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Genetically determined organ specific responses to the teratogenic action of 6-aminonicotinamide in the mouse†

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It is now well recognized that the frequency of malformations following treatment of experimental animals with a teratogen varies with the genetic constitutions of the mother and embryo (Kalter & Warkany, 1959; Fraser, 1962). Little attention has been paid, however, to the question of whether the genes involved act by altering the animal's general susceptibility to the given teratogen, or by producing variations in response of the specific organ systems showing the malformations.

Kalter & Warkany (1957), using maternal treatment with the riboflavin antagonist galactoflavin, found that cleft palate, absent oesophagus and open eye (as well as several other defects) occurred with different frequencies in the four inbred mouse strains DBA, 129, A/Jax and C57BL. In two strains (DBA and 129) relatively high frequencies of malformations were produced by doses which caused very few, if any, malformations in the other two, indicating that there is a genetically controlled difference in general susceptibility to the drug. At a higher dose level, the relative frequencies of the different malformations varied from strain to strain—for instance, the frequency of cleft palate was high in the DBA and low in the A/Jax strain, absent oesophagus was frequent in both the A/Jax and DBA strains, and the frequency of open eye was high in the A/Jax and low in the DBA strain. This shows that there are also genetic differences in response of specific organ systems to the teratogen.

The present study makes use of a teratogen, 6-aminonicotinamide, and a method allowing precisely timed exposure of the embryo, to provide further evidence that the genetic basis for a strain difference in frequency of induced malformations differs for different organs in animals of the same genotype, and to show that these differences are in part cytoplasmic.

6-aminonicotinamide is a structural analogue of nicotinamide and was shown to be a competitive inhibitor of nicotinamide by Johnson & McColl (1955, 1956) and Dietrich *et al.* (1958). Johnson & McColl (1955, 1956) also showed that nicotinamide, nicotinic acid and, to a lesser extent, tryptophan, protect against its lethal effects.

The teratogenic nature of 6-aminonicotinamide has been demonstrated in chickens (Landauer, 1957; Murphy, Dagg & Karnofsky, 1957; Dagg & Karnofsky,

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1958), in rats (Murphy, Dagg & Karnofsky, 1957; Dagg & Karnofsky, 1958), and mice (Pinsky & Fraser, 1958, 1960). When nicotinamide was injected at the same time no malformations were induced, suggesting that the analogue interferes with development by producing a relative deficiency of nicotinamide. Pinsky & Fraser (1960) demonstrated that the nature of the malformations induced varied sharply with the gestational stage at which the drug was given. By administering nicotinamide, in a quantity shown to prevent any further teratogenic action of 6-aminonicotinamide, 2 hr. after the initial dose of 6-aminonicotinamide, they were able to focus the teratogen on a very precisely defined stage of pregnancy.

1. MATERIALS AND METHODS

Mice of two highly inbred strains, A/Jax and C57BL/6J, subsequently referred to as A and C, were obtained from the Jackson Memorial Laboratory, Bar Harbor, Maine, at 6–7 weeks of age and maintained on Purina Lab Chow and water *ad libitum*, supplemented with milk, bread and lettuce once a week. The males were placed in cages with nulliparous females for one night. The females were examined for vaginal plugs and weighed the next morning, and examined for pregnancy by palpation on the day of treatment. The day on which a vaginal plug was found was called day 0.

The following types of mating were carried out, the female parent being written first in each case: intra-strain—A × A, C × C; inter-strain—A × C, C × A; and back-cross to the A strain—(AC) × A and (CA) × A. Pregnant females were treated on one of the following days of gestation: 8·5, 9·5, 10·5, 11·5, 12·5, 13·5, or 14·5. The treatment consisted of an intramuscular injection of 6-aminonicotinamide in aqueous solution (19 mg./kg.) followed 2 hr. later by an injection of nicotinamide in aqueous solution (7·3 mg./kg.) which had previously been shown to prevent further teratogenic action of the antimetabolite (Pinsky & Fraser, 1960). Control observations were made on the offspring of untreated females and on the offspring of females given an injection of water followed 2 hr. later by nicotinamide on day 9·5 in A × C and C × A, day 10·5 in A × A and day 12·5 in C × C (these were the days when the embryos were most susceptible to the teratogenic effects of the treatment). No differences were observed between the control series in litter size or malformation frequency; thus the experimental procedure had no teratogenic effect.

