Cholesterol deficit but not accumulation of aberrant sterols is the major cause of the teratogenic activity in the Smith-Lemli-Opitz syndrome animal model

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Abstract **Low cholesterol and high 7-dehydrocholesterol (7DHC) levels are associated with a blockade of Δ7-reductase in the Smith-Lemli-Opitz syndrome (SLOS) and in the animals treated with the inhibitor AY9944. The impact of the cholesterol deficit and of the accumulation of 7DHC on the embryo were investigated in AY9944-treated pregnant rats receiving an enriched cholesterol or 7DHC diet. Sterol profiling was performed under the various nutritional conditions. AY9944 caused a severe decrease in the maternal and embryo cholesterol. The deficit in the embryo was sustained by the embryonic uptake of the inhibitor. A cholesterol-rich diet was efficient in restoring the maternal and embryonic cholesterol and phenotype but a 7DHC-rich diet did not modify the sterol status compared with dams treated with only AY9944. The offspring phenotype remained deleterious whether or not the dams received 7DHC-rich diet. Over 80% of the 7DHC was absorbed, as was cholesterol, which was not quantitatively influenced by AY9944. When cholesterol and 7DHC were simultaneously administered, a competition for intestinal absorption enhanced the lowering cholesterol effect of AY9944. Whether or not the dams received a 7DHC dietary supplement, the offspring's phenotype became normal when the diet was supplemented with cholesterol. Under conditions in which the ratio of cholesterol/7DHC is substantially varied, the normal development of embryos can be achieved as long as the cholesterol is sufficient. The phenotype is reversed in vivo by cholesterol which contrasts with the irreversible effects manifested in vitro by oxidized 7DHC by-products.**—Gaoua, W., C. Wolf, F. Chevy, F. Ilien, and C. Roux. **Cholesterol deficit but not accumulation of aberrant sterols is the major cause of the teratogenic activity in the Smith-Lemli-Opiz syndrome animal model.** *J. Lipid Res.* **2000.** 41: **637–646.**

Supplementary key words cholesterol synthesis inhibitor AY9944 • embryo • dietary supplementation of the dam

The Smith-Lemli-Opitz syndrome (SLOS) is an autosomal recessive syndrome (1) characterized by facial and brain malformations, growth retardation, limb defects, and male genital abnormalities. SLOS has an estimated incidence of 1 in 20,000 (2). A low plasma and tissue cholesterol level and a large increase in the precursor, cholesta-5, 7-dien-3β-ol (7-dehydrocholesterol or 7DHC) are indicative (3) of SLOS which is caused by a deficit in 7DHC Δ7-reductase. The deleterious mutations in both alleles of the gene for 7DHC Δ7-reductase that result in blockade of the ultimate step of cholesterol synthesis have been published (4, 5).

The morphological and biochemical features of SLOS are closely related to the defect in synthesis induced by AY9944 (*trans*-1,4-bis(2-chlorobenzyl-amino-methyl) cyclohexane dihydrochloride) in rat offspring. AY9944 is known to inhibit both 7DHC Δ7-reductase and Δ8,7-sterol isomerase (6). This distal inhibitor is shown to be highly teratogenic when administered as a single dose on gestational day 3 (gd 3) (7). AY9944 induces a variety of holoprosencephalic (HPE) phenotypes from severe cyclocephaly with cyclopia and proboscis to isolated pituitary agenesis (8). A single dose of 75 mg/kg on gd 3 results in a gradual decrease in the maternal cholesterol level reaching a minimum level by gd 9. The teratogenic effect increases sharply below a threshold concentration of 30 mg/dl for the combined maternal sterolemia (cholesterol and aberrant sterols) measured when cholesterol and various 3β-hydroxylated aberrant sterols are assayed simultaneously by a routine enzyme kit. This level was considered as the teratogenic threshold as pituitary agenesis, a sensitive index, was found constant (≈97%) below this concentration of 30 mg/dl (7, 8). Indeed, the HPE phenotype is initiated in explanted embryos much earlier than gd 10, even before the somite stage at gd 7 (9). It results in defective ventral forebrain development which was recently related to a deficit in the developmental protein Sonic

Abbreviations: 7DHC, 7-dehydrocholesterol; GC–MS, gas chromatography–mass spectrometry; SLOS, Smith-Lemli-Opitz syndrome; HPE, holoprosencephalic.

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Hedgehog (SHH) (the cholesterol-dependent activation of SHH is discussed below). Feeding the cholesterol-rich diet to dams concurrently with AY9944 suppresses HPE (10). As shown in the present investigation, the diet increases the maternal cholesterol level but also reduces the aberrant sterol level by the down-regulation exerted by cholesterol. Similar observations were also reported for cultured embryos enveloped within the yolk sac visceral leaflet maintained in high cholesterol-containing rat serum (11).

GC-MS profiling of serum from AY9944-treated rats showed modifications similar to those of SLOS: low cholesterol and accumulation of so-called "aberrant" sterol precursors and by-derivatives such as 7DHC, 8DHC, nortrienol, and 8-lathosterol (11–13). Another documented inhibitor of 7DHC Δ7-reductase, BM15766, produced identical malformations along with similar sterol modifications (14–16). Interestingly, triparanol, a chemical not related to AY9944 which targets sterol Δ24 reductase, induced malformations similar to AY9944 (17) but led to accumulation of desmosterol. This points to shortage of cholesterol during embryogenesis as the common critical factor for the teratogenic activity of Δ7-reductase inhibitors and does not support the notion of a specific and toxic role of oxidized 7DHC derivatives, as described for cultured embryos.

