Potential host-defense role of a human milk vitamin B-12–binding protein, haptocorrin, in the gastrointestinal tract of breastfed infants, as assessed with porcine haptocorrin in vitro\(^1-3\)

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**ABSTRACT**

**Background:** Limited information exists on the biological role of a vitamin B-12–binding protein, haptocorrin, in human milk. The expression of haptocorrin by human mammary epithelial cells and its presence in human milk suggest a potential physiologic function in breastfed infants.

**Objective:** We investigated the extent to which haptocorrin could withstand proteolytic degradation and exert antimicrobial activity under in vitro conditions designed to simulate the gastrointestinal tract of breastfed infants.

**Design:** An in vitro model that simulates infant gastric and intestinal digestion was developed. The structural stability of porcine haptocorrin after exposure to digestive enzymes (pepsin and pancreatin) was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, Western blot analysis, column chromatography, and matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). The antimicrobial activity of haptocorrin was determined by incubating haptocorrin with enteropathogenic *Escherichia coli* O127 strain 2348/69 and monitoring bacterial growth.

**Results:** The structural analysis of haptocorrin exposed to enzymes did not show a decrease in molecular weight, which indicated that haptocorrin can survive proteolytic degradation. Both haptocorrin exposed to digestive enzymes and undigested haptocorrin inhibited the growth of enteropathogenic *E. coli* and did so to a similar extent. Thus, haptocorrin in vitro not only retains its structure after exposure to proteases but also exhibits antimicrobial activity.

**Conclusion:** These results suggest that haptocorrin may exert a host-defense function against pathogens in the gastrointestinal tracts of breastfed infants.

**KEY WORDS** Vitamin B-12, cobalamin, enteropathogenic *E. coli*, haptocorrin, human milk proteins, human milk host-defense factors, breast milk, breastfed infants, breastfeeding

**INTRODUCTION**

It has been well established that human milk contains numerous proteins that benefit the newborn infant. Proteins in human milk not only provide amino acids but also bind to and facilitate the absorption of nutrients, stimulate the growth and development of the intestinal epithelium, and aid in the digestion of other nutrients (1). In addition, breast milk has been shown to contain antimicrobial and antiviral components. The antimicrobial agents include lactoferrin (2), lysozyme (3), secretory immunoglobulin A (4), kappa-casein (5), and oligosaccharides and glycoconjugates (6, 7). The antiviral agents in human milk include mucins from the milk fat globule membrane (8) and lactoferrin (9). It was suggested that the presence of these defense components in human milk contribute to the lower incidence and shorter duration of infections in breastfed infants than in their formula-fed counterparts (10).

For the breastfed infant, dietary vitamin B-12 is supplied by human milk exclusively bound to a vitamin B-12–binding protein, haptocorrin (11). Haptocorrin has a molecular weight of \(\approx 68 \text{kDa}\) and is heavily glycosylated; \(\approx 34\%\) of its molecular weight consists of carbohydrates (12). There is a high binding capacity for vitamin B-12 in human milk; concentrations of vitamin B-12-unsaturated haptocorrin (apo-haptocorrin) are much higher than are those of vitamin B-12-saturated haptocorrin (holo-haptocorrin) (11, 13–15). Apart from its high affinity for vitamin B-12, the exact function of haptocorrin is not well understood. Because human milk haptocorrin is expressed by human mammary epithelial cells and is not passively transferred from the maternal circulation (16), we speculate that haptocorrin has some physiologic function in the neonatal gastrointestinal tract. Previous studies have proposed a bacteriostatic function for haptocorrin, but direct experiments with pathogenic bacteria have not been performed (17–19).

In order for human milk haptocorrin to exert a physiologic function in the gastrointestinal tracts of breastfed infants, haptocorrin must first be able to survive the passage through the intestine. In the present study, we simulated in vitro the con-
ditions of the neonatal stomach and intestine and determined the structural stability of haptocorrin against the proteolytic activity of the digestive enzymes. Because human haptocorrin is not commercially available, porcine haptocorrin was used; it is similar to human haptocorrin with regard to amino acid composition, amino-terminal sequence, antigenic properties, and carbohydrate composition. Porcine haptocorrin has been well characterized for use in several studies (14, 20–25). We also assessed its ability to inhibit the growth of a strain of *Escherichia coli* known to cause diarrhea in infants. We theorized that if haptocorrin exhibited this function in vitro, it could have a similar effect in the gastrointestinal tracts of breastfed infants.

