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Retinoic acid-induced spina bifida: evidence for a pathogenetic mechanism

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Summary

Treatment of C57Bl/6J mice with three successive doses of all-*trans* retinoic acid (28 mg kg^{-1} body weight) on 8 day, 6 h (8d,6h), 8d,12h, and 8d,18h of gestation resulted in a high incidence (79 %, 31/39 fetuses) of spina bifida with myeloschisis (spina bifida aperta) in near term fetuses. Twelve hours following the last maternal dose (9d,6h), the caudal aspects of treated embryos, were abnormal, with eversion of the neural plate at the posterior neuropore, as compared to its normal concavity in comparably staged control specimens. This eversion persisted in affected embryos through the time that the posterior neuropore should normally close. The distribution of cell death in control and experimental embryos was determined using vital staining with Nile blue sulphate and with routine histological techniques. Twelve hours following the maternal dosing regimen, experimental embryos showed evidence of excessive cell death, predominantly in the mesenchyme associated with the primitive streak and in the endoderm of the tail gut, both of which are readily identifiable sites of physiological cell death at this stage of development. In addition, the presumptive trunk neural crest cells located in the dorsal midline, cranial to the posterior neuropore, exhibited a marked amount of cell death in the experimental embryos. We propose that the major factor in the generation of spina bifida in this model is excessive cell death in the tail gut and mesenchyme ventral to the neuroepithelium of the posterior neuropore. This causes a disparity in growth between the ventral and dorsal regions of the tail causing the relatively faster growing dorsal region (the neural plate) to evert, preventing caudal neural tube closure. Mesenchymal deficiencies resulting from excessive cell death in the gastrulating mesoderm would also account for the lack of tail formation and the abnormally formed lumbosacral vertebrae observed in this model. Previous investigations have suggested that the pathogenesis of a number of retinoic acid-induced malformations is related to excessive cell death in regions of physiological cell death. The current investigation explores the role of this phenomenon in the pathogenesis of experimental spina bifida.

Key words: spina bifida, pathogenesis, retinoids, mouse, cell death.

Introduction

Spina bifida is a common human malformation with an incidence ranging from 1/1000 in Wales and the Republic of Ireland to 1/2000 in England, The Federal Republic of Germany and the USA (Lorber, 1986). In many western countries, the rate of appearance of this malformation has shown a gradual decline, attributable in part to antenatal diagnosis and termination of pregnancy and possibly also to an improvement in antenatal nutrition (Lorber, 1986). In spite of the significance of caudal neural tube defects in terms of their incidence and consequences, relatively little data exist concerning the cellular basis for their genesis. Non-closure of the neural folds (von Recklinghausen, 1886) or rupture of the closed neural tube (Morgagni, 1769) are the major hypotheses for the pathogenesis of these malformations. While various investigators have championed one or the other hypothesis, it would seem reasonable that each is involved in the generation of different types of spinal anomalies (reviewed by Brocklehurst, 1971). Experimental models have shown that an insult at the neural plate stage of development may prevent closure (Peters and Dormans, 1981; Wiley, 1983), while a later insult could reopen a closed neural tube (Padmanabhan, 1984).

Current controversy regarding the non-closure phenomenon concerns the question as to what embryonic tissue(s) is initially affected. Peters and Dormans (1981) state that it is an accepted view that the neuroepithelium is primarily affected and that associated mesenchymal (vertebral) abnormalities are secondary effects. However, they (Peters and Dormans, 1981) concluded that mesenchymal changes, in the absence of neuroepithelial changes in the presumptive lumbosacral region during neurulation, can cause spina bifida aperta. Additionally, Marin-Padilla (1966) and Morriss (1972) have suggested that hypervitaminosis A-induced cranial neural tube defects (exencephaly) are related to a mesenchymal affect. This idea has been extended to

the pathogenesis of all dysraphic disorders (Marin-Padilla and Marin-Padilla, 1981).

In the present investigation, we have studied the pathogenesis of retinoic acid-induced spina bifida. The retinoids are potent teratogens, with excessive gestational exposure resulting in a variety of developmental defects in humans and other vertebrates (reviewed by Geelen, 1979; Howard and Wilhite, 1986; Rosa *et al.* 1986). Among the defects seen in rodents exposed to retinoic acid (RA) are anomalies of the lower vertebrae and spinal cord or spina bifida (Shenefelt, 1972; Marin-Padilla and Marin-Padilla, 1981; Wiley, 1983). Although spina bifida is not a recognized feature of retinoic acid embryopathy (Lammer *et al.* 1985), its association with excessive retinoid exposure in humans has been reported (Rosa *et al.* 1986; Happle *et al.* 1984).

