Considerable variation in the concentration of osteopontin in human milk, bovine milk, and infant formulas¹

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ABSTRACT

Osteopontin (OPN) is a multifunctional bioactive protein that is implicated in numerous biological processes such as bone remodeling, inhibition of ectopic calcification, and cellular adhesion and migration, as well as several immune functions. Osteopontin has cytokine-like properties and is a key factor in the initiation of T helper 1 immune responses. Osteopontin is present in most tissues and body fluids, with the highest concentrations being found in milk. In the present study, ELISA for human and bovine milk OPN were developed and OPN concentration in human breast milk, bovine milk, and infant formulas was measured and compared. The OPN concentration in human milk was measured to approximately 138 mg/L, which corresponds to 2.1% (wt/wt) of the total protein in human breast milk. This is considerably higher than the corresponding OPN concentrations in bovine milk (~18 mg/L) and infant formulas (~9 mg/L). Moreover, bovine milk OPN is shown to induce the expression of the T helper 1 cytokine IL-12 in cultured human lamina propria mononuclear cells isolated from intestinal biopsies. Finally, the OPN concentration in plasma samples from umbilical cords, 3-mo-old infants, and pregnant and nonpregnant adults was measured. The OPN level in plasma from 3-mo-old infants and umbilical cords was found to be 7 to 10 times higher than in adults. Thus, the high levels of OPN in milk and infant plasma suggest that OPN is important to infants and that ingested milk OPN is likely to induce cytokine production in neonate intestinal immune cells.

Key words: osteopontin, human milk, bovine milk, infant formula

INTRODUCTION

Milk is an excellent source of nutrition, vitamins, minerals, and energy and it contains several bioactive proteins that play important roles in infant immunity. During the first phase of life, when infants are immunologically immature, breast-feeding provides an effective protection against infections (Lawrence and Pane, 2007); breast-fed infants have a reduced frequency of infectious episodes compared with formula-fed infants (Huffman and Combest, 1990; Cunningham et al., 1991; Dewey et al., 1995). This protective effect of human milk is likely achieved through the numerous bioactive components contained in the milk, which include proteins and peptides with antimicrobial and immune-stimulating properties such as lactoferrin, lactoperoxidase, lysozyme, and IgA (Hanson et al., 2003; Field, 2005). However, a significant protein compositional dissimilarity exists between human milk and that of bovine milk and infant formulas (Hambreus et al., 1977). Human breast milk is evolutionarily optimized to fulfill the needs of the developing infant; hence, efforts have been made to improve the composition of infant formulas to mimic that of breast milk. This is achieved by purifying milk fractions and individual milk proteins and supplementing these to infant formula as is done, for instance, with lactoferrin (Tomita et al., 2002). A less well-characterized bioactive protein that could potentially play a role in neonate and infant immunity and development is the cytokine osteopontin (**OPN**).

Osteopontin is a multifunctional protein that is implicated in a wide number of biological processes including cell survival, bone remodeling, inhibition of ectopic calcification, and immune modulatory functions (Sodek et al., 2000). The protein is expressed in most tissues and body fluids including blood, urine, bile, and milk (Sodek et al., 2000). Osteopontin is a highly acidic phosphorylated glycoprotein containing an integrin-binding Arg-Gly-Asp (RGD) sequence. Bovine milk OPN contains 27 phosphoserine residues and 1 phosphothreonine (Sørensen et al., 1995); in human milk, up to 34 phosphoserines and 2 phosphothreonines have been

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identified (Christensen et al., 2005). The phosphorylations are arranged in clusters of 3 to 5 phosphoresidues and are located in the target sequence of the mammary gland casein kinase or casein kinase II (Sørensen et al., 1995; Christensen et al., 2005). This arrangement of phosphorylations is identical to the distribution of phosphate groups on the major milk proteins, the caseins, (Mercier, 1981) as well as component PP3 (Sørensen and Petersen, 1993a). Furthermore, bovine and human milk OPN contain 3 and 5 O-glycosylated threonines, respectively. In milk, OPN is present as an intact protein or in several proteolytically generated N- and C-terminal fragments (Senger et al., 1989; Sørensen and Petersen, 1993b; Sørensen et al., 2003). An N-terminal fragment, which can be generated by proteolytic cleavage close to the RGD sequence (Senger et al., 1989), has been shown to increase its cell binding properties both through the RGD sequence and through exposure of a cryptic integrin binding site (Sodek et al., 2000).

