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Genetics of Susceptibility to 6-Aminonicotinamide-Induced Cleft Palate in the Mouse: Studies in Congenic and Recombinant Inbred Strains

JILL KAROLYI, ROBERT P. ERICKSON, AND SHARON LIU
Departments of Human Genetics, Pediatrics and Communicable Diseases, University of Michigan, Ann Arbor, Michigan 49109-0618

ABSTRACT In a search for genetic differences in susceptibility to cleft palate, congenic and recombinant inbred strains of mice were treated with 6-aminonicotinamide or control injections. Of six loci tested, only the chromosome segment marked by N-acetyl transferase was found to affect susceptibility to 6-aminonicotinamide-induced cleft palate. This chromosome segment is known to affect glucocorticoid-induced cleft palate and phenytoin-induced cleft lip with or without cleft palate in these strains of mice.

The A/J and C57BL/6J inbred strains of mice differ markedly in their susceptibility to teratogen-induced cleft palate (CP) and in their susceptibility to spontaneous and teratogen-induced cleft lip with or without CP [CL(P)] including the induction of isolated CP by 6-aminonicotinamide (6-AN) (Pinsky and Fraser, '59; Goldstein et al., '63; Verrusio, '68). Previous genetic studies of this difference between the susceptible A/J and the resistant C57BL/6J strains concluded that the results were compatible with control by three loci, with a suggestion that one might be associated with the brown (*b*) locus (Biddle, '77). We have previously used recombinant inbred (RI) strains between these two strains of mice to study the genetics of susceptibility to hydrocortisone-induced CP (Liu and Erickson, '86) and phenytoin-induced CL(P) (Karolyi et al., '87). We have now applied this powerful tool of genetic analysis to 6-AN-induced isolated CP.

MATERIALS AND METHODS

Mouse maintenance and drug administration

A/J, C57BL/6J, A.BY/SnJ, and B10.A mice were purchased from the Jackson Laboratory, Bar Harbor, ME. RI strains of A/J and C57BL/6J were supplied by Dr. Muriel Nesbitt at the University of California (Nesbitt and Skamene, '84). The mice were then bred and maintained in our mouse colony. Timed females of the parental strains, A/J (A background, *H-2*^{a}) and C57BL/6J (B background, *H-2*^{b}), of related *H-2* congenic strains A.BY/SnJ (A background, *H-2*^{b}) and B10.A (B background, *H-2*^{a}), and of the RI strains were injected intraperitoneally with 6-AN on day 13 of pregnancy. The dose was 9 mg per kg. Control injections of sterile distilled water (the drug solvent) were administered in a similar manner. The fetuses were examined on day 17 and were scored for CP. The day the plug was found was designated as day 0.

Statistical analyses

The nonparametric Fisher's exact probability test statistic (Siegel, '56; Dixon and Massey, '69) was chosen for the incidence data because it does not presuppose a large sample, in contrast to the chi-square test. Numbers of litters with CP and litters with no CP were contrasted with the test alleles in a 2 × 2 table to derive the test statistic. Confidence limits for percentages were interpolated from the tables published by Rohlf and Sokal ('69).

RESULTS

Table 1 summarizes the CP response to 6-AN in the mouse strains studied. The sporadic levels of CP presented are combined results from females who had either been injected with this drug solvent, or who had received no injections, or who had been in-

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This paper is dedicated to the memory of Robert M. Pratt, whose untimely death has deprived cleft palate research of one of its leaders.

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TABLE 1. Cleft palate (CP) response in parental, H-2 congenic, and RI strains

Strain	6-Aminonicotinamide-induced CP				Sporadic CP			
	Average % CP/ litter	95% Confidence limits	No. of litters	No. of fetuses	Average % CP/ litter	95% Confidence limits	No. of litters	No. of fetuses
A/J	55.3	47.78–67.73	13	101	0.6	0.30–2.41	66	449
C57BL/6J	12.0	5.24–16.94	16	115	0.3	0.00–0.99	39	301
A.BY/SnJ	50.2	38.78–57.32	19	117	1.6	0.30–2.45	73	431
B10.A	20.6	12.96–28.75	13	108	2.0	1.03–3.65	57	457
A×B2	6.3	1.14–15.85	16	55	0	0.00–1.48	41	202
A×B6	21.8	15.15–30.25	20	123	0.9	0.10–4.01	27	162
A×B12	59.3	49.89–73.24	12	71	0	0.00–3.86	14	76
A×B15	32.5	24.27–42.69	13	107	1.2	0.52–5.23	23	183
A×B17	15.6	13.59–30.16	23	102	1.4	0.56–4.49	40	204
B×A10	16.4	13.03–28.65	18	110	2.2	0.61–8.60	18	98
B×A14	79.5	77.59–92.15	13	99	0	0.00–0.82	49	366
B×A15	40.2	26.36–56.52	10	44	0	0.00–3.70	12	63

jected intramuscularly with PBS (phosphate-buffered saline) or by the intraperitoneal route with 40% propylene glycol in 10% ethanol (two controls used for other teratogens) since there is no significant difference between these four controls ($P = 1.000$) (Liu and Erickson, '86).