**MATERIALS AND METHODS**

**Sample preparation**

Porcine haptocorrin (21 800 U/mg protein, as specified; 1 U will bind 1 × 10⁻⁹ g vitamin B₁₂; Sigma Chemical Co, St Louis) was resuspended in 1 mL phosphate-buffered saline (PBS: 120 mmol NaCl/L, 2.7 mmol KCl/L, 10 mmol NaPO₄/L, pH 7.6). Porcine haptocorrin was used in these experiments because of its biochemical and immunologic similarity to human haptocorrin (14, 20, 22, 23, 25), which is not commercially available, and because the amount of haptocorrin required for conducting the experiments in this study greatly exceeded the amount that can be isolated by our purification methods (16). The resuspended haptocorrin was divided into two 0.5-mL aliquots (=0.46 mg/mL) representing apo-haptocorrin and holo-haptocorrin. The holo-haptocorrin was formed by incubating apo-haptocorrin with 20 μL of a 1 mg/mL solution of cyanocobalamin for 1 h at room temperature. Free vitamin B₁₂ was removed by application of the sample to a PD-10 desalting column (Amersham Pharmacia, Piscataway, NJ). Reaction volumes for apo-haptocorrin and holo-haptocorrin were brought up to 1 mL with the addition of either PBS or human milk. Human milk was collected at 2 mo postpartum from a healthy donor. For the PBS control reaction, human serum albumin (1 mg/mL) was resuspended and brought up to a final volume of 1 mL with PBS.

**In vitro digestion of haptocorrin**

The following is a modification of the procedure described by Rudloff and Lönnerrdal (26). To mimic conditions in the infant stomach, apo-haptocorrin (0.23 mg/mL) and holo-haptocorrin (0.23 mg/mL) in PBS or human milk and serum albumin in PBS were adjusted to pH 3.5 with 1 mol/L HCl (≈9 μL for samples in PBS and ≈13 μL for samples in human milk). Approximately 2 μL 2% pepsin in 0.001 mol/L HCl (wt:vol, 3100 U/mg solid, Sigma Chemical Co) was added to all the samples, which were then placed in a shaking incubator for 30 min at 37°C. The enzyme-to-substrate ratio (wt:wt) was between 1:20 and 1:30, depending on the protein content of the samples. Then, to simulate conditions in the infant intestine, the pH of the samples was increased gradually to 7.0 with 0.5 mol/L NaHCO₃ (≈9 μL for samples in PBS and ≈13 μL for samples in human milk), which subsequently inactivates pepsin activity. Next we added 4 μL of a 0.4% pancreatic solution in 0.1 mol/L NaHCO₃ (wt:vol, 8 × USP specification, Sigma Chemical Co). The amount of pancreatic enzymes added was similar to that found in the duodenal juices of human infants (27). Samples were then placed in a shaking incubator for 1 h at 37°C. The pH of the samples was rechecked before placing the samples in a 90°C water bath for 3 min to inactivate the enzymes. The experiments were repeated 5 times.

**Sodium dodecyl sulfate–polyacrylamide gel electrophoresis**

For the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), apo-haptocorrin and holo-haptocorrin samples in PBS or human milk were diluted 1:1 in sample buffer (0.0625 mol tris-HCl/L (pH 6.8), 2% SDS, 25% glycerol, and 0.01% Bromphenol Blue). Approximately 20 μg protein was applied to each well of a 10–12% gradient mini-gel (BioRad, Hercules, CA). Gels were run for 40 min at 200 constant volts in tris-glycine buffer (0.025 mol tris-HCl/L, pH 8.3; 0.192 mol glycine/L, 0.1% SDS). For the detection of proteins, gels were stained overnight with Coomassie Brilliant Blue R (Sigma Chemical Co). Destaining with acetic acid:ethanol:water (10:25:65, v:v:v) was stopped when the background became clear.

