Major Effects on Teratogen-Induced Facial Clefting in Mice Determined by a Single Genetic Region

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Manuscript received February 12, 1990
Accepted for publication May 29, 1990

ABSTRACT

A major correlation has been found between the incidence of glucocorticoid-induced cleft palate and the chromosome *8* segment identified by *N*-acetyl transferase in mice. The resistant strain became fully susceptible while the susceptible strain became resistant when this chromosomal region, representing <0.7% of the genome, was transferred from one strain to the other by the construction of congenic strains. 6-Aminonicotinamide-induced cleft palate and phenytoin-induced cleft lip with or without cleft palate are also influenced by this genetic region but not as strongly. In both cases the susceptible strain became quite resistant to the teratogen-induced clefting when the N-acetyl transferase region of chromosome *8* was transferred. However, this chromosomal region does not make the resistant strain susceptible to these two teratogens.

HUMAN cleft palate (CP) is usually thought to be multifactorially inherited and genetically distinct from cleft lip with or without cleft palate [CL(P)] (CHING and CHUNG 1974; WHO Scientific Group 1970). However, a major gene effect (MARAZITA, SPENCE and MELNICK 1984; CHUNG *et al.* 1986) and a relationship of the two birth defects (CHABORA and HOROWITZ 1974; BONAITIO *et al.* 1982) have also been claimed. The analysis of mouse models of teratogen-induced CP or CL(P) (FRASER and FAINSTAT 1951; GOLDMAN *et al.* 1982), provides an opportunity to test such divergent hypotheses. We have studied the A/J and C57BL/6J inbred strains of mice which differ markedly in their susceptibility to teratogen-induced CP and spontaneous and teratogen-induced CL(P) (PINSKY and FRASER 1959; GOLDSTEIN, PINSKY and FRASER 1963; VERRUSIO, POLLARD and FRASER 1968). We previously used recombinant inbred (RI) strains between these two strains of mice to study the genetics of susceptibility to glucocorticoid-induced CP (LIU and ERICKSON 1986a), 6-aminonicotinamide (6-AN)-induced CP (KAROLYI, ERICKSON and LIU 1988) and phenytoin-induced CL(P) (KAROLYI, LIU and ERICKSON 1987). The strong correlation of these teratogenic effects with the chromosome *8* segment marked by *Nat* (*N*-acetyl transferase) found in the 6-AN and phenytoin studies has now been tested with *Nat* congenic strains. We also studied these congenic strains for susceptibility to glucocorticoid-induced CP and examined the data from previous studies of glucocorticoid-induced CP in RI strains (LIU and ERICKSON 1986a) for the *Nat* correlation. A major correlation has been found between the incidence of glucocorticoid-induced CP and this chromosome *8* segment with a weaker correlation found for 6-AN-induced CP. A possible relationship of phenytoin-induced CL(P) with this chromosome segment is still unclear.

MATERIALS AND METHODS

Mouse maintenance and drug administration: A/J (Nat^s) and C57BL/6J (Nat^r) mice were purchased from The Jackson Laboratory. A.B6-Nat^r (A/J background, Nat^r) and B6.A-Nat^s (C57BL/6J background, Nat^s) congenic strains were constructed in our mouse colony by the backcross system, using whole blood *N*-acetyl transferase activity to determine genotype. Animals at the twelfth backcross generation were brother-sister mated (MATTANO *et al.* 1988). Nat^r is the symbol for the rapid acetylator allele and Nat^s is the symbol for the slow acetylator allele. The segment of chromosome *8* transferred in the production of these congenic strains is expected to be between 12 and 20 cM (MATTANO *et al.* 1988).

For the study of glucocorticoid-induced cleft palate, 2.5 mg of hydrocortisone in 0.05 ml volume were administered to females by intramuscular injection with hydrocortisone sodium phosphate (Hydrocortone, Merck Sharp & Dohme) on days 11–14 of pregnancy. Control injections of 0.05 ml of Dulbecco's phosphate-buffered saline were administered in a similar manner.

6-AN treated mice were injected intraperitoneally with 9 mg per kg of 6-AN on day 13 of pregnancy. Control injections consisted of sterile distilled water.

Phenytoin treated mice were injected intraperitoneally with 60 mg per kg of phenytoin (Dilantin, Parke Davis) on day 10 of pregnancy. The control injection for phenytoin consisted of 40% propylene glycol in 10% ethanol (the drug solvent).

The fetuses were examined on day 17 and were scored for clefting. The day the plug was found was designated as day zero.

