Inositol prevents folate-resistant neural tube defects in the mouse

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Clinical trials demonstrate that up to 70% of neural tube defects (NTDs) can be prevented by folic acid supplementation in early pregnancy, whereas the remaining NTDs are resistant to folic acid. Here, we show that a second vitamin, myo-inositol, is capable of significantly reducing the incidence of spinal NTDs in curly tail mice, a genetic model of folate-resistant NTDs. Inositol increases flux through the inositol/lipid cycle, stimulating protein kinase C activity and upregulating expression of retinoic acid receptor β, specifically in the caudal portion of the embryonic hindgut. This reduces the delay in closure of the posterior neuropore, the embryonic defect that is known to lead directly to spina bifida in curly tail embryos. Our findings reveal a molecular pathway of NTD prevention and suggest the possible efficacy of combined treatment with folic acid and inositol in overcoming the majority of human NTDs.

Vitamin status is a key factor in determining the susceptibility of human pregnancies to neural tube defects (NTDs). Multivitamin supplementation in early pregnancy prevents a significant percentage of recurrent NTDs (ref. 1) and the Medical Research Council trial suggested that the active ingredient in the multivitamin preparation is folic acid6. Apparent prevention of the first occurrence of NTDs has also been observed using a multivitamin preparation containing folic acid1. The clinical trials indicate that up to 70% of NTDs can be prevented by folate administration, leaving a significant number of NTDs that do not respond. There is currently no preventative therapy for these folate-resistant defects.

The curly tail mutant mouse is a genetic model of folate-resistant NTDs. Defects in this strain closely resemble human NTDs in form and structure, axial location, sex bias among anencephalics and association with elevated levels of amniotic fluid alpha-fetoprotein6. Moreover, as in humans, genes and environmental factors interact to determine the incidence and severity of the NTDs in curly tail mice46. It is clear, however, that NTDs in curly tail mice are not prevented by folic acid or several of its metabolites4. Treatment of pregnant females with folic acid, folinic acid4 or methionine12 at various stages of early pregnancy failed to alter the incidence of NTDs. Thus, curly tail may correspond, in terms of its etiology and/or pathogenesis, to the folate-resistant subcategory of human NTDs.

myo-Inositol is a water-soluble vitamin that plays a vital role in the inositol/lipid cycle, a source of metabolic intermediates for processes including signal transduction, steroid synthesis and intracellular calcium regulation8. Cranial NTDs occur in cultured rat embryos when inositol is omitted from the culture medium, whereas the absence of other vitamins causes only growth retardation11. Inositol deficiency also elevates the normally low incidence of exencephaly in curly tail mice, with the mutant embryos requiring a higher dose of exogenous inositol to protect against the defects10. Pregnancies in both diabetic humans31 and rodents32 exhibit an elevated frequency of NTDs, and rodents can be protected against these defects, both in vivo and in vitro, by exogenous inositol or arachidonic acid41.

Despite the considerable evidence linking inositol status with NTDs, it is not clear whether exogenous inositol may protect against genetically determined NTDs, as seems most relevant to the human situation. In the present study, we demonstrate a specific preventive effect of exogenous inositol on the development of spinal NTDs in curly tail mouse embryos, both in vivo and in vitro, and show that this action is mediated by stimulation of protein kinase C (PKC) and upregulation of retinoic acid receptor β (RARβ), a gene already known to be expressed at reduced levels in the hindgut of mutant embryos31. This study provides strong evidence for the role of a hitherto untested vitamin in preventing folate-resistant NTDs.

Inositol reduces frequency of spina bifida

Sellers reported preliminary data suggesting that exogenous inositol (at both 250 and 400 mg/kg) reduces NTD frequency in curly tail mice. We repeated and extended this study, administering one or two intraperitoneal injections of 400 mg/kg inositol to pregnant females at various times during the critical phase of neural tube closure. The frequency of spina bifida, the most severe NTD observed in curly tail mice, was reduced by inositol in all treatment groups (Fig. 1), with a trend toward the greatest reduction in NTD frequency (by 70%) in embryos treated with a single injection of inositol in the afternoon of embryonic day E9.5. Treatment with a higher dose of inositol (2000 mg/kg) produced no further reduction of NTD frequency nor any adverse effects (data not shown). Mean litter size and frequency of resorptions did not differ significantly for inositol-treated and control litters, indicating that selective mortality of embryos with spina bifida is very unlikely to account for the preventive effect of inositol.