The mechanism by which the cholesterol deficit induced a teratogenic effect has been considered recently in relation to the role of the developmental gene *shh* (Sonic Hedgehog). The *shh*−/− mutation leads to severe HPE (18). In humans, an alteration in only one allele of *shh* causes HPE (19). Because *shh* codes for a protein requiring cholesterol-dependent processing (20) its direct relevance to SLOS is questionable. HPE-like developmental defects have been demonstrated in embryos lacking the normal supply of cholesterol due to a deficiency in megalin-dependent endocytosis (21). As a result of mutations in megalin, the cholesterol transfer to the yolk sac and the uptake by the neuro-ectoderm were severely restricted besides no accumulation of aberrant sterols occurred. Similarly, in the apolipoprotein B−/− mouse fetus, the embryonic absorption of maternal cholesterol is blocked at the yolk sac envelope (22) and leads to cerebral malformations (23).

Besides the cholesterol shortage which is due to the Δ7-reductase blockade, toxicity of 7-DHC-oxidized by-products in embryo development was recently detected in cultured embryos (24). 7DHC-oxidized derivatives worsen growth retardation in AY9944-treated embryos by reducing the compensatory cholesterol influx supplied to the culture medium. The growth defect in vitro in the presence of 7DHC becomes irreversible even in the presence of a large excess of cholesterol. Therefore 7DHC was assumed to contribute to the constant antenatal retardation characteristic in SLOS in spite of a normal heterozygous maternal cholesterol level. In the experiments conducted in vitro, the beneficial effect of vitamin E on the embryo's growth rate confirmed that the negative impact of 7DHC was related to the oxidation of the aberrant Δ5,7 diene sterol. To delineate the role of cholesterol shortage and/or 7DHC accumulation on embryonic development, we performed in the present study investigations in vivo with dams treated with AY9944. The drug inhibits the conversion of 7DHC to cholesterol. AY9944-treated rats were supplemented with a cholesterol- and/or 7-DHC-enriched diet in order to determine whether the nutritional status modulates the phenotype.

MATERIALS AND METHODS

Animal maintenance

Wistar rats (this strain has been found to be more sensitive to the teratogen than the Sprague-Dawley strain) weighing approximately 200 g (Iffa Credo, France) were housed under standard conditions with a 12-h light/dark cycle. After a 15-day adaptation, females were mated with males. The day sperm was found in vaginal smears was designated as day 0 of gestation (gd 0).

Chemicals and reagents

AY9944 (the hydrophobic *trans*-1,4-bis(2-chlorobenzylaminoethyl) cyclohexane was used in the water-soluble dihydrochloride form) was a gift from Ayerst Laboratories (New York, NY). Cholesterol, 7DHC, and epicoprostanol (5β-cholestan-3α-ol) were obtained from Sigma (St. Louis, MO). Solvents for extraction were of analytical (HPLC) grade from Prolabo (France). [1α, 2α-^{3}H]cholesterol was obtained from Amersham Pharmacia. [1α, 2α-^{3}H]7-dehydrocholesterol was a generous gift from Dr. C. Hétru (IBMC, Strasbourg) and was re-purified by TLC.

Treatment

Animals were fed a stock diet (Union de l'alimentation rationnelle AO3) with the following composition: 3100 cal/kg, 12% water, 20% protein, 4% lipid (25% from animal origin, 75% from vegetable origin), 54.5% carbohydrate, 4% cellulose, and a 5.5% salt mixture. The vitamins in the diet were vitamins A, B, D, K, E, and other vitamin cofactors. The diet does not contain appreciable amounts of cholesterol (estimated below 10 mg/day). The dams were separated into four different groups according to the various treatments: group A, dams receiving a unique oral dose of AY9944 (75 mg/kg) on gd 3; group B, dams receiving AY9944 (75 mg/kg) on gd 3 and a dietary cholesterol supplementation (500 mg/kg/day) from gd 3 to gd 14; group C, dams receiving AY9944 (75 mg/kg) on gd 3 and a 7DHC supplementation (500 mg/kg/day) from gd 3 to gd 14 and group D, dams receiving AY9944 (75 mg/kg) on gd 3 and simultaneous supplementation with cholesterol and 7-DHC, each sterol given at 500 mg/kg/day from gd 3 to gd 14.

Cholesterol and/or 7-dehydrocholesterol were dissolved at 40°C in olive oil and administered by daily esophagus intubation. AY9944 was dissolved in distilled water and administered orally to groups A–D (75 mg/kg) on gd 3.

Maternal blood samples were collected at gd 11 and gd 14. Serum was stored at −20°C. Embryos were extracted on gd 11 or gd 14 for sterol profiling by GC-MS. Finally, fetuses extracted on gd 21 (a day before the expected delivery) were weighed, examined externally, and fixed in Bouin's fluid for internal dissection.

Lipid extraction and gas chromatography–mass spectrometry (GC-MS)

Embryos were transferred to chloroform–methanol 2:1 (v/v: 5 ml) with epicoprostanol added as internal standard (100 μg). Five repeated cycles (each 15 seconds) of sonication (20 kHz:

100 mW) were used for tissue homogenization. Five embryos at gd 11 and one embryo at gd 14 were required for quantitative sterol profiling (the size of embryos is considerably increased at gd 14 compared to gd 11). Lipids were extracted as indicated (24), saponified for 15 min, and silylated. The trimethylsilylether derivatives of sterols were separated by GC (Hewlett-Packard 5890) on a medium polarity (65% diphenyl-methylsiloxane) capillary column (RTX-65, length 30 m, diameter 0.32 mm, film 0.25 μ from Restesk [Evry, France]). Sterols were identified by comparison of the mass spectra with the NIST library or with published spectra (12). The positive fragment ions were produced in the electron impact mode at 70 eV (Nermag R10-10 C mass spectrometer) as described previously (12). The sterols were quantified by selective monitoring of the prominent characteristic ion after normalization with an internal standard (epicoprostanol) and calibration with weighed standards of cholesterol and 7DHC.

