Journal of Clinical Investigation
Vol. 43, No. 11, 1964

Prolonged Neonatal Unconjugated Hyperbilirubinemia Associated with Breast Feeding and a Steroid, Pregnane-3(Alpha), 20(Beta)-Diol, in Maternal Milk That Inhibits Glucuronide Formation *In Vitro* *

IRWIN M. ARIAS, LAWRENCE M. GARTNER,† SAM SEIFTER, AND MATHILDA FURMAN

(From the Departments of Medicine, Pediatrics, and Biochemistry, Albert Einstein College of Medicine, and Bronx Municipal Hospital Center, New York, N. Y.)

Prolonged neonatal jaundice has been associated with breast feeding (1–4), but the clinical syndrome has not been fully described nor has the mechanism been elucidated. We have studied a syndrome of severe and prolonged unconjugated hyperbilirubinemia associated with breast feeding in seven full-term, unrelated, newborn infants. This report describes the clinical aspects of this syndrome and studies of its pathogenesis.

The transient unconjugated hyperbilirubinemia observed in all newborn infants during the first 5 days of life is usually referred to as physiologic hyperbilirubinemia and is believed to result from delayed development of the hepatic glucuronide conjugating system (5–7), particularly glucuronyl transferase (8). Glucuronyl transferase catalyzes the transfer of glucuronic acid from uridine diphosphate glucuronic acid (UDPGA) to bilirubin and other receptors to form the corresponding glucuronides. Inhibition of glucuronyl transferase activity could theoretically result in unconjugated hyperbilirubinemia. The clinical observations suggested a relationship between mother's milk and the pathogenesis of the syndrome. We therefore studied the effect of milk from mothers of the seven jaundiced infants, from control mothers, and from pregnant and postpartum cows on glucuronyl transferase activity *in vitro*.

* Submitted for publication April 27, 1964; accepted July 2, 1964.

Supported by grants AM-02019 and A03172 of the U. S. Public Health Service and a grant from G. D. Searle Co., Inc.

Presented in part at the annual meeting of the American Society for Clinical Investigation, Atlantic City, N. J., May 19, 1963.

† Postdoctoral trainee, U. S. Public Health Service training grant 2A-5291.

Methods

Seven full-term, breast-fed infants with severe, prolonged, and unexplained jaundice and their anicteric mothers were studied. Three cases were studied at the Bronx Municipal Hospital Center. Clinical and laboratory data for the four other patients were provided by referring pediatricians. Data regarding prenatal, perinatal, postpartum, and neonatal examinations, treatment, and medications were obtained from appropriate physicians and hospital records.

By conventional methods, the following examinations or tests were performed in each infant to exclude known causes of prolonged neonatal jaundice: hematocrit, hemoglobin, erythrocyte and leukocyte counts, peripheral blood morphology, Coombs test, and blood grouping; serum cephalin cholesterol flocculation, thymol turbidity, glutamic oxaloacetic and glutamic pyruvic transaminase activities, and estimations of the concentration of albumin and globulin; serologic examinations for syphilis; and blood cultures. Erythrocyte glucose-6-phosphate dehydrogenase activity was estimated in four infants, and the urinary sediment was studied in three cases for evidence of cytomegalic inclusion body disease.

Serum total and direct-reacting bilirubin concentrations were estimated according to the method of Malloy and Evelyn (9). In five cases, plasma bile pigments were coupled with diazotized sulfanilic acid, and the resulting dipyrrol azopigments were characterized chromatographically according to Schmid (10).

Mother's milk was collected manually or with the aid of a breast pump and was frozen until examined. Forty-eight specimens of milk and three specimens of colostrum were obtained from the mothers of the seven jaundiced infants from postpartum days 2 to 35. Ninety-nine specimens of milk were obtained from 71 women whose infants did not demonstrate prolonged jaundice.

Thirty-two specimens of milk were freshly obtained from ten Guernsey, eight Jersey, and six Holstein cows during various stages of pregnancy and the postpartum period. These specimens were also frozen until examined.

Adult male Wistar rats, New Zealand white rabbits, and Hartley guinea pigs were obtained from a single supplier and killed as needed by decapitation; the livers

were removed rapidly and placed in ice-cold isotonic KCl before tissue preparations were made. Human liver was obtained at surgery for diseases not involving the liver or biliary tract and was treated identically as the animal liver. All subsequent operations were performed at 0 to 4° C. Liver was blotted and slices were prepared with a Stadie blade. Twenty-five to 50 mg of liver slices was placed in each flask for incubation. Ten per cent liver homogenates were prepared in 0.25 M sucrose in a glass homogenizer with a motor driven Teflon pestle. Microsomal fractions were prepared according to Hogeboom's technique (11).

Frozen human and cow's milk was thawed to 20° C and mixed manually with a loosely fitting glass homogenizer. If necessary, the pH was adjusted to 7.3 to 7.4 with 0.1 N NaOH or 0.1 N HCl. The effect of milk on glucuronide formation was estimated *in vitro* using *o*-aminophenol and bilirubin as glucuronide receptors. *o*-Aminophenol (1.25×10^{-3} M in 0.01 M ascorbic acid), 0.2 ml; 0.5 M Tris buffer (pH 8.0), 0.1 ml; 0.5 M $MgCl_2$, 0.1 M; UDPGA (1.1×10^{-3} M), 0.15 ml; human or cow's milk, 0.2 ml; and 10% liver homogenate or microsomes derived from 200 mg liver, 0.2 ml, were incubated in a total volume of 1.72 ml. Incubation was in duplicate with shaking at 90 cycles per minute in air for 30 minutes. *o*-Aminophenol glucuronide formation was estimated according to Levvy and Storey (12). Direct-reacting bilirubin formation by slices and homogenates of guinea pig, rat, rabbit, and human liver was estimated in quadruplicate according to the technique of Lathe and Walker (13) with and without the addition of human or cow's milk (0.2 ml). UDPGA was added in excess when homogenates and microsomes were studied but was not added to liver slices. Inhibition was estimated by comparing the formation of *o*-aminophenol glucuronide or direct-reacting bilirubin by tissue preparations in the presence and absence of human or cow's milk *in vitro*.