The biological role of OPN in milk is not clear; however, several functions could be hypothesized. Osteopontin has been reported to be involved in mammary gland development and differentiation (Nemir et al., 2000), and high levels of OPN expression have been observed in the mammary gland in early lactation (Rittling and Novick, 1997). Osteopontin has also been reported to interact with the milk proteins lactoferrin, lactoperoxidase, and IgM through electrostatic and affinity interactions (Azuma et al., 2006). This prompted the hypothesis that OPN could potentially act as a transporter of these immunomodulating and antibacterial proteins to their site of action. Furthermore, the highly anionic nature of the protein could enable OPN to form soluble complexes with calcium ions and thereby inhibit unintentional calcium crystallization and precipitation in milk (Gericke et al., 2005). Supporting an inhibitory function of OPN in ectopic calcification, an *in vivo* model using OPN-deficient mice showed diminished calcification upon exogenous addition of the protein (Ohri et al., 2005). In addition, OPN is involved in the urinary tract's defense against the formation of renal stones because OPN can inhibit growth and aggregation of calcium oxalate monohydrate crystals (Asplin et al., 1998).

Osteopontin has been shown to play a pivotal role in the development and maintenance of immune responses because it influences the function of immune cells such as macrophages, dendritic cells, and T cells (Wang and Denhardt, 2008). The T cells are known to differentiate into 2 major subpopulations, T helper 1 (**Th1**) and T helper 2 (**Th2**) cells, differing in the type of cytokines secreted upon antigen stimulation. The most important inducers of the Th1 response are IL-12, IFN- γ , and type 1 interferon, whereas IL-4 and IL-10 are important

inducers of the Th2 response (Santana and Rosenstein, 2003). The generation of a Th1 response is essential for the clearance of intracellular pathogens such as viruses and certain bacteria, but it is also linked to autoimmune diseases when response is excessive. Osteopontin has been shown to be an important early regulator of Th1-mediated immunity by inducing secretion of the Th1 cytokine IL-12 and inhibiting the production of the Th2 cytokine IL-10 (Ashkar et al., 2000). Moreover, several studies have indicated that OPN enhances host resistance to infections (Patarca et al., 1989; Nau et al., 1999; Rollo et al., 2005) and recently we have shown that OPN acts as an opsonin that enhances bacterial phagocytosis (Schack et al., 2009).

Measurements of early and mature human milk showed OPN concentrations of approximately 1,500 and 900 mg/L, respectively (Nagatomo et al., 2004). These values correspond to up to 10% of the total protein amount in human breast milk, which seems surprisingly high. In the present study, a human OPN ELISA developed in-house and 2 commercial ELISA were used to measure the OPN concentration in human breast milk samples. It was shown that the commercial ELISA, which was used in the aforementioned study (Nagatomo et al., 2004), significantly overestimated the OPN concentration. An ELISA for bovine milk OPN was also developed and used to measure the OPN content in bovine milk and select infant formulas. We demonstrated that the OPN concentration in human milk is considerably higher than in bovine milk and infant formulas and that OPN levels in infant plasma were 10-fold higher than in adults. Finally, we showed that milk OPN was able to induce IL-12 secretion from human lamina propria mononuclear cells (**LPMNC**) isolated from gut biopsies. Together, these data could suggest that OPN is involved in the infant immune response.

MATERIALS AND METHODS

Purification of Human and Bovine Milk OPN

Osteopontin was purified from human milk essentially as described (Senger et al., 1989). However, instead of the final reversed-phase HPLC step, the supernatant resulting from the barium chloride and sodium citrate precipitation was dialyzed against 0.1% formic acid overnight at 4°C, centrifuged (31,000 $\times g$, 25 min), and the resulting supernatant was lyophilized. To separate full-length OPN (~90%) from the N-terminal fragment (~10%), OPN was dissolved in 0.1 M ammonium bicarbonate and subjected to gel-filtration chromatography on a Superdex 200 HR10/30 (GE Healthcare, Uppsala, Sweden). The peak corresponding to full-length OPN

was collected and lyophilized. The purity of the OPN (+98%) was verified by SDS-PAGE, reversed phase-chromatography, and N-terminal sequencing.