GC-MS assay of AY9944 in embryos

AY9944 was extracted from embryos with ethyl ether (5 ml/embryo) in the presence of aqueous sodium hydroxide (1.5 N: 1 ml). The drug was separated by GC on a non-polar capillary column (CP-SIL5, 25 m, 0.25 mm ID, film 0.25 μm [Chrompack, Varian SA, Courtaboeuf, France]) and quantified by selective ion monitoring of the fragment *m/z* 391 with ammonia as the reagent for chemical ionization. The internal standard acetopromazine (10 Fg: *m/z* 327) was used for normalization.

Intestinal absorption of tritium-labeled cholesterol and 7DHC

Rats were separated into three groups according to the various treatments and the feces of each animal within each group were collected and weighed: Control, rats receiving a high cholesterol diet (500 mg/kg/day) for 5 days; Group B, rats receiving a unique oral dose of AY9944 (75 mg/kg) on the first day and cholesterol supplementation (500 mg/kg/day) during 5 days; Group C, rats receiving a unique oral dose of AY9944 (75 mg/kg) on the first day and 7DHC dietary supplementation (500 mg/kg/day) for 5 days; Group D, dams receiving AY9944 (75 mg/kg) on the first day and a supplementation of cholesterol (500 mg/kg/day) and 7DHC (500 mg/kg/day) for 5 days. On day 6, either [1α, 2α (n)-^{3}H]cholesterol or [1α, 2α (n)-^{3}H]7DHC (50 μCi) was added to the cholesterol or 7DHC supplementation dissolved in 2 ml of oil (see above). Feces were collected 24 h after the administration of radioactive sterols. Radioactive cholesterol and 7DHC were extracted from the feces and quantified by scintillation counting.

Statistics

Data are reported as mean ± SEM. The morphological parameters of the gd 21 fetuses were analyzed with the χ^2 test. The biochemical parameters of the different groups were evaluated by the Mann-Whitney test and significance was accepted at the level of $P < 0.05$.

RESULTS

Morphological observations

The morphological defects of fetuses extracted on gestational day 21 from dams treated with a single dose of AY9944 (75 mg/kg) given on gd 3 (group A) are reported in **Table 1**. Fourty-six out of 49 fetuses displayed pituitary agenesis, the minor form of holoprosencephaly, characteristically reported in the offspring of dams treated with a distal cholesterol synthesis inhibitor (25). The size of the brain was constantly reduced with a narrow third ventricle. All embryos displayed severe growth retardation. Embryos presented various facial malformations highly reminiscent of the human SLO syndrome such as a bilateral cleft palate (n = 5), monorhinia (n = 3), or mandibular hypoplasia (n = 11). Thirteen out of 49 fetuses had an internal hydrocephalus. The fetuses were frequently microcephalic with a dome-shaped or a vaulted crania and a short "pointed" nose (embryo A, **Fig. 1**). A cholesterol diet of 500 mg/kg/day from gd 3 to 14 restored normal development in AY9944-treated offspring (10) (embryo B, Fig. 1).

The influence of 7-dehydrocholesterol (7DHC) dietary supplementation was then investigated after giving a high dose of AY9944 on gd 3; this treatment prevented the interconversion of 7DHC to cholesterol. The offspring (group C (n = 46)) presented similar malformations and growth retardation as compared to group A (Table 1 and Fig. 1). The higher frequency of internal hydrocephalus correlated with the narrowness of the third ventricle, which causes a blockade in the circulation of the cerebrospinal fluid that is considered to be a consequence of the specific abnormal pattern induced by distal cholesterol inhibitors. The severe phenotypes in group C indicated clearly that dietary 7DHC unlike dietary cholesterol did not play a beneficial role in spite of their chemical analogy. The different effects of the sterols observed in vivo contrast with their similar activities observed for *Sonic Hedgehog* cleavage (26). When AY9944-treated dams were exposed to cholesterol plus 7DHC dietary supplements (group D), normal growth rate with no malformation was observed (embryo D, Fig. 1). The presence of 7DHC in the diet did not influence development,

TABLE 1. Frequency of defects observed in fetuses

	n	Abnormal Face	Cleft Palate	Hydrocephalus	Pituitary Agenesis	Adrenal Hypoplasia	Abnormal Embryos
		%	%	%	%	%	%
AY9944 (75 mg/kg) on gd 3	49	26	10	26	95	95	100
AY9944 (75 mg/kg) on gd 3 plus supplementation[a]	46	17	4	63[b]	91	91	91

The frequency of characteristic defects observed in fetuses extracted at gestational day 21 from AY9944-treated rat dams (group A, n = 49) is compared to AY9944-treated dams receiving 7-dehydrocholesterol dietary supplementation from gd 3 to 14 (group B, n = 46).

[a] Supplementation with 7DHC (500 mg/kg) from gd 3 to 14.

[b] Significance for hydrocephalus is given by the Chi-square test.

