Presence and changes in the concentration of vitamin D-binding protein throughout early lactation in human and bovine colostrum and milk

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Synthesis of vitamin D-binding protein (DBP) by explants of ovine mammary gland, and the changes in the concentration of this protein in human and bovine colostrum and milk throughout early lactation have been studied. The changes in the concentration of this protein were similar in human and bovine species. The highest concentration of DBP was found in the first milking (250 μg/mL and 111 μg/mL for bovine and human colostrum, respectively). The levels of DBP then decreased sharply during the first days of lactation, reaching stable values during the second week postpartum (6 μg/mL and 16 μg/mL, respectively). Milk and plasma DBP were immunologically identical by immunodiffusion and there was no cross-reaction between the two species. DBP synthesis in mammary gland explants could not be detected. The ratio of DBP to albumin in mature milk and especially in colostrum was much higher than in plasma. This could be due to the existence of a specific mechanism in the mammary gland cells for the transference of DBP from plasma to milk.

Keywords: Vitamin D-binding protein; DBP; milk; colostrum; lactation; mammary gland

Introduction

The vitamin D-binding protein (DBP), also called Group Specific Component (Gc) is a glycoprotein present in the plasma of most vertebrates and it has a molecular weight of about 52,000 in humans.[1] Since its discovery in 1947,[2] it has been known to be a highly polymorphic protein and up to now, more than 120 genetic variants have been recorded.[3] It is the main carrier protein for vitamin D and its hydroxylated metabolites in the plasma of vertebrates, showing the highest affinity for 25-hydroxy-cholecalciferol.[4] In addition, DBP binds actin with high affinity, causing its depolymerization or preventing the polymerization of actin released into the blood.[5]

This protein has a strong structural homology with albumin and α-fetoprotein, both of which are genetically related to DBP,[6,7] and consequently DBP shares some of their functional properties, such as the capacity to bind fatty acids,[8,9] particularly palmitic and oleic acids. Moreover, it has been reported that arachidonic acid can affect the binding of vitamin D by DBP in vitro.[10] As well as being present in the circulation, DBP has been detected on the surface of several cell types such as cytotrophoblasts isolated from human placentae, yolk sac endodermal cells, and some T- and B-lymphocytes. In B-cells, DBP seems to participate in the linkage of surface immunoglobulins.[11]

DBP is known to be synthesized in significant amounts only by the liver and secreted into the blood, though mRNA has also been found in many tissues (kidney, testis, abdominal fat, fetal yolk sac), but in levels 100–1000-fold less than in the liver.[12]

DBP has also been detected in the mature milk of several species,[13] but in levels much lower than in serum; the level in human milk is only about 2% that of serum.[14,15] Interestingly, the vitamin D activity in fresh human and bovine milk is found in the whey and not in the fat,[16] but following secretion of the milk, the vitamin D activity migrates to the lipid phase.[14]

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The origin of this protein in milk remains unclear, and its concentration throughout lactation has not been determined. The levels of milk proteins vary depending on the stage of lactation. Milk proteins derived from blood such as albumin, α_2-macroglobulin, or immunoglobulins, as well as those synthesized in mammary gland such as β-lactoglobulin, α-lactalbumin, or lactoferrin[17,18] are present in higher concentration in colostrum than in mature milk. Some of these proteins play important physiological roles in the newborn, such as immunoglobulins that confer immunity, or carrier proteins that supply oligoelements and vitamins required for the appropriate development.

The aim of this work has been to study the concentration of this protein in colostrum and milk throughout early lactation in both humans and cattle, and also to study the synthesis of the same protein by explants of ovine mammary gland. Such information might give a clue to its relation to the presence of the vitamin D activity in the whey fraction.[14] Furthermore, as a comparison, albumin levels also have been determined in the same samples.

Materials and methods

Samples of human colostrum and milk were collected from several healthy donors during the 12 first days of lactation (one milking per day). Bovine colostrum and milk, kindly supplied by a local farm (Tauste Ganadera, Zaragoza, Spain), were obtained from 10 Holstein cows throughout the lactation period. Samples were taken during the first 4 days postpartum (two milkings daily) and during the following 3 weeks (one milking per week). Ovine colostrum samples were obtained from sheep during the first 4 days of lactation. Samples were skimmed by centrifugation (2000 × *g* for 10 min at 4° C) and whey was obtained by chymosin (EC 3.4.23.4) coagulation followed by centrifugation. All samples were stored frozen at −25° C until analysis.

