The effect of 5-bromodeoxyuridine on mouse embryos during neurulation in vitro[1]

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Summary. Mouse embryos explanted at various stages during neurulation were cultured for 20-28 h in the presence of 25–900 μg/ml of 5-bromodeoxyuridine (BUdR). BUdR strongly inhibited closure of the cranial neural tube, which was found to be stage-dependent. When mouse embryos were exposed to BUdR after development of the concave curvature in the neuroepithelium of the midbrain to the upper hindbrain regions, they became insensitive to the drug-induced open cranial neural tube. Histological observations showed that BUdR interfered with interkinetic migration and cytokinesis of the neuroepithelial cells. These cellular abnormalities were not dependent on the morphological development of the cranial neural folds. The ^{3}H-BUdR experiment confirmed that the label was mostly incorporated into the DNA fraction.

Key words. Mouse embryo explants; bromodeoxyuridine; neurulation in vitro; interkinetic migration; cytokinesis; neuroepithelial.

One of the halogenated pyrimidine analogs, 5-bromodeoxyuridine (BUdR), is incorporated into DNA in place of thymidine[12]. This substitution has been examined from the point of view of the differentiation of specialized cell types. In a number of differentiated cell types, including myoblast[6], chondrocyte[2] and erythrocyte precursors[22], BUdR can suppress the expression of differentiated characteristics at an appropriate concentration without apparent cytotoxicity. It has also been documented that the analog interferes with developing embryos. In preimplantation mouse embryos, BUdR affects normal morphological development at low doses or causes death at high doses[8, 10, 29, 37, 39]. In sensitive periods during organogenesis, BUdR produces various types of abnormalities in mice[38], rats[26], hamsters[32, 36] and chicks[16–18]. This kind of a teratogenic effect is specific to the developmental stage of embryos. When the analog is introduced at an early phase of organogenesis, an open cranial neural tube is a major abnormality observed[17, 26, 32, 38]. Skalko et al.[38] have suggested in their experiments with pregnant mice that exencephaly occurs during the period of open anterior neuropore but no longer occurs once the neuropore is closed. It is of interest to test whether such a stage-specificity in the formation of this abnormality is observed when mouse embryos are directly exposed to BUdR in culture. The aim of the present study is to examine the full potential of BUdR-induced open cranial neural tube in relation to the morphological development of the cranial neural folds in mouse embryos.

Materials and methods. Collection of embryos. All animals were maintained in the light from 06.00–18.00 h. 7–8-week-old female mice of the STD:ddY strain (obtained from Shizuoka Laboratory Animal Center, Shizuoka) were mated with 10–20-week-old males of the same strain from approximately 17.00–09.00 h of the following morning. The presence of a vaginal plug at approximately 09.00 h was regarded as an evidence of successful mating. This day was designated as day 0.5 of pregnancy.

Embryos of approximately 6–8, 10–11, 13–14 and 16–17 somite stages were examined in this study. Embryos of these stages or 1 to 2 somite younger were collected, since their development differed within and between litters of the same gestation age. At approximately 04.00–18.00 h on day 8 of pregnancy, the animals were sacrificed by cervical dislocation and the uteri were removed and placed in Hanks balanced salt solution (HBBS). Under a dissecting microscope, the Reichert's membrane was torn open leaving the visceral yolk sac and ectoplacental cone intact[28]. The embryos thus obtained were divided into different developmental stages by external shape and morphological development of the cranial neural folds. 6–8 somite embryos were characterized by optic pit invagination on both sides of the forebrain neural folds (fig. 1A). At the 10–11 somite

