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Prevention of spinal neural tube defects in the mouse embryo by growth retardation during neurulation

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Summary

Homozygous mutant curly tail mouse embryos developing spinal neural tube defects (NTD) exhibit a cell-type-specific abnormality of cell proliferation that affects the gut endoderm and notochord but not the neuroepithelium. We suggested that spinal NTD in these embryos may result from the imbalance of cell proliferation rates between affected and unaffected cell types. In order to test this hypothesis, curly tail embryos were subjected to influences that retard growth *in vivo* and *in vitro*. The expectation was that growth of unaffected rapidly growing cell types would be reduced to a greater extent than affected slowly growing cell types, thus counteracting the genetically determined imbalance of cell proliferation rates and leading to normalization of spinal neurulation.

Food deprivation of pregnant females for 48 h prior to the stage of posterior neuropore closure reduced the overall incidence of spinal NTD and almost completely prevented open spina bifida, the most severe form of spinal NTD in curly tail mice. Analysis of embryos earlier in gestation showed that growth retardation acts by reducing the incidence of delayed neuropore closure. Culture of embryos at 40·5°C for 15–23 h from day 10 of gestation, like food deprivation *in vivo*, also produced growth retardation and led to normalization of posterior neuropore closure. Labelling of embryos *in vitro* with [^{3}H]thymidine for 1 h at the end of the culture period showed that the labelling index is reduced to a greater extent in the neuroepithelium than in other cell types in growth-retarded embryos compared with controls cultured at 38°C. Moreover, the incidence of cell death is increased in the neuroepithelium to a greater extent than in other cell types. These results are consistent with the hypothesis that spinal NTD in curly tail embryos result from a genetically determined growth imbalance between neuroepithelium and gut endoderm/notochord.

Key words: embryo, mouse, culture, neural tube defects, growth retardation, spinal cord, curly tail mutant.

Introduction

Spinal neural tube defects (NTD) arise in 60% of mouse embryos homozygous for the curly tail (*ct*) mutation due to delay of neural fold closure at the posterior neuropore (Copp, 1985). In the preceding paper, we identified a cell-type-specific cell proliferation abnormality that affects *ct/ct* embryos developing spinal NTD but not their normally developing littermates (Copp *et al.* 1988). The findings of this cell proliferation analysis suggest that defective spinal neurulation in curly tail embryos may result from an imbalance of cell proliferation rates within the posterior neuropore region. That is, growth of the neuroepithelium which is unaffected in *ct/ct* embryos is out of balance with growth of the gut endoderm and notochord, which proliferate abnormally slowly in *ct/ct* embryos.

If this hypothesis is correct, then spinal NTD should be prevented if a balance can be re-established between proliferation rates of the slowly growing gut endoderm and notochord, and the unaffected neuroepithelium. One way of achieving this may be to retard embryonic growth which, by analogy with cytotoxic chemotherapy (Bruce *et al.* 1966), should have the greatest effect on the most rapidly growing cells in the embryo. Thus, in *ct/ct* embryos affected by the genetically determined cell proliferation imbalance, growth retardation should diminish the absolute magnitude of the imbalance and lead to normal

spinal neurulation. In the present paper, we describe experiments designed to test this hypothesis in which curly tail embryos were subjected to influences that retard growth, both *in vivo* and *in vitro*.

Materials and methods

Curly tail mice were maintained and embryos were recovered from pregnant females as described in the accompanying paper (Copp *et al.* 1988).

Growth retardation in vivo

Pregnant curly tail females were deprived of food (with water freely available) for periods of 24 or 48 h, starting and ending at 10 a.m. on each day, between days 8 and 12 of gestation (day 1 = day of finding a copulation plug). Pregnant females were killed either on day 11 of gestation, when embryos were assessed for neuropore category (Copp, 1985) and somite number, or on day 13 when embryos were assessed for viability (on the basis of general appearance and presence of heart beat), presence of NTD, somite number and crown–rump length. Protein contents were determined by the method of Lowry *et al.* (1951) for ten of the day-13 embryos selected at random from each treatment group.