**Western blot analysis**

After protein separation by SDS-PAGE, proteins were electrobotted onto a nitrocellulose membrane (Schleicher and Schuell, Keene, NH) in tris-glycine buffer at a constant current of 60 mA for 1 h at 4°C. The membrane was blocked overnight in 4°C in 4% bovine serum albumin in PBST (PBS, pH 7.4, 0.05% Tween-20) to reduce nonspecific binding of antiserum to the membrane. After the blocking step, the membrane was washed 3 times for 15 min with PBST and then incubated with rabbit anti-human haptocorrin (provided by Dr K Kobayashi, Hokkaido University, Hokkaido, Japan) at 1:10 000 dilution for 1 h at room temperature. The membrane was washed 3 times for 15 min with PBST and subsequently incubated with donkey anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Dako, Carpinteria, CA) at 1:10 000 dilution in PBST for 1 h at room temperature. The membrane was again washed 3 times for 15 min with PBST and the bound antibody was detected with an enhanced chemiluminescent system using horseradish peroxidase/hydrogen peroxide catalyzed oxidation of luminol (Amer- sham Pharmacia).

**Column chromatography**

Apo-haptocorrin and holo-haptocorrin samples in PBS before and after exposure to proteases (200 μL) were injected into a fast-protein liquid chromatography system with a Superose 12 gel filtration column (Amersham Pharmacia). Proteins were eluted with PBS at a flow rate of 0.25 mL/min. Protein peaks were monitored at 280 nm.

**Matrix-assisted laser desorption ionization–time of flight mass spectrometry**

Mass spectrometry analysis was performed at the Analytical Chemistry Division, Nutrition Research, Wyeth Nutritional International (Collegeville, PA). Apo-haptocorrin and holo-haptocorrin samples in PBS before and after exposure to proteases were desalted by using a C₁₈ silica resin Zip Tip (Millipore, Bedford, MA) and mixed with sinapinic acid matrix (1:1; Hewlett-Packard, Wilmington, DE) before crystallization on a gold-coated 10-positi on probe. Mass spectra were acquired by using a Hewlett-Packard G2025A linear time of flight mass spectrometer operated in positive ion mode.
FIGURE 1. Structural stability of apo-haptocorrin (apo-HC) and holo-haptocorrin (holo-HC) in human milk before and after exposure to proteolytic enzymes: an in vitro digestion experiment was carried out to mimic the conditions of the infant stomach and small intestine. A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10–12% gel) showed a 68-kDa band corresponding to haptocorrin after exposure to proteolytic enzymes. B) Western blot analysis showed positive immunoreactivity to haptocorrin, which suggested that haptocorrin was structurally intact. SDS-PAGE confirmed that sufficient quantities of enzymes were used in this experiment, because human milk/lactalbumin (14 kDa), caseins (20–40 kDa), and serum albumin (64 kDa) (which co-migrates with haptocorrin) were virtually absent after proteolytic digestion (n = 5).

**Antimicrobial activity of haptocorrin**

Enteropathogenic *E. coli* (EPEC) O127 strain 2348/69 (provided by Dr Jim Nataro, Center for Vaccine Development, University of Maryland School of Medicine, Baltimore) was cultivated overnight in 40 mL Bacto Synthetic Broth AOAC, pH 7.1 (Becton Dickinson, Sparks, MD) containing 0.1% dextrose under anaerobic conditions at 37°C. EPEC was used because it is one of the most common causes of gastrointestinal infection with diarrhea in infants (28). Apo-haptocorrin and holo-haptocorrin samples in PBS before and after exposure to proteases (2, 5, and 10 μg/mL) were incubated in a 96-well plate (Nunc, Naperville, IL) with 10^7 viable EPEC per well that was obtained by adding 0.2 mL of a 1:100 dilution of the overnight culture. The concentration range of haptocorrin used in this assay correlates with the vitamin B-12-binding capacity of human milk haptocorrin (29–32). Samples were incubated for 0.5–28 h at 37°C. Growth of EPEC was monitored at 620 nm at all time points by using a Multiskan Ascent enzyme-linked immunosorbent assay plate reader (Labsystems, Helsinki). Growth of EPEC in the presence of haptocorrin was compared with the vitamin B-12-binding capacity of human milk haptocorrin (29–32). Samples were incubated for 0.5–28 h at 37°C. EPEC was monitored at 620 nm at all time points by using a Multiskan Ascent enzyme-linked immunosorbent assay plate reader (Labsystems, Helsinki).