In animal models, axial skeletal anomalies, such as spina bifida, are produced by early RA insults, with exposure occurring prior to the time of anterior neuropore closure, while appendicular skeletal anomalies (limb malformations) are produced by RA insults at later developmental stages (Shenefelt, 1972). This disparity in temporal vulnerability has prompted Wiley (1983) to remark that different mechanisms may generate the two types of defects. Vascular damage leading to abnormalities in the somitic mesoderm was initially proposed as the primary cause of RA-induced spina bifida (Wiley, 1983; Tibbles and Wiley, 1988), although more current work by this group suggests otherwise (Griffith and Wiley, 1989). Selective vulnerability of cartilage has been proposed as the primary basis for RA-induced appendicular skeletal defects (Kochhar, 1977 and 1985). Menkes *et al.* (1964) proposed that regions of physiological cell death are vulnerable sites for teratogenic insult. We suggest that this selective vulnerability could form a common basis for RA-induced axial and appendicular skeletal anomalies, as well as for malformations involving a variety of other organ systems. In previous studies in our laboratory (Sulik *et al.* 1988; Sulik and Dehart, 1988; Alles and Sulik, 1989) and that of Schweichel (1971), it has been demonstrated that the pathogenesis of RA-induced limb malformations, as well as those of the craniofacial region, involves excessive cell death in regions of physiological cell death. In the present study, we have extended this hypothesis to the pathogenesis of RA-induced spina bifida.

Materials and methods

C57Bl/6J mice purchased from The Jackson Laboratory, Bar Harbor ME, were paired for 1–2 h in the morning and, at the end of this time, animals with vaginal plugs were considered to be at 0 days and 0 h (0d,0h) of pregnancy. Pregnant mice were gavage-fed 3 successive doses of all-*trans* retinoic acid (RA, 28 mg kg^{-1} body weight) suspended in sesame oil on 8d,6h, 8d,12h and 8d,18h of gestation. This dose schedule was determined by preliminary studies using different doses to arrive at a schedule that caused a maximum impact on the conceptuses. Control animals were gavage-fed a comparable volume of vehicle (sesame oil) or were untreated; because there was no apparent difference between the control groups they are considered together.

Groups of treated and control mice were allowed to survive to day 18 of pregnancy, at which time they were killed and the litters harvested, with note being made of resorption sites and numbers of viable fetuses. The fetuses were examined for gross defects and then fixed in Bouin's fluid for histological examination. Additionally, treated mice were killed and the embryos removed 12 h after the administration of the last dose of RA (9d,6h). Control embryos were collected at the same time. Embryos were staged by somite number. Some embryos without extraembryonic membranes were supravitally stained with Nile blue sulphate (NBS; diluted 1:50000 in lactated Ringer's solution) for 30 min at 37°C (modified from Hinchliffe and Ede, 1973). Stained specimens were examined and photographed using a Nikon photomicroscope. Some embryos were fixed in Bouin's fluid and subsequently processed and embedded in paraffin (Ameraffin, American Scientific Products) or JB4 resin (Polysciences). Sections were cut, stained with methylene blue and acid fuchsin or haematoxylin and eosin, and examined for RA-induced histological changes. Mitotic indices were calculated for paramedian region of the neural plate in control and treated embryos. Mitotic and non-mitotic cells were counted in 10 μm parasagittal paraffin sections (at least 3 sections 40 μm apart per embryo). The mitotic index was calculated for each embryo and the mean mitotic indices of the control and treated group were compared using Student's *t*-test at the 0.05 level of significance. In addition, some control and experimental embryos collected on 9d,6h as well as on 9d,20h were fixed in 2.5% glutaraldehyde in Sorenson's buffer, dehydrated in ethanol, critical-point dried, and sputter coated with gold–palladium (Sulik and Johnston, 1982). These specimens were examined using a JEOL scanning electron microscope.

Results

Maternal treatment with retinoic acid on day 8 of gestation in mice results in the production of lumbosacral vertebral and spinal cord anomalies with varying degrees of severity (Tibbles and Wiley, 1988). In our study, treatment was commenced on 8d,6h (3- to 5-somite stage). A total of 3 control and 8 experimental litters of gestational day 18 fetuses were compared for gross malformations. A high incidence of resorption (44%, 31/70) and malformations in live fetuses (100%, 39/39) was observed in the treated animals while no

Table 1. *Retinoic acid-induced malformations in near term fetuses*

No. of near term litters		No. of implantation sites		No. of resorptions		No. of malformations		
C	Tr	C	Tr	C	Tr	C	Tr	
3	8	26	70	0	31	0	35	12: Ex, Sp 18: M, Sp, Eo, P 1: M, Sp 4: M, Eo, P 4: M

Legend: C=control; Tr=treated; M=microcephaly 69.2% (27/39); Sp=spina bifida 79.5% (31/39); Eo=exophthalmos 56.4% (22/39); Ex=exencephaly 30.8% (12/39); P=malformed pinna of external ear 56.4% (22/39).

resorptions (0/26) or malformations (0/26) were seen in controls. Among the surviving day 18 fetuses in this study, 79% (31/39) had rachischisis with myeloschisis, i.e. spina bifida aperta (Fig. 1). The other major malformations seen are detailed in Table 1.