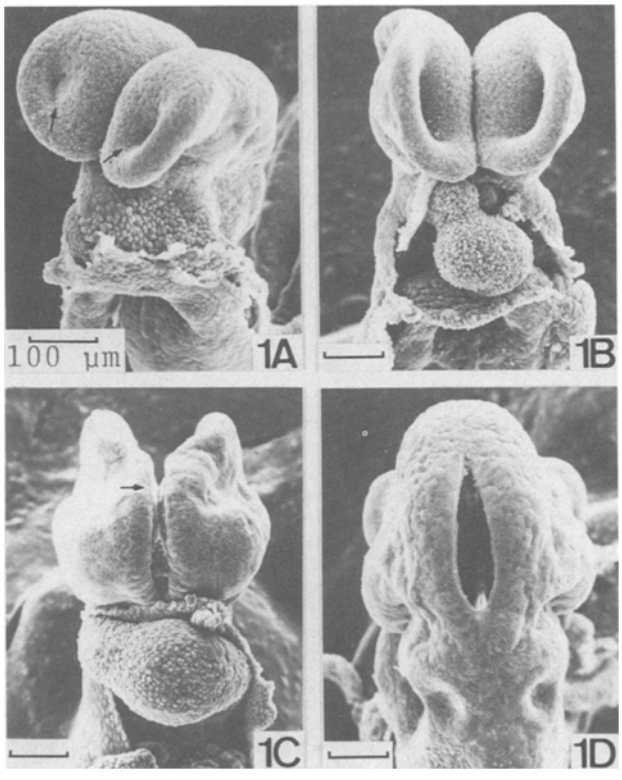

Figure 1. SEM of embryos at zero-time in culture. *A* Anterolateral view of an 8-somite embryo. The optic pits are invaginating on both sides of the forebrain neural folds (arrows). *B* Anterior view of a 10-somite embryo. The forebrain neural folds are approaching each other medially. *C* Anterior view of a 13-somite embryo. The cranial neural folds have developed to the stage just prior to fusion at the forebrain apposition point (arrow). *D* Posterior view of a 17-somite embryo. The anterior neuropore is closing to show a spindle-shaped opening overlying the upper hindbrain region.

stage, axial rotation was initiated in both the cranial and tail folds, with forebrain neural folds approaching each other medially (fig. 1B). At the 13–14 somite stage, the cranial neural folds developed to the stage just before fusion at the forebrain apposition point (fig. 1C). By the 16–17 somite stage, the anterior neuropore was closing to show a spindle-shaped opening in the midbrain to the upper hindbrain regions (fig. 1D).

Culture of whole embryos. The method used for culture of mouse embryos of various developmental stages was basically the same as reported by New et al.[28]. A culture vessel (No. 223746, Wheaton Scientific, Millvilli) was silicon-coated with Surfacil (Pierce Chemical Co., Rockford)[4]. Prior to culture each culture vessel containing 5.82 ml of medium was gassed with a gas mixture for 3 min under gentle stirring and then kept for approximately 30 min in an incubator at 38 °C until the medium gained pH stability. Then three embryos were placed in a culture vessel and rotated at 30 rpm at 38 °C. In the culture of the 6–8 and 10–11 somite embryos, $5\%O_2/5\%CO_2/90\%N_2$ was used until 10 and 6 h respectively after the treatment had been made, followed by $20\%O_2/5\%CO_2/75\%N_2$ until the termination of culture. In the culture of the 13–14 and 16–17 somite embryos, $20\%O_2/5\%CO_2/75\%N_2$ was used throughout the culture period. The gas phase was exchanged at approximately 18.00 h and 09.00 h.

Preparation of medium. 12-week-old females of the Wistar Imamichi rat strain (Imamichi Institute for Animal Production, Saitama) were used for sera preparation. All the animals were anesthesized with ether and the blood withdrawn from the dorsal aorta was immediately centrifuged at 3000 rpm for 5 min at 0 °C. The serum samples were then heat-inactivated at 56 °C for 30 min[40]. The medium consisted of 10 volumes of rat serum and 1 volume of Earle's balanced salt solution (EBBS). It was supplemented with glucose (final concentration 2.0 mg/ml), streptomycin sulfate (50 µg/ml) and phenol red (6 µg/ml). The osmolarity increase caused by addition of glucose was corrected by diluting the medium with distilled water. The amount of glucose added was calculated from the glucose assays of 10 samples of pure rat serum by the glucose oxidase method[23], which gave a mean glucose concentration of 1.35 mg/ml with a standard error of 0.04 mg/ml. The medium was filtered for sterilization (Type GS filter, pore size 0.22 µm, Millipore Corp., Bedford), frozen immediately and then stored for less than 2 weeks at −20 °C.

