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Teratogenic Effect of AY 9944 in Rats: Importance of the Day of Administration and Maternal Plasma Cholesterol Level (41842)

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Abstract. An inhibitor of cholesterol synthesis, AY 9944 (*trans*-1,4-bis(2-chlorobenzylaminomethyl) cyclohexane dihydrochloride) is teratogenic. A single dose of AY 9944 (50 mg/kg or 75 mg/kg) given to Wistar pregnant rats on the second, fourth, sixth, seventh, or eighth day of gestation induced malformations such as holoprosencephaly. They were often limited to isolated pituitary agenesis. The highest percentage of holoprosencephalic fetuses was found when AY 9944 was given on the fourth day of gestation. Whatever the dose and the day of administration, the lower the maternal plasma cholesterol level, the more frequent were holoprosencephalic fetuses. Therefore, it is suggested that the decrease in maternal plasma cholesterol level is at least one of the factors provoking holoprosencephaly.

We have previously reported that AY 9944 (*trans*-1,4-bis(2-chlorobenzylaminomethyl) cyclohexane dihydrochloride), an inhibitor of cholesterol synthesis, given to pregnant Wistar rats on the second, third, and fourth days of gestation at a dose of 50 mg/kg/day induced fetal abnormalities such as holoprosencephaly (1, 2). Pituitary agenesis, which can be considered a minor form of holoprosencephaly, was the most frequently observed abnormality. These abnormalities are initiated around the 10th day of embryonic development. We have also shown that this early administration of AY 9944 produced a significant decrease of maternal plasma sterol level on the 10th day of gestation. Pituitary agenesis was found only when maternal plasma sterol level was below 0.30 g/liter (3).

The present studies were conducted to delineate the relationships between fetal abnormalities and the day of treatment with AY 9944 or maternal plasma sterol level during gestation. A single dose of AY 9944 was therefore given on the second, fourth, sixth, seventh, or eighth day of gestation and maternal plasma sterol level was measured on various days of gestation. The nature of the sterols was determined by thin-layer chromatography.

Materials and Methods. Female virgin Wistar rats weighing about 200 g (Lessieux Elevage) were mated with males of the same stock overnight. Vaginal smears were examined in the morning. The presence of spermatozoa designated the first day of gestation. The animals were fed a stock diet (Union de l'Alimentation Rationnelle 103) with the following composition: 3100 cal/kg, 12% water, 20% protein, 4% lipid (25% from animal origin, 75% from vegetable origin), 54.5% carbohydrates, 4% cellulose, and 5.5% salt mixture. The vitamin content of the diet was composed of the major vitamins A, B, D, K, E, and other vitamin cofactors.

The females were assigned to three groups (according to dosage given) and 11 subgroups (according to day of treatment).

Group 1. Wistar control rats given 5 ml/kg of distilled water by intubation.

Groups 2 to 6. Wistar rats given by intubation a single dose of 50 mg/kg of AY 9944 in 5 ml/kg of distilled water, respectively, on the second, fourth, sixth, seventh, or eighth day of gestation.

Groups 7 to 11. Wistar rats given by intubation a single dose of 75 mg/kg of AY 9944 in 5 ml/kg of distilled water, respectively, on the second, fourth, sixth, seventh, or eighth day of gestation.

On Day 0 (before mating) and on the 10th and 21st days, blood was taken from the orbital sinus for analysis of plasma cholesterol concentration. Plasma was kept frozen at −20°C until assayed by an enzymatic method (4, 5)

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TABLE I. EFFECT OF AY 9944 ON FETAL MORTALITY AND ABNORMALITIES

Rats	Day of administration of AY 9944	Number of litters	Number of implantations	Number of live fetuses	Fetal mortality (%)	Number of fetuses with holoprosencephaly	Percentage holoprosencephaly among live fetuses
A							
Control		10	106	88	17.0	0	0
Treated by	2nd	5	50	43	14.0[a]	5	11.6***
50 mg/kg	4th	13	105	75	28.6*	29	38.7†
AY 9944	6th	11	103	61	40.8†	22	36.1†
	7th	12	105	72	31.4**	4	5.6[a]
	8th	14	172	138	19.8[a]	8	5.8[a]
B							
Control		10	106	88	17.0	0	0
Treated by	2nd	7	63	46	27.0[a]	22	47.8†
75 mg/kg	4th	13	103	53	48.5†	41	77.3†
AY 9944	6th	14	174	82	52.9†	36	43.9†
	7th	7	81	34	58.9†	6	17.6†
	8th	13	133	81	39.1†	6	7.4*

Note. Treated female Wistar rats received either 50 or 75 mg/kg of AY 9944 in 5 ml/kg distilled water by intubation at various days of gestation. Control female Wistar rats received 5 ml/kg distilled water by intubation.