Bovine OPN was purified from fresh pooled bovine milk as described previously (Sørensen and Petersen, 1993b). To increase purity, OPN was further purified by reversed-phase HPLC on a Vydac C₄ column (The Separations Group, Hesperia, CA). The purity and concentration of human and bovine OPN were determined by SDS-PAGE, Edman sequence, and AA analyses.

Polyclonal Antibodies

Immunizations and collection of antiserum were performed at Dako A/S (Glostrup, Denmark) and MedProbe (Oslo, Norway) for bovine and human OPN, respectively. The IgG fraction of the antiserum was purified on a Protein A Sepharose CL-4B column (Uppsala, Sweden). The specificities of the antibodies were checked and verified by Western blotting analyses of milk samples and purified OPN. No cross-reactivity with other milk proteins was observed.

For biotinylation, antibodies were dialyzed 3 times against 500 mL of 0.1 M sodium borate buffer (pH 8.8) overnight at 4°C. One-sixth (vol/vol) of 10 mg/mL of N-hydroxysuccinimide (BHNS) biotin in dimethylsulfoxide (DMSO) was added to the antibodies. After 4 h of incubation at room temperature, 20 µL of 1 M of ammonium bicarbonate per 250 µg of BHNS was added to the mixture and further incubated for 10 min at room temperature. Finally, the biotinylated antibodies were dialyzed twice against 500 mL of PBS overnight at 4°C and the antibody concentration was calculated by measuring the absorbance at 280 nm.

Milk and Plasma Samples

Milk from 29 mothers aged 22 to 37 yr (mean = 29.6 yr) was sampled 6 to 58 d postpartum (mean = 20.4 d postpartum) at the maternity ward at the University Hospital of Aarhus (Denmark). The milk was obtained by breast pumping and 2 mL from each milking was sampled and pooled to obtain whole-day milk. The cream was extracted by centrifugation ($3,000 \times g$, 5 min) and the skimmed milk collected, aliquoted, and stored at -20°C until analyses.

Pooled unpasteurized bovine milk samples were obtained at a local dairy over a period of 4 mo, pooled, and processed as described for human milk. Five commercially available infant formulas [XO1 and Premium XO (Nam Yang Dairy Products Co. Ltd., Seoul, South Korea), Nan 1 and Nidina (Nestlé, Copenhagen, Denmark), and Allomin 2 (Beauvais, Taastrup, Denmark)]

were prepared according to the manufacturers' instructions.

Blood samples were collected from umbilical cords (10 samples), from 3-mo-old infants (10 samples), from nonpregnant adults (10 samples), and from pregnant or postpregnant adults (7 samples) at Aarhus University Hospital, Skejby Sygehus (Denmark). The samples from the pregnant or postpregnant group were collected during the third trimester, at delivery, or 5 to 10 d after birth. The plasma was prepared immediately after blood collection and was stored at -80°C until analyses.