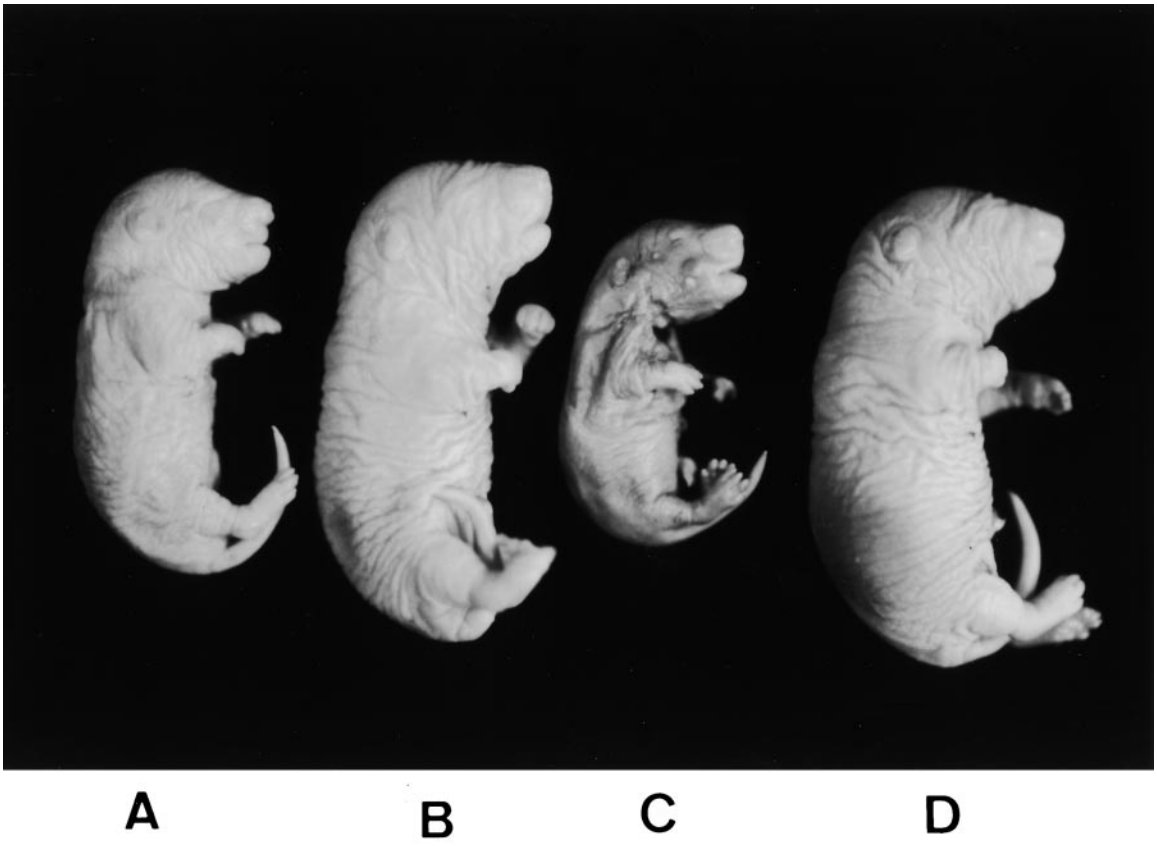

Fig. 1. Morphology of the rat fetus extracted on gestational day 21 as a function of the dietary supplementation of AY9944 -treated dams. A characteristic phenotype was selected from a litter and photographed 1 day before the time expected for delivery. Group A was exposed to AY9944 treatment (a single oral dose of 75 mg/kg) which was given to the dam on gestational day 3. Cebocephaly and hypotrophy were constant. In group B, dams received AY9944 on gd 3 and a cholesterol-enriched diet (500 mg/kg/day) from gestational day 3 to 14. The phenotype was normal. In group C, dams received AY9944 treatment on gd 3 and 7-dehydrocholesterol dietary supplementation (500 mg/kg/day) from gd 3 to 14. Multiple malformations were observed. Detailed anatomical examination in this group (n = 46) compared to group A (n = 49) is given in Table 1. Dams of group D received AY9944 treatment on gd 3 and a double dietary supplement with cholesterol and 7DHC (each at 500 mg/kg/day) from gd 3 to gd 14. The offspring were normal.

which contrasted with previous results for cultured embryos (24) where irreversible deleterious effects of 7DHC oxidized by-products could not be counteracted by substantial cholesterol supplementation of the growth medium.

Cholesterol and 7DHC levels in maternal serum

The influence of diet on maternal sterols after blockade of Δ7-dehydrosterol-reductase with AY9944 is shown in **Fig. 2** and **Fig. 3**. Serum cholesterol assayed by GC-MS decreased by 76% on gd 11 and by 56% on gd 14 in AY9944-treated dams (group A, Fig. 2). A slight increase in the cholesterol level is usually noted during the same period in control dams as compared to the level recorded before pregnancy (8).

7DHC increased from undetectable amounts in control rats (not shown) to 7.8 ± 2.5 mg/dl and 5.4 ± 1 mg/dl on gd 11 and gd 14, respectively, after AY9944 treatment (group A, Fig. 3). As reported previously (7), when administered as a single oral dose at gd 3, the drug developed its maximum inhibitory potency after 6 days. When AY9944-treated dams received cholesterol supplementation (group B, Fig. 2), the serum cholesterol was restored by 65% on gd 11 and reached the control level on gd 14. 7DHC (group B, Fig. 3) decreased on gd 11 (65%) and 14 (72%) as the result of the negative feedback exerted by cholesterol on its synthesis which produced the aberrant sterol under the conditions used in this study (27).

7DHC supplementation of AY9944-treated dams (group C) did not significantly change the sterol profile as compared to group A (Fig. 3). An oral dose of tritium-labeled 7DHC given on the sixth day of supplementation indicated that this aberrant sterol was readily absorbed through the maternal intestinal barrier (>80% of the given dose). The intestinal absorption of the precursor was similar to that of cholesterol. Noticeably, the intestinal absorption of cholesterol decreases slightly under the influence of AY9944 (group B) as regard to non-treated dams (**Fig. 4**). The constant level of 7DHC in the maternal serum suggested that the exogenous 7DHC exerted efficient down-regulation on endogenous synthesis. Negative feed-back exerted by 7DHC has been previously demonstrated in cultured SLO fibroblasts lacking Δ7 reductase (27).