Inositol prevents spinal neurulation defects

We used the method of whole-embryo culture30 to investigate in
in these treatment groups (Table 1), suggesting that growth retardation is unlikely to mediate the preventive effect of inositol. Moreover, addition of 200 μg/ml lithium chloride to the culture medium completely abolished the preventive effect of 50 μg/ml inositol (Fig. 3), without adversely affecting other developmental parameters. Lithium is a known inhibitor of inositol phosphate recycling that reverses many of the specific effects of inositol\(^1\). Thus, the effect of inositol appears to be mediated by a specific effect on the inositol/lipid cycle.

**Curly tail embryos are not deficient in uptake of inositol**

A striking finding was that addition of lithium did not exacerbate the delay of neuroepic closure in *curly tail* embryos, in the absence of exogenous inositol (Fig. 3). This suggested that the basic abnormality in *curly tail* embryos does not involve inositol deficiency. Indeed, uptake of \(^{[\text{H}]\text{inositol}}\) did not differ for unaffected *curly tail* embryos and their severely affected littermates (Fig. 4). Moreover, thin-layer chromatography demonstrated closely similar incorporation of \(^{[\text{H}]\text{inositol}}\) into phosphatidylinositol (PI) in affected and unaffected *curly tail* embryos (data not shown). These data support the idea that spinal NTDs in *curly tail* mice are not the result of a genetically determined defect in inositol uptake or incorporation. On the other hand, culture in 50 μg/ml inositol led to a twofold increase in \(^{[\text{H}]\text{inositol}}\) in the lipid-soluble phase and an eightfold increase in the water-soluble phase, compared with culture in 5 μg/ml inositol (Fig. 4). Thus, supranormal doses of inositol are needed to stimulate increased incorporation into inositol lipids in cultured embryos.

**Inositol action is not mediated by the arachidonic acid pathway**

We investigated whether arachidonic acid, a downstream product of inositol/lipid metabolism and the major precursor of prostaglandins, may also reduce the incidence of *curly tail* neurulation defects, as is reported for hyperglycemia-induced defects\(^2\). Culture of embryos in the presence of arachidonic acid, even at concentrations up to 50 times the effective dose in hyperglycemia, failed to alter the neuroepic length of *curly tail* embryos (Fig. 3). In fact, the preventive effect of 50 μg/ml inositol was abolished by coadministration of 50 μg/ml arachidonic acid. These findings strongly suggest that inositol is unlikely to be act-

### Table 1 Development of *curly tail* and nonmutant CBA/Ca embryos (all somite groups pooled) cultured in the presence of varying concentrations of inositol for 24 hours from embryonic day 9.5

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>No. of embryos</th>
<th>No. of somites</th>
<th>Neurupore length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Curly tail</em> embryos</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole serum</td>
<td>52</td>
<td>3.60 ± 0.07</td>
<td>28.73 ± 0.11</td>
</tr>
<tr>
<td>0 μg/ml inositol</td>
<td>67</td>
<td>3.49 ± 0.06</td>
<td>28.97 ± 0.16</td>
</tr>
<tr>
<td>20 μg/ml inositol</td>
<td>48</td>
<td>3.47 ± 0.07</td>
<td>28.96 ± 0.22</td>
</tr>
<tr>
<td>50 μg/ml inositol</td>
<td>78</td>
<td>3.46 ± 0.06</td>
<td>28.58 ± 0.18</td>
</tr>
<tr>
<td>CBA/Ca embryos</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole serum</td>
<td>14</td>
<td>3.39 ± 0.09</td>
<td>27.50 ± 0.67</td>
</tr>
<tr>
<td>0 μg/ml inositol</td>
<td>10</td>
<td>3.45 ± 0.09</td>
<td>27.64 ± 0.48</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m. See Fig. 2 for data on separated somite groups. Inositol concentrations added to dialyzed serum before culture.