BUdR treatment. BUdR (Sigma, St. Louis) was dissolved in EBBS at concentrations of 0.083-3%, filtered for sterilization and stored at −20 °C. No solution older than 2 weeks was used. In explantation of the 6–8, 10–11, 13–14 and 16–17 somite embryos, approximately 30 min after explantation was taken as zero-time to allow the embryos to adapt to the culture environment. However, for the 1–2 somite younger embryos, zero-time was when they had reached to the desired developmental stages (usually approximately 2–4 h after the explantation). During this pre-treatment period, embryos were discarded if injury became evident in the yolk sac and/or ectoplacental cone. At zero-time in culture, 180 µl of BUdR solution was directly added to the culture medium. In all control experiments medium with an equal volume of EBBS was added.

The treatments were continued for 20–28 h until at least the otic vesicles normally would have closed. On termination of culture, the embryos were transferred into a petri dish containing warm HBBS. Under a dissecting microscope, the heartbeat and visceral yolk sac circulation were examined. Then the embryos were cleaned of extraembryonic membranes, followed by assessment of the number of somites, closure of the cranial neural tube and otic vesicles, completion of axial rotation and malformations. Some of them were used for preparing samples for scanning electron microscopy (SEM) and for light microscopy. The rest of the embryos were used for measurement of protein content by the Lowry method[19]. The number of somites and protein content were statistically analyzed by Student's t-test. The samples for SEM were fixed by the method of Kaufman[15], dehydrated in graded alcohols, critical-point-dried and coated with gold in a sputter coater. They were subsequently examined with a JEOL JSM-35 C scanning electron microscope. The embryos at zero-time in culture were also prepared for SEM. The samples for light microscopy were fixed in a Carnoy's solution for 2 h, prepared as transverse sections in paraffin (3 µm thickness) and stained with Lillie-Mayer's hematoxylin and eosin.

Incorporation of ^{3}H-BUdR. To ascertain the incorporation of labeled BUdR, 10–11 somite embryos were cultured in the presence of 150 µg/ml of [^{3}H]BUdR ([6-^{3}H]5-bromodeoxyuridine, specific activity 4.54 GBq/mmol, Amersham, Arlington Heights). After 22 h they were removed from the culture medium and transferred into ice-cooled HBBS containing 500 µg/ml of cold BUdR to stop further incorporation of the label. The embryos were then cleaned of extraembryonic membranes, washed 3 times with HBBS and individually homogenized in distilled water. An aliquot of the homogenate was added to an equal volume of mouse liver homogenate (1% in wet weight) as a carrier, because the precipitate after adding trichloroacetic acid (TCA) was too small in volume for centrifugation. This homogenate solution was suspended in 10% TCA overnight at 4 °C. RNA and DNA were extracted by the method of Ruddick and Runner[31]. The supernatant after the hot perchloric acid treatment was defined to be a DNA fraction. An aliquot of each fraction was added to PCS-II cocktail (Amersham) to count activity on a liquid scintillation counter (Aloka Model LSC-556). Incorporation of the label was indicated by the mean for 9 embryos.

Results. In this series of experiments, the mouse embryos at stages ranging from approximately 6–8 to 16–17 somites were directly exposed to different concentrations of BUdR for 20–28 h. Table 1 shows the inhibitory effects of BUdR on these embryos. Regardless of the developmental stage of embryos at the time of treatment, the analog inhibited the heartbeat, visceral yolk sac circulation and erythrocyte and somite formation. The degree of these inhibitory effects was largely dependent on both the amount of BUdR added to the culture medium and the developmental stage of embryos at treatment. At low concentrations of BUdR, caudal so-

Table 1. Inhibitory effects of BUdR treatments on the in vitro development of mouse embryos at various stages during neurulation