On day 18·5 the females were killed. The positions of the embryos and resorption sites in the uterus were recorded, the embryos were removed, and, after gross examination, most of them were cleared for skeletal staining with alizarin (modified from Dawson, 1926).

2. RESULTS

A variety of malformations were produced, depending on the day the treatment was given, and these will be reported elsewhere. The present paper will deal with only two of them—vertebral fusions and cleft palate.

(i) *Vertebral fusions*

Treatment on day 8·5 and on day 9·5, but not on any other day tested, resulted in fusions between vertebral bodies in the thoracic and lumbar region. The frequencies are presented in Table 1.

Table 1. *Frequencies of vertebral fusions (VF) following maternal treatment with 6-aminonicotinamide on day* 8·5 *or* 9·5 *of gestation*

Cross	Day treated	Numbers of females treated	Numbers of embryos		Percent of embryos with VF
			Resorbed	Alive	
1. A × A	8·5	9	75	7	0
	9·5	10	37	55	89
	Control	18	34	111	0
2. C × C	8·5	12	27	64	9
	9·5	12	20	73	56
	Control	12	19	72	0
3. A × C	8·5	10	61	14	14
	9·5	10	16	49 (61)†	45
	Control	10	24	52	0
4. C × A	8·5	5	1	32	37
	9·5	10	7	52 (67)†	67
	Control	10	13	65	0

† Only the numbers of mice cleared for the examination of the skeletons are included. The numbers in brackets refer to the total number of embryos.

In crosses involving strain A mothers (1 and 3) treatment on day 8·5 caused such a high frequency of resorptions that no conclusions about the frequency of malformations can be drawn. In crosses involving strain C mothers (2 and 4) the frequency of vertebral fusions is lower in the C strain (9%) than in the F_1(37%) the difference being highly significant ($p < 0{\cdot}001$).

After treatment on day 9·5 the frequency of vertebral fusions is higher in offspring of the A strain (89%) than of the C57BL strain (56%), at the 0·1% level of significance. A patroclinous reciprocal cross difference is noted in the F_1 hybrids—67% in the offspring of C × A and 45% in the offspring of A × C crosses ($p < 0{\cdot}05$).

(ii) *Cleft palate*

Figure 1 represents the frequency of cleft palate following a 2-hr. treatment with 6-aminonicotinamide on the various days of gestation. All four crosses show definite peaks on day 13·5 which is thus the critical period for cleft palate production with 6-aminonicotinamide.

The results of treatment at the critical period are presented in Table 2.

The frequency of induced cleft palate in the A × A embryos (76·1%) was higher than in the C × C embryos (10·8%), the difference being highly significant ($p < 0{\cdot}001$), and was also higher than that in the A × C embryos (36·3%; $p < 0{\cdot}01$).

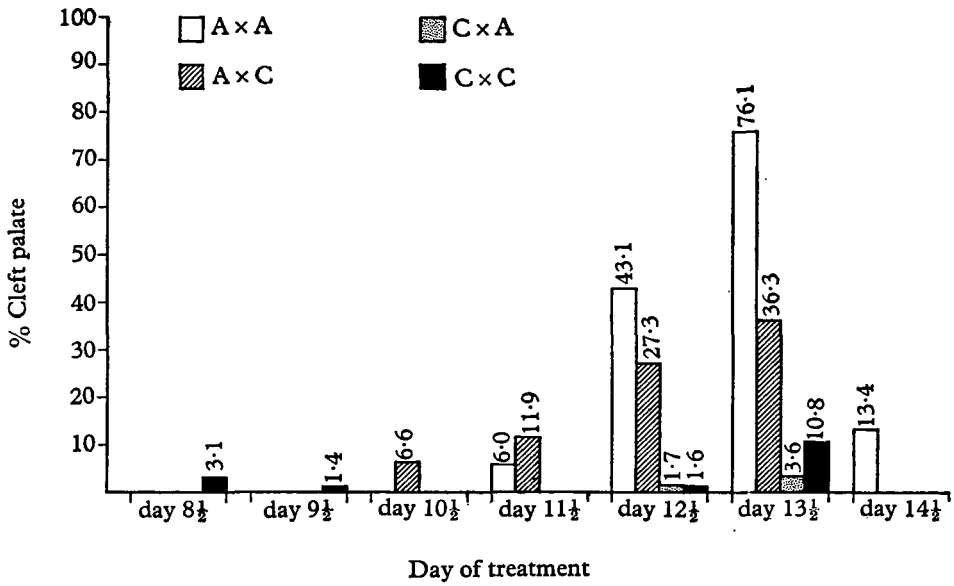

Fig. 1. Frequency of cleft palate following treatment on various days of gestation.