*Significant variation among neurupore lengths of *curly tail* embryos (Kruskal-Wallis one-way analysis of variance; \(H = 11.8; \ P = 0.008\)). Mann-Whitney rank sum tests shows significant differences between median values for 50 μg/ml inositol and 0 μg/ml \(P = 0.0396\) and between 50 μg/ml and 20 μg/ml \(P = 0.0001\). This overall difference results from a more marked difference at the 30–31 somite stage (see Fig. 2). There is no significant variation in crown–rump length or somite number for *curly tail* embryos cultured under varying conditions, or in any of the parameters for CBA/Ca embryos \((P > 0.05)\).
Fig. 2 Length of the posterior neuropore in curly tail embryos at the 30–31 somite stage is reduced by treatment with 50 μg/ml inositol, but not with 20 or 0 μg/ml, or in whole-serum culture. Neuropore length is reduced to a value that is known to lead to closure of the neuropore and to the development of a normal spinal cord in the majority of curly tail embryos\(^2\). Non-mutant CBA/Ca embryos are not affected by addition of exogenous inositol and exhibit normal neuropore closure at the 30–31 somite stage. Note that the rate of decrease in neuropore length is closely similar in CBA/Ca and in curly tail embryos treated with 50 μg/ml inositol, suggesting normalization of neuropore closure in the latter. Statistical analysis: Neuropore length in curly tail embryos with 30–31 somites treated with 50 μg/ml inositol (asterisk) is significantly less than in all other groups of curly tail embryos (t-tests, \(P < 0.001\) in each case). Numbers of embryos are given in Table 1.

Discussion

The semidominant curly tail mutation is a cause of predominately low spinal NTDs in the mouse\(^1\). The trait has been mapped by linkage analysis\(^2\) to a principal locus on distal chromosome 4 (equivalent to 1p36–pter in humans), with penetrance that is markedly affected by other loci. The strongest modifier appears to be mct1 (ref. 5) on chromosome 17, whereas interactions also occur with other NTD-related genes, such as Pax3 (ref. 22). Environmental factors are important, with alterations in penetrance and expressivity resulting from the treatment of pregnant curly tail females with agents including retinoic acid, mitomycin C, hydroxyurea and 5-fluorouracil\(^3\). Against this background of multiple interacting factors, it is particularly striking that folate and methionine have no effect on the incidence or type of NTDs in curly tail mice\(^4\).

In contrast, supplementation with inositol can reduce the incidence and severity of NTDs in the curly tail mouse, both in vivo and in vitro. This protective effect appears specific, because it is reversed by lithium treatment and is not mediated by generalized growth retardation, unlike the protective effect of cytoxic factors in the curly tail system\(^6\). Indeed, we find that relatively large doses of inositol, up to 100 times the serum level, are not teratogenic to the mouse embryo, although there is no further reduction in neuropore length in vitro at doses above 50 μg/ml

Inositol and TPA both cause upregulation of RAR\(\beta\) expression

Neural tube defects in the curly tail mutant result from a cell proliferation defect in the hindgut endoderm that is causally related to downregulation of RAR\(\beta\) expression\(^1\). In the present study, culture in 50 μg/ml inositol did not affect the overall level or pattern of expression of RAR\(\beta\) in curly tail embryos (Fig. 6, a and b). Significantly, however, the domain of RAR\(\beta\) expression in the hindgut extended caudally in inositol-treated embryos, so that expression could be clearly detected in the hindgut caudal to the hind limb bud (Fig. 6, d, g and i), whereas this expression was absent from embryos cultured in serum only (Fig. 6, e and h). Closely similar findings were obtained from embryos exposed to 500 nM TPA after 17 hours of culture (data not shown). There was no comparable effect of inositol or TPA on expression of the retinoic acid receptor γ (RAR\(\gamma\)) or the genes for the cellular retinoic acid-binding proteins CRABP-I and CRABP-II. Thus, prevention of spinal NTDs in curly tail embryos by inositol and TPA is accompanied by specific upregulation of RAR\(\beta\) expression in the hindgut.

![Graph showing the effect of inositol and TPA on neuropore length](image)

![Graph showing the effect of LiCl and arachidonic acid on neuropore length](image)