In group D, which received simultaneous cholesterol and 7DHC supplementation, an inhibitory effect on the intestinal absorption of cholesterol was manifested by the

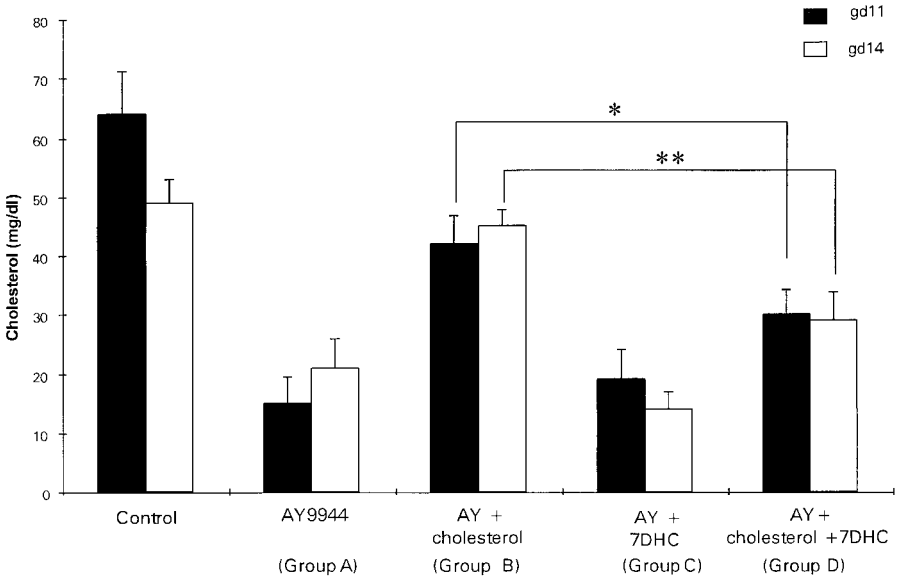

Fig. 2. Assay by GC-MS of maternal serum cholesterol on gestational day 11 (filled bar) and 14 (open bar). Cholesterol is identified by its retention time of 17.4 min on selective ion monitoring at *m/z* 329. Results are given for control and AY9944 (AY) treated dams (a single oral dose of 75 mg/kg given on gd 3) exposed to the various nutritional supplements (groups A–D are described in Fig. 1). Values are mean ± SEM. (n = 3–6 dams).* $P < 0.05$ for the difference between group B and group D of cholesterol level on gd 11; ** $P < 0.01$ for the difference between group B and group D on gd 14.

aberrant sterol (group D, Fig. 4). We assumed that intestinal absorption of the two sterols was competitive. However, the beneficial effect of cholesterol supplementation on the phenotype was not counteracted in group D by simultaneous 7DHC supplementation. In group D compared to group B, the cholesterol level was significantly lower on gd 11 and 14 whereas the 7DHC level was higher (Figs. 2 and 3). This resulted in a substantially increased ratio of 7DHC/cholesterol in group D with no consequence on the phenotype.

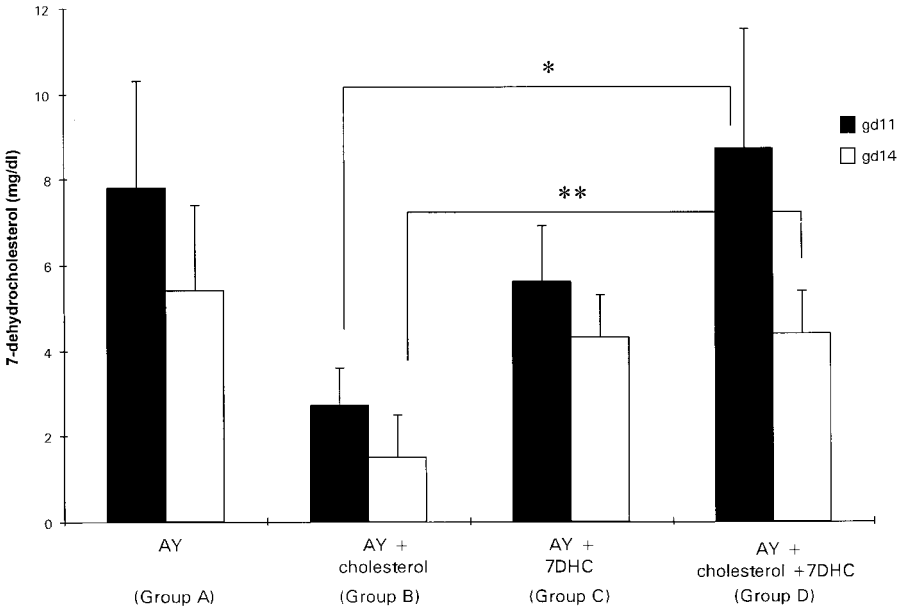

Fig. 3. Assay by GC-MS of 7-dehydrocholesterol in the maternal serum of AY9944-treated dams after a single oral dose (75 mg/kg) of the inhibitory drug on gd 3. Dams were subsequently given a supplementary diet with cholesterol and/or 7-dehydrocholesterol (500 mg/kg/day) from gd 3 to gd 11 or to gd 14. Values are mean ± SEM (n = 3–6 dams). * $P < 0.03$ and ** $P < 0.05$ indicate the significant difference between group C (cholesterol-supplemented) and D (cholesterol plus 7-dehydrocholesterol) on gd 11 (filled bar) and 14 (open bar).

