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STUDIES ON THE CLOSURE OF THE SECONDARY PALATE

V. ATTEMPTS TO STUDY THE TERATOGENIC ACTION OF CORTISONE IN MICE

by

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INTRODUCTION

An account of different ways of inducing congenital malformations has been given in a review by *Kalter & Warkany* (19). Cortisone has proved to be one of the most effective teratogenic substances as far as cleft palate without cleft lip in mice is concerned (11, 13, 14).

In autoradiographic and histochemical studies of mouse embryos, cortisone has been shown to interfere with the sulphomucopolysaccharide metabolism in the palatine shelves (22). The present writer suggested that this is one of the causes of development of cortisone-induced cleft palate (22), whereas *Kalter* suggested the protein-catabolic action of cortisone as the cause (16). I therefore considered that a study of the effect of a protein-anabolic steroid hormone on embryonic development would allow these theories to be tested in greater detail.

It has been reported that the incidence of cortisone-induced cleft palate can be reduced by administration of certain B vitamins (29). This has been further investigated, since it would have been valuable to study the sulpho-mucopolysaccharide me-

tabolism during development of the secondary palate, using the isotope technique described earlier, when the cleft palate-inducing action of cortisone was inhibited.

EXPERIMENTAL

Primiparous mice of the A/Jax strain (21) were used, as in earlier experiments (21, 22). An account of their diet and of the equipment of the animal room was given in a previous paper (20). They were mated as described earlier (21), the day on which vaginal plug was observed being taken as the zero day of pregnancy. (Some pregnant females not exhibiting plug were included, since the day of mating was known.)

Totally 97 mothers were used; their distribution in the 7 experimental groups is shown in Table I. They were given injections of one or several of the following solutions (for the doses, see Table I):

1. Cortisone acetate. 25 mg/ml. Cortodrin® [1])
2. Methyl androstenediol. 25 mg/ml. Neosteron® [2])
3. Methyl androstenediol-free vehicle used in Neosteron® [3])
4. Vitamin B_6. 0.1 mg/ml [4])
5. Vitamin B_{12}. 0.025 mg/ml. Hepagon ® [5])

All the solutions were administered daily at 10 a.m. on the 11th—14th day of pregnancy inclusively. They were injected intramuscularly in the thigh, with the exception of vitamin B_{12}, which was given as a subcutaneous injection in the back.

The mothers were killed by dislocation of the atlas at 10 a.m.

[1]) Kindly supplied by Astra AB, Södertälje, Sweden. (Composition: cortisone acetate 25 mg, sodium chloride 9 mg, benzyl alcohol 9 mg, sodium carboxymethyl cellulose and Tween 80 q.s., sterile water to 1 ml.)

[2]) Kindly supplied by AB Pharmacia, Uppsala, Sweden. (Composition: methyl androstenediol 0.025 g, dextrose 0.04 g, phenol 0.005 g, sodium phosphate and polyoxyethylene Sorbitan mono-laurate q.s., sterile water to 1 ml.)

[3]) Kindly supplied by AB Pharmacia, Uppsala, Sweden.

[4]) Obtained from Militärapoteket, Stockholm. (Composition: pyridoxine chloride 0.1 mg, sodium chloride solution to 1 ml.)

[5]) Kindly supplied by Astra AB, Södertälje, Sweden. (Composition: cyanocobalamin (vitamin B_{12}) 0.025 mg, sodium chloride 9 mg, benzyl alcohol 15 mg, hydrochloric acid diluted to pH 5—5.5, sterile water to 1 ml.)

on the 18th day of pregnancy. The embryos, lying in the uterus, were fixed in Bouin's fluid and then transferred to 70 % alcohol (33, 22). After removal of the lower jaw and tongue, the embryo was examined under a dissecting microscope (Hensoldt Wetzlar) at a magnification of 10 ×. Of the embryos that were judged to have been alive, those with a closed palate were assigned to one group, those with cleft palate without cleft lip to another (CP), and those with both cleft palate and cleft lip to a third group (CLP). Photographs illustrating these three types were published in an earlier paper (22). Finally, embryos with all degrees of resorption were collected in a separate group.

RESULTS

The results of examination of the embryonic material are shown in Table I. "Student's" *t* test (28) was used for a statistical comparison between the groups with respect to the incidence of cleft palate without cleft lip (CP) and the incidence of resorption; the results are given in Table II.

It was found that when A/Jax mice were given 1.25 mg of cortisone daily on the 11th—14th days of pregnancy, the incidence of cleft palate without cleft lip (CP) was 100 % (Group 1, Table I). In this group — as in Groups 3, 4, 5 and 6 — cleft palate combined with cleft lip (CLP) was also observed (in 14 of 600 living embryos = 2.3 %).

