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Genetic and Environmental Factors in Cortisone Induced Cleft Palate (40259)[1]

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Cleft lip and/or palate are the most frequently occurring birth defects in the United States, accounting for 13% of all reported anomalies (1). Genetic hypotheses for the etiology of these malformations have evolved slowly, gaining complexity with time. In the first extensive study in humans it was concluded that isolated cleft palate was inherited as a single dominant trait with greatly reduced penetrance (2). More recently, genetic models based on allelic restriction (3) and multifactorial threshold inheritance involving the effects of genetic and environmental factors (4–6) have been advanced. At the present time no clearcut evidence exists which discriminates in humans between the single locus and multifactorial models (7).

Over 20 years ago an animal model for cleft palate was developed in mice (8). It was shown that clefting of the developing secondary palate could be induced by the administration of glucocorticosteroids to pregnant mice between the 11th and 14th days of gestation. Further, it was found that various inbred strains of mice had different degrees of susceptibility to cortisone induced cleft palate (9, 10). Specifically, over 90% of the progeny of A/J and DBA/1 mice given 2.5 mg of cortisone developed cleft palate (C-P), whereas only 20–25% of the offspring of similarly treated C57BL/6 or CBA/J mice had the defect. More recently, it has been found that one factor determining susceptibility to cortisone induced C-P is associated with the major histocompatibility locus (H-2) of the mouse (11). Using congenic strains it was shown that mice of the H-2^a type were susceptible but mice identical genetically except for the fact that they carried the H-2^b or closely associated alleles were resistant. The H-2 complex is not the sole determinant of C-P sensitivity, however, for C3H/HeJ and CBA mice have the same H-2 genotype (H-2^k) but different susceptibilities to cortisone induced C-P (68% vs 12%, respectively) (12).

Studies of crosses between susceptible and resistant strains of mice have suggested that maternal inheritance and the maternal environment also are major contributing factors in determining susceptibility to cortisone induced C-P (10, 11). For example, clefting frequency in the congenic hybrid derived from the cross, B10.A × B10, (i.e., susceptible female × resistant male) was 64% but that in the reciprocal cross, B10 × B10.A, was 31% (11). Although studies of this type have consistently demonstrated a "maternal effect," it has not been clear to what extent the effect is determined by maternal sex-linked characteristics, cytoplasmic inheritance or maternal intrauterine environmental influences.

To sum, studies in mice have clearly disclosed that susceptibility to cortisone induced C-P is controlled by (a) H-2 and non-H-2 associated genes and (b) a genetically controlled maternal effect, one component of which appears to be associated with H-2 (11). In this paper we wish to present evidence demonstrating that (a) at least two separate H-2 associated loci are involved in determining susceptibility to cortisone induced C-P, (b) dietary factors can modulate susceptibility and (c) in certain strains of mice the frequency of C-P is significantly higher in females.

Materials and methods. All mice used in these studies were purchased from the Jackson Laboratory, Bar Harbor, Maine, and throughout the experiment they were fed either Purina Breeder Chow or Lab. Chow. The inbred strains and their H-2 haplotypes (H-2K, Ir, Ss, H-2D), (see Fig. 1) were A/J (k, k, d, d), DBA/1 (q, q, q, q), C57BL/6 (b, b, b, b) and C57BL/Ks (d, d, d, d); the congenic and recombinant congenic strains were C57BL/10 Sn (B10; b, b, b, b), B10.A

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(k, k, d, d), B10.A2R (k, k, d, b) and B10.A5R (b, b, d, d).

Coisogenic strains are defined as strains genetically identical one with another except for a difference at a single locus. True coisogenicity is achievable only when a mutation occurs in an inbred stock. An approximation to the coisogenic state can be produced by continued crossing of a gene or locus (e.g., H-2) from one stock onto the inbred and therefore isogenic background of another. In this case the foreign gene is always accompanied by and is part of a foreign chromosome segment. Other contaminant genes may also be present, but the length of the foreign chromosome segment and the number of contaminant genes will decrease as the number of generations of matings to the inbred parent strain increases. A strain derived in this manner, which approximates but may never fully achieve the true coisogenic state is referred to as a congenic strain. The inbred strain to which repeated crosses have been made (in this case, C57BL/10 Sn) is the inbred partner. Congenic strains derived from a crossover event within the major histocompatibility locus (H-2) have been designated as recombinant congenic strains. The congenic mice used in these studies have been crossed with the inbred partner a minimum of eleven times.