The highly susceptible parental A/J strain showed a 55.3% 6-AN-induced CP incidence. The *H-2* congenic strain which shares the A background (A.BY/SnJ) but has the $H\text{-}2^b$ allele of C57BL/6J showed a 50.2% incidence of 6-AN-induced CP. Four of the RI strains also showed a high incidence of 6-AN-induced CP. These are A×B12, A×B15, B×A14, and B×A15. The low susceptibility parental C57BL/6J strain showed an incidence of 12.0% 6-AN-induced CP. A similar low level of incidence was found also in a related congenic strain sharing the B background but the $H\text{-}2^a$ allele of A/J (B10.A), 20.6%, and in four of the RI strains: A×B2, A×B6, A×B17, and B×A10. Incidence levels of less than 25% were considered low. The *H-2* congenic strains were studied because *H-2* (the major histocompatibility locus) has been shown to affect susceptibility to CP induced by hydrocortisone in *H-2* congenic strains (Bonner and Slavkin, '75; Erickson et al., '79) but not in the RI strains (Liu and Erickson, '86). A test of correlation of the *H-2* locus and incidence of 6-AN-induced CP in the four *H-2* congenic strains shows no relationship ($P = .38$).

Table 2 indicates the association of six genetic markers with the incidence of CP among the parental and RI strains. Table 3 indicates these same genetic associations when calculated on the basis of RI strains only. We calculated the data both ways because including the parental strains influences the results as they were selected a priori for high and low levels of susceptibility to 6-AN and the most conservative handling of the data would exclude them. Table 4 shows the distribution of these markers in the strains studied. In this table, a and b alleles refer to the A/J allele (a) or C57BL/6J allele (b). These genetic markers identify chromosomal regions and not only the named locus. *H-2* was discussed above. *B2m* (β_2 microglobulin) has been found to influence susceptibility to hydrocortisone-induced CP in RI strains (Liu and Erickson, '86). *Gus* (β-glucuronidase) marks chromosome 5 for which a locus has been implicated in the genesis of glucocorticoid-induced CP (Vekemans et al., '81); *b* (brown pigmentation) has been suggested as one component of susceptibility to 6-AN-induced CP (Biddle, '77). *Nat* (N-acetyl transferase) is a marker for a gene strongly influencing susceptibility to glucocorticoid-induced CP (unpublished data) and susceptibility to phenytoin-induced CL(P) (Karolyi et al., '87). *H1t* (Histone-1 testes) was included as a control marker.

Because litter size had a strong effect on the incidence data when expressed as a percentage, litters were classified as either small (one to five fetuses) or large (six to 12 fetuses). Average litter size was 6.2. The results indicate that the chromosome segment marked by the *Nat* a gene increases susceptibility to 6-AN-induced CP in both small and large litters when calculated with or without the parental lines. The chromosome segment marked by the *H1t* a allele seems to increase susceptibility to CP in large litters when the

TABLE 2. 6-Aminonicotinamide-induced and sporadic CP association with a and b alleles of H-2, B2m, Gus, b, Nat, *and* H1t *in parental and RI strains*

Gene	Fisher's exact probability test statistic: Small litters	Fisher's exact probability test statistic: Large litters	Gene effect
6-Aminonicotinamide-induced CP			
H-2 (chromosome 17)	.38	.03	
B2m (chromosome 2)	.12	.21	
Gus (chromosome 5)	.64	.22	
b (chromosome 4)	.01	.57	
Nat (chromosome 8)	.002	.0000	*Nat* a↑
H1t (chromosome 13)	.14	.001	*H1t* a↑
Sporadic CP			
H-2	.32	.04	
B2m	.66	.24	
Gus	.73	.59	
b	.68	.52	
Nat	.64	.41	
H1t	.75	.56	

TABLE 3. 6-Aminonicotinamide-induced and sporadic CP association with a and b alleles of H-2, B2m, Gus, b, Nat, *and* H1t *in RI strains only*

Gene	Fisher's exact probability test statistic: Small litters	Fisher's exact probability test statistic: Large litters	Gene effect
6-Aminonicotinamide-induced CP			
H-2 (chromosome 17)	.35	.53	
B2m (chromosome 2)	.09	.08	
Gus (chromosome 5)	.64	.13	
b (chromosome 4)	.009	.03	
Nat (chromosome 8)	.005	.008	*Nat* a↑
H1t (chromosome 13)	.37	.05	
Sporadic CP			
H-2	.34	.05	
B2m	.64	.30	
Gus	.72	.49	
b	.67	.43	
Nat	.69	.51	
H1t	.82	.48	

