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HUMAN BREAST MILK STIMULATES BILE ACID SECRETION BY FETAL RABBIT LIVER IN ORGAN CULTURE*

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Fetal livers from rabbits at 30 days of gestation were grown in organ culture and the effect of human milk added to the culture medium on the ability of liver to excrete bile acids (cholylglycine) was examined. Human breast milk promoted a concentration related increase in cholylglycine accumulation in the medium. The factor(s) present in milk responsible for this effect appear to be non-protein in nature and is associated with the floating lipid fraction. Furthermore, milk enhances the integrity of liver explants, as established by light microscopy.

Bile acid synthesis develops late in gestation and is not completely mature at birth (1). In the newborn, the bile acid pool size and the rate of bile acid synthesis are reduced when compared to adults (2). After birth, bile acid synthesis develops markedly reaching adult levels by weaning (1,3). The factors involved in the maturation of bile acid synthetic pathways are not known.

Human breast milk has been shown to contain some biologically active growth factors (4-7). Premature weaning in animals to solid diets has been shown to cause changes in cholesterol homeostatic mechanisms some of which persist into adult life (8-9). This suggests that milk might contain factors essential for the normal development of a cholesterol catabolic pathway which is immature at birth (1). Recently, studies from our laboratory showed that human breast milk markedly stimulates the activity of hepatic cholesterol 7α-hydroxylase (rate limiting enzyme involved in cholesterol metabolism) in vitro (10).

In this study, the capacity of rabbit fetal liver in organ culture to excrete bile acids under the influence of milk and the histological aspects of the liver explants were evaluated and we show that milk stimulates the secretion of bile acids by fetal livers.

Human breast milk samples were obtained from volunteers in their early twenties during the 4th week of lactation. Milk was subjected to proteolysis by treatment with trypsin and chymotrypsin as described by Klagsbrun (3). Boiled milk was obtained by boiling samples for 30 min. Milk samples were also centrifuged at 3000 rpm for 30 min., and the infranatant was used for the assays (skimmed milk).

Fetal livers from New Zealand rabbits (Kingswheel Rabbitry, Mt. Vernon, OH) at 30 days of gestation (normal gestation 31-32 days) were dissected under sterile conditions, removed rapidly and placed in petri dishes with sterile Hank's Salt Solution (Gibco Company, Grand Island, NY) on

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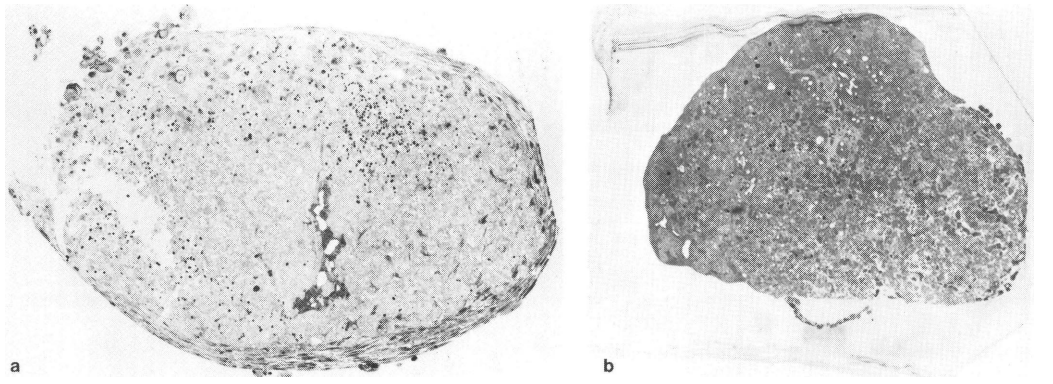

FIGURE 1: Histology of fetal rat liver after 7 days in organ culture in the absence (a) and presence (b) of milk (1%). Note in (a) proliferating cells in the periphery and occasional biliary canaliculi and hepatocytes in the center. Note in (b) the numerous hepatocyte plates and biliary canaliculi. Toluidine Blue, X100.

ice. Explants of liver, approximately 2 mm, were prepared, and three explants were maintained on a sterile, scored Falcon plastic petri dish with center well (Beckon, Dickinson and Co., Cockeysville, MD) with 2.0 ml of culture medium. The culture medium consisted of Basal Eagle Medium (39 ml%) (MA Bioproducts, Walkersville, MD) and Ham's F-12 medium (39 ml%) (Gibco Laboratories, Life Technologies Inc., Chagrin Fall OH), and was supplemented with fetal bovine serum (20 ml%) (Hyclone Laboratories, Logan, Utah), L-glutamine (1 ml%), gentamycin (0.1 ml%) and fungizone (1 ml%). The experimental culture medium also contained milk or its fractions. The medium was prepared after filtering its constitutents with a sterilization filter unit with pore size of 0.8 um (Nalge Company, Rochester, NY).

Dishes with explants and medium were covered in order to prevent evaporation. Incubations were carried out for periods up to 7 days in a chamber at 37°C in a 5% CO_2-95% air atmosphere. The medium was changed with sterile precautions at 24 hr and every 24 or 48 hr thereafter, according to the experiment.