DBP and albumin were isolated in our laboratory from human and bovine serum by Cibacron Blue-Sepharose chromatography, gel filtration, and DEAE-Sephadex chromatography.[9] Traces of albumin were removed from DBP by immunoadsorption with Sepharose-anti albumin. The purity of these proteins was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis and by immunoelectrophoresis. Antisera were raised in rabbits as previously described.[19]

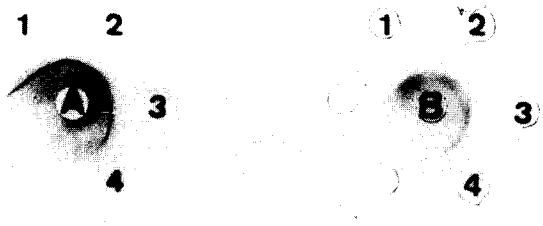

Figure 1 Double immunodiffusion. A) Antiserum against human serum DBP. 1) Purified human serum DBP; 2) human serum; 3) human colostrum whey; 4) human milk whey. B) Antiserum against bovine serum DBP. 1) Purified bovine serum DBP; 2) bovine serum; 3) bovine colostrum whey; 4) bovine milk whey.

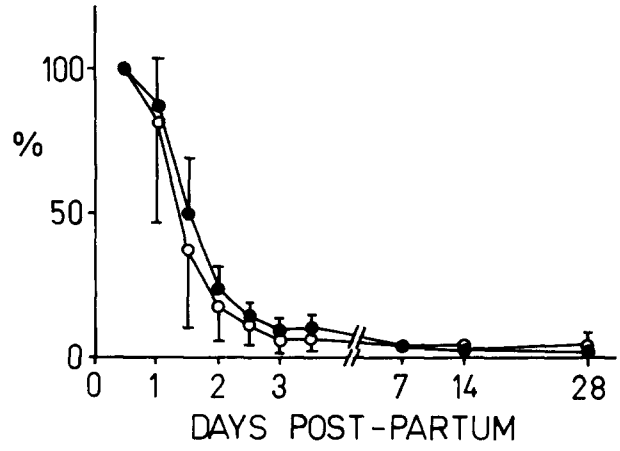

Figure 2 Changes in the concentration of DBP and albumin in cow's colostrum and milk during the first month of lactation. Values are expressed as a percentage of the first milking concentration (250 ± 40 mg/L and 3,129 ± 1,284 mg/L for DBP and albumin, respectively). Vertical bars indicate the standard deviation. ●-● DBP; ○-○ albumin.

DBP and albumin concentrations in whey samples were determined by quantitative immunoelectrophoresis according to Laurell[20] using purified proteins as standards. Proteins were quantified by the method of Lowry[21] using bovine serum albumin (fraction IV, Sigma Chemical Co. St. Louis, MO USA) as reference.

Immunological identity between serum and whey DBP was established by double immunodiffusion in agarose gels.[22]

DBP, albumin and lactoferrin mammary synthesis were determined in ovine mammary explants incubated with L-[3,4,5-^{3}H(N)]-leucine as described.[23] Ewe biopsies obtained 12-hours postpartum were incubated with [^{3}H]-leucine for 46 hours. Soluble synthesized proteins were passed through Sepharose-anti bovine DBP, albumin, or lactoferrin immunoadsorbents, which cross-react with their respective ovine proteins. The immunoadsorbents were prepared by coupling the appropriate antibodies to Sepharose 4B previously activated with CNBr.[24] Ovine mammary gland explants were chosen because ovine DBP reacts strongly with rabbit anti-bovine DBP. This antiserum was also used for the relative quantification (with respect to the serum level) of ovine DBP in colostrum samples.

Results

Figure 1 shows the existence of cross-reactivity by immunodiffusion between plasma and milk DBP. The precipitation lines formed against plasma and milk DBP were continuous indicating the immunochemical identity of these proteins. However, no cross-reaction was observed between human and bovine DBP.