Growth retardation in vitro

Embryo cultures (method of New *et al.* 1973) were initiated on the afternoon of day 10 of gestation. After a 2–4 h recovery period in culture at 38 °C, half of the embryos, chosen at random, were cultured for a further 15–23 h (mean 18·5 h) at 40·5 °C while the remaining embryos were maintained at 38 °C for the same period. Cultures were terminated when embryos were expected to have reached the 27–29 somite stage. Membranes were removed and embryos were assessed in the living state for somite number, crown–rump length and size of neuropore (see fig. 3 in Copp, 1985). Previous studies have shown that the majority of embryos with closed or small open neuropores (category 1/2) develop normally, those with moderately enlarged neuropores (category 3) develop tail flexion defects and those with severely enlarged neuropores (category 4/5) develop open spina bifida with tail flexion defects (Copp, 1985). DNA content of embryos was determined by the method of LaBarca & Paigen (1980).

Cell proliferation during growth retardation

Embryos were labelled for the final hour of culture by addition of 2 μCi ml^{-1} methyl[^{3}H]thymidine (2 Ci mmol^{-1}, Amersham, UK) to the culture medium. Embryos were fixed in Bouin's fluid and sectioned serially at 6 μm. Sections were exposed for 4 days to Kodak AR10 stripping film. At least 100 cells were scored in sections through the region of active neurulation for each cell type except notochord where all cells were counted within a block of six alternate serial sections. Background grain count was determined separately for each embryo and cells with above background grain count were scored as labelled. Dead cells were counted as described previously (Copp, 1978). Labelling index was calculated as the number of labelled nuclei divided by the total viable nuclear count (×100). Dead cell index was the number of dead cells divided by the total viable nuclear count (×100).

Results

Growth retardation in vivo

In an attempt to induce growth retardation in curly tail embryos developing *in vivo*, pregnant females were deprived of food for periods of 24–48 h at various times in gestation. Embryos were removed from pregnant females on day 13 in order to determine the effect of the treatment on the incidence of spinal NTD, which are well developed at this stage of gestation. Maternal food deprivation was compatible with continued embryonic development: embryos were viable as judged by general appearance (Fig. 1) and presence of heart beat. Food deprivation for 24 h did not produce significant retardation of growth (crown–rump length and protein content) or developmental stage (somite number) and the overall incidence of spinal NTD did not differ significantly from control levels (Table 1). Nevertheless, embryos treated for 24 h prior to posterior neuropore closure (day 9–10 group) developed fewer cases of spina bifida than controls (Table 1).

Females deprived of food for 48 h yielded embryos that were significantly retarded in growth and developmental stage (Table 1). Treatment of embryos prior to posterior neuropore closure (days 8–10 and 9–11), led to a significant reduction in the overall incidence of spinal NTD. Spina bifida was almost completely prevented in these treatment groups (Table 1).

The incidence of embryo resorption was not increased in any of the 48 h food-deprived groups by comparison with the control group (Table 1). Thus, the reduction in the proportion of embryos developing spinal NTD in food-deprived females cannot easily be explained by selective loss of affected embryos.

Embryos were recovered from a number of food-deprived females early on day 11 gestation in order to determine the effect of growth retardation on closure of the posterior neuropore. Of embryos with 27–29 somites, a significantly smaller proportion had enlarged neuropores by comparison with untreated control embryos (Table 2). This finding indicates that prevention of spinal NTD following food deprivation *in vivo* results from normalization of neural fold closure at the posterior neuropore.

Growth retardation in vitro

Curly tail embryos were cultured from day-10 gestation for 15–23 h at 40·5 °C, a treatment known to

produce growth retardation in midgestation rat embryos (Cockroft & New, 1978). Embryos cultured at 40·5 °C had fewer somites, smaller crown–rump lengths and smaller DNA contents than 38 °C control embryos (Table 3). The proportion of 27–29 somite embryos with abnormally enlarged neuropores at the end of the culture period was reduced significantly in the group cultured at 40·5 °C compared with the 38 °C