In the experimental embryos that were examined 12 h after the last maternal RA treatment, the neural plate at the posterior neuropore was everted. In control embryos of comparable developmental stages, the caudal neural plate grades from being flat to slightly

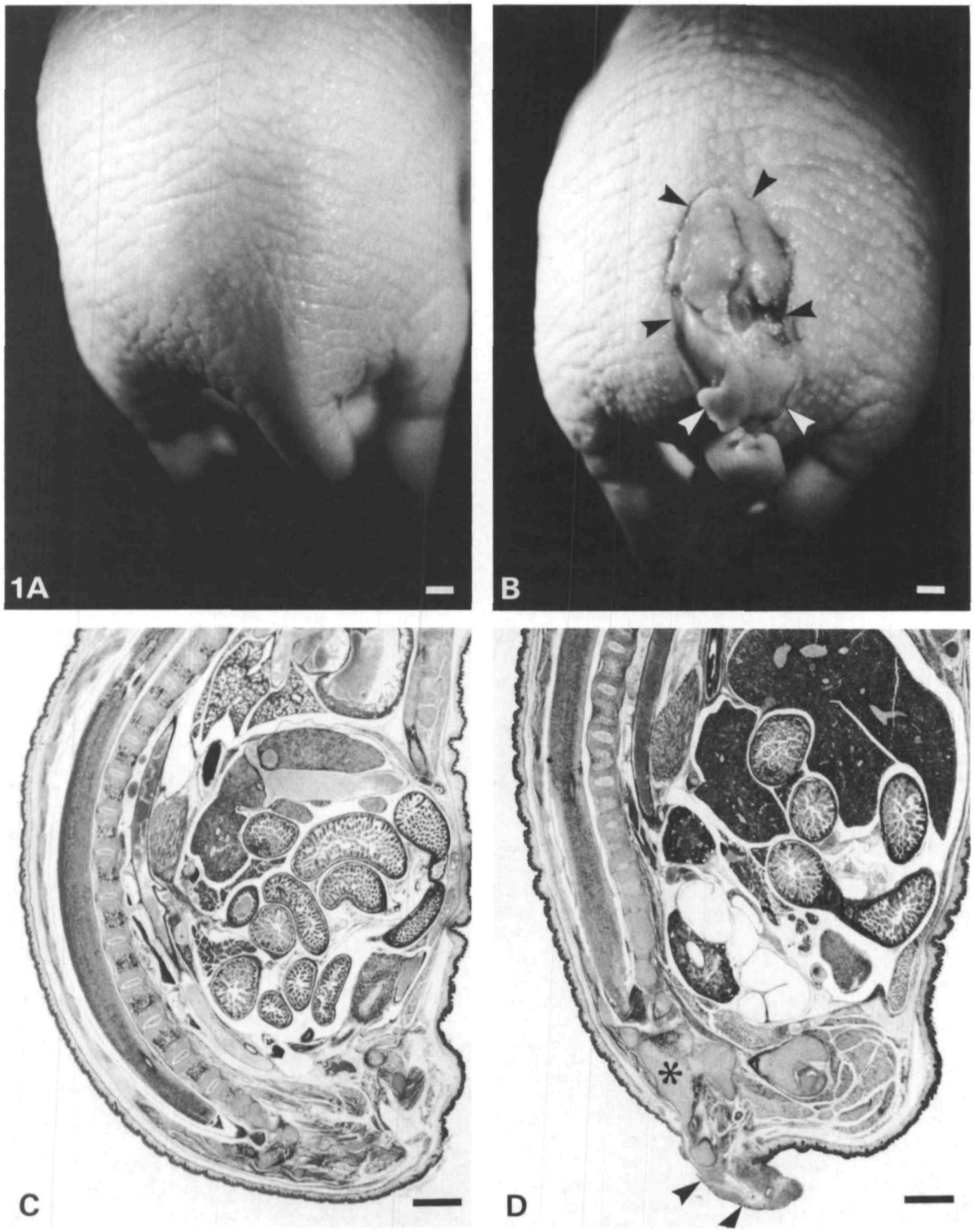
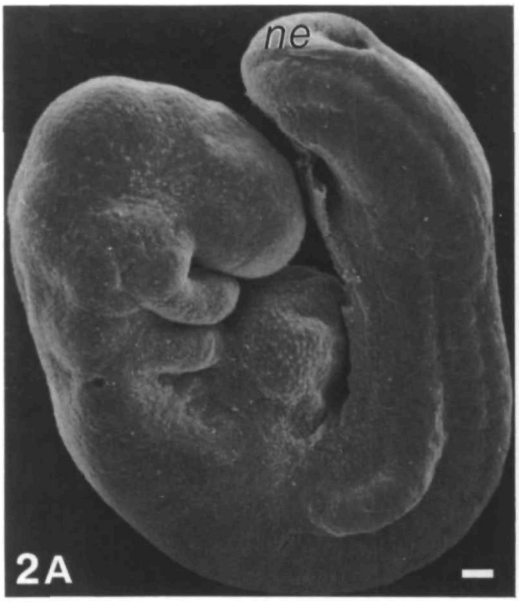