The effect of milk from three mothers of jaundiced infants and from three mothers of normal infants on *o*-aminophenol glucuronide formation by guinea pig liver homogenates was studied after storage of milk in the dark at 20° C for 6 weeks; after immersion of 10 ml of milk in a boiling water bath for 1 hour, cooling to room temperature, and gentle mixing with a loosely fitting glass homogenizer; and after dialysis in the dark at 4° C against flowing isotonic saline for 5 days.

From 75 to 450 ml of milk was separately collected from each of the following: *1*) three mothers of the seven jaundiced infants, *2*) two mothers whose milk was approximately 20% inhibitory and whose infants were normal, and *3*) three mothers whose milk was not inhibitory and whose infants were normal. Milk was stored at −20° C and slowly thawed. The cream layer was separated and saponified by heating in alkali (0.5 ml 60% KOH per g milk fat) at 100° C for 1 hour. The alkaline digest was repeatedly extracted with ether; the extracts were pooled, and the ether evaporated. The residue was taken up in a small volume of dry methanol with vigorous shaking and warming if necessary. From 100 to 300 μl of each sample was placed on separate, freshly prepared, thin layers of silica gel G spread on glass plates. Ascending chromatography was performed with chloroform/methanol/water (75/25/4, vol/vol/vol) as a developing system. Duplicate chromatograms were prepared, one of which was sprayed with sulfuric acid for identification of zones of organic material. From the other chromatogram 1.5- to 3.0-cm zones of silica gel were scraped starting at the solvent front. Each fraction was eluted with 1 ml of 90% methanol in water. The gel was centrifuged and washed twice with 0.5-ml portions of 90% methanol, and the washes were pooled with the eluates. Eluates from identical zones of several chromatograms were pooled, methanol was evaporated, and the eluates were further dried *in vacuo* over anhydrous $CaSO_4$ and paraffin shavings. The resulting crystalline material was identified by infrared spectrometry and gas-liquid and thin-layer

TABLE I

Clinical and laboratory data for infants

Infant	Sex	Birth wt	Maximal serum bilirubin concentration*			Return of serum bilirubin concentration to normal after cessation of breast feeding
			DRB	IRB	Day observed	
		g		*mg/100 ml*		*days*
A.Ml.	♂	3,250	1.9	18.2	18	6
S.B.	♂	2,740	1.7	15.5	19	12†
D.W.	♂	3,350	0.8	16.4	10	3
A.P.	♀	3,620	1.6	24.5	15	4
G.G.	♂	3,510	1.1	14.3	16	5
A.M.	♀	3,940	1.3	17.0	11	‡
L.M.	♀	2,880	1.3	15.7	10	§

* DRB = direct-reacting bilirubin; IRB = indirect-reacting bilirubin.
† Mother alternated breast and bottle feeding.
‡ Mother continued breast feeding despite intense jaundice in her infant (Figure 1).
§ Mother temporarily stopped breast feeding for 5 days (Figure 2).

TABLE II

Clinical data concerning the mothers of the seven jaundiced breast-fed infants

Mother	Age	Origin	Previous children	Breast-fed infants		Bottle-fed infants	
				Prolonged jaundice of undetermined etiology	No jaundice	Prolonged jaundice of undetermined etiology	No jaundice
Ml	31	Jewish	3	2			1
B	28	Italian	2		1		1
W	32	Italian	2	1			1
P	21	Jewish	1				1
G	27	Jewish	2	1			1
Mt	33	Negro	3	2			1
My	28	Chinese	0				
		Total	13	6	1	0	6

chromatography of the isolated material and known standards.[1] The crystalline material was also dissolved in 55% ethyl alcohol, and its effect on *o*-aminophenol glucuronide formation was studied *in vitro*. The ethyl alcohol concentration in the final incubation mixture was 0.28 M.

Kinetic studies were performed using *o*-aminophenol as a glucuronide receptor, guinea pig liver microsomes, and authentic pregnane-3(alpha), 20(beta)-diol and pregnane-3(alpha), 20(alpha)-diol. These steroids were dissolved in 55% ethanol and added to incubation flasks in a final concentration of 1.1×10^{-7} M. The resulting data were plotted according to the method of Lineweaver and Burke (14).

Serum was obtained from six of the seven mothers of jaundiced infants from days 10 to 26. The inhibitory effect of these sera on *o*-aminophenol glucuronide formation by guinea pig liver homogenates and direct-reacting bilirubin formation by rat liver slices was studied according to the techniques of Hsia, Dowben, Shaw, and Grossman (15) and of Lathe and Walker (13), respectively. The results were compared with those obtained with 17 samples of sera from 15 mothers of anicteric infants during the same postpartum period.

