**Crooked tail (Cd) models human folate-responsive neural tube defects**

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Genetic correlation of human neural tube defects (NTDs) with NTD genes identified in mouse may unravel predisposing complex traits for assessment of individual risk and treatment in clinical settings. Folic acid (FA) can reduce the recurrence of NTDs in human populations by as much as 50–70%, though the mechanism of this rescue is unknown. We examined whether Crooked tail (Cd), a mouse strain prone to exencephaly, could provide a genetic animal model for folate-responsive NTDs. The Cd locus was localized to a 0.2 cM interval of the Mouse Genome Database genetic map, identifying tightly linked markers for genotyping prior to phenotypic expression. In a controlled diet study, Cd was found to mimic closely the clinical response to FA. FA supplementation reduced the recurrence risk of Cd exencephaly by as much as 55%. This rescue was dose dependent and did not require subjects to be inherently folate deficient. Like the female predominance of NTDs in humans, female Cd embryos were most likely to display exencephaly and were more responsive than males to the FA rescue. Importantly, FA supplementation shifted the severity of Cd phenotypic expression from early embryonic lethality to longer survival, and reduced the incidence of NTDs. The Cd locus is distinct from the known genes associated with neurulation defects, and isolation of this gene will assist identification of biochemical, genetic and gender-dependent factors contributing to folate-responsive NTDs.

**RESULTS**

Features of the Cd/Cd exencephalic fetus help to define appropriate expression patterns for candidate genes. The mammalian neural tube closes in a zipper-like fashion, extending sequentially from four primary closure points (14). In Cd exencephalic embryos (Fig. 1), the neural folds in the mesencephalon, closure point 2, remain separated in the midline and splay over the skull base. Cd exencephalics surviving to the fetal period are of normal size, and the orbits and lower cranial structures are preserved (Fig. 1B). In addition, the digits are normally formed and, unlike Splotch NTD mice, there is no hypoplasia of limb muscles. Vascular markings outside of the exencephalic brain appear normal. Sagittal histological sections are also consistent with failure primarily of closure point 2 (Fig. 1D). The Cd/Cd mesencephalon is most distorted, pushed up and over the cortex, with markedly atrophic tectum. Cerebellum is relatively well preserved. Therefore, genes expressed predominantly in the mesencephalon

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will be favored candidates for \( Cd \). Moreover, mouse models producing exencephaly involving mesencephalic closure point 2 would be likely candidates for genetic interaction with \( Cd \).

The \( Cd \) mutation arose spontaneously in the A mouse strain, producing a crooked tail in heterozygotes (8). The appearance and distribution of \( Cd \) phenotypes are in agreement with those of Morgan. While as many as 20% of pre-implantation \( Cd/Cd \) embryos may be lost (8), after implantation, homozygous animals display a range of phenotypes. Approximately 30% are lost to early lethality, 20% exhibit exencephaly and the remaining animals successfully complete neural tube closure, but are runted (<40% of wild-type littermates by body weight, designated SMALL by Morgan), with more pronounced vertebral skeletal defects and reduced fertility. The tooth defect originally reported is no longer seen in the present colony, indicating that the dominant crooked tail trait, the recessive lethality, exencephaly, and the SMALL phenotypes result directly from the \( Cd \) mutation. Thus, the \( Cd \) genotype can be inferred from the phenotype, but genotype can be known unambiguously only in the visibly affected individuals of segregating crosses.

The penetrance of the \( Cd/+ \) phenotype varied with genetic background. A crooked tail was not expressed in \( Cd \) crosses with CAST/Ei (0 of 29 pups). The observed frequency of the crooked tail in heterozygous offspring from intraspecies crosses varied from 14 to 41% compared with the expected 50% for a dominantly inherited trait: 14% of \( Cd \times DBA \) pups (\( n = 1949 \)), 17% of \( Cd \times C57Bl6 \) pups (\( n = 564 \)), 32% of \( Cd \times 129Sv \) pups (\( n = 79 \)) and 41% of \( Cd \times A \) pups (\( n = 2430 \)). For fine mapping, DBA/2J mice were mated with \( Cd \) to generate \((Cd/+ \times DBA)F_1\) offspring. Because \( Cd \) is incompletely penetrant, only those \( F_1 \)s with visibly crooked tails were mated to A Jackson (A) mice to generate \([Cd/+\times DBA)F_1 \times A\]N2 offspring. Again, only those informative N2s with visibly crooked tails were haplotyped. Thus, only a small proportion of all offspring was used to create a recombination map of the \( Cd \) locus, ensuring that all individuals examined did in fact carry the \( Cd \) mutation.