Fig. 1. Control (A and C) and treated (B and D) 18d fetuses. Note the exposed open spinal cord (arrowheads) and disrupted vertebrae (asterisk) in the treated fetuses. Scale bar, 400 μm.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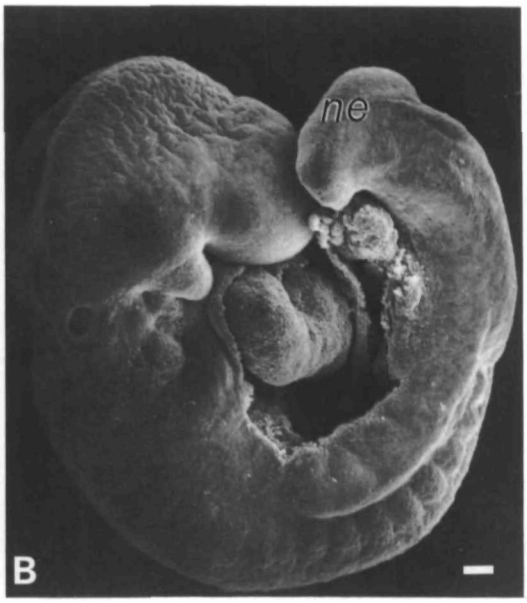
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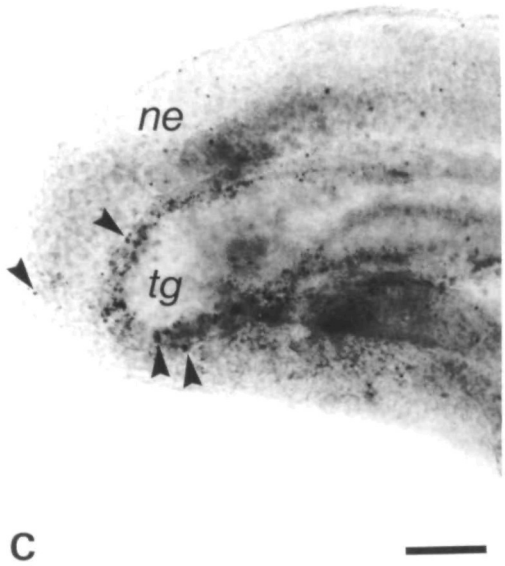
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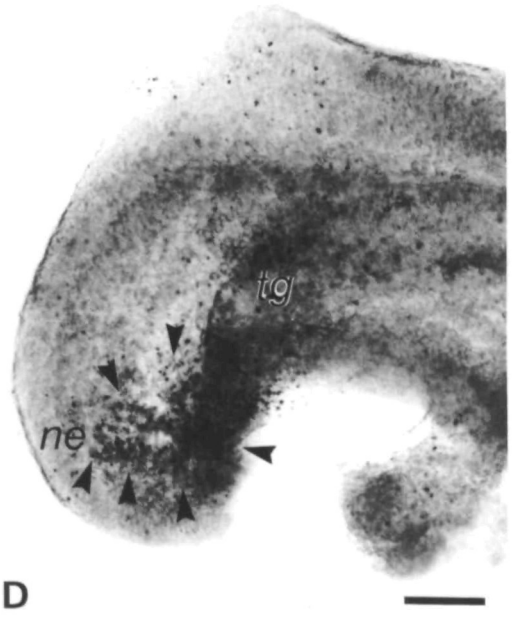
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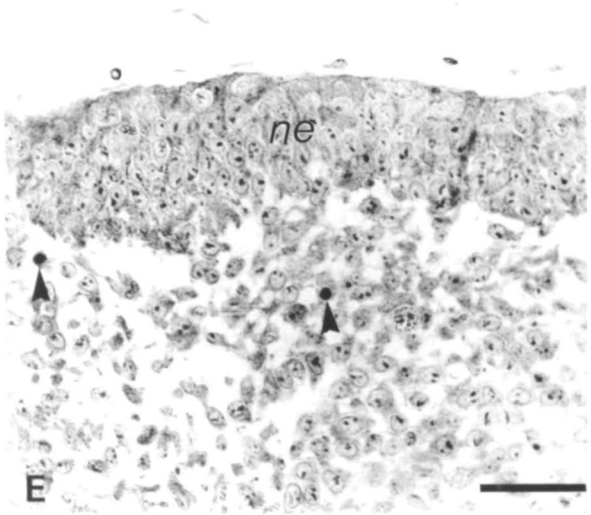
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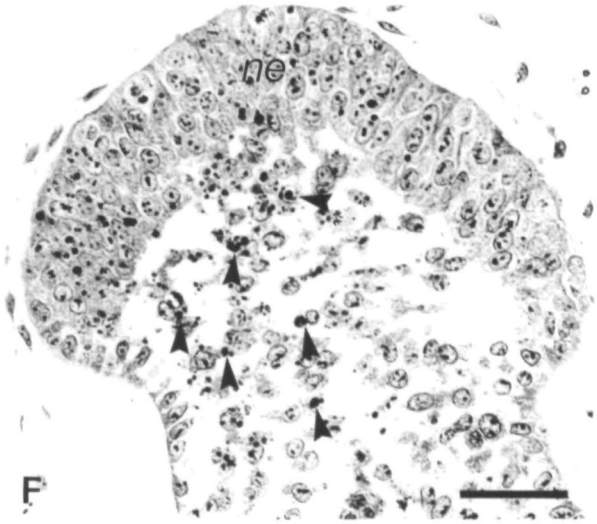
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Fig. 2. (A and B) Scanning electron micrographs of control (A) and treated (B) 9d,6h embryos. Note the abnormal everted appearance of neural epithelium (ne) in the treated embryo as compared to the control. (C and D) Whole-mount preparations of the caudal end of control (C) and treated (D) 9d,6h Nile blue sulphate-stained embryos showing an increased amount of cell death (arrowheads) in the tail gut (tg) and in the mesoderm ventral to the neural epithelium (ne) in the treated embryo. Note that the terminal end of the tail gut in the treated embryo is obscured by the surrounding cell death. (E and F) Transverse sections through the distal portion of the caudal end of control (E) and treated (F) embryos. Note the increased amount of cell death (arrowheads) in the treated embryo. Scale bars, 50 μm.