The ability of liver to excrete bile acids was evaluated by measuring the cholylglycine accumulation in the culture medium by specific radioimmunoassay (Abbott Laboratories, North Chicago, IL) using an Auto-Logic Gamma Counter (Abbott Laboratories). Bile acid secretion was expressed on the basis of protein content of the liver explants, measured by the method of Bradford (11).

For light microscopy, liver explants were fixed in 4% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate, trimmed into 0.5 mm cubes, washed twice in cacodylate buffer, and postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer at pH 7.4. Tissues were stained in block with 2% uranylacetate, dehydrated through ascending concentrations of ethylalcohol, transferred to propylene oxide, and embedded in Spurr's embedding resin. Sections were cut at 1 um and stained with 0.1% Toluidine Blue.

Control and experimental values were compared using the Student's t test, with p-values < 0.05 being considered as statistically significant.

Figure 1a shows the histological appearance of these liver explants after 7 days in culture. Healthy proliferating hepatocytes could be seen at the periphery. The center of the explant demonstrated occasional biliary canaliculi and scattered healthy hepatocytes as well as a certain degree of cell debris and granularity from degenerating cells. After the addition of milk (1%) (Figure 1b), hepatocyte plates and biliary canaliculi appeared more numerous. Numerous lipid droplets were also present within the explant.

TABLE I: Cholylglycine accumulation in fetal liver culture medium: effect of milk (1%)[a]

Milk added	(n)	Cholylglycine accumulation (ug/100 ug of liver protein) Days of incubation			
		1	3	5	7
None	4	1.69 ± 0.34	2.05 ± 0.31	2.68 ± 0.23	3.39 ± 0.20
Milk	4	1.92 ± 0.66	3.21 ± 0.81	5.79 ± 1.16*	8.85 ± 1.92*

[a]Expressed as mean ± SEM

*$p < 0.02$

Table I shows the cholylglycine accumulation with time in the medium until the 7th day of culture. Bile acid secretion increased progressively with time. Milk (1%) added to the medium promoted a gradual increase in cholylglycine secretion by the liver explants with nearly 100% increase by the 7th day of culture.

Table II shows that a proportionally linear increase in the secretion of cholylglycine by the liver explants related to the milk concentration was observed.

Further studies were done to examine the nature of factors in milk responsible for stimulating bile acid synthesis. Table III shows the cholylglycine accumulation in the medium between 24 and 48 hours of incubation as affected by the different milk fractions. Proteolysis and boiling of the milk did not change the stimulating effect of milk. However, skimmed milk, obtained by centrifugation (to remove floating fat), abolished the stimulatory effect of milk. This indicated that the factor(s) is non-protein in nature and is associated with the floating lipid fraction.

Human studies have suggested that milk has the potential to decrease serum

TABLE II: Cholylglycine accumulation in fetal liver culture medium. Effect of milk concentration[a]

Milk added (%)	(n)	Cholylglycine accumulation (ug/100 ug of liver protein) Hours of incubation		
		24	48	72
None	3	0.52 ± 0.15	1.93 ± 0.49	2.98 ± 0.78
Milk 1%	3	0.67 ± 0.15	3.45 ± 0.32*	6.14 ± 0.63*
Milk 2%	3	2.73 ± 0.74*	7.42 ± 0.65**	11.18 ± 0.79**

[a]Expressed as mean ± SEM

*$p < 0.05$ in comparison to controls

**$p < 0.01$ in comparison to milk 1%

TABLE III: Cholylglycine accumulation in fetal liver culture medium upon 24 hours of incubation: Effect of different fractions of the milk[a]

Milk or fraction added (1%)	(n)	Cholylglycine concentration in medium (ug/100ug of liver protein/24 hours)
None	3	1.24 ± 0.33
Milk	3	2.78 ± 0.26*
Proteolized Milk	3	2.13 ± 0.11*
Boiled Milk	3	2.46 ± 0.16*
Skimmed Milk	3	0.57 ± 0.08

[a]Expressed as mean ± SEM

*$p < 0.05$ in comparison to controls

cholesterol levels (12,13) although the mechanism of this effect is not completely clear. Studies by Bogulawski and Wrobel (14), Ward et al. (15) and Mann (12) suggested the presence of a factor in milk that inhibited the synthesis of sterols from radiolabeled acetate and mevalonate. In this laboratory it was also shown in vitro (10) that human breast milk stimulates rat liver cholesterol 7α-hydroxylase activity. The results of the present studies strongly support the concept that human milk stimulates the excretion of bile acid by the liver. This stimulation might also contribute to the hypocholesterolemic effect of milk.

This stimulating effect of milk is not likely to be due to an increased presence of substrate (cholesterol) in the medium since the amount of cholesterol present in the milk (measured by GLC) added to the medium was very small (64-86 ug%) compared to the cholesterol already present in the medium itself (6.40 mg%). Furthermore, milk itself contained undetectable amounts of bile acids, as measured by RIA.

Preliminary examination of the nature of the factor in milk indicated that boiling and proteolysis with trypsin and chymotrypsin did not alter the stimulating activity of milk. These facts indicate that the potential factor is not a protein. It is most likely that this factor may be a lipid associated with floating fat fraction of milk. Further studies to purify the active factor(s) are currently in progress.

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