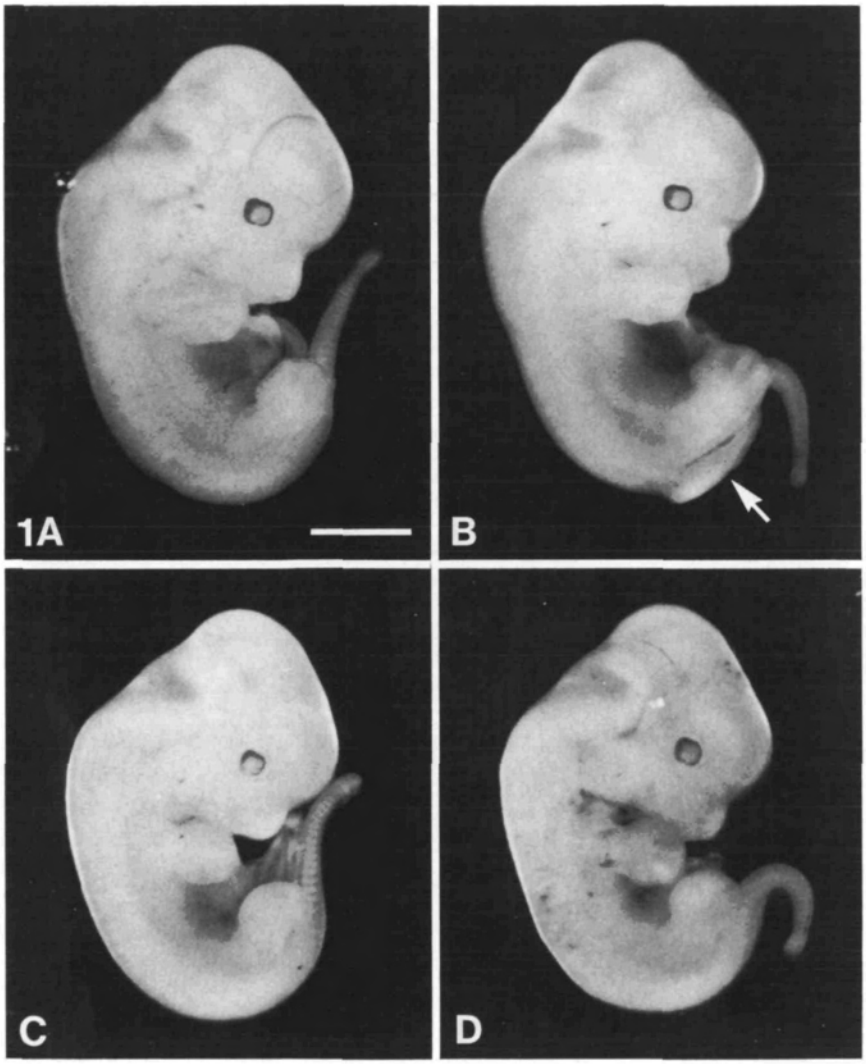

Fig. 1. Effect of maternal food deprivation on development of curly tail embryos recovered on day 13 gestation. (A,B) Control embryos show normal lower spinal development (A) and lumbosacral spina bifida (arrow) plus curly tail (B). (C,D) Embryos from females deprived of food for 48 h (day 8–10 gestation) are reduced in size by comparison with control embryos but otherwise appear normal. The growth-retarded embryos exhibit normal lower spinal development (C) and a curly tail (D). Bar, 2 mm.

Table 1. *Effect of maternal food deprivation on development of curly tail embryos*

Period of food deprivation					Developmental outcome						
					No. embryos showing						
Hours	Days gestation	No. litters	No. viable embryos	% Resorptions	Normal spine	Curly tail	Spina bifida	% NTD	No. somites†	Crown rump length (mm)†	Protein content (mg)†
0	–	7	36	18·2	14	14	8	61·1	53·7 ± 0·5	8·36 ± 0·11	3·5 ± 0·4
24	9–10	7	33	23·3	17	15	1	48·5	54·1 ± 0·5	8·50 ± 0·08	3·2 ± 0·4
	10–11	7	36	5·3	12	16	8	66·6	53·5 ± 0·4	8·21 ± 0·09	3·4 ± 0·2
	11–12	9	46	8·0	16	22	8	65·2	53·5 ± 0·3	8·45 ± 0·07	2·7 ± 0·2
48	8–10	7	37	17·4	25	12	0	32·4*	50·9 ± 0·3*	8·00 ± 0·06*	2·9 ± 0·1
	9–11	8	59	10·4	42	16	1	28·8*	49·4 ± 0·4*	7·54 ± 0·09*	1·6 ± 0·2*
	10–12	6	52	8·8	26	24	2	50·0	52·5 ± 0·3	8·21 ± 0·06	3·5 ± 0·2

† Values are means ± standard error of the mean.
* Differs significantly from control, $P < 0·05$ (Bonferroni t-tests).