convex. This difference in control and experimental specimens was especially evident in scanning electron micrographs (Fig. 2A and B) The changes that presage the craniofacial malformations observed on day 18, including absence of the second visceral arch (as previously reported by Webster *et al.* 1986) and delayed closure of the otic vesicle, were also noted (Fig. 2B).

When embryos were examined 12 h after maternal exposure to RA, there was no statistically significant difference between the mean somite number of control (20.23) and treated (18.77) embryos (Table 2). Because of the stage-dependent variation of physiological cell death, only embryos matched by somite number were used to assess the extent of cell death. Supravital NBS staining at this time revealed an excessive amount of stain uptake in the caudal extremity of experimental embryos (Fig. 2 C and D) which was most intense in the gastrulating mesoderm of the tail bud and the wall of tail gut. Staining was not excessive in the edges or surface of the everted neural plates of affected embryos except in the region of the primitive streak at the caudal-most extremity of the embryo. Compared to control embryos having equivalent numbers of somites, experimental specimens also had increased amounts of stain uptake in the dorsal midline of the trunk, extending to the posterior neuropore (Fig. 3) and in other sites such as the lateral lip of the otic vesicle.

Table 2. *Comparison of stage and malformations in embryos 12 h after retinoid exposure*

	Control	Treated
No. of embryos	26	31
Mean no. of somites	20.23 (S.D.=1.56)	18.77 (S.D.=3.86)*
No. of abnormal tail buds	0	31

*There was no difference at the 0.05 level of significance with Student's *t*-test.

As expected, histological sections through the caudal end of the embryos revealed that the regions with histological evidence of cell death corresponded with the regions of excessive NBS uptake seen in supravitally stained experimental embryos. In the tail bud, cellular debris was located ventral to the posterior neuropore, predominantly in the gastrulating mesoderm associated with the primitive streak and in the wall of the tail gut (Fig. 2 D and F). These are readily apparent sites of normal cell death in control embryos (Fig. 2 C and E). Mesenchymal cell populations appeared scant, as compared to those in the control specimens. Although not as extensive as in the aforementioned caudal tissues, some cellular debris was also seen in the neural plate close to the tip of the tail bud in experimental specimens. A remarkable amount of cell death was evident in the dorsal aspect of the trunk neural tube, obviously, to a large extent, affecting the trunk neural crest cell population.

There was no significant difference between the mitotic index of the neural plate in control and treated embryos (Table 3).