Virgin females 10–12 weeks old were used throughout. One male and two females were placed in each cage; the day a vaginal plug was detected was considered day 0 of pregnancy. On days 11 through 14 the mice were injected im with 2.5 mg cortisone acetate or an equal volume of diluent (Merck, Sharp and Dohme).

The mice were killed on the 18th day of pregnancy, and the number of living fetuses and resorptions were recorded (dead fetuses, regardless of the state of development were recorded as resorptions). Living fetuses were weighed and examined for gross external malformations. After decapitation, the fetal head was inspected for the presence of C-P or other anomalies, and the internal organs were examined for defects and to determine sex. The results were analyzed by Yate's χ^2 test (12).

Early results with mice fed Breeder Chow revealed a high frequency of C-P in the putative "resistant strains." Because of evidence indicating that mice maintained on Breeder Chow consistently show higher C-P frequencies than those maintained on certain other standard diets (14), parallel studies were performed with mice fed Purina Lab. Chow.

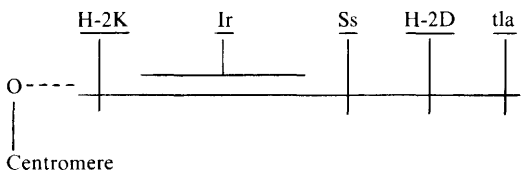

FIG. 1. A simplified map of the mouse major histocompatibility complex (H-2).

Results and discussion. When the mice were fed Lab. Chow before and during pregnancy, C57BL/6 (H-2^b) and B10 (H-2^b) strains had the expected low frequencies of C-P (21% and 36%, respectively; Table I), and all others were found to have significantly higher incidences (A/J, H-2^a, 100%; DBA/1, H-2^q, 94%; B10.A, H-2^a, 92%; C57BL/Ks, H-2^d, 65%; B10.A2R, 58%; B10.A5R, 50%). Viewed as a whole it is apparent that under these experimental conditions mice that bear the entire H-2^b complex are relatively resistant to cortisone induced C-P whereas those that have the H-2^a or H-2^q alleles are highly sensitive. Mice bearing H-2^k alleles on the H-2K side of Ss (B10.A2R) or H-2^d alleles in the region of H-2D (B10.A5R and C57BL/Ks) have intermediate frequencies of this anomaly (Fig. 1). These observations suggest the presence of two distinct loci associated with H-2 which can act in an additive fashion to determine relative susceptibility to cortisone induced C-P (i.e., H-2^a, (k, k, d, d) is thought to represent a recombination between H-2^k and H-2^d). However, it will require the testing of backcrossed populations to confirm this hypothesis and rule out the presence of contaminating closely associated non H-2 genes.

The frequencies of C-P increased significantly when C57BL/6, B10, and B10.A2R mice were fed Breeder Chow; no significant changes were noted among the other strains on this diet (the frequencies of resorption did increase, however, in A/J and DBA/1 mice). When the H-2 haplotypes of the former strains are examined, it is found that they share the H-2D^b allele suggesting that this

TABLE I. FREQUENCIES OF CORTISONE INDUCED CLEFT PALATE (C-P) IN INBRED AND CONGENIC STRAINS OF MICE FED PURINA MOUSE BREEDER OR LAB. CHOW.