[a] N.S. compared to control rats.

* $P < 0.05$ compared to control rats.

** $P < 0.02$ compared to control rats.

*** $P < 0.01$ compared to control rats.

† $P < 0.001$ compared to control rats.

(Kit Biomérieux) which tests both 7-dehydrocholesterol and cholesterol. The maternal plasma lipids were extracted according to the Folch's method. Thin-layer chromatographs have been assayed. One-half of the plates were dipped in 10% aqueous $AgNO_3$. The plates were then chromatographed in two solvent systems: the first half of the plate in chloroform/methanol/water, 65/25/4 (v/v/v); then in chloroform/di-isobutylketone, 90/10 (v/v). The sterols were separated in the silver nitrate part and seen after sulfuric acid treatment of the plates and read under UV light.

Females were killed on the 21st day of gestation by cervical dislocation. Fetuses were weighed, examined under a dissecting microscope, and fixed in Bouin's fluid. They were dissected, and their heads were sectioned according to the technique of Wilson (6).

Results. Administration of a single dose of AY 9944 to pregnant rats on different days of gestation resulted in high fetal mortality and teratogenesis (Table I, Fig. 1). The main malformations observed were of the holoprosencephalic type.

In the 50 mg/kg group, fetal mortality was significantly higher than in controls after administration on the fourth, the sixth, or the seventh day of gestation. The highest incidence was observed in the subgroup given the drug on the sixth day. Holoprosencephaly was observed in all groups. It was particularly frequent when the drug was given on the fourth or sixth days (39 vs 36%).

In the rats given 75 mg/kg on the fourth, sixth, seventh, or eighth day of gestation, fetal mortality was significantly higher than in controls; the highest rate was found in the sixth or seventh day subgroups. After administration on the eighth day of gestation, fetal mortality decreased to a level close to that of rats given the drug on the second day. Holoprosencephaly was observed in all groups. As in the 50 mg/kg group, the maximum effect was

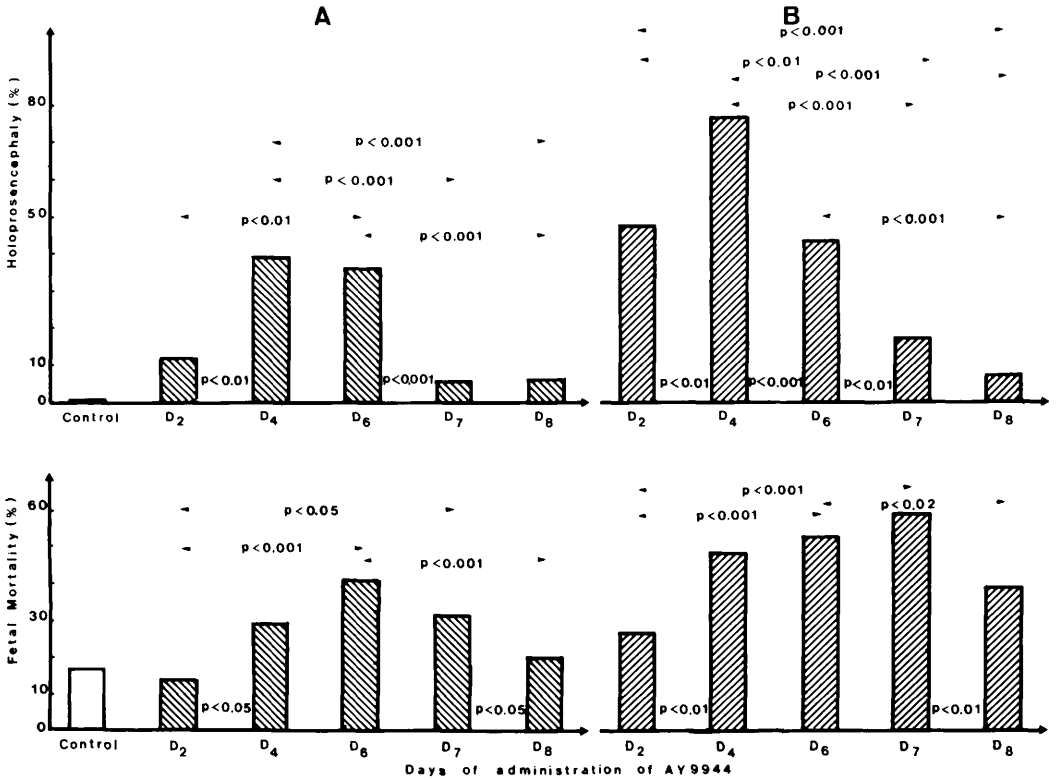

FIG. 1. Rate of holoprosencephaly and fetal mortality when a single dose of AY 9944 was given on the second, fourth, sixth, seventh, or eighth day of gestation. A, 50 mg/kg; B, 75 mg/kg.

found after administration on the fourth day of gestation: 77% of fetuses were holoprosencephalic.

