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Bilirubin binding proteins
bilirubin glucuronyltransferase
breast milk jaundice

Breast Milk Jaundice: *In Vitro* Inhibition of Rat Liver Bilirubin-Uridine Diphosphate Glucuronyltransferase Activity and Z Protein-Bromosulfophthalein Binding by Human Breast Milk

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Extract

Twenty-four samples of breast milk from nine mothers of infants suffering from breast milk jaundice were studied. Eight samples of milk from mothers of nonjaundiced infants, along with five formula milks enriched with polyunsaturated fatty acids, served as controls. Milks from mothers with jaundiced infants had no inhibitory effect when assayed immediately after thawing. However, after these milk samples were stored at 4°, they strongly inhibited bilirubin conjugation (80.3% inhibition of uridine diphosphate glucuronyltransferase (UDPGT) activity) and bromosulfophthalein (BSP) binding to cytoplasmic Z protein (dye binding inhibited 82.1%). There was no effect on BSP binding to Y protein (see Table 1). Heating the milk to 56° modified the results in the following manner; when the milk was heated immediately after thawing, no inhibitory effect was seen, even after storage for 96 hr. On the other hand, when the milk was first stored at 96 hr and then heated, it had the same inhibitory effects as the milks which were stored without heating. The present study shows that pathologic breast milk will inhibit BSP-Z protein binding only when stored under conditions that also cause the appearance of the capacity to inhibit bilirubin conjugation *in vitro*, as well as causing the liberation of nonesterified fatty acids. Thus, the appearance of this inhibitory capacity *in vitro* seems linked to the lipolytic activity particular to pathologic milks.

Speculation

By demonstrating that the *in vitro* inhibition of bilirubin conjugation by pathologic milks is due to the tying up of hepatic transport proteins, we have furnished the missing link in the sequence of events following ingestion of potentially inhibitory milk resulting in neonatal jaundice. We call the pediatrician's attention to the key role of ingested free fatty acids in the etiology of this condition.

A new variety of neonatal jaundice linked to maternal milk was reported nearly simultaneously by Newman and Gross (31) and by Arias *et al.* (4) and has since been confirmed by numerous authors (19, 22, 27 29). Gartner and Arias (16, 17) attributed this hyperbilirubinemia to the transmission of a thermo-stable factor in breast milk from the mother to the child. They identified this factor as being 3α,20β-pregnanediol, an isomer of a natural steroid, and further demonstrated that it was capable of inhibiting bilirubin conjugation *in vitro* as well as causing hyperbilirubinemia in normal infants. However, considerable doubt has been cast on this steroid's role in the etiology of this jaundice: its presence in milk considered "inhibitor" is not constant (36, 37) and its *in vitro* inhibitory effect on bilirubin conjugation is strongly contested (1, 2, 8, 21, 27), although it could have an inhibitory effect on the transport of conjugated bilirubin (2).

Recently, Levillain *et al.* (27) have established several facts: (*1*) *in vitro* inhibition of bilirubin conjugation is localized in the lipid fraction of inhibitory milks and (*2*) the ability to inhibit increases with storage time and is related to unusually high levels of free fatty acids, predominantly unsaturated. These inhibitory milks are further characterized by the ability to liberate normal fatty acids in large quantities, which is probably due to the presence of an abnormal or abnormally elevated thermolabile lipase activity. Shortly thereafter, Cole and Hargreaves (10) confirmed the role of storage time in inducing jaundice, whereas Bevan and Holton (6, 7,

18) showed the inhibition of glucuronoconjugation *in vitro* by fatty acids.