When 1.25 mg of methyl androstenediol and 1.25 mg of cortisone were given daily on the 11th—14th days of pregnancy to 5 mice, all the embryos were resorbed (Group 2, Table I). In Groups 3 and 4, in which methyl androstenediol only was given in daily doses of 1.25 and 2.5 mg, respectively, 8 of 130 living embryos had cleft palate without cleft lip (Table I). The higher dose produced a significant increase in the incidence of resorption ($p < 0.001$) as compared to the lower dose, whereas no difference was present between the incidence of CP (*cf.* Groups 3 and 4, Table II). CP was not observed in any of the embryos from the 5 mice given the methyl androstenediol-free vehicle used in Neosteron® on the aforementioned days of pregnancy, and the incidence of resorption was only 8.1 % in this group (Group 5, Table I). In a comparison between Groups 3 (1.25 mg of methyl

Table I

Survey of experimental groups: treatment, size, maternal weight, offspring.

Group	Daily injections to mothers: 11th—14th days of pregnancy	No. of litters	No. of implanted embryos	No. of living embryos	Embryos with cleft palate without cleft lip CP		Embryos with cleft palate + cleft lip CLP		Resorbed embryos		Mean maternal weight, g, 12th day of pregnancy
					No.	% of living —CLP	No.	% of living	No.	% of implanted	
1	Cortisone 1.25 mg	28	216	159	156	100	3	1.9	57	26.4	22.9
2	Cortisone 1.25 mg + methyl androstenediol 1.25 mg	5	29	0	0	—	0	—	29	100	21.4
3	Methyl androstenediol 1.25 mg	13	97	70	5	7.3	1	1.4	27	27.8	24.2
4	Methyl androstenediol 2.5 mg	15	123	60	3	5.2	2	3.3	63	51.2	23.8
5	Methyl androstenediol-free vehicle used in Neosteron® 0.1 ml	5	37	34	0	0	1	2.9	3	8.1	22.9
6	Cortisone 1.25 mg + vitamin B_6 10 μg	25	292	240	232	99.6	7	2.9	52	17.8	23.2
7	Cortisone 1.25 mg + vitamin B_6 10 μg + vitamin B_{12} 5 μg	6	46	37	37	100	0	0	9	19.6	22.7
	Total	97	840	600	433		14	2.3	240		23.2

androstenediol) and 5 (vehicle of methyl androstenediol), a significant difference ($p < 0.05$) could be demonstrated between the incidence of CP, whereas no such difference was present between Group 4 (2.5 mg of methyl androstenediol) and Group 5 (vehicle of methyl androstenediol); see Table II. Both groups showed a significantly higher incidence of resorption as compared to group 5 ($p < 0.01$ and $p < 0.001$, respectively; Table II).

Table II

Statistical comparison between groups listed in Table I with respect to incidence of cleft palate and of foetal resorption: results of »Student's« t test

Groups compared	Incidence of cleft palate: CP	Incidence of foetal resorption
	Significance[1]	Significance[1]
1—2	***	***
3—4	0	***
3—5	*	**
4—5	0	***
1—6	0	*
1—7	0	0
6—7	0	0

[1] 0 = not significant: * = almost significant, $p<0.05$; ** = significant, $p<0.01$: *** = highly significant, $p<0.001$.

When a daily dose of 10 μg of vitamin B_6 was injected concurrently with 1.25 mg of cortisone (11th—14th days of pregnancy), only one of 240 living embryos had a normally closed palate (Group 6, Table I). The incidence of resorption was significantly lower ($p < 0.05$) in this group than in Group 1 (1.25 mg of cortisone only), whereas no difference was present between Groups 6 and 1 with respect to the incidence of cortisone-induced cleft palate (Table II).

In Group 7, in which 5 μg of vitamin B_{12} were given on the 11th—14th days of pregnancy, in addition to daily intramuscular injections of 1.25 mg of cortisone and 10 μg of vitamin B_6, cleft palate was induced in 100 % of living embryos, and resorption in 19.6 % of implanted embryos. No significant difference in the incidence of cleft palate or of resorption could be demonstrated in a comparison with Group 1 (1.25 mg of cortisone) and Group 6 (1.25 mg of cortisone and 10 μg of vitamin B_6); see Table II.

DISCUSSION

The results of the present investigation do not substantiate the view that the teratogenic action of cortisone takes place by protein catabolism. They can, on the contrary, be cautiously interpreted as further evidence in support of the theory that congenital malformations are correlated to a decrease in sulpho-mucopolysaccharide metabolism. No reduction in the incidence of cortisone-induced cleft palate by vitamins B_6 or B_{12} could be observed.