	Inbred strains				Congenic strains			
Treatment	A/J k, k, d, d[i]	DBA/1 q, q, q, q	C57BL/6 b, b, b, b	C57BL/Ks d, d, d, d	B10 b, b, b, b	B10.A k, k, d, d	B10.A2R k, k, d, b	B10.A5R b, b, d, d
Breeder chow plus diluent								
Pregnancies		3	7		8	2	1	6
Embryos		28	52		41	19	7	39
% C-P		3%	0%		0%	0%	0%	0%
Lab. chow plus cortisone								
Pregnancies	8	15	18	16	14	18	18	21
Embryos	40[a]	72	100	97	91	130	117	113
% C-P total	100%	94%	21%[f]	65%[f, h]	36%[f]	92%[f]	58%[f]	50%[k]
males	100%	94%	23%[d]	64%	24%[e]	91%	56%	40%[e]
females	100%	95%	19%[d]	65%	47%[e]	94%	61%	60%[e]
Breeder chow plus cortisone								
Pregnancies	8	16	12	13	11	13	17	11
Embryos	9[b]	43[c]	73	84	90	99	125	85
% C-P total	100%	70%	62%[h]	73%[h]	71%[f]	98%[f]	97%[f]	51%[f]
male		65%	50%[d]	67%[d]	61%[e]	98%	93%	42%[d]
female		74%	70%[d]	78%[d]	80%[e]	98%	100%	58%[d]

[a] 38 resorbed.
[b] 50 resorbed.
[c] 66 resorbed.
[d] Male *vs* female: frequencies of C-P not significantly different (NS), Yates χ^2 test.
[e] Male *vs* female: $P < 0.05$.
[f] $P < 0.01$: B10 *vs* B10.A (Breeder and Lab. chow); B10.A2R *vs* B10.A54 (Breeder chow); C57BL/6 *vs* C57BL/Ks (Lab. chow); B10 *vs* B10.A2R (Lab. chow).
[k] $P < 0.05$: B10 *vs* B10.A5R (Lab. chow).
[h] NS:C57BL/Ks, Breeder *vs* Lab. chow and *vs* C57BL/6 (Breeder chow).
[i] H-2 haplotype (H-2K, Ir, Ss, H-2D).

TABLE II. DIFFERENCES IN THE CHEMICAL COMPOSITIONS OF THE TWO PURINA DIETS USED IN THESE STUDIES[a]

Nutrients	Lab. Chow	Breeder Chow
Fat, %	3.78	11.71
Fiber, %	4.86	1.94
Calcium, %	1.42	0.40
Zinc, ppm	60.64	19.82
Copper, ppm	18.20	8.21
Cobalt, ppm	0.35	0.09
Iodine, ppm	1.18	0.02
Vitamin A, I.U./g	12.00	30.00
Vitamin D, I.U./g	5.31	3.30
Alpha tocopherol, I.U./lb.	38.35	8.17
Thiamin, ppm	16.55	7.68
Niacin, ppm	98.34	53.33
Choline, ppm	1665.00	913.00
Folic Acid, ppm	6.46	1.62
Pyridoxine, ppm	5.00	2.69
Vitamin B-12, μg/lb.	15.89	—

[a] Data taken from "Purina Laboratory Manual" Ralston Purina Company, Checkerboard Square, St. Louis, Missouri.

dietary effect may be mediated by factors controlled by this region of the genome. As with other data presented here, the final proof of this will evolve from studies on F_2 and backcross populations.

The differences in chemical composition of the two Purina diets used in these studies are listed in Table II. Although the formulae for these diets differ markedly in many respects, preliminary evidence suggests that the level of Vitamin A (14) or its vehicle is the major if not sole factor which produced the variable degree of susceptibility noted in the "sensitive" strains. In experiments (ms in preparation) in which C57BL/6 mice were fed Breeder Chows that differed only in Vitamin A content, it was found that, in general, the frequency of C-P varied directly with the level of this vitamin. It should be noted that the standard Purina Breeder Chow contains 30 I.U./g; thus a mouse that eats 5 g/day will

consume 150 I.U. or approximately 6000 I.U./kg (this is equivalent to the ingestion of over 400,000 I.U. by a 70 kg man, a dose clearly in the toxic range (15)). The potential for toxic effects from the Vitamin A may be enhanced by the low levels of alpha tocopherol which antagonizes the actions of Vitamin A on membranes (15).