We felt it would be interesting and worthwhile to study the effects of inhibitory breast milk on the intrahepatic bilirubin transport system. The roles of Y and Z hepatic proteins have been clearly established (23, 24) and the fixation of certain fatty acids by Z protein has been demonstrated (15, 30, 32). Furthermore, insufficient development of Y protein is a probable factor in the pathology of neonatal jaundice (14, 25, 26)

MATERIALS AND METHODS

MILK SAMPLES

Twenty-four samples of breast milk from nine mothers of infants suffering from breast milk jaundice were studied. The criteria assessing the diagnosis of breast milk jaundice were: an unconjugated hyperbilirubinemia developing late after starting breast feeding in a healthy child without anemia or overt signs of hemolysis, no clinical or biologic signs of hepatic disease, and the disappearance of the jaundice a few days after weaning. One sample each was taken from four of the women, whereas the other five were sampled four times during the feeding period. Milk samples were immediately frozen and stored at −18°.

Eight samples of milk from six mothers of nonjaundiced infants, along with five formula milks enriched with polyunsaturated fatty acids, served as controls. One control mother was sampled three times.

Total milk lipids were measured according to the method of Folch *et al.* (13) and fatty acid content by the method of Duncombe (12).

MEASUREMENT OF BILIRUBIN UDPGT

Bilirubin and UDPGA were obtained from Sigma Chemical Company Ltd.; BSP from Serlabo; and all other reagents from Merck, Darmstadt. UDPGT activity was assayed in homogenates of livers from Sprague-Dawley rats (Sodelabo) using a modification of the method of Black *et al.* (9). Since the affinity of albumin for fatty acids (38, 40) could interfere with the demonstration of the inhibitory properties of the milks studied, bilirubin alone was used as a substrate instead of albumin-bilirubin. Assays were performed on 100-μl samples with distilled water serving as the blank control. Each milk sample was assayed four times: immediately upon thawing; after 96 hr of storage at +4°; after thawing, heating at 56° for 15 min, and then storing for 96 hr at 4°; and after thawing, storing for 96 hr and then heating at 56° for 15 min. Results are expressed in milligrams of conjugated bilirubin per g of liver per hr.

MEASUREMENT OF BSP BINDING BY Y AND Z HEPATIC CYTOPLASMIC PROTEINS

The technique of Levi (24) was used. The Y and Z fractions in the 105,000 × *g* supernate of Sprague-Dawley rat liver homogenates were separated by Sephadex gel filtration after labeling with BSP. An aliquot of supernate corresponding to 40 mg soluble protein (determined by the technique of Lowry *et al.* (29) was incubated for 10 min at room temperature with 1.5 μmol BSP, and in the presence of 100 μl milk or water (blank). Protein concentration was estimated by absorbance at 280 nm and protein-bound BSP by absorbance at 580 nm, after alkalinization with 1 N NaOH. A standard curve was established to convert OD into nanomoles of BSP. Results were expressed as nanomoles of BSP bound to each fraction per g of liver. The Student *t*-test was used for analysis of results.

RESULTS

Results are summarized in Table 1, except for the results of the heated samples, which have been left out for clarity (see text).

Under the *in vitro* conditions chosen, neither distilled water nor the control milks or the formula milks had any effect on the ability of Sprague-Dawley rat liver homogenates to conjugate bilirubin or the binding of BSP to the Y and Z proteins. Milks from mothers with jaundiced infants had no inhibitory effect when assayed immediately after thawing. However, after these milks were stored for 96 hr at 4°, they strongly inhibited bilirubin conjugation (80.3% inhibition of UDPGT activity) and BSP binding to cytoplasmic Z protein (dye binding inhibited 82.1%). There was no effect on BSP binding to Y protein.

Only one discrepancy was observed (and verified) among the four samples provided by the same mother. After storage, two of these samples provided the same activity, 80% and 50%, and inhibited BSP binding 50%, whereas the other two samples inhibited UDPGT activity 77% each but had no effect whatsoever on BSP binding by Z. These four samples were therefore left out in the calculation of results ($n = 20$).

Heating the milk to 56° modified the results in the following manner; when the milk was heated immediately after thawing, no inhibitory effect was seen, even after storage for 96 hr. On the other hand, when the milk was first stored for 96 hr and then heated, it had the same inhibitory effects as the milks which were stored without heating.