Results

Clinical and laboratory data pertaining to the seven jaundiced infants and their anicteric mothers are presented in Tables I and II. The mothers' pregnancies were considered normal; no known hepatotoxic drugs or hormones were administered, and deliveries were uncomplicated and occurred either without anesthesia (one case), with Demerol and scopolamine (three cases), or with promazine hydrochloride and nitrous oxide (three cases).

The four male and three female babies were full term (birth weights, 2,740 to 3,940 g), and each had an uncomplicated birth. Breast feeding began on the second or third day of life. Each baby thrived. Mild jaundice was noted in four cases during the first 4 days of life; serum bilirubin concentrations were not estimated, and a diagnosis of physiologic hyperbilirubinemia was made. Mothers and babies were discharged from the hospital between the fourth and seventh days. Progressively increasing jaundice was observed by each mother in her infant, and rehospitalization of five babies occurred on days 9 to 14. Two mothers and their infants were examined at the hospital but remained at home under medical care. Examination of the babies revealed intense jaundice of the skin and sclerae but no hepatomegaly, splenomegaly, fever, pallor, or neurologic changes. During the subsequent 4 to 8 days, breast feeding was continued, and appropriate laboratory studies were performed to determine the etiology of the jaundice. The results of these studies were normal. The maximal serum unconjugated bilirubin concentrations ranged from 14.3 to 24.5 mg per 100 ml and were observed on the tenth to the nineteenth days of life (Table I). The serum direct-reacting bilirubin concentration did not exceed 10% of the total bilirubin concentration in any case. In five cases, chromatographic study of the dipyrrol azopigments revealed only azopigment A, the unconjugated azopigment.

[1] Pregnane-3(alpha), 20(alpha)-diol and pregnane-3(alpha), 20(beta)-diol were obtained from Steraloids, Inc., Flushing, N. Y. The corresponding pregnanediol glucuronides and uridine diphosphate glucuronic acid (Sigma grade) were obtained from Sigma Chemical Co., St. Louis, Mo.

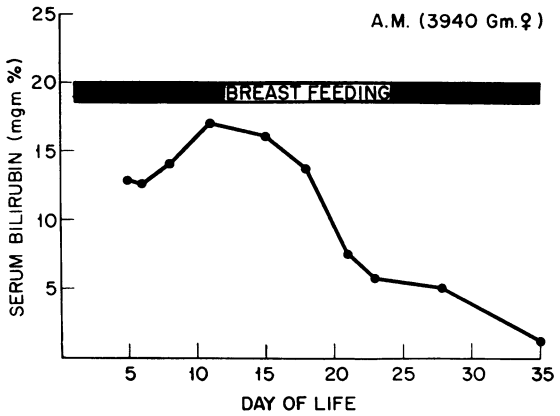

FIG. 1. COURSE OF HYPERBILIRUBINEMIA IN INFANT A.M., WHOSE MOTHER CONTINUED BREAST FEEDING.

Breast feeding was abruptly terminated in four infants (Table I). The serum bilirubin concentration was estimated at 1- to 3-day intervals and became normal in 3 to 6 days. One woman alternated breast and bottle feeding of her infant (S.B.), and the serum bilirubin concentration became normal in 12 days. The mother of baby A.M. continued breast feeding her infant despite intense jaundice (Figure 1). The serum bilirubin concentration was 17 mg per 100 ml on the eleventh day of life, 7.5 mg per 100 ml on the twenty-first day, and normal on the thirty-fifth day. The mother of baby L. M. breast fed her baby from the second to the ninth days, at which time the infant's serum bilirubin concentration was 16 mg per 100 ml (Figure 2). Breast feeding was discontinued for 4 days, evaporated milk was substituted, and the mother manually collected her milk. The infant's serum bilirubin concentration decreased to 5.5 mg per 100 ml within 4 days. On the thirteenth day of life breast feeding was reinstituted. The infant's serum bilirubin concentration remained at 5.0 to 5.8 mg per 100 ml for 5 days and was 2.5 mg per 100 ml on the twentieth day of life.

The seven mothers were unrelated and were of Jewish (three cases), Italian (two cases), Negro (one case), and Chinese (one case) ancestry (Table II). None of their mothers, sisters, or grandmothers had had infants with severe or prolonged jaundice clinically associated with breast feeding. The seven mothers had had thirteen previous children, seven of whom had been breast fed. Prolonged jaundice of unknown etiology had been observed in six of seven breast-fed infants but in none of six bottle-fed infants.

These clinical observations suggested a relationship between breast feeding and the unexplained, severe, and prolonged neonatal unconjugated hyperbilirubinemia.

Figure 3 presents the mean percentage of inhibition of duplicate estimates of glucuronyl transferase activity in guinea pig liver homogenates using *o*-aminophenol as a glucuronide receptor after the addition of milk from five mothers of the seven jaundiced infants and from control mothers. Three specimens of colostrum obtained from two mothers with jaundiced infants demonstrated 16 to 19% inhibition. Excluding these, the mean inhibition of glucuronyl transferase activity by milk from the mothers of the seven jaundiced infants was 79 ± 6.5% (SD). The mean inhibition of glucuronyl transferase activity by milk from control mothers was 11 ± 4.1% (SD). Inhibition exceeding 20% was observed with ten specimens of milk obtained from five mothers in the control group.