In control and experimental embryos examined at 9d,20h (approximately 24 h after the last RA treatment, a time at which the posterior neuropore is completing its closure), affected embryos showed varying degrees of abnormality with continued eversion of the posterior

Table 3. *Mitotic indices of treated and control embryos*

Control					Treated				
Embryo no.	No. of non-mitotic	No. of mitotic	Total	Mitotic index (%)	Embryo no.	No. of non-mitotic	No. of mitotic	Total	Mitotic index (%)
1	215	11	226	4.867	1	413	22	435	5.057
2	343	17	360	4.722	2	403	21	424	4.952
3	292	16	308	5.194	3	312	14	326	4.294
4	397	28	425	6.588	4	281	19	300	6.333
5	196	14	210	6.666	5	281	12	293	4.095
6	245	19	264	7.196	6	327	16	343	4.664
7	564	29	593	4.890	7	212	12	224	5.357
8	324	18	342	5.263	8	208	18	226	7.964
9	305	20	325	6.153	9	212	8	220	3.636
10	312	16	328	4.878	10	265	18	283	6.360
11	214	16	230	6.956					

n=11
Mean control mitotic index=5.761, S.D.=0.955

n=10
Mean treated mitotic index=5.271, S.D.=1.295

There was no difference in the mitotic indices of the two groups at the 0.05 level of significance with Student's *t*-test.

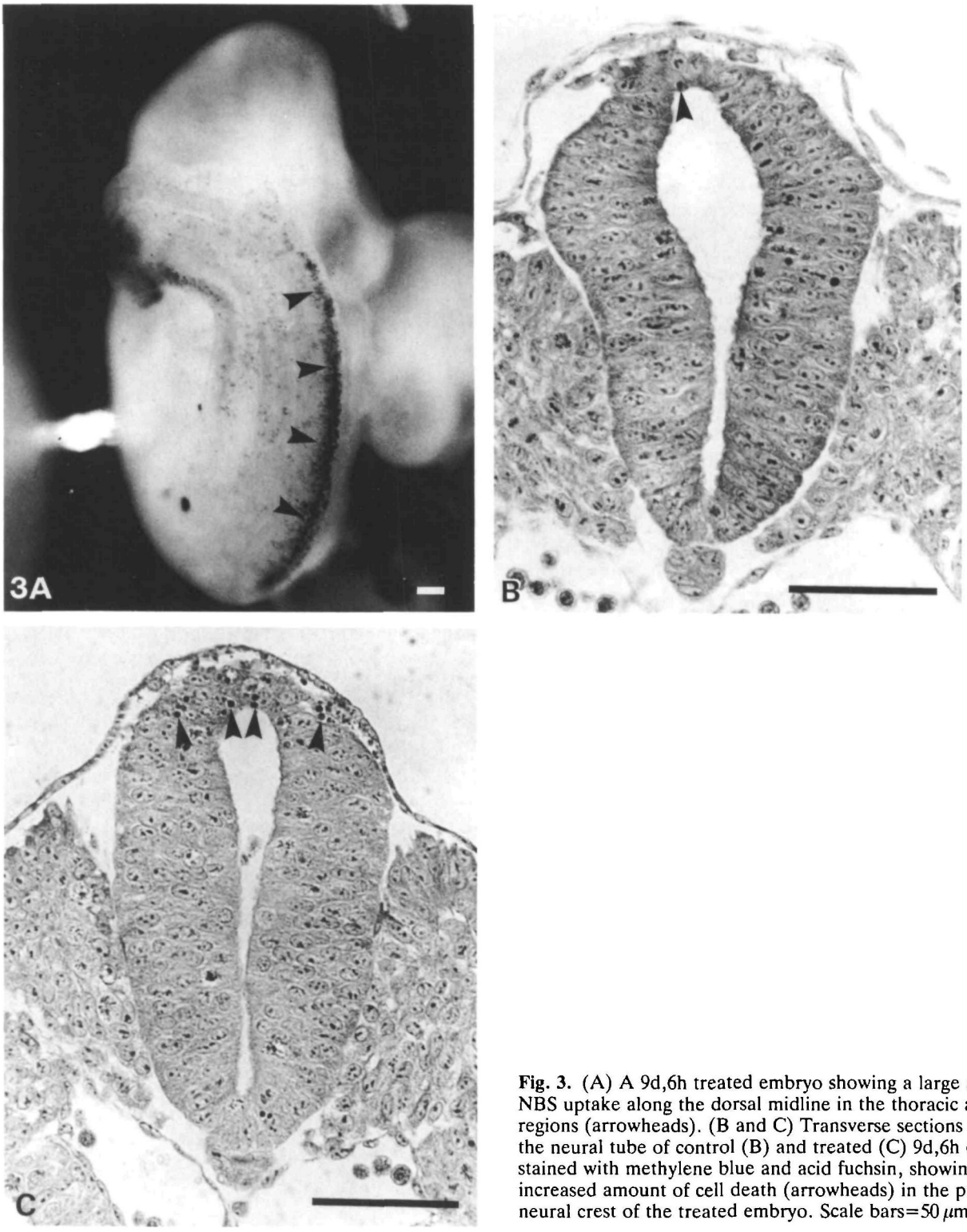

Fig. 3. (A) A 9d,6h treated embryo showing a large amount of NBS uptake along the dorsal midline in the thoracic and lumbar regions (arrowheads). (B and C) Transverse sections through the neural tube of control (B) and treated (C) 9d,6h embryos stained with methylene blue and acid fuchsin, showing an increased amount of cell death (arrowheads) in the presumptive neural crest of the treated embryo. Scale bars=50 μm.

neural plate and absence of the tail in those severely affected (Fig. 4).