ELISA Procedures

For our in-house human OPN ELISA, MaxiSorp immunoassay plates (Thermo Fisher Scientific, Roskilde, Denmark) were incubated with antibodies (1 µg/mL in 0.1 M of sodium carbonate, pH 9.8) overnight at 4°C and were subsequently washed extensively with PBS buffer and blocked with 3% BSA. Milk samples were diluted 10,000 to 30,000-fold in Tris-Tween buffer (0.1% Tween 20, 10 mM of Tris-HCl, 1 M of NaCl, 30 mM of CaCl₂, 2% BSA, pH 7.4), applied to the assay plate, and incubated for 1 h at room temperature, followed by extensive washing. Captured OPN was detected by incubation with 0.5 µg of biotinylated polyclonal antibody per well followed by incubation with 100 µL of horseradish peroxidase-conjugated streptavidin (diluted 1:8,000) for 1 h at room temperature. Color development was obtained using 1,2-phenyldiamine-dihydrochloride (OPD) tablets (Dako) and the reaction was stopped by addition of 1 M of H₂SO₄. Color intensity was measured at 490 nm using an ELISA reader (Bio-tek Instruments Inc., Winooski, VT). Full-length OPN purified from human milk was quantified by AA analysis and used as the standard. All analyses were performed in triplicate. Two commercial human OPN ELISA were purchased from R & D Systems (Abingdon, UK) and IBL (Immuno-Biological Laboratories; Gunma, Japan), respectively, and were used according to the manufacturers' descriptions. The OPN concentration in human milk was measured using the in-house human OPN ELISA as well as the ELISA from R & D Systems and IBL. The ELISA from R & D Systems was also used to measure the OPN concentration in plasma samples from umbilical cords, 3-mo-old infants, and pregnant and nonpregnant adults.

A bovine milk OPN ELISA was developed as described for the human milk OPN ELISA using antibodies raised against bovine milk OPN (Dako) and purified full-length bovine milk OPN as standard. The in-house bovine OPN ELISA was used to measure OPN concentration in bovine milk and in infant formulas. The

analyses of the infant formulas were based on 125 g/L of formula solutions and the result was calculated as milligrams of OPN per liter of ready-to-feed formula.

The protein concentration in the milk samples was measured by Bradford analyses with BSA as the standard using a Bio-Rad protein assay kit (Richmond, CA).

IL-12 Expression in Human LPMNC

Mucosal biopsies from colons of healthy adults were taken during colonoscopy. The biopsies were cultured in the presence of IL-2 and IL-4 as described (Agnholt and Kaltoft, 2001). This culture system promoted the growth of mucosal T cells, which showed preserved cellular features and phenotype of CD4⁺ T cells (Kelsen et al., 2005). High purity of CD4⁺ T cells was reached after 3 to 4 wk of culture; however, trace amounts of other cell types including monocytes were occasionally found. Culture plates were coated overnight at 4°C by incubation with 1 mL of PBS in the presence or absence of bovine milk OPN (1 mg/mL). The LPMNC were cultured (10⁶ cells/mL) for 24 h and, subsequently, aliquots of supernatants were sampled for analyses. Cytokine-matched antibody pairs were obtained from R & D Systems for determination of IL-12. The detecting antibodies were all biotinylated. A time-resolved fluorometric assay applying Europium (Eu³⁺)-labeled streptavidin and a Delphia 1234 fluorometer (Wallac, Turku, Finland) were used to determine the levels of IL-12. Obtained values were averages of 3 ELISA readings in triplicate experiments and were representative of 3 healthy persons.

Statistical Analysis

Statistical analysis of data was done by Student's *t*-test. Difference was considered to be statistically significant at *P* < 0.05.

Ethical Considerations

The study of human samples was approved by the local ethics committee of the County of Aarhus, Denmark. Blood samples were taken anonymously from a scientific blood bank containing blood from infants and umbilical cords.

RESULTS

OPN in Human Milk

The OPN concentration in 29 breast milk samples obtained 6 to 58 d postpartum was measured by a newly

developed in-house ELISA. Concurrently, the protein concentration in the samples was measured by Bradford analysis. The combined results are shown in Table 1. The OPN concentration in human milk ranged from 18 to 322 mg/L with an average value of 138 mg/L (SD = 79). The protein content of the milk samples varied from 1,834 to 15,430 mg/L, with an average value of 8,062 mg/L (SD = 3,680). Thus, according to these analyses, OPN constitutes an average of 2.1% (wt/wt; SD = 1.4) of the total protein in human milk. The OPN content in 14 of the milk samples was also measured with ELISA kits from R & D Systems and IBL (Table 2). The average OPN content for the ELISA from R & D Systems was 144 mg/L (SD 83), which is not significantly different (*P* = 0.45) from the value of 122 mg/mL (SD = 70) obtained with our in-house ELISA. In contrast, the OPN concentration measured with the ELISA from IBL was 1,175 mg/mL (SD = 1,137), which is significantly higher (*P* < 0.01) than the values obtained with the in-house ELISA. The results obtained with the 3 tests were well-correlated, with correlation coefficients squared in excess of 0.8 (Table 1). In conclusion, the OPN concentrations measured by the newly designed in-house ELISA were in close agreement with the data obtained with the ELISA kit from R & D Systems, whereas values measured with the IBL kit were approximately an order of magnitude higher.