Treatment Somites	Duration (h)	BUdR (μg/ml)	No. of embryos explanted	Somites[a]	Protein[a] (μg)	Percent of embryos showing Yolk sac circulation (–)	Rotation completed	Open cranial neural tube	Open ear vesicles
6–8	28	Control	15	24.7 ± 0.8	164.7 ± 15.8	0	100	0	0
		25	15	24.2 ± 1.3	147.3 ± 18.0	0	100	20	80
		50	15	20.9 ± 2.1*	141.8 ± 17.5***	0	100	60	100
		100	15	–[b]	131.2 ± 11.6**	0	40	100	100
10–11	22	Control	18	24.8 ± 0.7	170.9 ± 13.0	0	100	0	0
		50	18	23.4 ± 0.3*	155.6 ± 13.0	0	94.4	11.1	94.4
		150	18[c]	–[b]	121.4 ± 11.2*	0	100	66.7	100
		300	18	–[b]	117.8 ± 23.7*	0	94.4	100	100
		450	18	–[b]	116.1 ± 10.2*	27.8	61.1	100	100
13–14	20	Control	18	25.4 ± 0.5	195.1 ± 12.1	0	100	0	0
		150	18	23.3 ± 2.4*	164.4 ± 32.8	0	100	0	77.8
		450	18	–[b]	153.1 ± 20.8*	0	100	0	100
		900	18	–[b]	102.9 ± 19.9*	33.3	100	5.6	100
16–17	20	Control	18	27.9 ± 0.5	251.2 ± 18.5	0	100	0	0
		300	18	–[b]	152.5 ± 28.2*	0	100	0	61.1
		900	18	–[b]	141.0 ± 16.9*	0	100	0	100

[a] Values represent mean ± SE. [b] Could not be counted because of poor definition. [c] Including the isotope experiment. * Significantly different from control ($p < 0.001$). ** Significantly different from control ($p < 0.005$). *** Significantly different from control ($0.05 < p < 0.02$).

mites were most susceptible. However, at higher concentrations, it was difficult to count the number of somites because of poor definition. Of 4 groups of embryos examined, 6–8 somite embryos had the highest sensitivity to BUdR treatment. In contrast, 16–17 somite embryos remained alive in the presence of a high concentration of the drug as 900 μg/ml. BUdR also interfered with embryonic growth; treated embryos had a lower protein content than untreated controls.

The most remarkable inhibitory effect of BUdR was formation of the open cranial neural tube. The incidence of this abnormality was dependent on both the concentration of BUdR and the developmental stage of embryos at treatment. At the 6–8 and 10–11 somite stages, 100 and 300 μg/ml of BUdR respectively caused this abnormality in 100% of the embryos examined. The embryos were severely affected in the midbrain to the upper hindbrain regions; this resulted in poor development of the concave curvature in the neuroepithelium (fig. 2). The forebrain neural folds were also variably unfused. At the 13–14 and 16–17 somite stages, however, the embryos became insensitive to the induction of an open cranial neural tube by BUdR. After treatment of the 13–14 somite embryos with 900 μg/ml of BUdR, only 1 embryo had an open cranial neural tube in the rostral half of the midbrain region. This embryo was considered dead because the heartbeat was very feeble and blood particles did not circulate in the visceral yolk sac. The 16–17 somite embryos were free from the formation of this abnormality.

Another characteristic effect of BUdR was inhibition of closure of the otic vesicles. This inhibitory effect was not dependent on the developmental stage of embryos at treatment, but the degree of inhibition was largely dependent on the concentration of BUdR (fig. 3, A–D). Histological observations were made on the 10–11 somite embryos treated with 300 μg/ml of BUdR and 13–14 somite embryos treated with 150–900 μg/ml of BUdR in comparison with the respective controls. BUdR caused various types of abnormal cells to develop in the neuroepithelium. Affected neuroepithelial cells often had an enlarged nucleus, pyknosis and chromosomal abnormalities (fig. 3). In addition to these abnormal cells, mitotic figures were occasionally misplaced, although they were mostly located on the luminal side of the neuroepithelium (fig. 4). Furthermore, a number of cells protruded from the luminal side of the neuroepithelium. Most of them were mononuclear, while a part of them appeared to be binuclear (fig. 5). All the above mentioned abnormalities in the neuroepithelium occurred commonly in both the 10–11 somite embryos treated with 300 μg/ml of BUdR and 13–14 somite embryos treated with 900 μg/ml of BUdR. Table 2 shows the incorporation of the label into the

Table 2. Incorporation of the label into the fractions of the 10–11 somite mouse embryos after 22 h of culture in the presence of 150 μg/ml of ^{3}H-BUdR

Fraction	$\times 10^3$ dpm/embryo (mean ± SE)
TCA-soluble	11.9 ± 1.0
RNA	0.96 ± 0.06
DNA	48.9 ± 6.2