The duration of inhibition of *o*-aminophenol glucuronide formation *in vitro* was studied in milk from two mothers with jaundiced infants (Figure 4). The mother of baby A.M. continued to nurse her infant, whose serum bilirubin concentration slowly decreased to normal by day 35 (Figure 1), at which time inhibition of glucuronyl transferase activity by breast milk was 22%. The inhibitory activity of breast milk from the mother of baby L.M. persisted for 35 days, although jaundice disappeared in L.M. by the twentieth day of life (Figure 2).

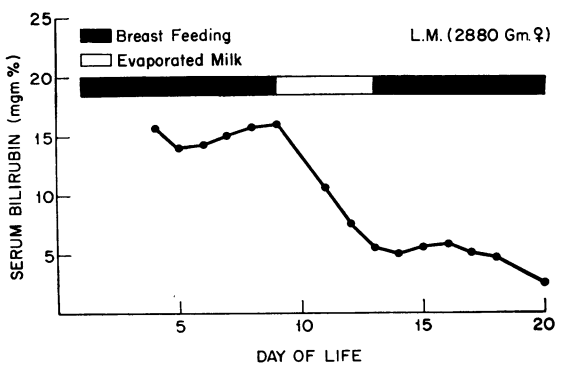

FIG. 2. COURSE OF HYPERBILIRUBINEMIA IN INFANT L.M., WHOSE MOTHER TEMPORARILY STOPPED BREAST FEEDING.

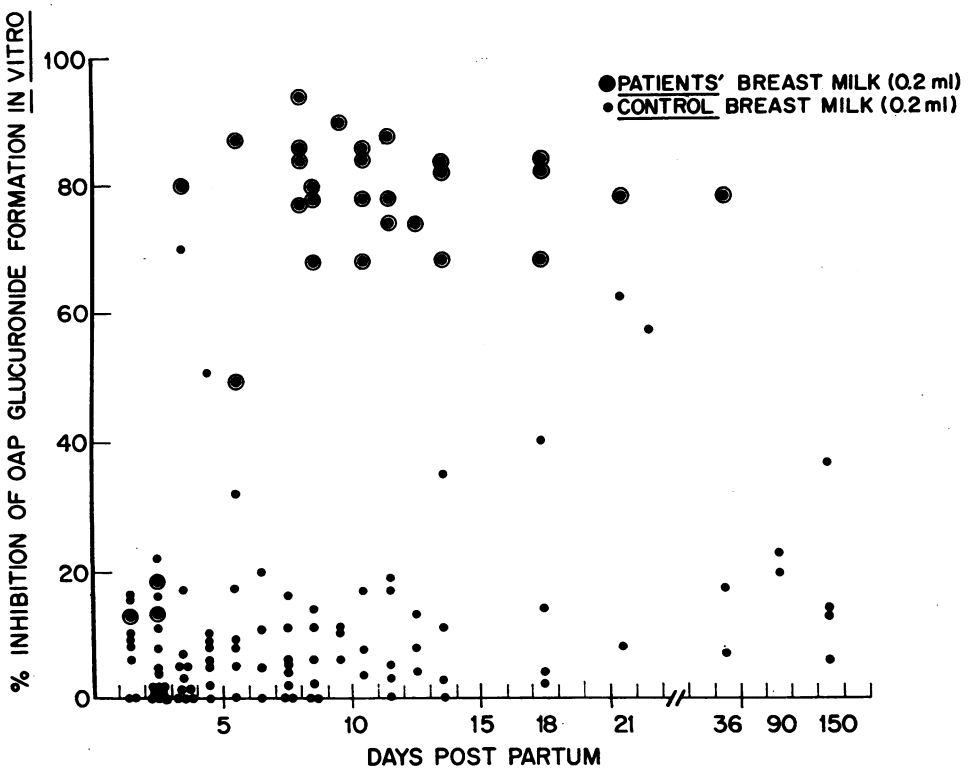

FIG. 3. INHIBITION OF GLUCURONYL TRANSFERASE ACTIVITY BY MILK FROM FIVE MOTHERS OF THE SEVEN JAUNDICED INFANTS AND FROM CONTROL MOTHERS IN VITRO. OAP = *o*-aminophenol.

The effect of milk from seven mothers with jaundiced infants and from control mothers on the formation of direct-reacting bilirubin by slices and homogenates of human, guinea pig, rat, and rabbit liver is presented in Table III. Direct-reacting bilirubin production by liver homogenates from each species exceeded that by slices of comparable wet weight from the same species. The

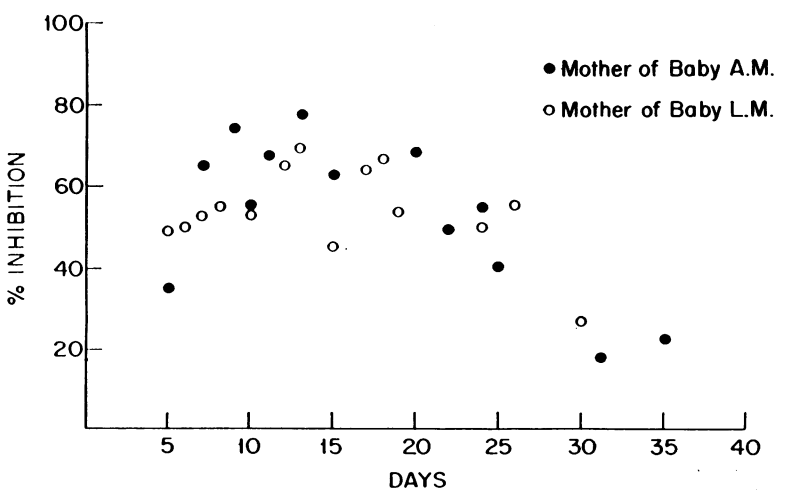

FIG. 4. COURSE OF INHIBITION OF GLUCURONYL TRANSFERASE ACTIVITY BY MILK FROM THE MOTHERS OF INFANTS A.M. AND L.M. IN VITRO.