Daily doses of 2.5 mg of cortisone on the 11th—14th days of pregnancy have generally been used earlier to produce cleft palate without cleft lip in mice (12, 22, 27, 29). A difference has been demonstrated between the incidence of cleft palate in different strains (13). In an attempt to study the ability of various substances to reduce the incidence of cortisone-induced cleft palate, I considered it appropriate to administer a "minimum dose" of cortisone, which would still result in a 100 % incidence of this malformation. In the A/Jax strain, a daily dose of 1.25 mg of cortisone acetate proved to be suitable (Group 1, Table I).

By means of autoradiography, it was demonstrated in an earlier study (22) that cortisone produces a marked decrease in the ^{35}S-labelled sulphate incorporation into the sulpho-mucopolysaccharides of the palatine shelves at the time of palatal closure in mice. It was concluded that the ground substance of the shelves was changed in this way, so that their upward movement into the horizontal plane was retarded, with cleft palate as a result (22).

In addition to cortisone (2, 3, 4, 24, 25, 31), other factors — hydrocortisone (35), salicylates (1, 35), A-avitaminosis (5), A-hypervitaminosis (10), and roentgen irradiation (6) — have been found in *in vivo* and/or *in vitro* studies to reduce the ^{35}S sulphate incorporation into the sulpho-mucopolysaccharides of various tissues. Since these factors have also proved to be teratogenic, it can be surmised that it is the actual effect on sulpho-mucopolysaccharide metabolism which is the cause of several different congenital malformations, among them cleft palate (15, 18, 19, 30, 34, 36).

Stimulation of sulpho-mucopolysaccharide synthesis in the palatine shelves would be a conceivable way of decreasing the incidence of cortisone-induced cleft palate. At present, the possibilities of such stimulation *in vivo* are limited. The growth hormone is one of the few substances which stimulate the synthesis of sulpho-mucopolysaccharides (7). In view of the tissue-stimulating action of the growth hormone, it has been administered to counteract the damaging effect of cortisone in experiments on chick embryos, and found to have a positive result (32). *Nishihara et al.* (27) gave the growth hormone to Webster-Swiss mice, concurrently with 4 daily doses of cortisone (2.5 mg), and achieved a decrease in the incidence of foetal resorption, but not in the incidence of cortisone-induced cleft palate.

The teratogenic effect of cortisone was suggested by *Fraser et al.* (12) to depend on protein catabolism. *Kalter* (16) subsequently elaborated a theoretical basis for this suggestion. It was therefore considered that the teratogenic action of cortisone would be counteracted by a protein-anabolic steroid hormone, which should not alone cause malformations in the offspring. Consequently, methyl androstenediol was tested in the present study, since it has been shown in other connexions to counteract protein catabolism by cortisone (8, 9). Concurrent injection of methyl androstenediol and cortisone (1.25 mg of each substance daily on the 11th—14th days of pregnancy) was, however, found to result in resorption of all the embryos in the 5 experimental litters. The embryonic damage was, in fact, considerably greater than when a corresponding amount of each steroid hormone was given separately (Group 4, Table I, and ref. 22, Table II). Testosterone propionate (12), progesterone (27), and chorionic gonadotropin (27) have also been shown to produce violent embryonic resorption or abortion, when given to pregnant mice concurrently with injections of cortisone. Moreover, the present investigation shows that cleft palate of the same type as that induced by cortisone can be produced by administration of a protein-anabolic steroid hormone. Consequently, there is further reason to doubt the existence of a relation between protein catabolism (by cortisone) and teratogenic effect.

The most promising results of attempts to reduce the incidence of cortisone-induced cleft palate have been obtained by *Peer et al.*

(29), with injection of some B vitamins in Swiss albino mice. When 2.5 mg of cortisone only were given daily on the 11th—14th days of pregnancy, the incidence of cleft palate was 85 %. When 10 μg of vitamin B_6 or 10 μg of folic acid were given concurrently with cortisone, the incidence was reduced to 43 % (5 litters) and 26 % (9 litters), respectively. In 3 litters given a concurrent supplement of both vitamin B_6 and folic acid, the incidence of cortisone-induced cleft palate was reduced to 15 %.

Peer et al. (29) discussed various ways in which the B vitamins could counteract the embryonic damage produced by cortisone. Summing up, they considered it probable that these vitamins protect the synthesis of proteins and nucleic acids, which are necessary for growth of the embryo. The B vitamins are, in fact, present as co-enzymes in a number of important processes.