\( Cd \) originally was mapped by two- and three-point coupling test crosses (15) to distal chromosome 6. A panel of 74 sequence tagged sites (STSs) on chromosome 6 was examined. Of these, 38 were polymorphic between the \( Cd \) strain and DBA/2J, and were used to haplotype 221 informative N2 offspring (Fig. 2). Four markers tightly linked to the \( Cd \) locus were identified (D6Mit13, D6Mit219, D6Mit135 and D6Mit111; LOD = 62.6; \( \chi^2 = 215 \), \( P < 0.000001 \); 0.0–1.4 cM at 95% confidence interval). The distal limit of the \( Cd \) critical region is D6Mit301, with a LOD score of 65.3 (\( \chi^2 = 209 \), \( P < 0.000001 \); 0.7–5.4 cM at 95% confidence interval).

Figure 1. Exencephaly in the \( Cd/Cd \) embryo. Normal (A and C) and exencephalic (B and D) littermates are shown. The E14.5 exencephalic littermate (B) reveals non-closure of the neural folds in the mesencephalic region [m in (D)], with splaying of brain tissue over the base of the skull. Orbits and lower cranial structures are intact. The size and morphology of the trunk and limbs are normal [compare with wild-type littermate in (A)]. The E18.5 exencephalic littermate (D) is shown in sagittal section, stained with hematoxylin and eosin [compare with wild-type (WT/WT) in (C)]. The mesencephalon (m) is most distorted, pushed up and over the cortex (ctx) with marked atrophy of the midbrain tectum (t). Cerebral cortex (ctx) is disorganized with loss of emerging lamination, whereas cerebellum (cb) is relatively spared. Note that brain is covered by developing skull vault [arrowheads in (C)] in the normal sibling while brain is exposed in the \( Cd/Cd \) fetus.
interval). These markers placed Cd within a 0.2 cM interval on the MGD chromosome 6 consensus map, or ~0.4 Mb on the MGD physical map (12,13). A marker panel consisting of D6Mit135, D6Mit13 and Ms6hm3 (13) was used for the genotypic identification of Cd embryos prior to phenotypic manifestation of the mutation. In >5000 individuals haplotyped with these three markers for the linkage, prenatal diet and on-going postnatal developmental studies, there was no inconsistency between order of loci or allele study and phenotype.

To assess the prenatal effects of FA supplementation, mating pairs of Cd/A Jackson mice were maintained on one of four defined diets differing only in the concentration of FA (0, 4, 7 and 10 mg FA/kg chow). Twenty litters per diet were examined between embryonic day (E)12 and 14; phenotypes were scored and DNA collected for genotyping and sex typing (n = 1105). Resorption, early lethality and exencephaly were seen only in Cd/Cd embryos. As dietary FA increased, the percentage of affected embryos decreased from 59% at 0 mg of FA to 30% at 10 mg of FA (χ² = 14.3, P < 0.005) (Fig. 3A). A dose-dependent phenotype shift was observed whereby lethality was more prevalent among Cd/Cd embryos receiving no folate, while on the 4 mg/kg diet, exencephaly became a more common outcome. With higher FA diets, most Cd/Cd embryos were viable and successfully closed their neural tubes. Thus, dietary FA rescued both the early lethality and exencephaly phenotypes of the Cd/Cd embryos. This rescue parallels human studies in which FA supplementation is effective even in the absence of folate deficiency (4,5,16).

Across all diets, female Cd/Cd embryos exhibited higher rates of lethality and exencephaly than their homozygous male siblings (overall, χ² = 72.5, P < 0.005). Dietary FA preferentially rescued female Cd/Cd embryos (up to 57%, all phenotypes, χ² = 96.8, P < 0.005) whereas the percentage of affected male Cd/Cd embryos was lower, with a similar FA response (Fig. 3B). Compared with the incidence on the 4 mg/kg FA diet, relative risk of exencephaly for the 10 mg/kg diet group was reduced 33% overall (χ² = 10.4, P < 0.005), and 55% in female embryos (χ² = 30.2, P < 0.005) (Fig. 3C). This gender difference is also observed in human NTDs, in particular in anencephaly, which has a female predominance of 2:1 (3,17).

Compared across conditions, adult mice differed only in the concentration of blood folate, not in fertility or weight gain (Fig. 3D–F). Blood folate levels increased with dietary FA levels (one-way ANOVA, F = 15.01, P < 0.01). Paternal weight did not vary according to diet, suggesting that FA did not affect adult nutritional requirements. Only females in the no folate group took significantly longer than all other groups to become pregnant after the birth of each litter (t = 6.4, P < 0.0001). Although some small amount of FA was required to maintain baseline fertility levels, additional FA supplementation did not enhance fertility further. Therefore, FA levels had a relatively selective effect on embryonic survival and neurulation, rather than conception.