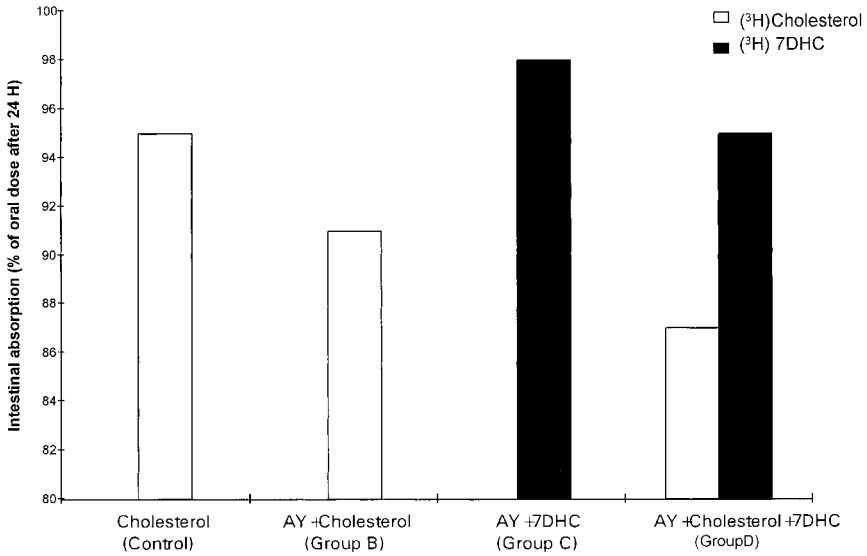

Fig. 4. Intestinal absorption of cholesterol (open bar) and 7-DHC (filled bar) after oral administration of a radioactive tracer dose (110×10^6 dpm) of 1α–2α tritium-labeled cholesterol or 7DHC. Rats were given a supplement for 5 days of cholesterol and on the sixth day the tracer dose was added to the sterol supplementation administered by oesophagus intubation. Feces were collected after 24 h and weighed, and labeled sterols were extracted and counted by scintillation counting.

Cholesterol and 7DHC accumulation in embryo tissues

The cholesterol level was 2.9 μg in control embryos on gestational day 11 but increased 89-fold in just 3 days to reach 258 μg on gd 14 (**Fig. 5**. Nota bene: the scale for embryos on gd 14 (open bar) is multiplied ×100). This sharp increase illustrates the very high rate of endogenous cholesterol synthesis and transfer from the maternal serum during normal development. The level of cholesterol in the embryo was reduced 80% after AY9944 treatment (group A, Fig. 5). The decrease was at least partially

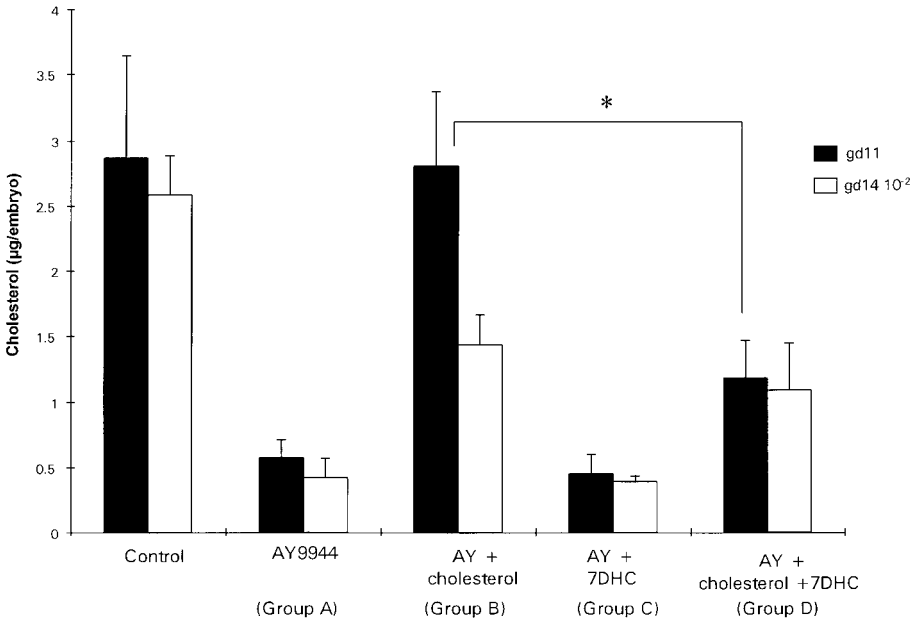

Fig. 5. The level of cholesterol in the embryo was assayed in the offspring of AY9944-treated rat dams exposed to cholesterol and/or 7-dehydrocholesterol dietary supplementation from gd 3 to gd 11 (filled bar) or gd 14 (open bar). Values are mean ± SEM (n = 20–30 embryos). The Mann-Whitney test shows the significance (* $P < 0.05$) of the cholesterol decrease at gd 11 in embryos exposed to 7-dehydrocholesterol and cholesterol supplements (group D) as compared to cholesterol alone (group C). Nota bene: the cholesterol level in embryos at gd 11 (filled bar) is given as μg/embryo and at gd 14 (open bar) is given as 100 μg/embryo.

explained by the uptake by the fetus of AY9944, which exerted a blockade in the synthesis in situ (11) of cholesterol. This was supported by the results of the GC-MS assay of the drug which showed accumulation in embryos between gd 11 (5 ng/embryo) and gd 14 (800 ng/embryo). A cholesterol-rich diet restored the level of cholesterol to the control level in gd 11 embryos (group B, Fig. 5). The partial restoration observed on gd 14 probably reflected that a very high cholesterol level was required around gd 11–14 which could not be entirely matched by the import of exogenous cholesterol from the mother (group B, Fig. 5). In spite of incomplete cholesterol restoration on gd 14 (≈143 μg/embryo vs. 258 μg/embryos) normal development was achieved, which showed that an increased compensatory maternal influx of cholesterol could substitute successfully for the synthetic defect.

The offspring of AY9944-treated dams receiving simultaneous cholesterol and 7DHC diets (group D, Fig. 5) had a lower cholesterol content on gd 11 as compared to the embryos exposed to only cholesterol supplementation (group B). Nevertheless, development remained normal (embryo D is shown in Fig. 1) in spite of a relatively low cholesterol content. In this group inhibition of synthesis was also successfully circumvented as in group B by efficient cholesterol transfer from the mother in contrast to that observed previously in vitro where the oxidized by-products of 7DHC suppressed the compensatory influx (24) and resulted in irreversible deleterious effects.