Kalter (17) could not verify this favourable effect of folic acid, even though it was given for a longer period and in larger doses, but he used a different strain of mice, as well as another mode of administration of the vitamin. Nor did he succeed in decreasing the incidence of cortisone-induced cleft palate by a supplement of riboflavin (17).

In view of the reduction in the incidence of cortisone-induced cleft palate achieved by *Peer et al.*, I considered it of value to test supplementary administration of vitamin B_6, as well as of vitamin B_{12}. The latter has been reported to counteract the protein-catabolic action of cortisone (26). Vitamin B_6 did not, however, reduce the incidence of cleft palate in my experiments, since only 1 of 240 living embryos had a normally closed palate (Group 6, Table I). It must, on the other hand, be regarded as probable that this vitamin actually had an effect, manifested as a decrease in the incidence of resorption (Group 6, Table I). Administration of vitamin B_{12} concurrently with vitamin B_6 produced no further reduction in the incidence of resorption, nor did it diminish the incidence of cortisone-induced cleft palate (Group 7, Table I).

Many highly divergent teratogenic substances can cause the same type of defect, *e.g.*, cleft palate (19). These substances can presumably interfere with the metabolism of different constituents and in different ways, but with the same visible develop-

mental anomaly as a result. It seems likely that better knowledge of the distribution and metabolism of the sulpho-mucopolysaccharides during different embryonic stages, as well as additional information on the effect of various substances on sulpho-mucopolysaccharide metabolism, would provide a firmer basis for discussion of the pathogenesis of numerous congenital malformations.

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SUMMARY

Cleft palate without cleft lip (CP) was induced in mouse embryos of the A/Jax strain by injecting cortisone into the mothers on the 11th—14th days of pregnancy. When a protein-anabolic steroid hormone, methyl androstenediol, was given concurrently with cortisone, all the embryos were resorbed. Administration of methyl androstenediol alone resulted in cleft palate of the same type as that produced by cortisone, but in only 5—7 % of the embryos judged to have been alive on the 18th embryonic day.

Administration of vitamin B_6 was found to reduce the incidence of resorption, but not of cortisone-induced cleft palate. Vitamin B_{12} gave no additional protection against the damaging effect of cortisone on the embryos. — The teratogenic mechanism of action of cortisone is discussed.

RÉSUMÉ

ETUDES SUR LA FERMETURE DU PALAIS SECONDAIRE.

V. Tentative d'étude de l'action tératogène de la cortisone chez la souris.

On a produit des divisions palatines sans bec-de-lièvre chez le foetus de la souris, dans une souche A/Jax, par des injections de cortisone chez des mères ayant une grossesse de 11—14 jours. Quand on a donné, en même temps que la cortisone, un stéroide protéinoanabolique, le méthylandrostendiol, tous les foetus se sont résorbés. On a pu aussi avec le méthylandrostendiol seul développer des divisions palatines du même type que celles obtenues par la cortisone, mais dans seulement 5—7 % des foetus qui ont été estimés vivants le 18ème jour de leur vie foetale.

La vitamine B_6 diminue la fréquence de résorption du foetus, mais non la fréquence d'induction de la cortisone sur les divisions palatines. La vitamine B_{12} n'a pas eu d'effet ultérieurement protecteur contre l'effet nocif de la cortisone. L'action tératogène de la cortisone fait l'objet d'une discussion.

ZUSAMMENFASSUNG

STUDIEN ÜBER DEN VERSCHLUSS DES SEKUNDÄREN GAUMENS.

V. Versuche über die teratogenetische Wirkung von Cortison an Mäusen.

Gaumenspalten ohne Lippenspalten konnten bei Mäuseembryos des Stammes A/Jax erzeugt werden, wenn man den Muttertieren während des 11.—14. Graviditätstages Cortisoninjektionen gab. Wurde gleichzeitig ein proteinanabolisches Steroid, Methylandrostendiol, gegeben, wurden sämtliche Embryos resorbiert. Alleine mit Methylandrostendiol konnten Gaumenspalten vom gleichen Typ wie mit Cortison hervorgerufen werden, jedoch nur in 5—7 % der Embryos, die am 18. Embryonaltag als lebend beurteilt wurden.

Vitamin B_6 verringerte die Frequenz der resorbierten Embryos, jedoch nicht die Frequenz der cortisoninduzierten Gaumenspalten. Vitamin B_{12} ergab keinen weiteren Schutz gegen die Cortisonschädigung. Der teratogene Wirkungsmechanismus des Cortisons wurde diskutiert.

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