The changes in the concentration of bovine DBP throughout early lactation are shown in *Figure 2*. The highest concentration of DBP was found in the first colostrum (250 μg/mL), which represents about 27% of the serum concentration. DBP levels fell sharply to about 22% of the initial value within the first 48 hours, and then declined slowly to 3.5% of the level in the

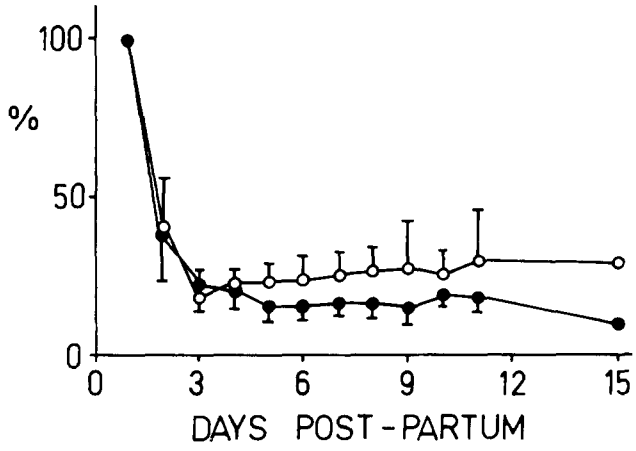

Figure 3 Changes in the concentration of DBP and albumin in human colostrum and milk during the first 15 days of lactation. Values are expressed as a percentage of the first milking concentration (112 mg/L and 1400 mg/L for DBP and albumin, respectively). Vertical bars indicate the standard deviation. ●-● DBP; ○-○ albumin.

first milking after 1 week postpartum. This level did not change significantly during the following 3 weeks of lactation. Albumin levels were also determined in the same whey samples, and similar changes in concentration were found. The highest concentration corresponded to the first colostrum (3.1 mg/mL) reaching a stable level (about 5% of the first milking) after 1 week postpartum.

A similar falling-off of the DBP level in the first days of lactation was also observed in sheep colostrum. Twenty-four hours after delivery, the level of DBP in colostrum was about 17% that of serum, and only about 2% at 96-hours postpartum.

Changes in the concentration of human DBP and albumin in colostrum and milk during early lactation are shown in *Figure 3*. The highest levels of DBP and albumin were also found in the first colostrum (0.11 and 1.4 mg/mL, respectively). Levels of both proteins fell to about 20% of their initial values within the first 48 hours of lactation. By day 4, definitive levels were reached (about 15% and 17% for DBP and albumin, respectively). Afterwards, these values remained approximately constant up to the end of the period studied.

Table 1 shows the concentration of human and bovine DBP and albumin found in serum, first colostrum, and mature milk. The ratio of DBP to albumin is also shown. This ratio is higher in milk or colostrum than in plasma in both species, being three and six times higher in bovine and human first colostrum than in their respective plasma.

Synthesis in vitro of DBP and albumin by culturing explants of ovine mammary gland in presence of [^{3}H]-leucine was not detected. The total radioactivity associated with synthesized soluble proteins was 2×10^6 cpm. After passing the total proteins through a Sepharose–anti-bovine DBP or anti-albumin column followed by exhaustive washing with 0.5 M NaCl, 0.1 M phosphate buffer, pH 7.4, no significant increase in radioactivity was recorded when the anti-DBP and anti-albumin immunoadsorbents were eluted with 0.1 M Tris-HCl pH 2.8 at 4° C. Positive control experiments using the same explants showed that labeled lactoferrin retained by the immunoadsorbent accounted for 68,000 cpm, which represents about 3.4% of the synthesized total soluble proteins.

Discussion

The results obtained in this work indicate that DBP levels in mammary secretion vary widely depending on the time of lactation. The highest concentration of DBP corresponds to the first milking postpartum in both bovine and human milk. Levels declined sharply within the first 2 days postpartum, followed by a slower decrease up to the end of the first week. They then remained stable during the period studied (3 weeks). The final concentration of DBP found in human milk is similar to that reported by Hollis,[15] about 12 μg/mL[15] and is higher than that found in definitive cow's milk, which is about 6 μg/mL. Both human and bovine DBP in milk were immunologically indistinguishable from their respective serum DBP.