Fig. 3 Neuropore length is significantly reduced in 30–31 somite embryos cultured in dialyzed serum containing 50 μg/ml inositol (second bar, \(n = 28\)) compared with those cultured in 0 μg/ml inositol (first bar, \(n = 24\)). Addition of 200 μg/ml lithium chloride (third bar, \(n = 22\)) or 50 μg/ml arachidonic acid (fifth bar, \(n = 20\)) does not alter mean neuropore length but the preventive effect of 50 μg/ml inositol is abolished by coadministration of lithium (fourth bar, \(n = 23\)) or arachidonic acid (sixth bar, \(n = 19\)). Statistical analysis: Mean neuropore length of embryos treated with 50 μg/ml inositol is significantly less than in embryos cultured in 0 μg/ml inositol, lithium chloride or arachidonic acid (t-tests, \(P < 0.005\) in each case).
Fig. 4 Uptake of \( ^{[\text{H}]} \)inositol (means ± s.e.m.) compared for a, unaffected (small neuropore, \( n = 15 \)) and affected (large neuropore, \( n = 9 \)) curly tail embryos cultured in whole rat serum (approximate inositol concentration, 5 \( \mu \text{g/ml} \)) and b, curly tail embryos cultured in dialyzed serum containing 5 \( \mu \text{g/ml} (n = 21) \) or 50 \( \mu \text{g/ml} (n = 22) \) exogenous inositol. \( ^{[\text{H}]} \)inositol at 2 \( \mu \text{Ci/ml} \) (approximate specific activity, 0.4 \( \mu \text{Ci/\mu g inositol} \)) was added to whole-serum culture media. Inositol was added to dialyzed serum culture media at specific activity 0.05 \( \mu \text{Ci/\mu g inositol} \) in both cases. Therefore, the rat serum cultures contained eight times the specific activity of \( ^{[\text{H}]} \)inositol as the dialyzed serum cultures, hence the higher uptake observed in rat serum cultures compared with dialyzed serum + 5 \( \mu \text{g/ml} \) insitol. Addition of 50 \( \mu \text{g/ml} \) exogenous inositol stimulates a fourfold increase in total uptake of \( ^{[\text{H}]} \)inositol compared with cultures containing 5 \( \mu \text{g/ml} \) inositol, but produces only a twofold rise in incorporation into the lipid-soluble fraction. Statistical analysis: a, Uptake does not differ significantly for any of the fractions for unaffected and affected curly tail embryos (\( P > 0.05 \)); b, Uptake differs significantly for 5 and 50 \( \mu \text{g/ml} \) exogenous inositol in all fractions (\( P < 0.0001 \), Mann-Whitney rank sum tests).

(datum not shown). Inositol appears to exert this protective action on NTD development by increasing flux through the inositol/lipid cycle. Supplementation with inositol enhances uptake and incorporation into inositol phospholipids, yet inositol uptake is not a limiting factor in curly tail embryos, indicating that inositol deficiency is not responsible for spinal NTDs in the curly tail mouse.

We investigated which of several possible downstream inositol-related pathways need to be stimulated in order for NTDs to be prevented. Although arachidonic acid can mimic the protective effect of inositol on hyperglycemia-related cranial NTDs, there was no preventive effect of arachidonic acid on neuropore closure in the present study, indicating that the pathway downstream of arachidonic acid pathway is unlikely to be involved in the prevention of spinal NTDs in curly tail embryos. It is interesting that arachidonic acid abolishes the protective effect of 50 \( \mu \text{g/ml} \) inositol. This may be related to the action of arachidonic acid in stimulating embryonic growth in vitro, a phenomenon that we observed (data not shown) and that also occurs in rat embryo culture. This effect might exacerbate the delay in posterior neuropore closure, in a manner opposite to the reduction in neuropore length that was observed previously in growth-retarded curly tail embryos.

We found that stimulation of PKC by short-term treatment with its agonist TPA is able to replicate the effect of inositol supplementation. This suggests that the mechanism of action of inositol may be mediated by increased production of diacylglycerol, a physiological activator of PKC, and this idea is supported by the ability of the specific PKC inhibitor, GF109203X, to block the protective effect of both inositol and TPA.

Activation of RAR in embryos in the pathway leading to prevention of spinal NTDs in curly tail embryos. In normal embryos, RAR is expressed strongly at rostral levels of the embryonic neuraxis but is largely excluded from tissues in the vicinity of the posterior neuropore. The exception is the hindgut located beneath the posterior neuropore, which is a site of intense expression. Significantly, affected curly tail embryos exhibit reduced expression of RAR in the hindgut, correlating with the diminished cell proliferative rate that has been demonstrated in this tissue. The effect of reduced hindgut cell proliferation is to cause enhanced ventral curvature of the caudal region, which mechanically inhibits closure of the neural folds at the posterior neuropore, leading to spinal NTDs (Fig. 7).

