with both extra digits and digital giantism.

and *foyer marginal V* (fMV) by Milaire (1976) were also examined. The post-axial zone (fMV) first appears late on the 13th day (324 h) in both ara-C treated and control embryos and is still present in both at 360 h, the latest time examined. The preaxial zone (fMI) first appears early on the 14th day (336 h) in treated and control embryos and is also visible in both 24 h later (360 h). No differences in the extent and intensity of stain uptake could be distinguished.

Polydactyly induction by FUdR

Figure 5 presents the incidence of rear limb polydactyly following *i.v.* administration of 50 mg/kg of FUdR at various times on day 11 or 12 of gestation. The optimal time of administration (283 h) was somewhat later in pregnancy than after cytosine arabinoside (276 h) (Scott, Ritter & Wilson, 1975). Polydactyly produced by cytosine arabinoside and FUdR was always preaxial, but after FUdR a number of individuals were affected only by increased size of the first digit. Enlargement of the first digit may be a different expression of the same changes producing preaxial polydactyly. The incidence of polydactyly appeared to be the same in male and female fetuses and in cases of unilateral involvement no predilection for sidedness was apparent.

Delay of ectodermal cell death by FUdR

Figure 6 compares the pattern of cell death in hindlimbs of embryos treated with FUdR at 283 h to appropriate controls. In control rats bred under this regime cell death begins to appear at 289 h, at which time dye uptake is usually restricted

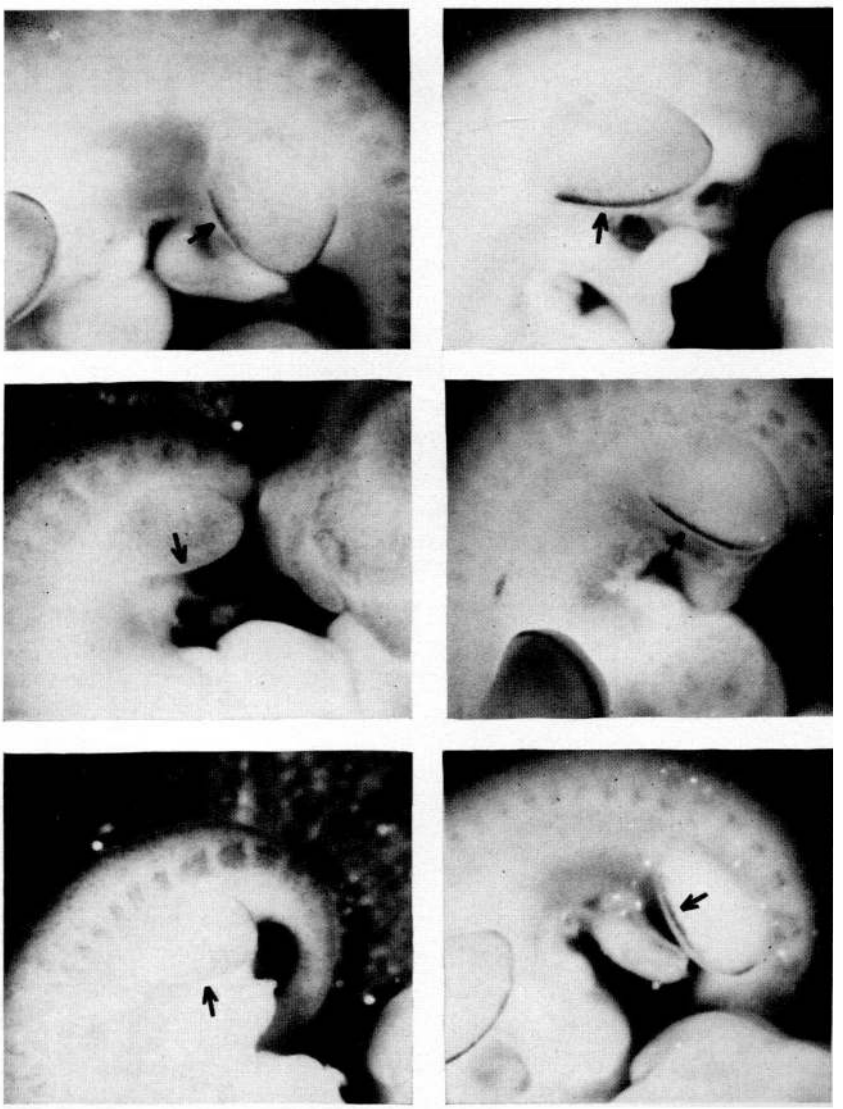

Fig. 6. Uptake of Nile blue indicating dead cells in hindlimb-buds of rat embryos. Control embryos on left, FUdR-treated embryos on right. Top, 298 h; middle, 307 h; bottom, 313 h. Arrow indicates preaxial surface.

to the postaxial ectoderm, while by 292 h uptake occurs both pre- and postaxially. Little change in this pattern is seen at 298 h, although the area of preaxial cell death seems somewhat extended. By 304 h a nearly continuous band of cell death exists in the hindlimb ectoderm and this pattern does not change much at 313 h.