Statistical analyses: The nonparametric Fisher's exact probability test statistic (SIEGEL 1956; DIXON and MASSEY

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TABLE 1

Facial clefting and resorption response in parental and *Nat* congenic strains

Strain	Average % CP (0.95 confidence intervals)	Average % CL (P) (0.95 confidence intervals)	Average % resorptions (0.95 confidence intervals)	No. of litters	No. of fetuses
Glucocorticoid-induced					
A/J(*Nat*^{s})	24.8 (15.9–33.7)	−2.7 (−6.1–0.8)	0.4 (−6.3–5.5)	21	174
C57BL/6J (*Nat*^{r})	1.0 (−0.9–2.9)	0.7 (−0.7–2.0)	−2.6 (−6.3–−0.3)	17	141
A.B6-*Nat*^{r}	10.6 (1.0–20.2)	−1.4 (−7.3–4.5)	2.2 (−1.0–5.4)	20	153
B6.A-*Nat*^{s}	30.3 (22.0–38.5)	0	0	12	98
6-Aminonicotinamide-induced					
A/J (*Nat*^{s})	41.5 (25.7–59.2)	9.5 (−7.6–26.5)	20.9 (4.3–37.5)	17	109
C57BL/6J (*Nat*^{r})	10.2 (1.6–18.7)	6.8 (−3.8–17.4)	5.6 (−1.7–12.9)	21	146
A.B6-*Nat*^{r}	1.6 (0.1–7.1)	1.7 (−6.3–9.7)	12.6 (0.8–24.4)	20	100
B6.A-*Nat*^{s}	2.9 (−1.6–7.3)	1.8 (−0.8–4.4)	6.5 (−2.2–15.1)	15	105
Phenytoin-induced					
A/J (*Nat*^{s})	6.8 (0.7–14.3)	41.9 (22.6–61.1)	5.0 (−2.3–12.2)	20	153
C57BL/6J (*Nat*^{r})	0.6 (−0.7–2.0)	1.1 (−0.5–2.6)	6.1 (−3.6–15.7)	28	180
A.B6-*Nat*^{r}	16.3 (2.7–30.0)	−6.9 (−9.3–−4.6)	18.1 (4.7–31.4)	23	134
B6.A-*Nat*^{s}	21.4 (4.4–38.4)	2.1 (−2.5–6.7)	0	12	95
Sporadic					
A/J (*Nat*^{s})	0.8 (−0.3–1.6)	6.1 (3.1–9.0)	6.7 (3.9–9.5)	72	497
C57BL/6J (*Nat*^{r})	0.3 (−2.9–8.6)	0	4.2 (1.6–6.9)	44	342
A.B6-*Nat*^{r}	1.9 (−0.9–4.6)	9.4 (3.6–1.5)	2.1 (−1.1–5.2)	15	111
B6.A-*Nat*^{s}	0	0	0	15	115

The facial clefting and resorption percentages with confidence intervals presented are the levels after sporadic percentages are subtracted. The sporadic levels presented are combined results from females who had either been injected with controls for each of these three teratogens or had received no injections since there is no significant difference between these four controls ($P = 1.000$) (LIU and ERICKSON 1986a).

1969) was used to analyze the incidence data. Numbers of litters with clefting and litters with no clefting were contrasted with the *Nat* allele in a 2 × 2 table to derive the test statistic.

RESULTS

Table 1 presents the results for the teratogen-induced clefting and resorption rates in the parental and *Nat* congenic strains. It is apparent from Table 1 that the transfer of the *Nat*^{s} region from the A/J strain to the C57BL/6J background has made the B6.A-*Nat*^{s} strain fully susceptible to glucocorticoid-induced CP. In addition, the corresponding congenic strain, A.B6-*Nat*^{r}, has an intermediate susceptibility closer to that of the *Nat*^{r} donor strain (C57BL/6J). This reflects a significant decrease in the susceptibility associated with the A/J background. Glucocorticoid treatment has shifted some of the sporadically occurring CL(P) or resorptions to isolated CP in three of the strains studied as indicated by the negative values.

6-AN treated mice again show a decrease in susceptibility causing CP in the A/J background when the *Nat*^{r} allele is moved onto the A/J background (A.B6-*Nat*^{r}) but not the increased susceptibility in the B6.A-*Nat*^{s} strain seen with glucocorticoid treatment. 6-AN treatment increases the incidence of CL(P) and resorptions in all four strains regardless of *Nat* allele.

The high susceptibility of the A/J background to phenytoin-induced CL(P) is markedly decreased in the A.B6-*Nat*^{r} strain with a shift from CL(P) to isolated CP and resorption in this strain. Again, however, the B6.A-*Nat*^{s} strain does not have the increased susceptibility seen in glucocorticoid treated mice. Isolated CP incidence is increased in all four strains with phenytoin treatment and resorption rates are increased in three of the four strains.