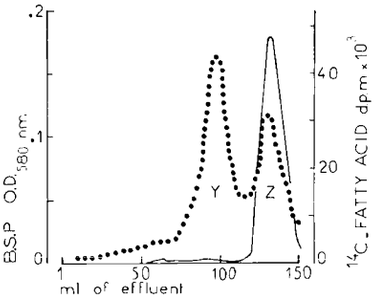

Fig. 1. Binding of sodium bromosulfophthalein (*BSP*) (● ● ●) and [^{14}C]oleic acid (——) to the proteins in Sprague-Dawley liver supernatant after chromatography on Sephadex G-75. The bindings shown are compiled from separate experiments in which only one ligand was added to liver supernatant.

Table 1. *Effect of various milks on bilirubin glucuronyltransferase activity and bromosulfophthalein binding to hepatic cytosolic Y and Z proteins*[1]

	Water (blank)		Formula milks		Noninhibitory milks (control)		Inhibitory milks		
	n	M ± SEM	*n*	M ± SEM	*n*	M ± SEM	*n*	M ± SEM	*P*
Glucuronyltransferase	5	1.22 ± 0.27	5	1.26 ± 0.06	8	1.28 ± 0.16	20	0.24 ± 0.04	<0.001
BSP binding									
Y	20	103.5 ± 0.9	5	113.00 ± 2.6	8	105.6 ± 5.2	20	108.5 ± 5.3	NS
Z	20	44.1 ± 2.2	5	46.4 ± 1.7	8	43.5 ± 2.0	20	7.8 ± 1.3	<0.001

[1] Results are expressed in milligrams of bilirubin conjugated per hr per g of liver for uridine diphosphate glucuronyltransferase activity; and in nanomoles of BSP bound per g of liver for BSP binding. NS: not significant.

Figure 1 shows the elution peaks of BSP when a mixture of the dye and soluble fraction is filtered on a Sephadex column, permitting the identification of the Y and Z proteins. Binding of ^{14}C-labeled oleic acid to Z protein is shown by the peak of radioactivity. The comparative binding of some fatty acids is given in Table 2. The diminution of the BSP-Z peak after incubation of the soluble fraction with either [^{14}C]oleic acid or an inhibitory milk is shown in Figure 2.

The total lipid content of the various milks was not modified by storage for 96 hr at 4°, whereas free fatty acid level in the inhibitory milks was highly increased compared with the control milks (Table 3). Figure 3 shows the relation between free fatty acid content and the inhibitory effect on protein Z-BSP binding of the milks after storage. There was a positive correlation (r = 0.86).

DISCUSSION

Our study confirms certain recently reported observations: milk from women whose breast-fed infants are jaundiced will acquire the capacity to inhibit bilirubin conjugation when stored under certain conditions (10, 18, 27, 35); the inhibition phenomenon is not observed when the milk is tested either immediately after thawing or when heated to 56° before storage, regardless of the ensuing storage conditions. Our results further show that these inhibitory milks are capable of inhibiting the *in vitro* binding of BSP to the hepatocyte Z protein without modifying the anion's affinity for the Y protein.

We feel that the competitive inhibition of the BSP-Z protein binding as well as the inhibition of bilirubin conjugation is due to abnormally high levels of fatty acids in inhibitory milks. Levillain *et al.* (27) and Odièvre (35) have shown that a strict correlation exists between breast milk fatty acid levels and the inhibition of bilirubin conjugation. The general inhibitory effect of fatty acids on bilirubin conjugation has furthermore been experimentally proved *in vitro* (8, 18, 27) On the other hand, the studies of Ockner (32 34) and Arias (30), confirmed in our own laboratory, show that the hepatic Z protein is capable of binding certain fatty acids (Table 2). In addition, certain fatty acids are capable of competing for BSP binding sites on the Z protein (13).