Discussion

Spina bifida aperta was induced in this study by exposure of mice to excessive amounts of retinoic acid. This malformation is comparable to that previously described by other investigators using hamsters or mice (Wiley, 1983; Tibbles and Wiley, 1988). However, the incidence of myeloschisis was higher in this study (79 % as compared to 8.4 %) than in the report by Tibbles and Wiley (1988) in which a single 80 mg kg^{-1} dose of all-*trans* retinoic acid was acutely administered to CD-1 mice on the eighth day of pregnancy. The fact that multiple low doses of RA can produce a higher inci-

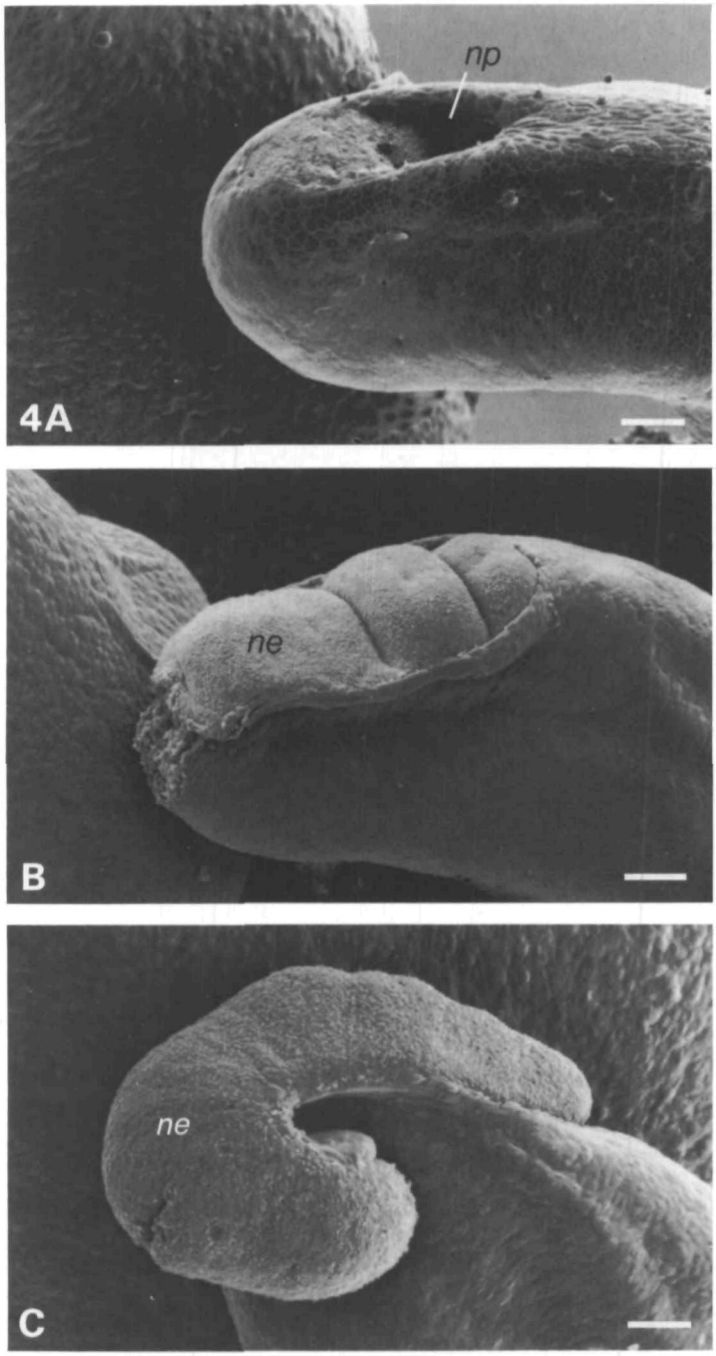

Fig. 4. Scanning electron micrographs of control (A) and treated (B and C) 9d,20h embryos (30 somites). Note the posterior neuropore (NP) in the control and the eversion of the neural epithelium (ne) in the treated embryos. The embryo in Fig. 4C, in particular, depicts the dramatic eversion of neural tissue. Scale bar=50 μm.

dence of malformation has also been demonstrated in other investigations (Kochhar and Penner, 1987). However, in the present study, the multiple dose regimen also resulted in a high incidence of resorptions. The ability to effectively induce a high incidence of malformation (79% of live fetuses) facilitated our investigation of pathogenesis.