Sporadic levels of CP, CL(P) and resorptions reflect the susceptibility of the A/J background to spontaneous CL(P) but no *Nat* allele correlation is seen.

Table 2 shows the statistical correlations for all three teratogens with the *Nat* allele in parental and congenic strains and also in previously studied RI strains. Previously published data on glucocorticoid-induced CP in RI strains is now analyzed for association with the *Nat* region in row 2, Table 2. Glucocorticoid-induced CP response data in RI strains are found in LIU and ERICKSON (1986a). *Nat* typings for the RI strains are found in MATTANO *et al.* (1988) Previously published statistical analyses for the RI strains treated with 6-AN and phenytoin are presented in Table 2 for comparison.

In large litters, increased susceptibility to glucocorticoid-induced CP is very significantly associated with the *Nat*^{s} allele in combined parental and congenic strains and in RI strains. Association of the *Nat*^{s} allele with susceptibility to 6-AN-induced CP is less striking. Phenytoin-induced CL(P) is weakly associated with *Nat*^{s} in the combined parental and congenic strains but is very strongly associated with the *Nat*^{r} allele in RI strains. In small litters, the *Nat* effect is masked in most cases. It is important to remember that the *Nat*

TABLE 2

Statistical analyses of teratogen-induced and sporadic clefting association with *s* and *r* alleles of *Nat*

Strain	Probability of difference occurring by chance		*Nat* allele associated with increased susceptibility
	Small litters	Large litters	
Glucocorticoid-induced CP			
Parental and *Nat* congenic strains	0.80	0.0000	Nat^s
RI strains	0.43	0.0002	Nat^s
6-Aminonicotinamide-induced CP			
Parental and *Nat* congenic strains	0.62	0.008	Nat^s
RI strains (12)	0.005	0.008	Nat^s
Phenytoin-Induced CL (P)			
Parental and *Nat* congenic strains	0.006	0.01	Nat^s
RI strains (13)	0.05	0.000	Nat^r
Sporadic CP			
Parental and *Nat* congenic strains	NA[a]	0.64	
RI strains (12)	0.69	0.51	
Sporadic CL (P)			
Parental and *Nat* congenic strains	0.73	0.11	
RI strains (13)	0.50	0.005	

The numbers represented are Fisher's exact probability test statistic. Because litter size had a strong effect on the incidence data when expressed as a percentage, litters were classified as either small (1–5 fetuses) or large (6–12 fetuses). Average litter size was 6.2. Because six genetic contrasts were made on the same data set in RI strains, a value greater than .008 is not significant (0.05/6 = 0.008). RI strains of A/J and C57BL/6J were supplied by Dr. Muriel Nesbitt at the University of California (NESBITT and SKAMENE 1984).

[a] Not available–test statistic could not be computed as there were no small litters with CP in this group.

gene identifies a chromosome *8* region and not only the *Nat* locus.

Potentially separate maternal effects and fetal effects (BIDDLE and FRASER 1976) are constant in our study since the fetuses and mothers in the strains studied are genetically identical (except *XX vs. XY*). Thus, we are studying gene effects that could be working through either mechanism.

DISCUSSION

Our previous work on the genetics of susceptibility to teratogens causing CP or CL(P) among RI strains revealed that a number of loci were found to influence the degree of susceptibility. That work used RI strains which, with their strain distribution patterns of marker genes, are valuable for clarifying the genetic complexity of teratogen-induced facial clefting. RI strains have proved to be powerful genetic tools in demonstrating genetic independence, linkage and pleiotropism (BAILEY 1971; SWANK and BAILEY 1973; TAYLOR 1978). It was of interest that the chromosome *8* region marked by the *Nat* locus influenced both 6-AN-induced CP (KAROLYI, ERICKSON and LIU 1988) and phenytoin-induced CL(P) (KAROLYI, LIU and ERICKSON 1987) although contrasting alleles correlated with susceptibility. This is not necessarily inconsistent since isolated CP is a different entity than CL(P). A number of other loci influencing phenytoin-induced CL(P) did not influence 6-AN or glucocorticoid-induced CP as strongly (LIU and ERICKSON 1986a). Our work on the genetics of susceptibility to glucocorticoid-induced CP in RI strains had not included an analysis of the chromosomal region marked by *Nat* since we had not yet discovered the association. We have now provided such an analysis and find a major effect of the *Nat* region on glucocorticoid-induced CP among these RI strains. Thus, this region of chromosome *8* influences all three forms of teratogen-induced facial clefting in RI strains.