The present study shows that pathologic breast-milk will inhibit BSP-Z protein binding only when stored under conditions that also cause the appearance of the capacity to inhibit bilirubin conjugation *in vitro*, as well as causing the liberation of nonesterified fatty acids. Thus, the appearance of this inhibitory capacity *in vitro* seems linked to the lipolytic activity particular to pathologic milks, as recently shown (18, 27, 35). However, the free fatty acid content in milk is perhaps not the only factor causing inhibition of bilirubin conjugation and/or protein Z BSP-binding because some milks are inhibiting but they liberate nonesterified fatty acids during storage to a lesser degree than others. This fact suggests that a qualitative factor which we have not studied presently could be implicated. The discrepancy observed from the four samples of one mother was not found again and the milk of the only control

Table 2. *Comparative binding of fatty acids to hepatic cytosolic Z protein from Sprague-Dawley rats*[1]

Fatty acid		Z protein binding, %
Arachidonic	C_{20}:4	18
Stearic	C_{18}:0	24
Oleic	C_{18}:1	60
Linoleic	C_{18}:2	40
Linolenic	C_{18}:3	30
Palmitic	C_{16}:0	12
Myristic	C_{14}:0	1
Lauric	C_{12}:0	0.4

[1] Cytosol (2 ml = 40 mg soluble proteins) incubated with labeled fatty acids (1 μCi) for 15 min at 20°. Filtered on Sephadex G-75 with phosphate buffer, 0.01 M, pH 7.4.

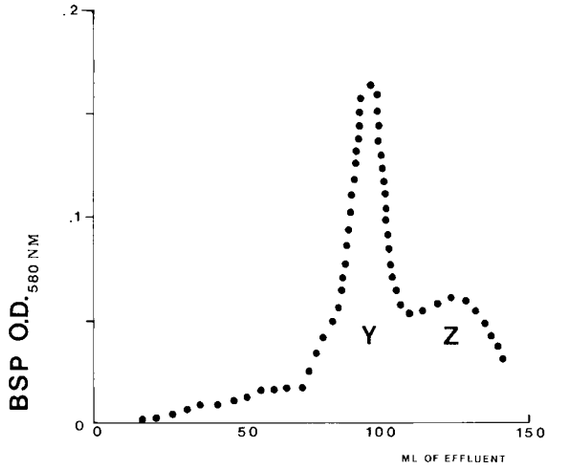

Fig. 2. Binding of sodium bromosulfophthalein (*BSP*) to the proteins in Sprague-Dawley liver supernatant after chromatography on Sephadex G-75 when incubated with oleic acid or inhibitory milk.

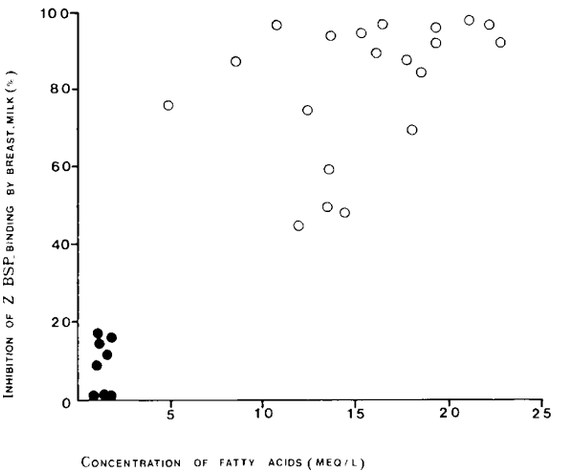

Fig. 3. Relation between inhibitory activity on protein Z-bromosulfophthalein (*BSP*) binding and free fatty acid content of breast milk. ●: control; O: inhibitory milks stored 96 hr at 4°.