To confirm the finding of an OPN concentration of 138 mg/L in human breast milk, purified full-length human milk OPN was quantified by AA analysis and a sample containing 10 ng of OPN was measured by the in-house ELISA and the 2 commercial ELISA. Contents of 10 ng and 13 ng were measured with the in-house ELISA and the R & D ELISA, respectively, whereas the IBL ELISA showed a content of 114 ng. This confirms our findings and emphasizes that the ELISA from IBL considerably overestimated the content of OPN in milk.

OPN in Bovine Milk and Infant Formulas

The level of OPN in bovine milk was measured by use of the in-house bovine OPN ELISA. The OPN concentration in bovine milk was measured as 18 mg/L and the total protein content was 35 g/L according to the literature. Consequently, the OPN content in bovine milk is 0.05% (wt/wt) of the total protein. Hence, the average OPN concentration related to total protein is significantly higher in human breast milk than in bovine milk (2.1 vs. 0.05%). This suggests that the OPN content in infant formulas, based on bovine milk, may also be considerably lower than the concentration found in human milk. Consequently, we measured the concentration of OPN in 5 commercially available in-

Table 1. Osteopontin (OPN) concentration in human breast milk from 29 women

Sample	Age of mother (yr)	Time postpartum (d)	OPN concentration ¹ (mg/L)	Total protein concentration ² (mg/L)	OPN/total protein (wt/wt, %)
1	28	58	66	8,550	0.77
2	35	13	257	8,327	3.09
3	28	12	201	13,268	1.51
4	25	6	65	11,868	0.55
5	31	9	46	15,430	0.30
6	25	12	22	11,442	0.19
7	30	33	102	10,000	1.02
8	31	39	114	10,157	1.12
9	30	17	67	10,328	0.65
10	23	12	212	10,936	1.94
11	30	10	105	12,576	0.83
12	31	27	157	5,376	2.92
13	30	17	185	4,459	4.15
14	31	37	103	5,568	1.85
15	30	10	239	12,628	1.89
16	22	15	100	6,664	1.50
17	27	26	100	9,706	1.03
18	30	14	102	3,963	2.57
19	35	24	322	9,660	3.33
20	32	15	18	2,077	0.87
21	32	22	47	1,834	2.56
22	35	28	171	5,219	3.28
23	28	28	192	4,602	4.17
24	30	17	245	8,832	2.77
25	27	22	154	3,426	4.50
26	30	11	206	4,069	5.06
27	29	15	175	4,738	3.69
28	37	15	40	6,520	0.61
29	27	28	203	11,562	1.76
Mean	29.6	20.4	138	8,062	2.1
SD	3.5	11.3	79	3,680	1.4

¹OPN concentration measured with an in-house-developed ELISA.

²Protein concentration determined by Bradford analysis.

fant formulas (Table 3) and found values in the range of 5.3 to 13.0 mg/mL in ready-to-use formulas.

OPN-Induced IL-12 Expression in Human LPMNC

Previous studies from our laboratory have shown that OPN is partly resistant to digestion by human neonatal gastric juice (Chatterton et al., 2004) and that a fraction of the ingested OPN is therefore expected to reach the intestine in intact form. To evaluate the potential immune modulatory effect of orally ingested OPN, LPMNC (which represent the first line of the immune system at the gut mucosal surface) were stimulated with OPN and the IL-12 secretion was measured. Upon stimulation of LPMNC with OPN, an IL-12 production of 16.6 pg/mL (SD 2.1) was observed, which is a significant increase ($P < 0.01$) compared with 3.3 pg/mL (SD 3.2) produced by the control cells (data not shown). We cannot define the exact cellular origin of the secreted IL-12. In the present culture system we obtained a purity of T cells of approximately 95%, but remaining monocytes may also contribute to the IL-12 production (Sato et al., 2005). In conclusion, we showed

that bovine milk OPN is able to induce expression of the potent Th1 cytokine IL-12 in human intestinal immune cells, indicating a role in the modulation of intestinal immunity.