To confirm this influence, we have extended this analysis to congenic strains for the chromosome *8* region marked by *Nat*. Congenic strains have proven useful in sorting out the importance of single gene effects. It was uncertain whether or not the genetic influence seen in RI strains would also be found in congenic strains. The *Histocompatibility-2* (*H-2*) region had been shown to have an influence on glucocorticoid-induced CP in congenic strains (BONNER and SLAVIN 1975; BIDDLE and FRASER 1977; ERICKSON, BUTLEY and SING 1979) but not in RI strains (LIU and ERICKSON 1986a; VEKEMANS, TAYLOR and FRASER 1981). However, the genetic influence of the chromosome *8* region is readily apparent in the *Nat* congenic strains. It has a very major influence on glucocorticoid-induced CP, being a stronger effect than was initially noted in congenic strains for the *H-2* complex. In fact, the B6.A-Nat^s congenic strain is more sensitive ($P < 0.15$) than the A/J strain, a finding which parallels *N*-acetyl transferase enzymatic activity in these two strains (HEIN *et al.* 1988).

Even though this chromosome *8* region has a smaller effect in congenic strains with 6-AN-induced CP and phenytoin-induced CL(P), the effects are present. This is in contrast to earlier work on 6-AN-induced CP which found no shared genetic susceptibility transmitted by males (BIDDLE and FRASER 1979) In both cases, the A/J background strain (A.B6-Nat^r) became quite resistant to the teratogen-induced clefting when the Nat^r region of chromosome *8* was introduced by constructing the congenic strains. However, this one chromosomal region from the A/J strain (Nat^s) does not make the C57BL/6J background strain (B6.A-Nat^s) susceptible to 6-AN or phenytoin even though it does make it very susceptible to glucocorticoid-induced CP. The effective locus, perhaps one close to *Nat*, could possibly have been lost in this backcross but not in the other.

We believe that it is likely that a single gene in this region of chromosome *8* is responsible for major effects on teratogen-induced clefting. The *Nat* locus itself is unlikely to be the gene responsible. Although

variations in metabolism of drugs have been suggested as a cause for differential susceptibility, *N*-acetyl transferase is not known to be involved in the metabolism of any one of these teratogens. In fact, genetic differences in phenytoin metabolism were previously found not to explain genetic differences among mice in susceptibility to this teratogen (ATLAS, ZWEIER and NEBERT 1980). Perhaps differences in drug receptors are related to the genetic differences in susceptibility but this is not true with glucocorticoid-induced CP. Although genetic differences in glucocorticoid receptor levels are found between the A/J and C56BL/6J strains, they are not related to differences in glucocorticoid-induced CP (LIU and ERICKSON 1986b). Thus, it seems more likely that some other genetic variation in this region is responsible for the differences in susceptibility.

The genetic effect of the *Nat* region on glucocorticoid-induced CP and 6-AN-induced CP seen in RI strains is confirmed in the *Nat* congenic strains. It is interesting that the *Nat* effect on phenytoin-induced CL(P) in RI strains is apparently reversed in the congenic strains. The Nat^r allele is strongly associated with greater susceptibility in RI strains while the Nat^s allele is very weakly associated with susceptibility in the combined parental and congenic strains. In fact phenytoin treatment seems to reduce the sporadic levels of CL(P) in the A.B6-Nat^r strain by shifting the incidence of CL(P) to isolated CP and resorptions. In the RI strains we had found a correlation between three loci (or chromosomal regions) that influence glucocorticoid-induced CP and phenytoin-induced and/or sporadic incidence of CL(P) but in all three cases it was a different allele that was associated with CP in the one case and with CL(P) in the other (KAROLYI, LIU AND ERICKSON 1987). In contrast the data in congenic strains shows the same allele of this chromosome *8* region affecting susceptibility to glucocorticoid-induced and 6-AN-induced clefting but a very weak correlation to phenytoin-induced clefting. These results argue strongly for the importance of the interaction of multiple, unlinked genes in the etiology of teratogen-induced clefting. Thus, congenic strains allow us to study the effects of one allele in isolation while RI lines "shuffle" many alleles at once.

The *Nat* defined region has the properties of a major gene affecting clefting. Given the large amount of linkage conservation between man and mouse (SEARLE *et al.* 1987) it will be worthwhile to see if the well known pharmacological variation in *Nat* in man (WEBER 1987) is related to human CP or CL(P).

We thank MURIEL NESBITT for the breeding stocks of the recombinant inbred strains, WILLIAM ERICKSON for statistical advice, ANN MOGAN and JUDY WORLEY for secretarial assistance, and National Institutes of Health for support (GM 27028).

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Communicating editor: R. E. GANSCHOW