Table 2. *Effect of maternal food deprivation on posterior neuropore closure in curly tail embryos*

Period of food deprivation				No. of embryos in posterior neuropore category:		
Hours	Days gestation	No. litters	No. embryos with 27–29 somites	1/2	3	4/5
0	–	5	15	4	5	6
24	9–10	6	23	9	10	4
	10–11	3	12	6	2	4
48	8–10	2	12	5	7	0
	9–11	7	21	7	12	2

Statistical analysis: distribution of posterior neuropore categories differs significantly only between control group and pooled 48 h (day 8–10 and day 9–11) treatment groups, $\chi^2 = 8{\cdot}6$, $P < 0{\cdot}05$, chi-square 2×3 contingency table.

Table 3. *Effect of culture at 40·5 °C on development of curly tail embryos*

Temperature of culture	No. embryos	% embryos in somite range: <27	27–29	>29	Crown–rump length (mm)*	DNA content (μg)*	No. of 27–29 somite embryos in posterior neuropore category:† Closed	1/2	3	4/5
38·0 °C	45	13·3	80·0	6·7	3·32 ± 0·06	17·5 ± 0·6	0	9	19	8
40·5 °C	50	28·0	70·0	2·0	3·12 ± 0·06	15·2 ± 0·6	3	17	11	4

* Mean ± standard error of the mean of all embryos, differs significantly (C–R length, $P < 0{\cdot}025$; DNA content, $P < 0{\cdot}01$; *t*-tests) between treatments.
† Distribution of neuropore sizes differs significantly ($P < 0{\cdot}025$; 2×4 contingency test) between treatments.

control group. Moreover, several embryos cultured at 40·5 °C exhibited premature neuropore closure (Table 3): the posterior neuropore is closed in normal embryos only from the 30-somite stage onwards (Copp *et al.* 1982).

Cell proliferation during growth retardation

In order to determine the effects of a growth-retarding influence on cell proliferation, embryos cultured at 40·5 °C were labelled with [^{3}H]thymidine for 1 h at the end of the culture period. Labelling index was reduced significantly in the neuroepithelium of embryos cultured at 40·5 °C compared with control embryos (Fig. 2A). This reduction in labelling index amounted to a decrease of 25 % in the proportion of cells entering S-phase during the labelling period. Other cell types showed smaller reductions of labelling index at 40·5 °C that were not significant statistically. Dead cells constituted less than 1 % of neuroepithelium, surface ectoderm and mesoderm cell populations in control embryos