TABLE III

Effect of milk from mothers of the seven jaundiced infants and from control mothers on direct-reacting bilirubin (DRB) formation by slices and homogenates of liver from four species in vitro

Species	Tissue preparation*	DRB formation without added milk	% inhibition of DRB formation by milk			
			Control mothers (10)†		Mothers of jaundiced infants (7)†	
			Mean	Range	Mean	Range
		μg conjugated/g/hr				
Human						
	Slice (6)	61 ± 11‡	5.1	0– 8	68	54–86
	Homogenate (7)	224 ± 21	6.4	0–15	59	52–71
Guinea pig						
	Slice (12)	27 ± 9	8.6	4–12	54	42–68
	Homogenate (15)	84 ± 6	4.1	0–15	64	52–75
Rabbit						
	Slice (5)	79 ± 11	6.8	0–21	61	41–90
	Homogenate (5)	94 ± 16	9.4	5–18	65	56–71
Rat						
	Slice (8)	47 ± 4	7.1	9–27	58	51–66
	Homogenate (10)	261 ± 18	6.2	4–11	77	72–81

* Figure in parentheses is number of animals.
† No. of milk specimens studied.
‡ Standard deviation.

mean inhibition of direct-reacting bilirubin production by milk from control mothers did not exceed 9.4% regardless of the tissue preparation or species. Milk from mothers of the seven jaundiced infants inhibited direct-reacting bilirubin formation by 41% or more regardless of the tissue preparation or species.

Serum obtained from six of the seven mothers of the jaundiced infants from postpartum days 10 to 26 inhibited *o*-aminophenol glucuronide formation by 7 ± 2.8% (SD) and direct-reacting bilirubin formation by 9 ± 4.6% (SD). Seventeen samples of sera obtained from 15 control mothers during the same postpartum interval inhibited *o*-aminophenol glucuronide formation by 9 ± 4.3% (SD) and direct-reacting bilirubin formation by 11 ± 3.6% (SD).

Milk obtained from 24 pregnant and postpartum Jersey, Guernsey, and Holstein cows inhibited *o*-aminophenol glucuronide formation by guinea pig liver homogenates by 4.2 ± 1.4% (SD).

Inhibition of *o*-aminophenol glucuronide formation by milk from three mothers of the seven jaundiced infants and three control mothers was unaffected by storage of milk at − 20° C for 2 weeks, immersion of milk in a boiling water bath for 1 hour, or prolonged dialysis against isotonic sodium chloride at 2° C.

Inhibition of *o*-aminophenol glucuronide formation by milk and milk extracts from the mother of baby A.M. is presented in Table IV. Each fraction was reconstituted with isotonic sodium chloride to its original volume and tested with controls. Thirty-five per cent of the inhibitory ac-

TABLE IV

Inhibition by milk and milk extracts from the mother of baby A.M. of o-aminophenol glucuronide formation by guinea pig liver homogenate in vitro

	Inhibition observed with 0.2-ml equivalents of milk	Remaining inhibition
	%	*% of original milk*
1 Original milk after thawing, mixing, and adjustment of pH to 7.4	65	
2 Combined ether extracts of 1 after evaporation of ether*	30	46
3 Combined eluates from appropriate zones on thin-layer chromatograms	23	35

* Resuspended in isotonic saline to reconstitute initial milk volume.