Treating dams with low-dose retinoic acid reduces the incidence of spinal NTDs in curly tail embryos, and this effect is preceded by a specific upregulation of RAR expression in the hindgut. These findings suggest a causative role between decreases in RAR and enhanced proliferation of hindgut epithelial cells. Indeed, it has been demonstrated that retinoic acid stimulates both growth and RAR expression in embryonic stem cells at low concentrations (10^{-8}–10^{-7} M), whereas higher concentrations of retinoic acid produce a decrease in RAR expression.
Fig. 6 Whole-mount in situ hybridization using the RARβ probe on curly tail embryos after 24-h culture from E9.5 in either 50 μg/ml inositol (a, d and g; n = 17) or in serum alone (b, e and h; n = 18). Intact embryos (a and b) show a closely similar overall pattern of expression of RARβ in the frontonasal region (F), neural tube (N), fore (L) and hind limb buds (H). Isolated caudal regions of three inositol-treated embryos (d and g) exhibit hybridization in a ventral, midline structure extending posteriorly beyond the hind limb bud (arrows in d and g), whereas this RARβ expression in the hindgut is absent from the three embryos cultured in rat serum alone (arrowheads in e and h). Transverse section of an inositol-treated embryo (i) confirms that the ventral midline hybridization signal is in the hindgut (G, arrows in i). Isolated caudal regions are viewed from the right side (c–e) or from the ventral surface (f–h). Anterior is to the left and posterior to the right in all cases. Note that the tail bud (TB) and posterior neuropore (PN) do not express RARβ and are not clearly visible in the whole mounts. Arrow in c indicates the direction of ventral view, as shown in (f–h). Dashed lines in the diagrams indicate the level of transverse section (i). Embryos treated with 500 nM TPA for 1h after 17 h of culture (n = 18, data not shown) yielded closely similar results to embryos cultured in 50 μg/ml inositol. Other abbreviations: HLB, hind limb bud; S, somites. Scale bars: a and b, 1 mm; d, e, g and h, 1.7 mm; and i, 0.2 mm.

RARβ expression and cell proliferation in the hindgut, thereby normalizing neuropore closure in the curly tail embryo.

Does our work have relevance for the prevention of human NTDs? The finding that NTDs in curly tail mice are resistant to prevention by folate or methionine suggests that curly tail is most likely to represent the folate-resistant subcategory of human NTDs. This is in contrast to the Axd mouse mutant in which NTDs are preventable by methionine, but not folic acid, and the Cart1 null mutant in which folate suppresses the development of anencephaly. We suggest that NTDs in humans comprise a heterogeneous group of conditions which, in some cases, involve folate-dependent pathways, whereas, in other cases, folate pathways are not involved and folate therapy is ineffective.

Inositol was not included in the multivitamin preparations used in previous intervention studies, so its effectiveness in preventing human NTDs has not been tested. However, two recent clinical trials have evaluated the effectiveness of inositol as a

Fig. 7 Summary of the developmental pathway linking the curly tail gene with development of spinal NTDs. Solid arrows indicate the events that mediate the protective effect of inositol, as demonstrated in the present study. Open arrows indicate steps in the pathway for which experimental evidence has been obtained previously, from the following studies: a, ref. 13; b, ref. 16, 25; c, ref. 39, 40; d, ref. 15. Dashed arrow shows a relation that is predicted, but not yet demonstrated experimentally.
treatment for depression and panic disorder. There were no reported side effects of inositol treatment at 12 g/day in these trials, and moreover, we found no adverse effects of 400–2000 mg/kg inositol during in vivo mouse embryonic development. Further studies of the teratogenic potential and pharmacokinetics of inositol during pregnancy will be necessary before a randomized, clinical trial can be contemplated. If inositol proves suitable for use during human pregnancy, a trial to compare the effectiveness of combined folate + inositol versus folate alone could lead to the development of a more complete strategy for preventing human NTDs.

Methods

Mouse strains. Curly tail mice are maintained as a closed, random-bred colony, on a light/dark cycle with the dark period extending from 01:00 h to 09:00 h. Females were paired with males at approximately 0830 h and checked for copulation plugs 3–4 h later. Mating was presumed to occur at the midpoint of this period (1000 h). Inbred CBA/Ca mice served as non-mutant controls, as this strain is closely related to the CBA/Gr strain that contributed largely to the curly tail background during the origin of the strain. CBA/Ca mice were maintained under the same light/dark-cycle conditions as the curly tail mice, but experimental litters were generated by overnight matings in order to obtain earlier fertilization and therefore compensate for the fact that CBA/Ca embryos are developmentally delayed compared with curly tail embryos. For both curly tail and CBA/Ca mice, the day of finding a copulation plug was designated E0.5 days post coitum.