In rats treated with 50 mg/kg of FUdR at 283 h, the first signs of ectodermal cell death in the hindlimb became evident 15 h later, when a few embryos of one

litter had a small amount of dye uptake postaxially. The remaining embryos in this litter and all the embryos of two other litters examined at this time had no evidence of ectodermal cell death. Drug-induced mesodermal cell death on the other hand was prominent in all embryos. By 304 h all embryos showed dye uptake postaxially, although none showed any preaxial cell death. Mesodermal cell death was still prominent. At 307 and 310 h some embryos began to show dye uptake preaxially. Mesodermal cell death due to FUdR was still evident. By 313 h all treated embryos showed a continuous heavy band of cell death in the ectoderm. Evidence of drug-induced mesodermal cell death was minimal by this time.

DISCUSSION

Results from the present study clearly indicate that polydactyly-inducing regimes of ara-C and FUdR retard the initiation of physiological cell death in the preaxial ectoderm of the hindlimb. This could account for the earlier demonstration (Scott *et al.* 1975) that the preaxial ectoderm covering and adjacent to the polydactylous region in treated embryos was thickened, hence presumably 'active', over a greater portion of the preaxial surface than in control limbs. Milaire (1971) and Rooze (1977) have similarly demonstrated a delay in ectodermal cell-death patterns in heterozygous dominant hemimelia mouse embryos, which often have a triphalangous hallux or preaxial polydactyly. These studies suggest a pathway of polydactyly induction in which delayed ectodermal cell death leads to an abnormally strong preaxial inductive effect, resulting in preaxial tissue-excess deformity. This hypothesis implies that inductive activity is not an all-or-none phenomenon, and that ectodermal cell death acts during normal limb development to diminish inductive activity of the AER.

Recently Milaire (1976) and Rooze (1977) have shown that zones of physiological necrosis in the mesoderm are also altered in situations leading to preaxial tissue-excess deformities. Likewise, in this study, the 'fpp', a zone of deep preaxial mesodermal necrosis, is almost always absent in ara-C treated embryos. Milaire (1976) has postulated that the function of this zone in normal development is to reduce the size of digit 1. The results of the present study give added credence to that idea. Little or no change could be detected in two marginal zones of mesodermal necrosis, fMI and fMV, in the present study.

Alteration of normal cell-death patterns as a suggested mechanism for producing polydactyly is not new. Hinchliffe & Ede (1967) have suggested that polydactyly in the talpid mutant of the fowl results from the demonstrated absence of cell death in the superficial mesenchyme of the anterior (ANZ) and posterior (PNZ) necrotic zones. Presumably these areas of cell death in non-mutant embryos restrict the spread of the apical ectodermal ridge, limiting the inductive influence and thereby producing the characteristic number of digits.

The general theory of limb development known as the Saunders–Zwilling model postulates that a mutual interaction between mesoderm and ectoderm is necessary

for normal limb morphogenesis. The above-quoted studies suggest that polydactyly arises because of excess ectodermal inductive activity following the failure of one cell type or another to die on proper schedule. Similar conclusions were drawn from the present results, in which the onset of normal ectodermal cell death was delayed 6–12 h although an unequivocal increase of morphologically demonstrable preaxial ectodermal ridge was not present until later in the ara-C syndrome (315–318 h) (Scott *et al.* 1975).

Although no evidence was presented regarding physiological cell-death patterns in ectoderm, Kameyama, Hayasaka & Hoshino (1973), Kameyama, Hoshino & Hayasaka (1974), Kameyana, Hayashi & Hoshino (1975), and Forsthoefel & Williams (1975) have noted hypertrophy of the apical ectodermal ridge in mouse embryos treated with polydactyly-inducing regimens of fluorinated pyrimidines, and Yasuda (1975) has demonstrated an abnormal extension and delayed involution of the preaxial apical ectodermal ridge in polydactylous human embryos. Thus by invoking the Saunders–Zwilling model of limb development polydactyly of diverse origin can be explained by a hyperactive apical ectoderm. The basis of this hyperactivity can vary and in the present report is attributed to treatment-induced delay of the normal degenerative program.

Observations from the present studies also provide clues as to the underlying basis for programmed cell death in ectodermal cells. Although both agents used here are considered to be 'pure' DNA synthesis inhibitors and to produce this effect rapidly, the optimal times for administering them in pregnancy for induction of polydactyly is somewhat different, 276 h for ara-C compared to 283 h for FUdR. Coinciding closely with this difference in optimal administration time is the onset of mesodermal cytotoxicity produced by these agents, FUdR being much more rapid in this respect than ara-C. Within 9 h FUdR produces a moderate cytotoxic response while ara-C requires 15 h to produce a roughly comparable effect. When these numbers are summed (276 + 15 or 283 + 9), their total coincides closely with the expected time of onset of physiological cell death in the ectoderm of control embryos. Perhaps the mesodermal cells that are killed would normally produce a substance lethal to the ectodermal cells, so that, in the absence of the mesodermal cells, the ectodermal cells continue to live and to influence digital development.

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