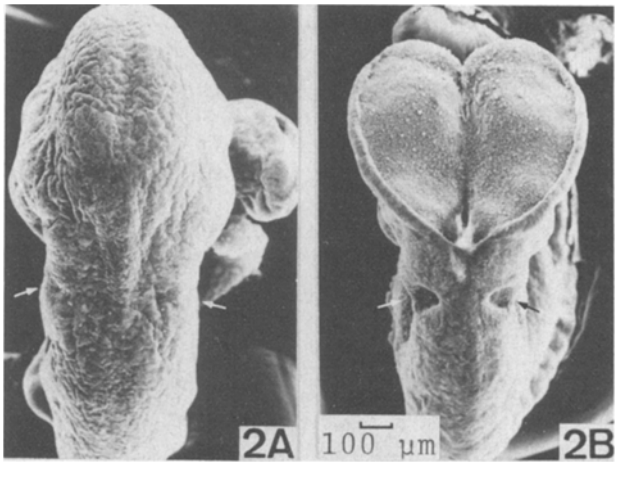

Figure 2. SEM of embryos explanted at the 10–11 somite stage and cultured for 22 h. *A* A control embryo. The otic vesicles (arrows) have closed. *B* An embryo treated with 300 μg/ml of BUdR. The neuroepithelium in the midbrain to the upper hindbrain regions is severely affected to show poor development of the concave curvature. The otic vesicles (arrows) are also open.