Figure 6 illustrates that 7DHC accumulation in embryos explanted from AY9944-treated dams was similar under the different nutritional conditions. 7DHC amounting up to 42% of the cholesterol content in group A (on gd 11) did not increase further when the dams were exposed to an additional 7DHC-enriched diet (groups C and D, Fig. 6). The increased 7DHC level on gd 14 compared to gd 11 observed in every group (Fig. 6) illustrated the lasting inhibitory activity of AY9944 in embryos while the maternal level declined during the same period of time (Fig. 3). Therefore, we assumed that 7DHC was derived, at least partially, from in situ production resulting from the block in the cholesterol synthesis pathway by AY9944 accumulated in embryonic tissues.

DISCUSSION

Two findings relevant to therapy of SLOS are presented in this study: *i*) the prevention of the teratogenic activity of a distal cholesterol inhibitor by dietary supplementation correlates with the restoration of cholesterol in maternal serum and in embryo tissues, and *ii*) the harmlessness of accumulated 7-dehydrocholesterol in vivo in the presence of restored levels of cholesterol contrasts with the embryotoxicity of the precursor observed in vitro.

Prevention of the teratogenic activity of cholesterol synthesis inhibitors by supplementation of the dams with alimentary cholesterol was demonstrated previously (10). However, this diet simultaneously restored the cholesterol level and down-regulated precursor accumulation. Therefore no clear-cut information could be obtained in vivo as to a putative toxic role for 7DCH. The toxicity of 7DHC-oxidized derivatives on cultured embryos was irreversible after cholesterol supplementation of the medium, which seriously abrogated the possibility of relieving the SLO condition of fetuses which synthesize abundant amounts of 7DHC (24). The present investigation aimed to delineate the role of cholesterol shortage and that of the aber-

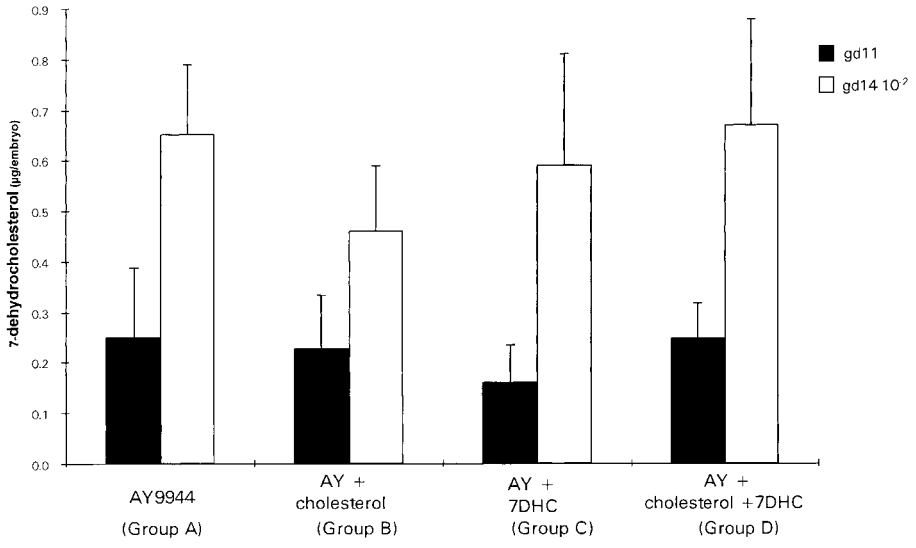

Fig. 6. The 7-dehydrocholesterol level was assayed by GC-MS in embryos from AY9944-treated dams exposed to cholesterol and/or 7-dehydrocholesterol dietary supplements (500 mg/kg/day) from gd 3 to 11 or 14 (AY9944 was given as a single oral dose on gd 3). The amount of 7DHC per embryo is indicated as μg/embryo for the pups extracted on gd 11 (filled bar) and as 100 μg/embryo on gd 14 (open bar). Values are mean ± SEM (n = 20–30 embryos).

rant sterol in vivo and to obtain information which should help develop prenatal therapy.

GC-MS showed high concentrations of AY9944 in embryo tissues on gd 11 and 14 that is to say 8–11 days after dams where treated on gd 3. The assay confirmed that the drug reached an inhibitory level as compared to that obtained in vitro after 10 ng/ml AY9944 was added to culture medium (11). A sustained accumulation of 7DHC between gd 11 and 14 whereas the maternal level of 7DHC declined at the same time confirmed that the aberrant sterol was produced in situ and not entirely imported from the mother serum. AY9944 uptake increased 160-fold during this period of time which is reminiscent of the uptake of the drug by the liver or the adrenals which act as reservoirs (C. Roux, unpublished results). The accumulation of the drug in sterol-rich tissues contrasts with its fast disappearance, within 24 h, from the serum (C. Roux, unpublished results). A delayed exchange between lipid-rich compartments should be envisioned from the GC-MS data of this study which show that the lipophilic drug accumulates in the fetus.

The cholesterol-enriched diet prevents the accumulation of 7DHC as a result of the negative feedback exerted on HMG-coenzyme A reductase. The data show that the restoration of the cholesterol level can be complete in the mother and in the embryos. Enriched supplementation of the diet should be considered knowing that there is practically no cholesterol in the diet of the animal. Efficient transfer of cholesterol from maternal plasma succeeds in maintaining the normal cholesterol level in embryos and down-regulates aberrant sterol synthesis which may be toxic.

A number of investigations have focused on the mechanism of transfer of sterols. Maternal lipoproteins, mostly HDL in rodents, transfer easily through the Reichert's membrane and bind to receptors of the apical microvilli of the visceral yolk sac endoderm. Cholesterol re-packaged in apoB-containing lipoproteins produced in the endoderm leaflet is secondarily secreted into the developing yolk sac circulation (22). The candidate receptors on the yolk sac epithelium for HDL in rodents are megalin and cubilin, which agrees with the teratogenic activity of antibodies raised against these multiple ligand receptors (P. Verroust, personal communication).