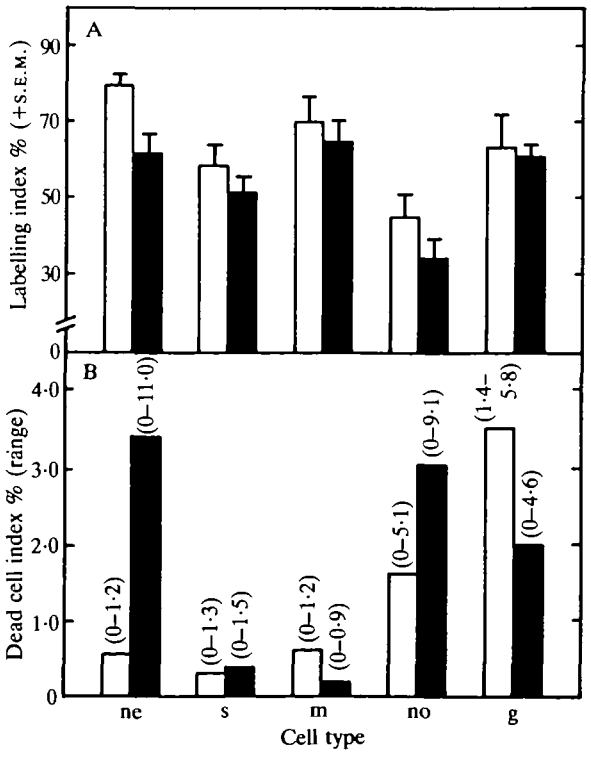

Fig. 2. Effect of high temperature culture on (A) labelling index and (B) dead cell index of resident cell types in the posterior neuropore region of curly tail embryos. Open bars, embryos cultured at 38 °C ($n = 5$); solid bars, embryos cultured at 40·5 °C ($n = 8$). Cell types: ne, neuroepithelium; s, surface ectoderm; m, mesoderm; no, notochord; g, gut endoderm. Statistical analysis. Mean labelling index values differed significantly between 38 °C and 40·5 °C for neuroepithelium ($P < 0{\cdot}05$, *t*-test) but not for other cell types. Since dead cell indices were not normally distributed, the range of individual values is given in the figure. Mean dead cell index differed significantly between 38 °C and 40·5 °C for neuroepithelium ($P < 0{\cdot}001$, χ^2 comparison of two proportions) but not for other cell types.

whereas the frequency of cell death was 1·5% in notochord and 3·5% in gut endoderm (Fig. 2B). Culture at 40·5°C produced a significant increase (more than sixfold) in the incidence of cell death in the neuroepithelium whereas other cell types were not affected significantly (Fig. 2B).

Discussion

60% of *ct/ct* embryos develop spinal NTD as a result of delayed neural fold closure at the posterior neuropore. The extent to which neuropore closure is delayed determines the type of NTD that results: embryos with severe delay develop open spina bifida plus tail flexion defects whereas embryos with moderate delay develop tail flexion defects alone (Copp, 1985).

In the present study, we found that influences producing embryonic growth retardation *in vivo* and *in vitro*, when present immediately before closure of the posterior neuropore, lead to normalization of neural fold closure at the posterior neuropore in *ct/ct* mouse embryos. In the presence of growth retardation, the incidence of severely delayed neuropore closure (category 4/5 neuropores) was reduced markedly and open spina bifida, the most severe form of spinal NTD in curly tail mice, was prevented almost completely. The incidence of moderately delayed neuropore closure (category 3 neuropores) was reduced to a smaller extent and the incidence of tail flexion defects was reduced less than open spina bifida.

Mechanism of spinal NTD prevention by embryonic growth retardation

Our results suggest that normal neurulation at the posterior neuropore depends on a balance between the longitudinal growth rates of dorsal (neuroepithelium) and ventral (gut endoderm and notochord) embryonic structures. Affected *ct/ct* embryos are imbalanced in growth due to a cell proliferation defect that specifically affects ventral structures (Copp *et al.* 1988). Stress forces appear to arise within the embryonic tail bud as a consequence of this growth imbalance since embryos undergoing delayed neuropore closure exhibit marked ventral curvature of the neuropore region (F.A.B. & A.J.C., unpublished data). Ventral curvature, by analogy with neurulation in the cranial region of the mouse embryo (Jacobson & Tam, 1982), may be directly responsible for delay of neural fold closure at the posterior neuropore of curly tail embryos.

We found that culture of embryos at 40·5°C diminishes cell proliferation and increases the incidence of cell death, to a greater extent in the neuroepithelium than in other cell types. This reduction of cell accumulation counteracts the gut endoderm/notochord cell proliferation defect and, we suggest, by this mechanism leads to normal spinal neurulation in *ct/ct* embryos. The finding that neuroepithelial growth inhibition coincides with normalization of neurulation argues against an alternative hypothesis of spinal NTD prevention (Seller & Perkins, 1986) based on catch-up growth, such as that described following cytotoxic insults (Snow & Tam, 1979).