TABLE 4. Genotypes of lines studied

Strain	*H-2* allele	*B2m* allele	*Gus* allele	*b* allele	*Nat* allele	*H1t* allele
A/J	a	a	a	a	a	a
C57BL/6J	b	b	b	b	b	b
A.BY/SnJ	b	a	a	a	a	a
B10.A	a	b	b	b	b	b
A×B2	a	b	a	a	b	b
A×B6	b	a	a	a	b	b
A×B12	a	a	a	b	b	b
A×B15	a	a	NA[1]	b	a	a
A×B17	a	a	a	a	b	b
B×A10	b	a	a	NA	b	NA
B×A14	b	b	b	b	a	a
B×A15	b	a	a	NA	a	b

[1]NA = not available.

parental strains are included in the analysis but this correlation disappears when the RI strains are analyzed separately. The other investigated gene relationships show no correlation in these strains. Since six statistical tests were performed on each data set, the significance level for an individual contrast must be $<.008$ (.05/6) to achieve an experiment error rate of $<.05$ (Bancroft, '68).

DISCUSSION

Six-aminonicotinamide has been a popular model of teratogenic action. The classic studies of Landauer ('60) on its effect in chicks greatly helped to clarify the phenocopy concept. Studies of its action at the biochemical level have provided both examples of synergism (Smithberg and Runner, '63) and antagonism, both with expected competitors such as nicotinamide (Chamberlain and Goldyne, '70) and unexpected antagonists such as ether (Hamly et al., '70). However, there have been few studies of the genetics of susceptibility to the effects of this teratogen, the paper by Biddle discussed in the beginning of this paper being the exception. RI strains of mice provide a powerful tool to study genetic effects on susceptibility to teratogen action. These strains, with their strain distribution patterns of genes marking chromosomal regions, are valuable for identifying genetic associations (Bailey, '71; Swank and Bailey, '73; Taylor, '78). Since the RI strains are homozygous at all loci, large numbers of identical mice are available for testing. We have now investigated the effect of six loci on the incidence of CP in the presence or absence of 6-AN treatment in the A/J, C57BL/6J, relevant *H-2* congenic strains (C57BL/6J and C57BL/10J are related but not genetically identical), and eight RI strains. These strains have been typed for the six genetic markers we selected. Interestingly, only the chromosomal region marked by *Nat*, or *Nat* itself, has a significant effect on the incidence of 6-AN-induced CP when analyzed both with and without data from the parental strains. This effect was significant both in large and small litters. The *H1t* effect was found only in the analysis which included parental strains and not in the more stringent analysis including RI strains only. Although we have presented both analyses, we believe the more stringent analysis is appropriate since the parental strains were chosen for their high or low clefting incidence. The brown locus, *b*, which Biddle had found to have an effect, did not have a statistically significant effect in our set of recombinant inbred strains, although a nearly statistically significant effect was found in small litters. These results are in marked contrast to our finding with glucocorticoid-induced CP (Liu and Erickson, '86) and phenytoin-induced cleft lip with or without CP (Karolyi et al., '87). In each of these cases, multiple genes were found to affect susceptibility to CP. Of course, these loci identify chromosomal regions which represent a small sampling of the mouse genome and it is possible that many other loci could be found that determine part of the susceptibility to 6-AN-induced CP. This is indeed suggested by Biddle's analysis, which suggested the involvement of at least three loci. Although glucocorticoid and phenytoin induce two different forms of CP which are classically considered to be etiologically different, some of the same loci were found to affect both kinds of teratogen-induced clefting (Karolyi et al., '87; Liu and Erickson, '86). However, it is different alleles at the loci that cause susceptibility to glucocorticoids as compared to phenytoin. It is interesting that the one gene, *Nat*, or the chromosomal region marked by *Nat* which was found to affect susceptibility to 6-AN, also affects susceptibility to the other two teratogens.

It is interesting to ask if the locus or chromosomal region marked by *Nat* corresponds to the major gene determining liability to spontaneous cleft lip in the mouse which is being studied by Biddle and Fraser ('86) and Juriloff ('86). The results with the AXB6 RI line, with *Nat* b and high spontaneous CL(P), suggests a negative answer (Liu and Erickson, '86). Congenic lines are being produced for *Nat* (Mattano et al., submitted) which will allow a more specific test of the effects of this region. It has recently been argued that a major gene also determines liability to cleft lip with or without CP in human populations (Marazita et al., '86). To date, the only association of human CP with a single gene is a syndrome of CP and ankyloglossia with an X-linked marker (Moore et al., '87). It would seem worthwhile to determine whether *Nat* variation in humans (Weber and Hein, '85) associates with spontaneous CP given the homology of linkage between mouse and man (Buckle et al., '84).

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