Table 2. *Frequencies of cleft palate (CP) following maternal treatment with 6-aminonicotinamide on day* 13·5 *of gestation*

Cross	Day treated	Number of females treated	Numbers of embryos		Percent of embryos with CP
			Resorbed	Alive	
1. A × A	13·5	10	19	71	76·1
	Control	18	34	111	0
2. C × C	13·5	12	28	74	10·8
	Control	12	19	72	2·8
3. A × C	13·5	12	13	80	36·3
	Control	9	14	31	0
4. C × A	13·5	10	7	56	3·6
	Control	11	7	64	0
5. AC × A	13·5	9	1	76	23·7
6. CA × A	13·5	11	5	73	5·5

There was a matroclinous reciprocal cross difference in the F_1 hybrids, the A × C embryos having a higher frequency (36·3%) than the C × A embryos (3·6%; $p < 0{\cdot}001$). Such a maternal effect could be a reflection either of differences in the embryo's environment, or of strain differences in factors transmitted through the egg cytoplasm. To distinguish between these possibilities the two types of F_1

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mothers (A × C and C × A) were backcrossed to strain A males, and treated with 6-aminonicotinamide on day 13·5. (These females had not been exposed to 6-aminonicotinamide when they themselves were embryos.) There was a significant difference in cleft palate frequency in the two types of backcross, with 24% for AC × A and 6% for CA × A, suggesting that the teratogenic response to 6-aminonicotinamide is influenced by factors transmitted through the egg cytoplasm.

3. DISCUSSION

A number of interesting features emerge from these data. Following treatment on day 9·5 of gestation the frequency of induced vertebral fusions is higher in the A strain (89%) then in the C strain (56%), showing that there are genetically determined differences in response to the teratogen. The complicated nature of these differences is revealed by the reciprocal cross difference in the frequency of induced vertebral fusions in the F_1 hybrids of crosses between the two strains. The frequency was higher in the C × A hybrids (67%) than in the A × C hybrids (45%); that is, the F_1 resembled the paternal strain. Since the genetic contributions of the parental strains to the F_1 (except for the Y chromosome) are presumably the same in the reciprocal crosses, the patroclinous effect may have been the result of (*a*) random variation, (*b*) a factor for resistance to the teratogenic effects of 6-aminonicotinamide transmitted by the sperm but not the egg—a highly unlikely interpretation—(*c*) a maternal uterine or cytoplasmic factor for resistance to the teratogenic effects of 6-aminonicotinamide, which was stronger in the A than the C strain, interacting with a genetic factor for resistance stronger in the C than in the A strain. Thus the A strain could be postulated to carry genes for relative susceptibility and a maternal uterus or cytoplasmic factor for relative resistance of the vertebral anlage to the effects of 6-aminonicotinamide, with the converse situation in the C strain. The F_1 hybrids would be genetically intermediate, but the maternal or cytoplasmic factors would result in a lower frequency of defects in the A × C than in the C × A offspring. The existence of cytoplasmic differences causing patroclinous reciprocal cross differences in vertebral number has been demonstrated by ova transplantation experiments in the mouse (Green & Green, 1959).

A fourth possibility (*d*) would be that the differences reflect differences in the rates of development of the two types of hybrid, as already demonstrated in the case of palate closure (Trasler & Fraser, 1958). If the C × A hybrids were being treated at, say, the point of maximum sensitivity of the vertebral anlage to the teratogen, and the A × C hybrids were developing a little faster, or (as in the case of the palate) slower than the C × A hybrids, so that they were being treated at some stage other than that of maximum sensitivity, they would have a lower frequency of defects. In this case the patroclinous nature of the difference, as measured by the frequency of vertebral fusion, might actually be a matroclinous effect (as with cortisone-induced cleft palate), when considered in terms of embryonic developmental rates.