Several drugs have been shown to prevent spinal NTD in mutant mice. Maternal administration of vitamin A reduces the incidence of spinal NTD in the curly tail mutant (Seller *et al.* 1979) and in homozygous Splotch (*Sp*) mice (Kapron-Bras & Trasler, 1985). Hydroxyurea and mitomycin C also reduce the incidence of spinal NTD in curly tail mice (Seller & Perkins, 1983, 1986). Vitamin A, hydroxyurea and mitomycin C all have the effect of inducing cell death and/or slowing the cell cycle in the neuroepithelium of rodent embryos (Langman & Welch, 1967; Herken, 1980; Snow & Tam, 1979). The simplest explanation, in the light of our results, for the prevention of spinal NTD by these agents in curly tail embryos appears to be a realignment of growth schedules.

In humans, there is circumstantial evidence that early embryonic growth retardation may also be associated with a reduced prevalence of spinal NTD. For instance, twin pregnancies are associated with growth retardation early in gestation (Secher *et al.* 1985) and show a reduced prevalence of spinal NTD by comparison with singletons (Hay & Wehrung, 1970; Layde & Erickson, 1980; Windham & Sever, 1982). Recent clinical trials have indicated that periconceptional administration of a multivitamin preparation is effective in reducing the recurrence rate of NTD (Smithells *et al.* 1981, 1983). We speculate elsewhere (Copp & Polani, 1988) that the preventive effect of vitamins with respect to spinal NTD in humans may be mediated through a mechanism based on embryonic growth retardation.

Relationship between embryonic growth retardation and congenital malformation

Spiers (1982) suggested that retardation of embryonic growth early in gestation predisposes to the development of major malformations, especially NTD. This idea is fully supported by evidence from studies of cranial NTD in rodents: growth-retarding teratogens produce cranial NTD (exencephaly) in nonmutant rodent strains (review: Kalter, 1968) and such teratogens increase the incidence of exencephaly in curly tail mice (Seller *et al.* 1979; Seller & Perkins, 1983, 1986). On the other hand, the findings of the present paper show that influences producing embryonic growth retardation have a quite different effect on the

development of spinal NTD. Far from predisposing embryos to spinal NTD, embryonic growth retardation can act to prevent the development of these malformations. Other examples have been described of a preventive effect of embryonic growth retardation on congenital malformations. For instance, preaxial polydactyly developing in mouse embryos homozygous for the mutation polydactyly Nagoya (*pdn*) can be reduced in severity and, in some cases prevented, by administration of cytosine arabinoside to pregnant females at the appropriate stage of gestation (Naruse & Kameyama, 1986). Polydactyly appears to arise in *pdn*/*pdn* embryos as a result of a failure of programmed cell death at the preaxial margin of the limb bud. Cytosine arabinoside, by inducing cell death in the embryo, can compensate for the genetically determined lesion thereby minimizing the mutant phenotype.

Thus, influences that produce embryonic growth retardation in some cases exacerbate, and in other cases prevent, the development of genetically determined malformations. It seems likely that the differing effect of growth retardation on the development of particular congenital malformations reflects the varied nature of the pathogenetic mechanisms in different developing systems.

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References

Bruce, W. R., Meeker, B. E. & Valeriote, F. A. (1966). Comparison of the sensitivity of normal hematopoietic and transplanted lymphoma colony-forming cells to chemotherapeutic agents administered in vivo. *J. natn. Cancer Inst.* **3**, 233–245.

Cockroft, D. L. & New, D. A. T. (1978). Abnormalities induced in cultured rat embryos by hyperthermia. *Teratology* **17**, 277–284.

Copp, A. J. (1978). Interaction between inner cell mass and trophectoderm of the mouse blastocyst. I. A study of cellular proliferation. *J. Embryol. exp. Morph.* **48**, 109–125.

Copp, A. J. (1985). Relationship between timing of posterior neuropore closure and development of spinal neural tube defects in mutant (curly tail) and normal mouse embryos in culture. *J. Embryol. exp. Morph.* **88**, 39–54.

Copp, A. J., Brook, F. A. & Roberts, H. J. (1988). A cell type-specific abnormality of cell proliferation in mutant (curly tail) mouse embryos developing spinal neural tube defects. *Development* **104**, 000–000.