**DISCUSSION**

The Cd locus is distinct from the known null mutations that predispose to NTDs, and the Cd strain provides a new opportunity to identify the biochemical, genetic and gender-dependent factors contributing to folate-responsive NTDs. Over the 45 years since the strain was first reported, only the tooth defect has been lost in the Cd line and crosses presented here. The appearance of exencephaly, the crooked tail defect and the SMALL phenotype have remained, suggesting that these arise from mutation at a single locus. The present study demonstrates that phenotypic expression of Cd is pleomorphic, incompletely penetrant and is modulated

![Figure 2. Linkage map of the Cd locus. (A) Position of Cd on the chromosome 6 consensus maps of the MGD (13). Comparison of the recombination distances (in cm) and physical map (in Mb) of STS markers used to determine linkage in the Cb×DBA backcross. Loci in the linkage groups on the right appear in the genetic order reported in the MGD. Anchor loci determined by the MGD are marked on the WC6 YAC clones (right) and indicate good correlation between genetic and physical distances in this region. Anchor loci D6Mit135 and D6Mit301 (underlined) are polymorphic in the backcross and flank the Cd locus. (B) Recombination map for Cd. Twenty-five of the 38 STSs found to be polymorphic between Cd and DBA are shown. The position of each STS on the MGD map is indicated (left). The order of loci in the backcross corresponds well to that reported in the MGD. Linkage defines a critical region between D6Mit135 and D6Mit301 and a recombination breakpoint for Cd between D6Mit111 and D6Mit301.](image-url)
This variability reflects the complexity of multigenic effects that underlie neural tube closure (14,18,19). This study reveals that NTDs in Cd are ameliorated by dietary FA supplementation. Human studies suggest that prenatal dietary FA supplementation can reduce the recurrence of NTDs as much as 70% in some populations (5,6). However, the mechanism of this rescue is unknown and it is not possible to assess which women are likely to benefit from FA supplementation. Like Cd, it is not necessary that human subjects be inherently folate deficient in order to observe an effect. A predisposing genetic background, which is likely to be complex, has yet to be identified. Thus, Cd provides an important new model for unraveling genetic factors leading to human NTDs.

Dietary FA supplementation has enabled modeling of the human clinical experience and is significant for several reasons. First, Cd is one of the few strains that expresses folate-responsive NTDs and is the first shown to respond to dietary FA administration. Previous attempts to reverse NTDs in animal models have met with mixed success and relied either on i.p. injection of FA (20,21) or FA supplementation of the medium used to culture embryos in vitro (22). In studies relying on i.p. injection, many NTD mouse models were found not to be affected by FA, including Axial deformity (Axd) (20), loop-tail (lp) (22) and curly tail (ct) (19,21). However, Axd, ct and other strains have responded to agents such as methionine or retinoic acid (20,21). Recent studies of Splotch embryos bearing Pax3 mutation indicated that exposure to FA in culture medium, or in utero at E8–9,
could reduce the expected rate of spina bifida and exencephaly (22). One other mouse model, a knockout of the cartilage regulatory gene Cart1, has been shown to respond to i.p. FA administration (23). However, this mutation produces acrania, a phenotype that is distinct from that of Splotch or Cd. Assuming that Splotch will also respond to dietary FA, the Pax3 and Cd gene products may well function in the same or interacting pathway affecting cranial neurulation. These mutant mouse strains provide an opportunity to examine metabolic pathways involving FA and to examine Cd as a possible mediator of neurulation downstream of the Pax3 transcription factor.

Secondly, the present study most closely simulates conditions used in clinical settings and has revealed striking parallels between the Cd mutant and human NTDs. The Cd phenotype is pleiomorphic, incompletely penetrant and can be altered by genetic background, indicating that expression of the mutation can be influenced by genetic and environmental factors, like NTDs in humans. In addition, exencephaly in Cd is exacerbated by insulin administration (24), which may be relevant to the increased risk of NTDs in pregnancies of diabetics. Moreover, Cd phenotypes are rescued by FA to a degree reminiscent of the 50–70% reduction of NTDs in humans by FA supplements. Finally, the skewed sex ratio seen in Cd exencephalics mirrors the higher rate of NTDs in human female fetuses (3).

Thirdly, this dietary FA study provides several insights into the observations of human studies. It has been debated whether the clinically observed amelioration of NTDs by folate is due to a true rescue or rather to an increase in early loss of NTD fetuses (7). Data presented here clearly indicate that FA supplementation can rescue from early lethality. They further indicate that FA-deficient populations may see a lower incidence of NTDs due to increased early fetal loss. Moreover, this is the first NTD model demonstrated to show a gender difference in response to FA. Therefore, Cd provides an experimental model in which to examine gender-dependent factors associated with the observed female predominance of human anencephaly.