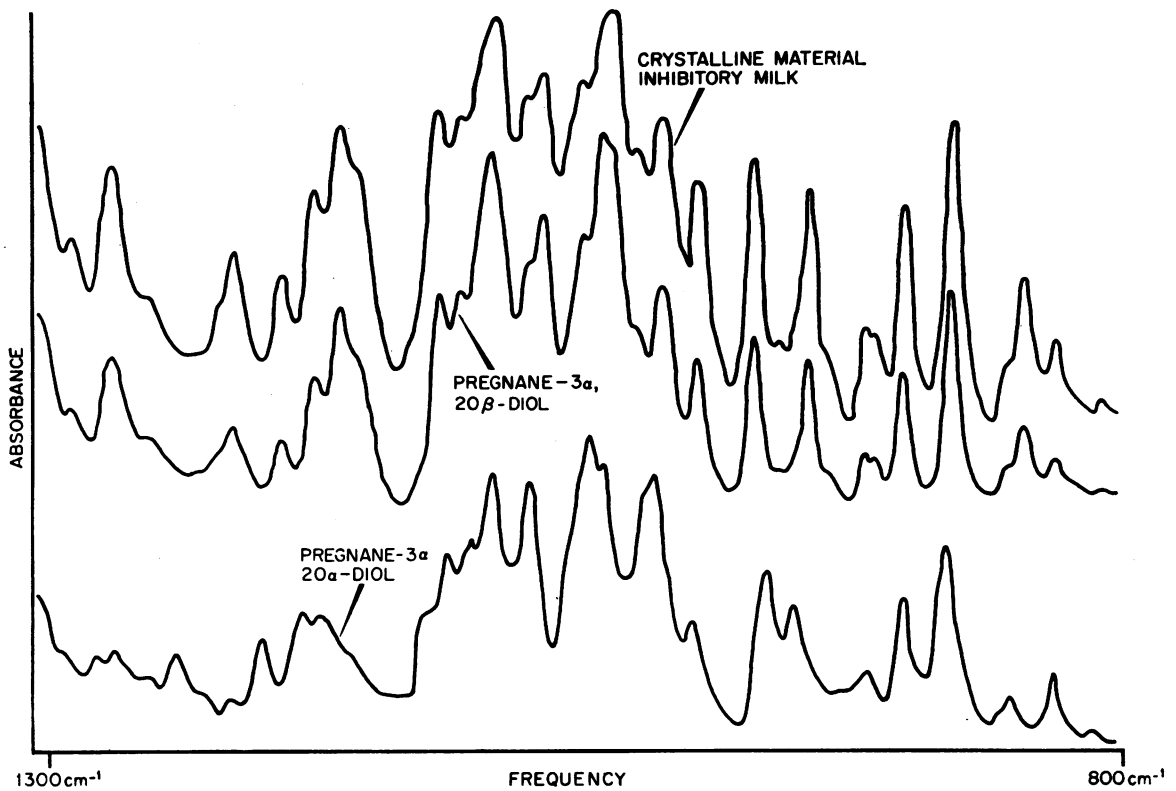

FIG. 5. INFRARED SPECTRUM OF CRYSTALLINE MATERIAL ISOLATED FROM INHIBITORY HUMAN MILK COMPARED WITH PURE PREGNANE-3(ALPHA), 20(BETA)-DIOL, AND PREGNANE-3(ALPHA), 20(ALPHA)-DIOL (potassium bromide dispersion, sodium chloride prism).

tivity in the original milk was recovered after the eluates of thin-layer chromatography zones having inhibitory activity were combined and assayed *in vitro*. No inhibition was observed with corresponding pooled extracts of noninhibitory milk from the five mothers whose infants were not icteric.

In each study of milk obtained from three mothers of the seven jaundiced infants, crystalline material was obtained from the eluates of inhibitory zones by slow evaporation of methanol. Approximately 50 to 100 mg of crystalline material was isolated from 75 to 400 ml of milk. The crystals were hexagonal and in polarized light demonstrated birefringence. The infrared spectrum of the isolated crystalline material was identical to that of pregnane-3(alpha), 20(beta)-diol and significantly differed from that of pregnane-3(alpha), 20(alpha)-diol (Figure 5). Gas-liquid chromatography of the isolated crystalline material was consistent with this identification (Table V). Figure 6 is a representative thin-layer chromatogram of the ether extract of the alkaline digest of an inhibitory breast milk from the baby A.M. The chromatogram was sprayed with sulfuric acid to locate organic material. The inhibitory zone of the patient's milk eluate demonstrates a spot that migrates the same as pregnane-3(alpha), 20(beta)-diol.

In Figure 7, the effects of equimolar amounts of authentic pregnane-3(alpha), 20(alpha)-diol

TABLE V

Gas-liquid chromatography of crystalline material isolated from milk, pregnane-3(alpha), 20(beta)-diol, and pregnane-3(alpha), 20(alpha)-diol *

	Column I	Column II
1 Crystalline material isolated from milk	0.67	1.69
2 Pregnane-3(alpha), 20(beta)-diol	0.67	1.69
3 Pregnane-3(alpha), 20(alpha)-diol	0.79	1.81

* Chromatography was performed on two columns; I) Gas Chrom P coated with 3% SE 30 and II) Gas Chrom P coated with 3% QF 1. The relative retention time is compared to cholestane.

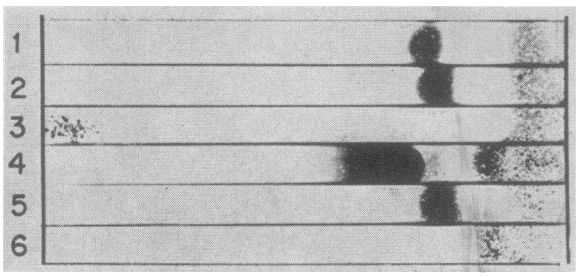

FIG. 6. THIN LAYER CHROMATOGRAM OF 1) PREGNANE-3(ALPHA), 20(ALPHA)-DIOL; 2) PREGNANE-3(ALPHA), 20(BETA)-DIOL; 3) PREGNANE-3(ALPHA), 20(ALPHA)-DIOL GLUCURONIDE; 4) THE ETHER EXTRACT OF THE NONSAPONIFIABLE SOLID FRACTION OF INHIBITORY BREAST MILK FROM THE MOTHER OF BABY A.M.; 5) THE CRYSTALLINE MATERIAL ISOLATED FROM THIS MILK; AND 6) CHOLESTEROL. The chromatogram has been sprayed with sulfuric acid.

and pregnane-3(alpha), 20(beta)-diol on glucuronyl transferase activity by guinea pig liver microsomes are plotted according to Lineweaver and Burke's method at five different substrate concentrations of *o*-aminophenol with an excess of UDPGA. Inhibition of glucuronyl transferase activity by equimolar amounts of the two steroids seems identical. The kinetic characteristics are those of competitive inhibition.