Thus, the fetal mortality and the percentage of live fetuses with holoprosencephaly depended on the day of administration of AY 9944 for both doses. As a whole, 52% of the 73 rats treated with a single dose of AY 9944, at any of the doses tested, gave abnormal fetuses.

The variations in maternal plasma sterol level as a function of the number of days elapsed after administration of AY 9944 are presented in Fig. 2. As plasma sterol level was measured on the 10th and 21st days of gestation, the number of days that elapsed after administration of the drug varied according to the day it was given. The lowest values of maternal plasma sterol level on the 10th day of gestation were obtained 6 days after treatment in the 50 mg/kg group and 6 to 8 days after treatment in the 75 mg/kg group. After this time, plasma sterol level returned rapidly to normal values. (Assays repeated every 2 days in five animals given 75 mg/kg on the 4th day of gestation showed the same curve.) Moreover, the lower the maternal plasma sterol level on the 10th day, the more frequent was holoprosencephaly in fetuses. Figure 3 shows the relationship between the incidence of fetal holoprosencephaly and maternal sterol concentration. Figure 4 shows that the thin-layer chromatography allowed us to point out the following fact: 7-dehydrocholesterol was one major sterol present among the plasma sterols in treated rats. Therefore, the enzymatic assay giving the sum of both 7-dehydrocholesterol and cholesterol, the decrease of maternal plasma sterol level can be considered as a decrease of maternal plasma cholesterol level.

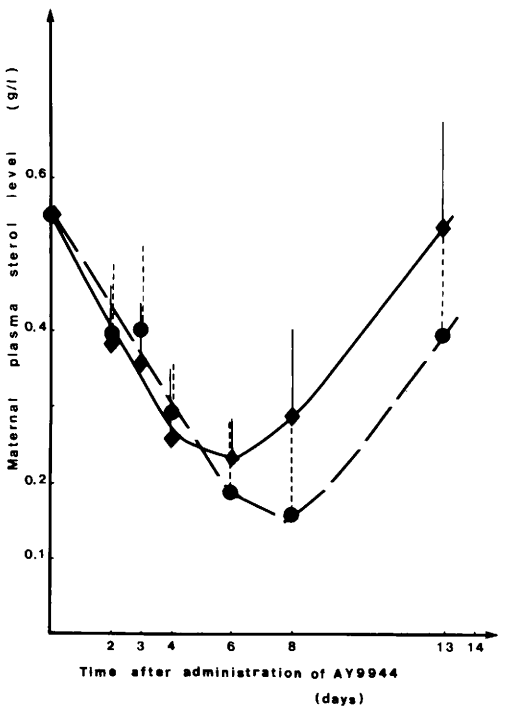

FIG. 2. Maternal plasma sterol level measured on various days after administration of a single dose of AY 9944. —♦—, 50 mg/kg; —●—, 75 mg/kg.

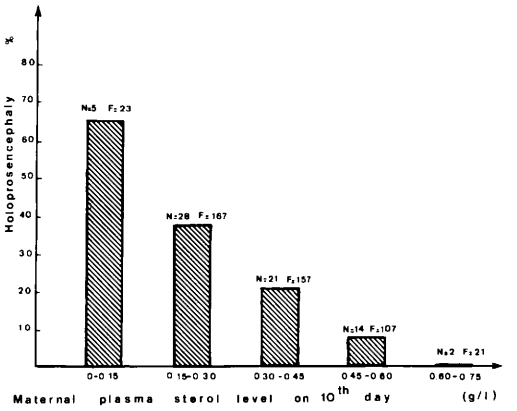

FIG. 3. ▨, Global rate of holoprosencephaly as related to maternal plasma sterol level measured on the 10th day of gestation ranged in classes of 0.15 g/liter. N, number of litters; F, number of live fetuses. Correlation coefficient $r = -0.971$ ($P < 0.01$).

Thus, a strong inverse correlation was found between maternal plasma cholesterol level on the 10th day of gestation and the percentage of holoprosencephaly which occurred.