Efforts are being launched world-wide to provide FA supplementation to women of child-bearing age with the goal of reducing NTD incidence. Observations in Cd suggest that epidemiological follow-up of human populations will be required to ensure that sufficient doses of FA are supplied to affect the desired NTD rescue, rather than shifting the population toward less severe neurological developmental disorders. Correlation of human genetic studies with mouse models such as Cd ultimately will enable accurate assessment of individual risk and tailored prevention of NTDs for families at risk.

MATERIALS AND METHODS

Mouse colony

Mice were housed in plastic cages with wood-chip bedding, with free access to water and either lab chow (Purina, St Louis, MO) for linkage crosses or a specialty chow with defined FA concentration (P.J. Noyes, Lancaster, NH) for FA diet studies. The room was maintained at 22°C on a 12–12 h light–dark cycle. Timed pregnancies were generated by housing a 6- to 10-week-old female with a male overnight, and removing the male the next morning; the presence of a vaginal plug signified day 0 of gestation.

Breeding crosses and analyses

Linkage. Interspecies crosses could not be used because outcrossing to Spretus (Spret/Ei) yielded no pups and to Castaneous (CAST/Ei) resulted in loss of the Cd phenotype. Several intraspecies crosses were then tested between Cd and 129 Sv, C57Bl6, DBA or A. Of these, DBA crosses were reasonably fertile, retained the crooked tail phenotype and yielded sufficient polymorphism for linkage mapping. Cd/+ animals were mated with those from the DBA/2J strain (Jackson Laboratory, Bar Harbor, ME) to produce crooked tailed F1 offspring, which were then mated to animals of the A strain (Jackson Laboratory). Data were generated only from crooked tailed N2 offspring to avoid false negatives due to incomplete penetration. Recombination frequencies were determined from DNA of 221 fully informative N2 offspring using the MapManager program for genetic mapping [© K. Manly (25)].

Prenatal phenotypes. At weaning, Cd/A females were assigned randomly to one of four diet conditions (0, 4, 7 or 10 mg FA/kg chow; P.J. Noyes), and maintained on it for 1 month prior to timed matings with Cd/A males. Between E12.5 and E14.5, embryos were dissected and phenotyped as resorption, lethal, exencephalic or normal. Tissues were taken for DNA extraction for genotyping and sex typing.

Parental growth and fertility. At 6 weeks of age, Cd/A animals were maintained as breeding pairs, and were assigned randomly to one of five experimental diets (0, 2, 4, 7 or 10 mg FA/kg chow) and maintained on that diet until 2 months passed without the birth of pups. Data were collected as to date of birth, litter size and parental weights taken weekly.

Polymorphism detection

High molecular weight DNA was prepared from tissue samples using the Puregene DNA Isolation kit (Gentra Systems, Minneapolis, MN). Recombination events in the ([Cd/+×DBA]F1×A]N2 generation were detected by PCR using a series of polymorphic markers from chromosome 6 (Research Genetics, Huntsville, AL) employing standard protocols (26). Forward primers were 5’ end-labeled with 33P using T4 polynucleotide kinase (New England Biolabs, Beverly, MA); labeled primers were used for PCR with 28 ng genomic DNA/5 µl reaction: 94°C for 1 min, then 30 cycles of 94°C for 30 s, 56°C for 50 s, 72°C for 50 s, then final extension at 72°C for 6 min. Products were visualized on autoradiography film after separation on 6% denaturing polyacrylamide gels. Alternatively, non-radioactive PCR reactions were run using 84 ng of genomic DNA/15 µl reaction, using the same reaction conditions, and visualized by ethidium bromide staining after separation on 5% MetaPhor (FMC BioProducts, Rockland, ME) gels. The above protocol was also used to genotype parents and embryos for the diet study, using markers D6Mit135, D6Mit111 (Research Genetics) and Ms6hm3 (27).
Sex typing
Embryos were sex typed using X chromosome- (DXMit43; Research Genetics) and Y chromosome-specific [DYBish6 (28)] markers. PCRs were run using 84 ng of genomic DNA/50 µl reaction and the recommended protocol (Research Genetics). Products were visualized by ethidium bromide staining after separation on 5% MetaPhor (FMC BioProducts) gels.

Blood folate levels
Blood levels of FA were determined from red cell lysates taken from adult animals maintained on each of the five experimental diets (0, 2, 4, 7 and 10 mg FA/kg chow) (n = 6–12 individuals/diet group). FA was measured using the Immulite Folic Acid chemoluminescent enzyme immunoassay (Diagnostic Products, Los Angeles, CA).

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