Copp, A. J. & Polani, P. E. (1988). Pathogenesis and prevention of spinal neural tube defects: evidence from a mouse model system. *N. Engl. J. Med.* (Submitted).

Copp, A. J., Seller, M. J. & Polani, P. E. (1982). Neural tube development in mutant (curly tail) and normal mouse embryos: the timing of posterior neuropore closure in vivo and in vitro. *J. Embryol. exp. Morph.* **69**, 151–167.

Hay, S. & Wehrung, D. A. (1970). Congenital malformations in twins. *Am. J. hum. Genet.* **22**, 662–678.

Herken, R. (1980). Cell cycle phase specificity of hydroxyurea and its effects on the cell kinetics in embryonic spinal cord. *Teratology* **21**, 9–14.

Jacobson, A. G. & Tam, P. P. L. (1982). Cephalic neurulation in the mouse embryo analyzed by SEM and morphometry. *Anat. Rec.* **203**, 375–396.

Kalter, H. (1968). *Teratology of the Central Nervous System.* Chicago: University of Chicago Press.

Kapron-Bras, C. M. & Trasler, D. (1985). Gene-teratogen interaction and its morphological basis in retinoic acid-induced mouse spina bifida. *Teratology* **32**, 87–92.

Labarca, C. & Paigen, K. (1980). A simple, rapid, and sensitive DNA assay procedure. *Anal. Biochem.* **102**, 344–352.

Langman, J. & Welch, G. W. (1967). Excess vitamin A and development of the cerebral cortex. *J. comp. Neurol.* **131**, 15–26.

Layde, P. M. & Erickson, J. D. (1980). Congenital malformations in twins. *Am. J. hum. Genet.* **32**, 69–78.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurements with the folin phenol reagent. *J. biol. Chem.* **193**, 265–275.

Naruse, I. & Kameyama, Y. (1986). Prevention of polydactyly manifestation in polydactyly Nagoya (*pdn*) mice by administration of cytosine arabinoside during pregnancy. *Teratology* **34**, 283–289.

New, D. A. T., Coppola, P. T. & Terry, S. (1973). Culture of explanted rat embryos in rotating tubes. *J. Reprod. Fertil.* **35**, 135–138.

Secher, N. J., Kaern, J. & Hansen, P. K. (1985). Intrauterine growth in twin pregnancies: prediction of fetal growth retardation. *Obstet. Gynecol.* **66**, 63–68.

Seller, M. J., Embury, S., Adinolfi, M. & Polani, P. E. (1979). Neural tube defects in curly-tail mice. II. Effect of maternal administration of vitamin A. *Proc. R. Soc. Lond.* B **206**, 95–107.

Seller, M. J. & Perkins, K. J. (1983). Effect of hydroxyurea on neural tube defects in the curly-tail mouse. *J. Craniofac. Genet. dev. Biol.* **3**, 11–17.

Seller, M. J. & Perkins, K. J. (1986). Effect of mitomycin C on the neural tube defects of the curly-tail mouse. *Teratology* **33**, 305–309.

Smithells, R. W., Seller, M. J., Harris, R., Fielding, D. W., Schorah, C. J., Nevin, N. C., Sheppard, S., Read, A. P., Walker, S. & Wild, J. (1983). Further experience of vitamin supplementation for prevention of neural tube defect recurrences. *Lancet* **i**, 1027–1031.

SMITHELLS, R. W., SHEPPARD, S., SCHORAH, C. J., SELLER, M. J., NEVIN, N. C., HARRIS, R., READ, A. P. & FIELDING, D. W. (1981). Apparent prevention of neural tube defects by periconceptional vitamin supplementation. *Archs Dis. Childh.* **56**, 911–918.

SNOW, M. H. L. & TAM, P. P. L. (1979). Is compensatory growth a complicating factor in mouse teratology? *Nature, Lond.* **279**, 555–557.

SPIERS, P. S. (1982). Does growth retardation predispose the fetus to congenital malformation? *Lancet* **i**, 312–314.

WINDHAM, G. C. & SEVER, L. E. (1982). Neural tube defects among twin births. *Am. J. hum. Genet.* **34**, 988–998.

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