Discussion. As the mammalian embryo is entirely dependent on its mother, all modifications of the maternal milieu can affect its development. Numerous authors (7–9) have shown that maternal deficiencies can induce fetal malformations. Therefore, administration of drugs producing maternal deficiencies can result in fetal abnormalities. Currently, it is well known that early rat embryos have a limited capacity to synthesize cholesterol from acetate (10, 11) and most embryo cholesterol

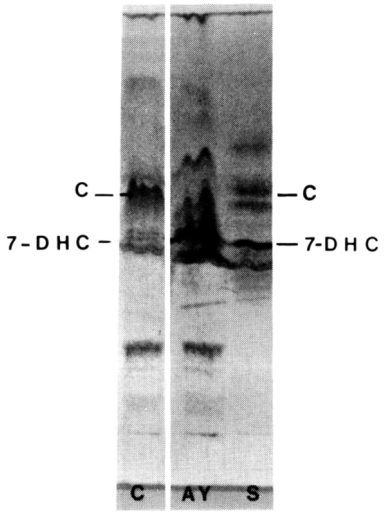

FIG. 4. Thin-layer chromatography of lipid extracts of maternal plasma on the 10th day of gestation. Bands: C, free cholesterol; 7-DHC, 7-dehydrocholesterol. Columns: C, control maternal plasma; AY, maternal plasma of Wistar rat treated by one single dose of AY 9944: 75 mg/kg on the 4th day of gestation; S, Standards of different sterols.

is of maternal origin until the 13th day of gestation. Thereafter, the ability to synthesize cholesterol increases progressively (11).

Consequently AY 9944, which inhibits cholesterol synthesis and induces a decrease of maternal plasma cholesterol level, is embryolethal and teratogenic, producing holoprosencephalic fetuses when given during the first few days of gestation. The results reported here demonstrate that the efficiency of a single dose of the drug depends on the concentration and day of administration.

The rates of malformation observed in 7th and 8th day subgroups were low and statistically nonsignificant when compared with controls, but they must be taken into account, since this kind of malformation is never observed spontaneously in rats.

The strong inverse correlation between the rate of holoprosencephaly and the concentration of maternal plasma sterol level on the 10th day of gestation suggests that decrease of maternal sterol level is at least one of the factors provoking holoprosencephaly. Chromatographic assay of the lipid extract from maternal plasma allowed us to confirm that the relative accumulation of 7-dehydrocholesterol implied a decrease of maternal plasma cholesterol.

Other previously reported results support this hypothesis. We have shown that AY 9944 teratogenesis and embryo lethality could be prevented by administration of hypercholesterolemia-provoking diet (12). In rats given AY 9944 on the 2nd, 3rd, and 4th days and fed a hypercholesterolemia-provoking diet before and during gestation, maternal plasma cholesterol level was higher than normal on the 10th day of gestation and fetuses were not malformed. These results led to the hypothesis that its teratogenic action is correlated with the decrease of maternal plasma cholesterol level.

These quantitative modifications are not the only ones to be considered. Concomitant qualitative modifications of sterols and lipids in mothers and fetuses must also play an important role. It is known that, after administration of AY 9944, the nature of synthesized sterols is modified. There is a clearcut decrease of cholesterol and an accumulation of 7-dehydrocholesterol (13–17). It can be hypothesized that the replacement of cholesterol by 7-dehydrocholesterol in cell membranes could play a role in the perturbation of differentiation where cell–cell interactions are known to be very important.

Furthermore, preliminary work has indicated the complex nature of the lipid modifications occurring after administration of AY 9944. In addition to sterols, phospholipids and sphingolipids were also found modified. Since they are essential constituents of the cell membrane, these changes could also modify its fluidity and, therefore, cell–cell interactions.

In addition, it can be hypothesized that a direct action of the drug could also play a role in provoking holoprosencephalic malformations. In fact, *in vitro* experiments have demonstrated a significant decrease in membrane viscosity, which was too rapid to be due to complex lipid modifications and must be attributed to a direct action of the drug on cell membranes (unpublished data).

In conclusion, in pregnant rats given a single dose of AY 9944, we observed a strong inverse correlation between the rate of fetal holoprosencephaly and the decrease of maternal plasma cholesterol level. It is suggested that the mechanisms of AY 9944 teratogenesis are complex.

In addition to a quantitative effect on cholesterol, modification of the nature of the sterols, as well as the phospholipids and sphingolipids in embryo cell membranes, could play a role in producing such abnormalities. A direct action of the drug itself may also be possible.

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