When the frequencies of C-P among male and female fetuses were compared, it was found that females of the B10 (on Breeder and Lab. Chow) and B10.A5R (on Lab. Chow only) strains had significantly higher incidences of C-P (although the frequencies were generally higher among females of the other strains, the differences were not significant). There is no clear evidence from these data that H-2 associated loci mediate this effect. The mechanism for the sexual dimorphism also is obscure, although a contributory factor may be the fact that palatal closure may occur somewhat earlier in males (16).

In summary, susceptibility to cleft palate in the mouse appears to be under multigenic control. The evidence suggests that perhaps two factors determining the degree of susceptibility to cortisone induced cleft palate are associated with the major histocompatibility complex (H-2) and certain dietary factors can modulate this susceptibility via a gene(s) which tentatively maps in the region of H-2D. In addition, genetically controlled maternal effects and sexual dimorphism have been noted. The mechanism(s) by which the degree of susceptibility is determined is not clear, although it has been suggested that at least one of the H-2 linked genes may control the number and/or avidity of cytoplasmic cortisone and corticosterone receptors (11, 12, 17, 18) as they do plasma testosterone levels (19) and membrane receptors for insulin and glucagon (20).

It is anticipated that data obtained from studies of this type eventually will allow us to predict the degree of C-P susceptibility of a given mouse strain or cross on the basis of H-2 type, dietary background and, perhaps, numbers and avidity of cytoplasmic cortisol receptors. Hopefully, this would allow for the development of simple tests to screen for human parents most at risk to bear children with cleft palate or other anomalies.

1. Pruzansky, S., Clifford, E., Converse, J. M., Johnson, M. C., Langman, J., Lis, E. F., Slavkin, H. C., and Warkany, J. Report of the ad hoc advisory committee on program strategies for research on craniofacial anomalies, July 1973. National Institute of Dental Research, Bethesda, Maryland 20014.
2. Fogh-Anderson, P. Inheritance of Harelip and Cleft Palate. Copenhagen 1942, Nyt Nordisk Forlag, Arnold Busck A/S, 266 (1942).
3. Melnick, M., and Shields, E. D., The Lancet, **i**, 176 (1976).
4. Gruneberg, H., J. Genet. **51**, 95 (1952).
5. Fraser, F. C., Proceedings 2nd Internat. Cong. Human Genet. *3*, Rome, September 1961, Instituto G. Mendel, vol. 1882 (1963).
6. Ching, G. H. S., and Chung, C. S., Amer. J. Hum. Genet. **26**, 162 (1974).
7. Chung, C. S., Ching, G. H. S., and Morton, N. E., Amer. J. Hum. Genet. **26**, 177 (1974).
8. Fraser, F. C., and Fainstat, T. D., Pediatrics **8**, 527 (1951).
9. Walker, B. E., and Fraser, F. C., J. Embryol. Exp. Morphol. **5**, 201 (1957).
10. Kalter, H. *in* "Teratology: Principles and Techniques" (J. G. Wilson and J. Warkany, eds.), vol. 57. University of Chicago Press, Chicago, **57** (1965).
11. Bonner, J. J., and Slavkin, H. C., Immunogenetics **2**, 213 (1975).
12. Goldman, A. S., Katsumata, M., Yaffe, S. J., and Gasser, D. L., Nature (London) **265**, 643 (1977).
13. Langley, R., Practical Statistics, Dover Publications, Inc., New York, p. 285 (1971).
14. Miller, K. K., Teratology **15**, 249 (1977).
15. Anderson, O. R., Roels, O. A., and Pfister, R. M., Nature (London) **213**, 47 (1967).
16. Hanker, J. S., Duncan, G. H., and Ambrose, W. W., J. Dent. Res. **56**, 203B (1977).
17. Salomon, D. S., and Pratt, R. M., Nature (London) **264**, 174 (1976).
18. Bonner, J. J., and Slavkin, H. C., Develop. Biol., in press.
19. Ivanyi, P., Hampl, R., Starka, L., and Mickova, M., Nature New Biol. **238**, 280 (1972b).
20. Meruelo, D., and Edidin, M., Proc. Nat. Acad. Sci. U.S.A. **72**, 2644 (1975).

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