Turning to the results of treatment on day 13·5 of gestation we find that the A strain embryos have a higher frequency of induced cleft palate than the C embryos,

and again there is a reciprocal cross difference in the F_1, but in this case it is matroclinous. The frequencies are: A × A, 76%; C × C, 11%; A × C, 36%; and C × A, 4%.

Since the A × A and A × C embryos both have A mothers but different genotypes, and have different frequencies of induced cleft palate, the embryo's genotype is important in determining its response to the teratogen. On the other hand, since the A × C embryos have a higher frequency of cleft palate than the C × A embryos, even though they are (except for the Y chromosome) genetically identical, the teratogenic effect is also influenced by the nature of the mother. In the backcross to the A strain, in which both the maternal and foetal genotypes are identical but any maternal cytoplasmic factors would be different, the (AC) × A embryos are significantly more susceptible to cleft palate (23·7%) than the (CA) × A embryos (5·5%) ($p = 0{\cdot}01 - 0{\cdot}001$). Since these crosses differ in the origin of their maternal cytoplasm, this suggests that there are factors in the A cytoplasm which increase the embryo's susceptibility to the teratogenic action of 6-aminonicotinamide on day 13·5.

An alternative, though less likely, interpretation is that the uterine environment provided by the mother is influenced by the uterine environment in which she herself developed. Additional backcrosses are being carried out to investigate this problem further. At present we shall assume, as a working hypothesis, that the differences are cytoplasmic in nature. This differs from the situation reported for cortisone by Kalter (1954), who found that the susceptibility to cortisone-induced cleft palate in the same two strains of mice was determined by an interaction between maternal and embryonic genotypes, but that maternal cytoplasmic factors were not involved. If the embryonic genotype and maternal cytoplasmic factors were the only determinants of susceptibility to cleft palate production by treatment on day 13·5, one would expect the cleft palate frequency to be higher in the (AC) × A embryos than in the A × C embryos since the former contain more genes from the susceptible A strain than the latter. In fact, however, the cleft palate frequency is, if anything, lower in the (AC) × A cross (24%) than in the A × C cross (36%) though the difference is not significant ($p = 0{\cdot}10$–$0{\cdot}05$). Since the cleft palate frequency in the (AC) × A cross did not show the expected increase above the A × C value, it is concluded that the maternal constitution must also play a role in determining susceptibility to the treatment, i.e. the palate of an embryo appears to be more resistant to 6-aminonicotinamide when it is growing in an A × C uterus than in an A uterus.

The reciprocal cross differences demonstrated in malformation frequency induced by 6-aminonicotinamide were patroclinous in the case of vertebral fusions and matroclinous for cleft palate. Cytoplasmic factors influencing the embryos' response to the teratogen appeared to be active in the case of cleft palate, and may exist for vertebral fusions, but if so they must act in the opposite direction to the ones for cleft palate. In any case, the fact that the reciprocal cross differences were in opposite directions for vertebral fusions and cleft palates, respectively, shows that the strain differences in response to the teratogen are organ-specific.

4. SUMMARY

1. A two-hour relative deficiency of nicotinamide, produced by maternal treatment with 6-aminonicotinamide, resulted in a maximum frequency of vertebral fusions following treatment on day 9·5 of gestation, and a maximum frequency of cleft palates following treatment on day 13·5.

2. Both defects appeared with higher frequencies in the A/Jax than in the C57BL/6J inbred strain.

3. The frequency of induced vertebral fusions in F_1 embryos from crosses between the strains was higher when the father was from the A/Jax strain than when the mother was—a patroclinous reciprocal cross difference.

4. The frequency of induced cleft palate in F_1 embryos from crosses between the strains was higher when the mother was from the A/Jax strain than when the father was—a matroclinous reciprocal cross difference.

5. Since the reciprocal cross differences in frequency of vertebral fusions and for cleft palates were in opposite directions, the hereditary factors influencing susceptibility to the teratogen appear to differ for the respective organ anlage. These differences appear to be, in part, cytoplasmic.

6. The frequency of induced cleft palate in the offspring of backcrosses of (untreated) F_1 female hybrids to A/Jax males differed according to the cytoplasmic origin of the F_1 mothers. Thus the susceptibility of an embryo to the teratogen appears to be influenced by factors transmitted through the cytoplasm.

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