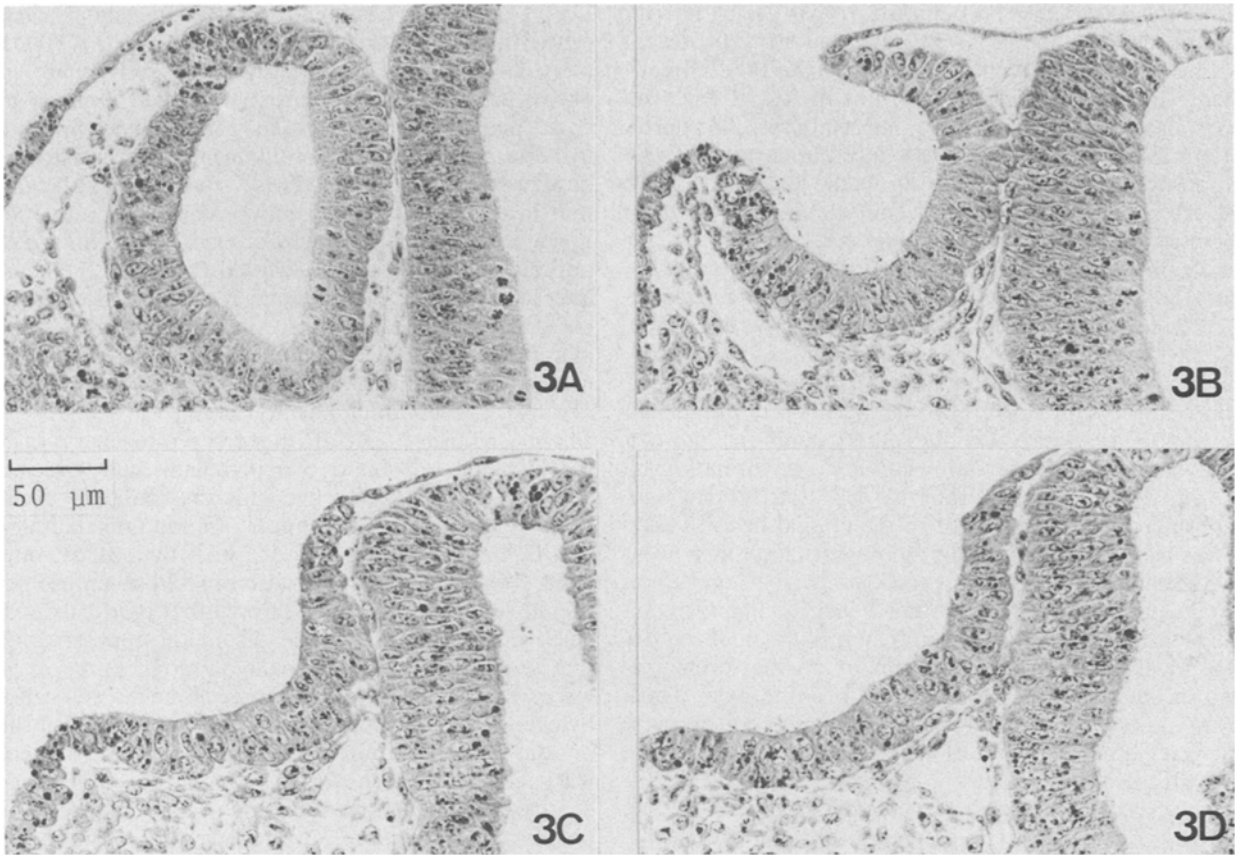

Figure 3. Transverse sections through the otic vesicles of embryos explanted at the 13–14 somite stage and cultured for 20 h. *A* A control embryo. *B* An embryo treated with 150 µg/ml of BUdR. *C* An embryo treated with 450 µg/ml of BUdR. *D* An embryo treated with 900 µg/ml of BUdR. Nuclear enlargement and pyknosis are evident in the neuroepithelium.

fractions when the 10–11 somite embryos were cultured for 22 h in the presence of 150 µg/ml of ^{3}H-BUdR. It was found that most of the label was incorporated into the DNA fraction.

Discussion. The present study showed the inhibitory effects of BUdR (25–900 µg/ml, i.e. 8.1×10^{-5} to 2.9×10^{-3} M) on mouse embryos at various stages of development during neurulation. Regardless of the developmental stage of embryos at treatment, BUdR inhibited the heartbeat, visceral yolk sac circulation, erythrocyte and somite formation, closure of the otic vesicles and embryonic growth. The degree of inhibition was largely dependent on both the concentration of BUdR in the culture medium and developmental stage of embryos at treatment. Nakashima and Fujiki[27] have reported similar effects on early somite mouse embryos.

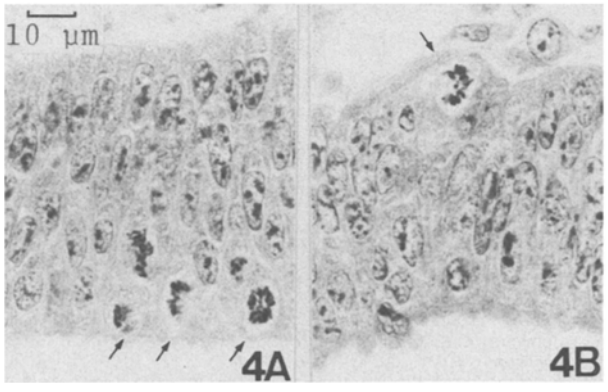

Figure 4. Neuroepithelium in the midbrain region of embryos explanted at the 10–11 somite stage and cultured for 22 h. *A* A control embryo. Mitotic figures (arrows) are located on the luminal side of the neuroepithelium. *B* An embryo treated with 300 µg/ml of BUdR. A mitotic figure (arrow) is misplaced.

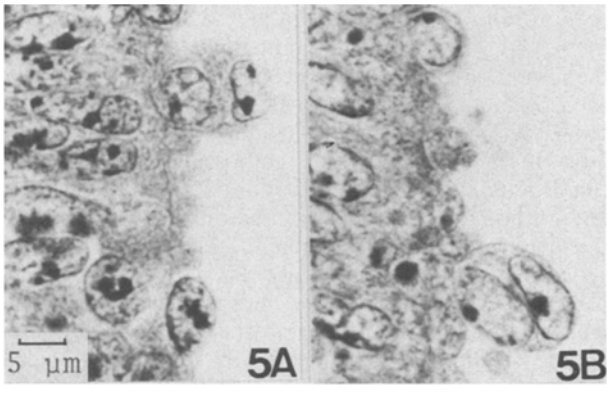

Figure 5. Protrusion of cells from the luminal side of the neuroepithelium having one or 2 nuclei. *A* A 10–11 somite embryo treated with 300 µg/ml of BUdR. *B* A 13–14 somite embryo treated with 900 µg/ml of BUdR.

From these studies, it was confirmed that during neurulation, mouse embryos at an advanced stage of development virtually became refractory to the BUdR treatment. Such a stage-dependent effect of BUdR has also been shown in cleaving sea urchin embryos. At appropriate concentration BUdR produced abnormal blastulation in sea urchin embryos up to the 8-cell stage. But BUdR at a 1000 µg/ml level had a minimal effect on them at the 100-cell stage or older stages[11,41].