Discussion

An association between mothers' milk and the clinical syndrome is suggested by repeated occurrence of severe and prolonged neonatal unconjugated hyperbilirubinemia not due to known causes in breast-fed infants of the seven mothers; by rapid disappearance of hyperbilirubinemia after cessation of breast feeding; by absence of severe jaundice in bottle-fed siblings; and, in one case, by rapid decrease in hyperbilirubinemia when artificial feeding was temporarily substituted for breast feeding and subsequent prolonged hyperbilirubinemia when breast feeding was reinstituted. Drugs or acquired diseases are unlikely etiologic factors because the syndrome was observed in 13 of 14 breast-fed infants of the seven mothers over an 8-year period. The role of inherited factors in the pathogenesis of the syndrome is uncertain because the seven mothers represent four different ethnic groups, and none of their sisters, mothers, or grandmothers had children with severe and prolonged jaundice clinically associated with breast feeding.

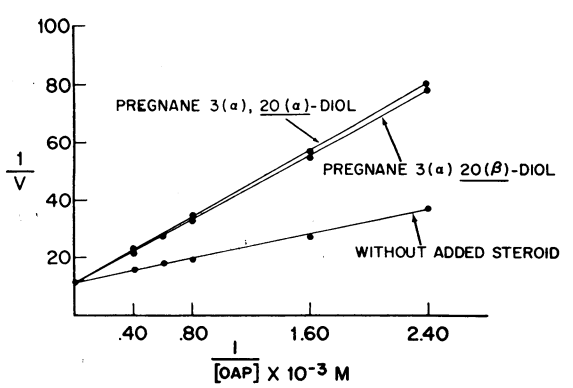

FIG. 7. INHIBITION OF GLUCURONYL TRANSFERASE ACTIVITY BY EQUIMOLAR AMOUNTS OF PREGNANE-3(ALPHA), 20(ALPHA)-DIOL AND PREGNANE-3(ALPHA), 20(BETA)-DIOL IN VITRO.

Jaundice in each infant was minimal during the first 5 days of life and was attributed to physiologic hyperbilirubinemia. Severe clinical icterus was not apparent until the seventh to tenth days. Whether physiologic hyperbilirubinemia persisted in these infants is not known, as serum bilirubin levels were not serially estimated and the cases were discovered in retrospect. Despite subsequent serum unconjugated bilirubin concentrations of 14.7 to 24.5 mg per 100 ml, neither signs nor symptoms of kernicterus were seen, probably because the blood brain barrier was already impermeable to unconjugated bilirubin (16).

Milk obtained from the mothers of the seven jaundiced infants after the third postpartum day consistently inhibited glucuronyl transferase activity *in vitro,* whereas three specimens of colostrum did not. Apparently the inhibitor is not present in colostrum and appears when true lactation begins on the third to fifth postpartum days. This observation may account for the apparent absence of severe hyperbilirubinemia during the first 5 days of life.

In etiology, this syndrome differs from other recognized conditions of severe, prolonged, nonhemolytic, unconjugated hyperbilirubinemia in full-term infants. An inherited homozygous deficiency in hepatic glucuronyl transferase results in the Crigler-Najjar syndrome (17–19). In the latter condition, severe jaundice occurs within hours after birth and is lifelong; the serum unconjugated bilirubin concentration is approximately 35 to 50 mg per 100 ml; kernicterus almost always

occurs, and there is no correlation with breast feeding. Another condition, transient familial neonatal hyperbilirubinemia, is manifested by transient nonhemolytic unconjugated hyperbilirubinemia within hours after birth, is unrelated to breast feeding, and occurs in most siblings (20). The serum unconjugated bilirubin concentration ranges from approximately 25 to 65 mg per 100 ml during the first 4 days of life, and kernicterus is frequently observed. Beginning in the second trimester of pregnancy, mothers of infants with this syndrome have in their plasma high titers of an unidentified substance (or substances) that inhibits glucuronyl transferase activity *in vitro* (21). In the present study, serum obtained from mothers of the seven jaundiced infants did not inhibit glucuronide formation *in vitro* more than was observed with sera from mothers of normal infants.

In our study, ten specimens of milk from five mothers whose infants were normal inhibited glucuronyl transferase activity *in vitro* in excess of 20%. The factor or factors responsible for the occasional inhibition observed in milk from mothers of anicteric infants are not known. Attempts to isolate an inhibitory factor in milk from two of these mothers and from three noninhibitory milk specimens were unsuccessful. These observations suggest that the difference between milk from mothers of the seven jaundiced infants and from control mothers may be qualitative, although the presence of small amounts of inhibitory steroid in normal milk cannot be excluded by these methods.

Saponification and other procedures used in the isolation of pregnane-3(alpha), 20(beta)-diol from milk do not hydrolyze pregnanediol glucuronide (22); therefore, the steroid is probably secreted in milk in the unconjugated form.

It was not possible to quantitate the amount of pregnane-3(alpha), 20(beta)-diol in serial daily specimens of milk. From the amount of steroid isolated from mothers' milk, we estimate that approximately 1 mg of pregnane-3(alpha), 20(beta)-diol is excreted per day in milk. In some cases, as illustrated in Figure 1, the infant's serum bilirubin concentration decreased in spite of continued breast feeding. Whether decreased hyperbilirubinemia is due to increased amounts of glucuronyl transferase or decreased amounts of inhibitor cannot be answered at this time.

Mothers' milk was equally inhibitory to direct-reacting bilirubin formation by slices of human and rat liver *in vitro*. Holton and Lathe (23) have demonstrated that pregnane-3(alpha), 20(alpha)-diol inhibits direct-reacting bilirubin formation by rat liver slices but not by slices of human liver *in vitro*. It is difficult to make any direct comparison between the results of these authors and the present study, as different biologic materials were used as sources of inhibitory activity.