Table 3. *Effect of storage on lipid content of breast milk*[1]

	Noninhibitory milks (n = 8)			Inhibitory milks (n = 20)		
	a	*b*	*P*	*a*	*b*	*P*
Total lipid, g/liter	32.8 ± 4.4	32.0 ± 3.4	NS	33.1 ± 3.3	35.6 ± 3.5	NS
Free fatty acids, mEq/liter	1.14 ± 0.07	1.24 ± 0.06	NS	1.26 ± 0.08	15.42 ± 1.03	<0.001

[1] *Samples a* are assayed immediately after thawing; *samples b* are assayed after storage 96 hr at 4°. Results are expressed as mean ± SEM. NS: not significant.

woman sampled three times did not exhibit any inhibitory effect or high free fatty acid content. Bilirubin, although difficult to utilize in the experimental conditions chosen (3, 5, 23–25), behaves undoubtedly similar to BSP, at least in regard to the overall competition of fatty acids observed *in vitro*. A study utilizing radioactive bilirubin is underway.

The *in vitro* demonstration of the inhibition phenomenon in pathological milks is not a proof of its existence *in vivo*: it allows us, however, to speculate on the sequence of events following the newborn's ingestion of potentially inhibitory milk which leads to breast milk jaundice, by furnishing the "missing link." The development of lipolytic activity in certain milks with the ensuing release of nonesterified fatty acids (35) in either the lumen or intestinal cells is perhaps the essential pathologic phenomenon for this disease. It produces either a quantitative increase of fatty acids absorbed, a liberation of an abnormal fatty acid, or a qualitative disequilibration of free fatty acids.

Indeed, there are a variety of ways in which abnormal increased lipolysis could ultimately have an inhibitory hepatic effect and there is by no means a strict relation between them. However, the newborn infant seems to be susceptible to conditions which increase intracellular hepatic levels of fatty acids, whether these be normal or pathologic. It is possible that a low esterification rate due to the immaturity of intestinal esterases coupled with the low concentration of biliary salts favors the passage of free fatty acids towards the portal blood flow in the newborn (11, 20). Differences normally existing in the esterification of fatty acids (35) would thus be accentuated; Z protein in the intestinal cells would favor absorption of fatty acids, for which it has a high affinity (15, 33), and thereby facilitate the passage of uncommonly large quantities of one or more free fatty acids derived from inhibitory milks towards the hepatocyte via the portal flow. The well known increase of plasmatic free fatty acids attributed to fasting in the newborn could, in part, add to the accrued absorption of certain ingested fatty acids. The possibility that these conditions intervene in the etiology of immature jaundice of the newborn has not been explored; it would therefore be interesting to compare levels of plasmatic and alimentary fatty acids with this point in mind. After freely traversing the cell membrane, the massive influx of fatty acids would quickly saturate all Z protein binding sites within the hepatocytes. This protein, present from birth, normally transports bilirubin, BSP (14, 25, 26), and, undoubtedly, fatty acids. Saturation would result in the following two consequences. (1) An overall decrease of bilirubin transport in the hepatocyte, especially since the capacity of the Y protein is reduced at this stage (4, 25, 26), would result in a diminution of bilirubin substrate for the UDPGT reaction. (2) Certain fatty acids could play a more important role than others: a good correlation exists between the strong inhibitory action of oleic acid (27), its high affinity for the hepatic Z protein (Table 2), and its good intestinal absorption, as shown by Ockner (34). Of the two consequences mentioned above, fatty acid inhibition of bilirubin conjugation is undoubtedly the more important, since maturation of the hepatic Y protein in the newborn will progressively increase the intrahepatic transport capacity of bilirubin. Consequently, breast milk jaundice appears later and lasts longer than the jaundice of immaturity, even after Y protein is fully matured. This would tend to indicate that the fatty acid inhibition of bilirubin conjugation is qualitatively more important than Z protein competition, as long as UDPGT is not fully mature. Finally, another hypothesis could be considered; if, in fact, it is the bilirubin-Z protein complex which serves as the substrate for the UDPGT reaction, then the fatty acids would be reducing substrate levels by tying up Z protein, as well as acting directly on the conjugation reaction.