OPN in Plasma

The high concentration of OPN in human milk suggests that it could play a role in the development of the infant. To examine potential differences in OPN content in blood from adults and infants, plasma samples from umbilical cords, 3-mo-old infants, nonpregnant adults, and pregnant or postpregnant adults were analyzed. The OPN concentration in plasma from adults was measured to be 35 ng/mL (Figure 1), which is in agreement with previously reported blood values (Agnholt et al., 2007; Sennels et al., 2007). No difference was observed between the adult nonpregnant and the pregnant or postpregnant groups. In comparison, the OPN concentrations in plasma from 3-mo-old infants and from umbilical cords were 342 ng/mL and 263 ng/mL, respectively (Figure 1), which is significantly higher than the levels in adults ($P < 0.01$).

Figure 1. Osteopontin (OPN) concentrations in plasma from umbilical cords, 3-mo-old infants, adults, and pregnant or postpregnant women, measured by ELISA. The number of samples analyzed for each group is indicated and standard errors are represented with vertical bars. *The OPN content in plasma from 3-mo-old infants was significantly higher than that from umbilical cords ($P < 0.05$). **The OPN content in plasma from umbilical cords and from 3-mo-old infants were both significantly higher than that from adults and pregnant or postpregnant women ($P < 0.01$).

DISCUSSION

Osteopontin is a multifunctional protein that is implicated in numerous cellular and physiological processes (Sodek et al., 2000). It is present in high concentrations in milk, where it can be hypothesized to be involved in the development and maturation of the infant immune system. Surprisingly high concentrations of OPN were found in human breast milk by use of an OPN ELISA from IBL. Osteopontin concentrations in early milk (3–7 d) and in mature milk (1 mo postpartum) were reported at 1,493.4 mg/L and 896.3 mg/L, respectively (Nagatomo et al., 2004). Though it has previously been reported that the OPN concentration in milk is quite high (Senger et al., 1989; Sørensen et al., 2003), these values, which suggest that OPN should constitute more than 10% of the total protein in breast milk, seemed to be overestimated.

To estimate the concentration of OPN more precisely, we designed an in-house OPN ELISA based on antibodies raised against highly purified full-length milk OPN. Subsequently, we analyzed milk samples from 29 women and found an average OPN concentration of 138 mg/mL (SD 79; Table 1). The values measured in this study comprise the natural mixture of full-length (>80%) and cleaved OPN that is present in milk. Depending on the degree of proteolysis taking place in the milk, OPN concentration may deviate slightly from the reported values. For example, the value will be slightly higher if a smaller OPN fragment is present in milk and not recognized by the antibodies used in this ELISA. Conversely, the concentration might be slightly lower if smaller fragments or peptides are recognized and converted to the mass of the full-length protein

Table 2. Osteopontin (OPN) concentrations in milk samples analyzed with an in-house-developed ELISA and ELISA from R & D Systems and IBL¹

Sample	OPN concentration (mg/L)		
	In-house	R & D ²	IBL ³
1	66	86	582
2	257	309	4,075
3	201	182	1,962
4	65	71	394
5	46	66	250
6	22	25	172
7	102	152	601
8	114	227	1,342
9	67	63	203
10	212	267	2,810
11	105	98	255
12	157	144	1,356
13	185	182	1,447
14	103	141	996
Mean	122 ^a	144 ^a	1,175 ^b
SD	70	83	1,137

^{a,b}Mean values within a row with unlike superscript letters are significantly different ($P < 0.01$).

¹Regression equations: In-house versus R & D: $1.07x + 13.88$ ($r^2 = 0.81$); in-house versus IBL: $14.9x - 638.30$ ($r^2 = 0.85$); R & D versus IBL: $12.65x - 640.11$ ($r^2 = 0.85$).

²R & D Systems, Abingdon, UK.