Progestational agents have been suggested as factors in the etiology of neonatal nonhemolytic unconjugated hyperbilirubinemia. Lathe and Walker (13) reported inhibition of bilirubin conjugation in rat liver slices by serum from pregnant women and demonstrated a similar effect with progestational steroids *in vitro*. Pregnane-3(alpha), 20(alpha)-diol was isolated from pooled pregnancy serum and demonstrated to be a competitive inhibitor of glucuronyl transferase *in vitro* (15). A variety of synthetic progestational steroids inhibit glucuronyl transferase *in vitro*. This action may play a role in the jaundice associated with their administration, although a major functional effect on hepatic excretory transport has also been demonstrated (24–25). Neither progesterone nor pregnanediol have been previously associated with jaundice.

Equimolar amounts of pregnane-3(alpha), 20(alpha)-diol and pregnane-3(alpha), 20(beta)-diol equally and competitively inhibit glucuronyl transferase activity *in vitro*. These steroids may act in a similar fashion *in vivo*. Normally occurring delayed development of hepatic glucuronyl transferase activity in the newborn guinea pig appears to limit the over-all transfer of bilirubin from blood to bile *in vivo* (26). If the same occurs in human infants, a suitable environment is created for an inhibitor of glucuronyl transferase to produce severe and prolonged jaundice. This postulate is supported by the observation that oral administration of pregnane-3(alpha), 20(beta)-diol (1 mg per day) to two full-term infants beginning on the sixth and eighth days resulted in reversible unconjugated hyperbilirubinemia unrelated to hemolysis or liver damage (27). There appears to be nothing unique about the infants of mothers who secrete pregnane-3(alpha), 20(beta)-diol in their milk, as hyperbilirubinemia will prob-

ably develop in any newborn infant after administration of the steroid. Normally occurring delayed development of glucuronyl transferase activity and competitive inhibition *in vivo* appear to be major mechanisms in the pathogenesis of the syndrome.

Milk from pregnant and postpartum cows was not significantly inhibitory *in vitro*. This is consistent with the observation that less than 0.06% of administered progesterone-C^{14} is excreted in milk from pregnant cows in 48 hours (28).

The source of pregnane-3(alpha), 20(beta)-diol in the mothers' breast milk is unknown. Progesterone formation is believed to cease with expulsion of the placenta (29). There was no clinical evidence of retention of placental tissue in the mothers studied. Pregnanediol excretion in the urine normally decreases rapidly after delivery and virtually disappears by the third postpartum day (30). Milk remained inhibitory for more than 4 weeks in the two women who continued to nurse their babies.

Pregnane-3(alpha), 20(beta)-diol has not been demonstrated in human tissues, although it is a major progesterone metabolite in guinea pigs and cattle. Review of the literature reveals that the presence of this isomer in human tissues has not been intensively investigated.

At the same time as the mothers' milk strongly inhibited glucuronyl transferase activity *in vitro*, their sera were not more inhibitory than normal postpartum sera. These observations suggest that the serum concentration of pregnanediol is not greatly increased. Whether pregnane-3(alpha), 20(beta)-diol is synthesized in the breast before lactation and subsequently slowly released, or is synthesized in nonmammary tissue and concentrated in the breast either before or during lactation, is not known. Radioautographic studies in rabbits and goats demonstrate marked uptake of progesterone-C^{14} or its metabolites in lactating mammary glands (31).

Summary

A syndrome of severe and prolonged unconjugated hyperbilirubinemia has been described in association with breast feeding in seven unrelated full-term infants.

Milk obtained from the mothers of the seven infants consistently inhibited glucuronyl transferase activity *in vitro* when compared with 99 specimens of milk obtained from 71 women whose infants did not demonstrate this syndrome.

A steroid that competitively inhibits glucuronyl transferase activity *in vitro* has been isolated from inhibitory but not from noninhibitory human milk. The steroid has been identified as pregnane-3(alpha), 20(beta)-diol.

The precursor of pregnane-3(alpha), 20(beta)-diol, the mechanisms responsible for its presence in human milk, and the significance of the unusual isomer are unknown and require further study.

Acknowledgments

The authors gratefully acknowledge the kind cooperation of the following physicians who referred patients for this study: C. Bauman, R. Harris, G. Hershel, P. Mao, G. Wilkinson, D. Williams, and C. Wilson. The assistance of many members of the Departments of Obstetrics and Gynecology and Pediatrics of the Bronx Municipal Hospital Center, White Plains Hospital, Babies Hospital in the City of New York, and New Rochelle Hospital is gratefully appreciated. We are indebted to Beatrice S. Gallagher for infrared spectrometry and to Dr. Robert S. Rosenfeld for gas-liquid chromatography, both of Montefiore Hospital; to Mrs. J. Ben Ezzer and Miss Gayla Schildhaus for technical assistance; to members of the Department of Surgery of the Bronx Municipal Hospital Center for the human liver specimens; to Drs. M. Davidson, M. Cornblath, and C. Glassman and the milk banks at the University of Chicago, Boston Lying-In Hospital, and the Delaware Hospital for providing milk specimens for study; to Dr. Eric Bloch of the Department of Obstetrics and Gynecology for advice regarding isolation of the steroid; to Dr. Mervin Silverberg of Montreal for early discussions of this syndrome; and to Mr. S. Wildonney, who generously supplied milk specimens from his purebred cows.

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