SUMMARY

A study of twenty-four milk samples from nine mothers of infants with breast milk jaundice has shown that they are able to inhibit bilirubin conjugation *in vitro* as well as BSP binding to hepatic Z protein in Sprague-Dawley rat liver homogenates, whereas normal control milk or formula milk does not affect these parameters. The inhibitory activity is maximal after storage at +4° for 96 hr and is destroyed by heating at 56° for 15 min. *In vivo*, increased liberation of fatty acids by abnormal lipolytic activity of inhibitory milks along with facilitation of their passage into the portal blood flow because of low intestinal esterase activity and the presence of an intestinal Z protein all lead to increased hepatic intracellular levels of fatty acids. These, then, interfere by two mechanisms: the major one is a direct inhibition of bilirubin conjugating enzyme activity by competition with bilirubin for hepatic Z protein, thus reducing the conjugation substrate.

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41. We wish to thank Miss Thérèse Fournier for her technical assistance.
42. Since this paper was submitted, one of the authors has undertaken a prospective study in a maternity hospital; 1,000 women will be sampled. The detection of pathological milks is done with a stained reaction using Nile blue (Luzeau R., Levillain P., Odièvre M., and Lemonnier A.: Dépistage des laits maternels inhibiteurs de la glucuroconjugaison de la bilirubine par une réaction colorée. Arch. Franç. Pédiat., *30:* 573 (1973)).The study's purpose is to see whether some inhibitory milks will be found in the absence of jaundice.
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Homocystinuria
kidney
liver
5,10-methylenetetrahydrofolate reductase deficiency
plasma
urine

Morphologic Studies in a Patient with Homocystinuria due to 5,10-Methylenetetrahydrofolate Reductase Deficiency

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Extract

Biochemical and morphologic studies on a patient with homocystinuria due to a deficiency of 5,10-methylenetetrahydrofolate reductase (EC. 1.1.1.68) were performed.

The concentrations of homocystine in the patient's plasma and urine were 2.97 μmol/dl and 44.67 μmol/24 hr, respectively. Activities of 5,10-methylenetetrahydrofolate reductase (expressed as nanomoles of formaldehyde formed per hr per mg of protein) in cultured skin fibroblasts and liver tissue were 0.53 (control: 5.14) and 0.00 (control: 13.80), respectively.

The major abnormalities were found in the arterial bed, consisting of intimal hyperplasia, fragmentation, and disruption of elastic lamellae and subcellular changes in the endothelial cells. Extensive thrombosis was observed. The brain and the liver also showed widespread pathologic changes. In the former, neuronal loss and cellular damage were prominent and extensive. Diffuse demyelination with moderate astrocytosis was found; but demyelination was out of proportion to the vascular changes. Hirano bodies in the cortical neurons and crystalline and lamellar bodies in the Purkinje cells were observed. In the liver, there were fatty change and mild to moderate portal fibrosis. Bizarre, giant mitochondria and membrane-bound multivesicular bodies were found. Mild pathologic changes were also observed in the striated muscles and the kidneys. Focal fragmentation, disruption, and smearing of the Z discs and disorganization of the myofilaments were found in the skeletal muscles. The kidneys showed shrunken glomeruli, thickened basement membranes, and swelling of epithelial as well as endothelial cells.

Speculation

The morphologic abnormalities under light or electron microscopy in this patient with 5,10-methylenetetrahydrofolate reductase deficiency were strikingly similar to those reported in patients with cystathionine-β-synthase deficiency and with N^5-methyltetrahydrofolate homocystine methyltransferase deficiency. The common denominator in all these disorders is homocystinemia. It is postulated that the widespread vascular lesions are produced by the "toxic" effect of homocystine and that the pathologic changes in other organs are the result of ischemia and thrombosis as well as a possible direct effect of homocystine.