Administration of inositol during pregnancy. Pregnant curly tail females received one or two intraperitoneal injections of inositol dissolved in PBS (either 400 or 2000 mg/kg) or PBS alone, in the morning (0900–1000 h) or evening (1700–1800 h) of E9.5 or in the morning of E10.5. Females were killed at E12.5, and embryos were removed and scored for the presence of spina bifida aperta and/or tail defects. Analysis was performed without knowledge of the treatment group to which the embryos belonged.

Whole-embryo culture. Immediately centrifuged, heat-inactivated rat serum was extensively dialyzed against a balanced salt solution and supplemented with 1.75 mg/ml d-glucose, MEM (modified Eagle's medium) essential amino acids, 200 mM glutamine and MEM vitamins, excluding inositol (all from Sigma Chemical Co.). myo-Inositol, lithium chloride, arachidonic acid, TPA (all from Sigma) and PKC inhibitor GF109203X (Calbiochem, La Jolla, CA) were added immediately before culture. Curly tail embryos were explanted for culture early on E9.5 of gestation in Dulbecco's modified MEM (Gibco, Grand Island, NY) containing 10% fetal calf serum. Three embryos were cultured in 3 ml of serum in a roller bottle incubator at 38 °C (ref. 14). Cultures were initially gassed with 20% O2, 5% CO2, 75% N2 and gasified after 17 h with 40% O2, 5% CO2, 55% N2. After 24 h, embryos were assessed for yolk sac function, then removed from culture, and dissected from their extraembryonic membranes. The number of somites, crown-rump length, neuroepithelium area and neuroepithelium length (measured from caudal extremity to the rostral point of neuroepitrochlear closure) were determined. Embryos were then either frozen at −70 °C for biochemical analysis or fixed in fresh 4% paraformaldehyde in PBS for whole-mount in situ hybridization.

Uptake and incorporation of [3H]inositol. The method was adapted from Estibeiro et al. In [3H]inositol was added to the culture medium in proportion to the total amount of added inositol (see Fig. 4). Following 24-h culture, each embryo was rinsed twice in ice-cold PBS, resuspended separately in 200 μl of methanol, disrupted by sonication for 20 s, spun in a vortex mixer and kept on dry ice. Two 5-μl samples were assayed for protein content with the use of the bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL). Two further 5-μl samples were added to 2 ml scintillant (UniverSol, ICN, Costa Mesa, CA), and the β counts were taken to give a measure of "total uptake." The homogenate was extracted with chloroform:methanol (2:1) and, following centrifugation, the pellet was analyzed as the "insoluble fraction." The supernatant was then extracted with 200 μl of 0.73% sodium chloride. The upper "water-soluble fraction" was kept, whereas the lower phase was reextracted twice with 200 μl of the upper phase from an 8:4:3 mixture of chloroform:methanol/0.58% aqueous sodium chloride. The reextracted lower phase was analyzed as the "lipid-soluble fraction," whereas the upper phases were pooled with the water-soluble fraction. Recovery of [3H] from total embryo homogenate was approximately 96%. The water-soluble phase contains free inositol and inositol phosphates, the lipid-soluble phase contains inositol phospholipids, and the chloroform/methanol extract contains intact inosoluble tissue residue (cytoskeleton, DNA and protein material). In some cases, the lipid-soluble fraction was analyzed further by thin-layer chromatography, by using a PI standard, as described previously.

Whole-mount in situ hybridization. cDNA probes for RARα, RARβ, CRABP-I and CRABP-II (ref. 36, 37; kindly provided by Pierre Chambon) were transcribed, and embryos were processed for whole-mount in situ hybridization, as described previously. Intact embryos were cleared in glyceral before photography on a Zeiss SV11 stereomicroscope (Zeiss, Jena, Germany). After dehydration and embedding in paraffin wax, embryos were sectioned at thickness of 20 μm, and sections were photographed on a Zeiss Axioskop microscope.

Acknowledgments

We are grateful to Patricia Ferretti and Deborah Henderson for critical reading of the manuscript. N.D.E.G. was a Wellcome Trust Ph.D. student and A.J.C. was a Wellcome Trust senior clinical research fellow. The work was also supported as part of a Multicenter Agreement for Studying Neural Tube Defects in Mutant Mice funded by the US National Institute of Child Health and Human Development through Cooperative Agreement HD 28882.

RECEIVED 12 SEPTEMBER; ACCEPTED 25 NOVEMBER 1996