The use of vital staining to illustrate sites of cell death was extremely useful, as it provided a comprehensive map, which would have been hard to appreciate without laborious three-dimensional reconstruction of histological sections. The fact that maternal retinoic acid administration resulted in excessive amounts of cell death in selected cell populations was readily apparent in both the vitally stained specimens and in those examined histologically. The regions of excessive cell death of particular interest for the current investigation were those in the dorsal neural tube, in the gastrulating mesoderm, and in the tail gut.

The affected cell population in the dorsal region of the trunk neural tube appeared, based upon location, to be presumptive neural crest. There was no morphological evidence of cell death in the presumptive neural crest associated with the posterior neuropore (the edges of the neural folds). Although the extensive amount of cell death that occurs in the presumptive trunk neural crest might be expected to result in the reopening of the closed neural tube in the trunk, no evidence of this phenomenon was observed. The neural tube defects noted in this study did not extend cranially into upper lumbar and thoracic levels. Cell death in the trunk neural crest may produce defects in its derivatives and further investigation along this line is needed. The susceptibility of some neural crest cell populations (primarily cranial neural crest cells) to RA teratogenesis has been described previously (Webster *et al.* 1986; Wiley *et al.* 1983). Webster *et al.* (1986) noted that the teratogenic insult may occur while the neural crest precursors are still within the neural folds. Studies by Dencker (1987, and personal communication) have shown specific accumulation of retinoic acid and localization of its cellular binding protein in the neural epithelium of mouse embryos. This is particularly evident in the transitional zone between the surface ectoderm and neural epithelium in the cranial region, i.e., the region of presumptive neural crest. It will be of interest to determine whether nuclear receptors for RA (Petkovich *et al.* 1987) localize to neural crest cell populations, and also to determine whether the other sites of excessive cell death noted in the present investigation co-localize with cellular binding protein and/or nuclear receptors for RA. A role for the excessive cell death noted in this study in the presumptive neural crest cell populations in the pathogenesis of spina bifida is not readily apparent. However, other effects on this population at the level of the posterior neuropore, and on the posterior neural plate as a whole, cannot be ruled out.

As in previous investigations of retinoid teratogenicity and cranial neural tube defects (Marin-Padilla, 1966; Morriss, 1972), our results indicate that a major causative role for non-closure of the caudal neural tube lies in deficiencies in subjacent non-neural tissues. We propose that the excessive cell death observed in the tissues that underlie the posterior neural plate plays a significant role in the genesis of spina bifida aperta. The eversion of the neural plate, which is evident as early as 12 h following maternal RA administration, appears to

result from deficiencies and subsequently delayed growth in the mesenchymal tissues in the region of the primitive streak and the tail gut, concurrent with continued growth of the neural plate. The absence of a significant change in the mitotic index of the neural plate in experimental embryos precludes the idea that the malformation is caused by an increased mitotic rate in this structure. Growth discrepancy in retinoid-exposed embryos has previously been pointed out by Morriss (1972). Copp *et al.* (1988*a* and *b*) have shown that a growth imbalance between the ventral and dorsal tissues in the tail causes caudal neural tube defects in the Curly Tail mutant mouse. One could predict based on our model that a compound like RA would increase the incidence of spina bifida in this mutant. In fact, retinoid exposure on day 8 of gestation has an exacerbating effect on posterior neuropore defects in this mutant (Seller and Perkins, 1982 and Seller *et al.* 1979).

The basis for the selective vulnerability of the gastrulating mesoderm and the tail gut cells presumably lies in their ability to bind and/or respond to retinoids, as discussed above. It is noteworthy that these are also readily apparent sites of natural or physiological cell death. Zwilling (1942) has described normal cell death in the tail gut and undifferentiated mesenchyme in the tail of the chick. Previous investigations in this laboratory have shown that the pathogenesis of a variety of retinoid-induced malformations is related to excessive cell death in regions of physiological death (Sulik *et al.* 1988; Sulik and Dehart, 1988; Alles and Sulik, 1989).

The recent proposal that the retinoids are naturally occurring morphogens (Tickle *et al.* 1985) has prompted us to speculate that retinoic acid might be involved in regulating physiological cell death. The fact that the retinoic acid receptor belongs to the steroid receptor superfamily (Petkovich *et al.* 1987) and that steroids can mediate physiological cell death (Wyllie and Morris, 1982) lends support to this idea. Our hypothesis is consistent with that of Saunders, who, as early as 1966, stated that 'the hormones that initiate cell death and those that stimulate growth and differentiation are the same, the selective effect being the property of the target organ' and that 'administration of hormones can cause extension of normal foci of necrosis into adjoining regions which would not normally regress...'.

On the basis of our results, we propose that excessive cell death in the gastrulating mesoderm and tail gut of the embryo plays a significant role in the formation of retinoic acid-induced spina bifida aperta.

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