³IBL (Immuno-Biological Laboratories), Gunma, Japan.

using the standard curve. The results obtained with our in-house ELISA corresponded well with those obtained with the ELISA from R & D Systems, whereas values approximately 10 times higher were measured with the ELISA from IBL (Table 2). This is in line with the results of recent comparisons of commercial OPN ELISA, which also showed substantial variation in the OPN concentration in plasma depending on the type of ELISA used (Vordermark et al., 2006; Plumer et al., 2008). For example, an approximately 10-fold higher

Table 3. Osteopontin (OPN) concentrations in selected infant formulas compared with bovine and human milk

Item	OPN concentration ¹ (mg/L)
Infant formula ²	
XO1	7.9
Premium XO	13.0
Nan 1	10.3
Nidina 1	5.3
Allomin 2	6.4
Milk	
Bovine milk	18
Human milk	138

¹The OPN concentration was measured in ready-to-feed formulas based on 125 g/L of powder.

²XO1 and Premium XO manufactured by Nam Yang Dairy Products Co. Ltd. (Seoul, South Korea); Nan 1 and Nidina 1 manufactured by Nestlé (Copenhagen, Denmark); Allomin 2 manufactured by Beauvais (Taastrup, Denmark).

concentration of human full-length OPN was measured with the IBL ELISA compared with 3 other ELISA (Plumer et al., 2008).

The high OPN values measured with the IBL ELISA may be explained by the fact that this ELISA had not been validated for measurements in milk. In contrast, our in-house ELISA was based on polyclonal antibodies raised against the human milk protein and utilized purified human milk OPN as the standard. Neither the epitopes recognized by the antibodies used in the R & D ELISA nor the nature of the OPN used for the immunizations were disclosed. However, the assay has, among others, been validated for quantitative determination of OPN in human milk, according to the manufacturer's instructions. Based on the highly consistent values obtained with our in-house ELISA and the R & D ELISA, we concluded that the concentration of OPN in human breast milk was approximately 138 mg/L, with considerable individual variation. Likewise, we determined the OPN concentration in bovine milk and 5 selected infant formulas to be 18 mg/L and an average of 8.6 mg/L, respectively (Table 3). Thus, the concentration of OPN in bovine milk and infant formula was considerably lower than that in human milk.

In this study, we measured the OPN plasma levels in umbilical cords, infants, pregnant adults, and pregnant or postpregnant adults and found a surprisingly high OPN level in the umbilical cord and infant plasma (Figure 1). Thus, the OPN plasma level varies considerably from infancy to adulthood. High plasma OPN levels have been reported in several pathological processes, including Crohn's disease (Sato et al., 2005; Agnholt et al., 2007), multiple sclerosis (Vogt et al., 2003), rheumatoid arthritis (Ohshima et al., 2002), and especially in several types of cancer (Rodrigues et al., 2007), in some of which OPN has also been suggested as a clinical marker of metastatic disease. The high level of OPN in infant plasma suggests that OPN plays a natural role in infant development and that a high plasma concentration of OPN is not linked to pathologic conditions but rather can be a natural part of infant development.

The present data show that infant formulas and bovine milk have a significantly lower content of OPN than human breast milk. This could be important for infant immunity because OPN has been shown to possess a key role in the development of Th1 immunity as well as to enhance host resistance to infections (Nau et al., 1999; Ashkar et al., 2000). We showed that OPN induced the production of the Th1 cytokine IL-12 in cultured LPMNC (which represent the first line of the immune system at the gut mucosal surface). Thus, the intestinal immune system may be modulated by ingested OPN. This is further supported by the observation that milk OPN is partially resistant to proteolysis by

neonatal gastric juice (Chatterton et al., 2004). Thus, part of the ingested OPN survives the passage through the infant's stomach and reaches the immune cells present in the intestine. The ingested OPN might further act directly against pathogenic bacteria in the gastrointestinal tract; we have recently shown that OPN is able to opsonize bacteria, which leads to an enhanced phagocytosis (Schack et al., 2009). Supplementation of infant formula with OPN could therefore potentially provide the infant with an important immunological factor.

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