In a number of developing embryos including mouse[38], rat[26], hamster[32,36] and chick[17], the teratogenic effects of BUdR are stage-specific. Skalko et al.[38] have suggested in their experiments with pregnant mice that exencephaly is produced during the period when the anterior neuropore is still open, but no longer occurs once the neuropore is closed. On the other hand, the present study showed a close relationship between formation of the open cranial neural tube by BUdR treatment and morphological development of the cranial neural folds. It has been reported that morphological development of the cranial neural folds is expressed by the number of somites in mouse[9,15,42], rat[24] and hamster[42] embryos. In mouse embryos, the neuroepithelium in the midbrain to the upper hindbrain regions remains in the form of a convex surface until about the 10–11 somite stage. Then by about the 13–14 somite stage, the neuroepithelium in these regions rapidly develops a concave curvature, although the anterior neuropore is still open. It is at this stage that mouse embryos become insensitive to the BUdR-induced open cranial neural tube. Therefore, the present results suggest that BUdR exerts its effect by interference with development of a concave curvature in the neuroepithelium. In addition, the results also suggest that the process of neural tube closure at the spindle-shaped opening posterior to the forebrain apposition point is insensitive to the analog, and so is the actual fusion process mediated by interdigitating cell processes.

Microfilament contraction has been reported to play an important role in bringing about the wedge-shape of cells[5,14], cell motility[13,30], and cytokinesis[33,34]. Cytochalasins (B and D), which are known to disrupt the microfilament bundles reversible, strongly inhibit closure of the neural tube in explanted rat embryos during neurulation[25]. Interkinetic migration is known to occur in the cells of the forming neural tube and almost all mitotic figures are located on the luminal side of the neuroepithelium[7,20]. Messier and Auclair[21] have proposed that such cell motility produces localized expansion of the outer zone of the neuroepithelium. In the neuroepithelium of chick[21] and mouse[43] embryos exposed to cytochalasin B, interkinetic migration is strongly inhibited and mitotic figures are distributed throughout the entire thickness of the neuroepithelium. The histological observations in the present study showed occasional misplacement of mitotic figures and protrusion of mononuclear or binuclear cells from the luminal side of the neuroepithelium, independently of the morphological development of the cranial neural folds. Again, these cellular abnormalities closely resembled the abnormal cells in the neuroepithelium of 10-day mouse embryos treated with cytochalasin B[43], although the effects were more pronounced than those of BUdR reported here. Lee et al.[18] have also reported similar cellular effects other than inhibition of cytokinesis in the BUdR-treated neuroepithelium of explanted early chick embryos. Moreover, they showed with their electron microscopic observations that the microtubules appeared to be normal but the microfilaments were not formed into prominent bundles. These considerations suggest that in the BUdR-treated mouse embryos poor development of the concave curvature in the midbrain to the upper hindbrain regions is, at least in part, the consequence of disruption of interkinetic migration and/or microfilament bundles. However, it still remains unexplained why BUdR is insensitive to the process of neural tube closure at the spindle-shaped opening posterior to the forebrain apposition point.

The mechanism of BUdR-induced teratogenesis is not well understood. In vivo experiments with pregnant animals showed that cell death was found in organs that would later be malformed[32]. In addition, Bannigan and Langman[3] were unable to show that BUdR interfered with cellular differentiation in 12-day mouse neuroepithelium. Instead, they showed that BUdR caused a delay in the cell cycle time. From these observations they speculated that exencephaly in mouse embryos following treatment on day 8 of development was caused by excessive cell death and the resultant cell deficit during the time of closure of the neural tube. On the contrary, it has been shown that in mouse embryos cell death is not an important feature in the pathogenesis of polydactyly induction[35]. Moreover, Wise and Scott[44] have indicated that incorporation of teratogenic levels of BUdR prevents physiological cell death in the foyer primaire preaxial. They supported a hypothesis based on these observations that BUdR-induced teratogenesis is a result of the antidifferentiative effects of the drug on cells of developing embryos. It has been reported that in early somite mouse embryos the induction of an open cranial neural tube by BUdR is effectively inhibited in the presence of excess thymidine[27]. Therefore, at present, it may be reasonable to conclude that incorporation of the analog into DNA in place of thymidine is a mechanism leading to an open cranial neural tube, although other mechanisms such as interference with interkinetic migration and/or microfilament bundles may also be involved.

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