7DHC dietary supplementation is not efficient in preventing malformations. However, 7DHC does not significantly worsen the embryotoxic effects of AY9944 in contrast to that observed previously in vitro. 7DHC does not counteract the beneficial effects of cholesterol supplementation as it does in cultured embryos. Despite an intestinal absorption which is similar to cholesterol, 7DHC does not accumulate in maternal serum, which suggests that it exerts compensatory down-regulation on its own synthesis. Noticeably, 7DHC might have a negative effect on residual cholesterol biosynthesis of embryos intoxicated with low doses of AY9944; in this case down-regulation would contribute to the low activity of HMG-CoA reductase. In the present study conducted with high doses of AY9944, malformations were not different in AY9944-treated embryos exposed to a 7DHC-enriched diet (group C) compared to embryos treated with AY alone (group A) except for the extreme narrowing between the mid- and hind-brain which probably contributed in severely affected embryos to the higher incidence of internal hydrocephalus (Table 1). Hydrocephaly was also reported earlier for the most severe phenotypes of apoB−/− knock-out or BM15.766-treated rodents (23, 28).

The competitive inhibition of the intestinal absorption of cholesterol exerted by 7DHC (detected in group D) might impair the beneficial effect of limited supplementation with cholesterol, which was not observed here due to the large excess of administered cholesterol. In group D, the cholesterol level amounts to only 2-fold the level of cholesterol in the AY9944-treated non-supplemented group A. However, cholesterol maintained above 40% of the control is sufficient to prevent the malformations. Noticeably, the low threshold for the cholesterol level required to induce malformation holds true even in the presence of high precursor concentrations in embryonic tissues. This conclusion was reached in the light of the results of group D, which suggest that supplementation of the diet of pregnant women of affected fetus may have a preventive activity. Under physiological conditions a "safe" maternal excess in cholesterol exists for most mammalian species. This explains why the mouse is resistant to the teratogenicity at 10-fold higher doses of AY9944 than the rat or than the hamster (17). In humans the cholesterol level is much higher than in the most sensitive animal model (Wistar rat). However, no information on the efficiency of the cholesterol transfer is yet available and the unknown quantitative requirement during the first 2 months of pregnancy, critical for the expression of SHH, impairs quantitative comparison to animal models.

In vivo, 7DHC cannot be responsible for the enhanced activity of AY9944 provided that the cholesterol level exceeds the threshold level. In vitro, 7-DHC toxicity was considerably enhanced by oxidation and accordingly it was reduced by the addition to the growth medium of vitamin E (24). The oxidative status in vivo is favorably balanced and the oxidation rate of 7DHC is expected to be much lower in the embryo than in an aerated vessel used for culture. We assume also that oxidized by-products of 7DHC are counteracted in vivo by the antioxidant defenses of the fetus (29).

7DHC competes with cholesterol for the transport through the intestinal barrier, as it also disturbs the cholesterol influx through the embryonic envelopes from the culture medium (24). Inhibition of the cholesterol absorption in the presence of 7DHC may be compared to plant sterols or cholestanol influence which significantly lowers absorption (30). In the present study, another possibility is that 7DHC instead of cholesterol adversely modifies the rat intestinal brush border membrane and limits the absorption of lipids. Indeed, we show that AY9944 reduces slightly the absorption of cholesterol as demonstrated previously for the distal inhibitor of cholesterol synthesis HCG-917 which induces enterocyte membrane alterations (31).

We assume that the critical factors responsible for the activity of cholesterol inhibitors are severe cholesterol shortage combined with the limited transportation of cholesterol from the mother during the early embryogenesis (gd 7). This assumption agrees with observations made of megalin knock-out mice which exhibit severe holoprosencephaly (21).

A role for cholesterol in the activity of the developmental gene *shh* during the early embryogenesis is expected from: *i*) the shh null phenotype of mice which mimics AY9944 treatment (18); *ii*) the cholesterol-dependent cleavage of the SHH protein (20); and *iii*) an alteration in the signaling pathway of SHH by a poor cholesterol environment (26, 32). However, a variety of possibilities exists to explain the relationship of cholesterol shortage and reduced SHH activity. In the absence of cholesterol, the N-terminal sequence with the signaling activity cannot be cleaved from the inactive precursor. However, 7DHC can substitute for cholesterol in the cleavage reaction of SHH in vitro (26). In neural tube explants, AY9944 was also assumed to inhibit directly the intracellular transport of cholesterol along with similar class II inhibitors (26). Treatment with AY9944 results in a decrease in the expression of SHH (33). Different possibilities have been described above to explain the mechanism by which this decrease occurs but none have been definitively identified. However, the condition is found to be reversible after cholesterol supplementation of the dam (unpublished results). In addition, the deficit in membrane microdomains that are enriched in cholesterol and act as a concentrator platform for sterol-linked SHH was recently reported (34). We assume that the role of membrane heterogeneity in the form of microdomains enriched with sphingolipids and the saturated species of glycerophospholipids can also help to better understand the teratogenicity of cholesterol shortage (34).

In conclusion, the data obtained from animal models suggest that in humans the normal maternal level of cholesterol of the heterozygous mother is not sufficient to suppress the abnormalities of SLO-affected embryos. This leads us to suggest that the elevation of circulating cholesterol levels in the mother during early pregnancy or the by-passing of placental transfer via surgically mediated delivery of cholesterol to the fetal circulation might reduce abnormal development. The early detection of mutations in 7-dehydrocholesterol Δ7 reductase during pregnancy (4, 5) and the absence of a deleterious effect of 7DHC in vivo, as illustrated in the present study, support the possibility of performing pre-natal treatment.

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