The pattern of change in the concentration of hu-

Table 1

	Bovine		
	DBP (μg/mL)	Albumin (μg/mL)	DBP/ALB
Serum	946 ± 360	34600 ± 7370	1/37
First colostrum	250 ± 40 (27)	3100 ± 1280 (8)	1/12
Definitive milk	6 ± 2 (1)	170 ± 108 (0.5)	1/20
	Human		
	DBP (μg/mL)	Albumin (μg/mL)	DBP/ALB
Serum	403	35000	1/88
First colostrum	111 (28)	1400 (4)	1/13
Definitive milk	16 (4)	395 (0.7)	1/23

DBP and albumin levels and ratio of DBP to albumin in human and bovine serum, first colostrum, and definitive milk.
Values are the mean ± SD of 10 cows, and the mean of two specimens in human samples. Figures in brackets are the percentage in relation to serum.

man, bovine, and ovine DBP throughout early lactation is similar to that observed for other milk proteins derived from blood such as IgG,[25] ribonuclease,[26] transferrin,[17] and α_2-macroglobulin[17] as well as for other milk proteins synthesized in the mammary gland such as β-lactoglobulin and α-lactalbumin[23] or lactoferrin.[17]

The origin of milk DBP is still unknown. We have not observed synthesis of DBP or albumin by explants of ovine mammary gland, and this method would be sensitive enough to detect the synthesis of about 10% of the DBP or albumin found in colostrum. Thus, the main bulk of milk DBP is not synthesized by the mammary gland. However, the ability of mammary cells to synthesize DBP cannot be discounted because DBP mRNA has been detected by the polymerase chain reaction in several tissues besides the liver.[12] Nevertheless, all the extrahepatic sources of DBP described up to now express this protein at very low levels.

It is known that DBP possesses only one affinity binding site for vitamin D and that in serum less than 5% of these binding sites are occupied by vitamin D sterols,[27] but nothing is known about the saturation of milk DBP. From our data on DBP levels and those reported for the concentration of vitamin D sterols in definitive milk,[14,28-30] it can be estimated that more than 95% of binding sites in milk DBP would remain free of sterols, as occurs in plasma, even assuming that all the milk vitamin D is in the whey fraction. This last supposition is supported by the results of Hartman and Dryden,[16] who found that the biological activity of human and bovine milk is associated with the whey, and more recently by the results of Hollis et al.,[14] who showed that the content of vitamin D in freshly drawn milk is the same as that of fresh whey, indicating that vitamin D is found in the aqueous phase of fresh milk and not in the lipid phase. However, when milk is stored after collection for several hours, vitamin D migrates from DBP to fat globules. This fact should be taken into account when milk samples are processed to analyze the content of vitamin D.

Ratios of DBP to albumin in mature milk and colostrum are higher than those found in blood serum, particularly in human milk. This fact suggests the existence of a specific mechanism in the mammary gland that permits a greater transference of DBP than of albumin from plasma to milk. Nevertheless, given the apparently similar saturation with vitamin D of the milk and serum DBP, this mechanism would not be specific enough to distinguish between the holo and apo forms of the DBP.

DBP has been reported to be associated to the surface of several cell types,[11] if so, some cells, such as lymphocytes and desquamation mammary epithelial cells, could be responsible for the transference of DBP into the milk. However, because DBP is thought to be tightly bound to the cell membranes, cell-associated DBP would be discarded during the obtention of the whey samples.

Lakdawala and Widdowson[31] determined the concentration of vitamin D, as sulphate, in the aqueous phase of human whey collected at different stages of lactation by a chemical method. Interestingly, the time-course pattern of this vitamin D-sulphate is similar to that reported here for the concentration of DBP throughout early lactation. They obtained high levels in the first days of lactation, a sharp decline during the first week, and stable levels thereafter. However, it has been shown that this compound possesses little or no biological activity.[32]

The concentration of DBP in human or bovine colostrum is about 10 times higher than that of definitive milk. Thus, colostrum could be a rich source of vitamin D associated with DBP and could play an important role in the intake of vitamin D sterols in the newborn. However, levels of DBP in milk remain high only during the first days postpartum, and the hypothetical content of vitamin D sterols associated with this protein would be far below the recommended intake of vitamin D sterols for infants of 400 IU (10,000 ng) vitamin D per day.[33]

Given the various properties attributed to DBP other than transport of vitamin D sterols,[5,8,9,11] it is possible that its presence in milk could be related to other physiological roles. Further investigations will be required to elucidate these points.

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