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**Results**

**Structural stability of haptocorrin**

In vitro digestion of haptocorrin was carried out in the presence of human milk, not only because this would most closely resemble what newborn infants consume, but also because some human milk proteins susceptible to proteolytic degradation (eg serum albumin, α-lactalbumin, and caseins) would serve as indicators that sufficient amounts of enzymes were added. SDS-PAGE showed a 68 kDa protein which is present after in vitro digestion in the presence of human milk (Figure 1A). SDS-PAGE also showed that serum albumin (64 kDa), which co-migrates with haptocorrin, caseins (20–40 kDa), and α-lactalbumin (14 kDa) are virtually absent after proteolytic digestion, which confirms that sufficient amounts of enzymes were added to the reaction mixture. Positive immunoreactivity exhibited by Western blot analysis for both apo-haptocorrin and holo-haptocorrin after they were subjected to pepsin and pancreatic proteases shows that haptocorrin has the capability to resist degradation by proteolytic enzymes (Figure 1B) and that the decrease in the width of the band in the 60-kDa range on SDS-PAGE is a result of degradation of serum albumin and not haptocorrin.

To assess the extent to which haptocorrin can resist proteolytic degradation in the absence of human milk, both apo-haptocorrin...
and holo-haptocorrin were subjected to in vitro digestion in PBS. SDS-PAGE did not reveal a decrease in the molecular weight of either apo-haptocorrin or holo-haptocorrin after exposure to proteolytic enzymes compared with that of the unexposed haptocorrin (data not shown). Also, Western blot analysis showed positive immunoreactivity to haptocorrin incubated in PBS after exposure to proteolytic enzymes; these results were similar to the results for haptocorrin incubated in human milk. Furthermore, we observed complete degradation of the positive control, human serum albumin dissolved in PBS, signifying that sufficient amounts of enzymes were used (data not shown).

The structural integrity of holo-haptocorrin in PBS after exposure to proteases, as assessed by gel filtration column chromatography, is shown in Figure 2. The elution profile for haptocorrin exposed to proteolytic enzymes is very similar to that for the unexposed haptocorrin sample, except for an additional peak that eluted at ≈19 mL. SDS-PAGE revealed that this peak corresponded to enzymes used in the reaction mixture. Similar results were observed for apo-haptocorrin (not shown); n = 4. V₀, void volume; Vₜ, total bed volume.

Antimicrobial actions of apo-haptocorrin and holo-haptocorrin

To measure the antimicrobial effects of apo-haptocorrin and holo-haptocorrin after exposure to proteolytic enzymes, EPEC was cultured in the presence of haptocorrin. A growth-inhibitory effect of apo-haptocorrin and holo-haptocorrin on EPEC after in vitro digestion was seen at all concentrations of haptocorrin (2, 5, and 10 μg/mL; Figure 4A and B). At 10 h, we observed significant (P < 0.001) reductions in EPEC growth of 9%, 24%, and 27% with 2, 5, and 10 μg apo-haptocorrin/mL, respectively, compared with growth of the control (0 μg apo-haptocorrin/mL). At 17 h, EPEC growth was reduced by 26%, 39%, and 38% with 2, 5, and 10 μg apo-haptocorrin/mL, respectively (Figure 4A; P < 0.001). For holo-haptocorrin at 10 h, reductions in EPEC growth of 21%, 21%, and 11% occurred with 2, 5, and 10 μg holo-haptocorrin/mL, respectively (Figure 4B; P < 0.001). Further reductions in EPEC growth of 35%, 33%, and 27% were observed at 17 h with 2, 5, and 10 μg holo-haptocorrin/mL, respectively, compared with growth of the control (Figure 4B; P < 0.001). Differences in the reduction in EPEC growth among the 3 holo-haptocorrin concentrations were not significant (P > 0.05). Similar inhibitory effects on EPEC growth were observed for both apo-haptocorrin and holo-haptocorrin samples not exposed to proteases (data not shown). The antimicrobial effect of haptocorrin was also observed when the EPEC mixture containing 5 μg holo-haptocorrin/mL was plated on trypticase soy agar plates. Growth of EPEC was drastically reduced, by ≈70%, in the presence of haptocorrin at both time points (ie, 10 and 21 h) (Figure 5).

DISCUSSION

The antiinfectious properties of human milk are well established. Specific host-defense factors in milk, such as secretory immunoglobulin A and lactoferrin, resist proteolytic degradation and survive passage through the gastrointestinal tract of breastfed infants (33, 34), subsequently exerting their physiologic function in the infants (35, 36). The exact function of haptocorrin has not been elucidated. It has been proposed that human milk haptocorrin has the capability to withhold vitamin B-12 from pathogens that require this vitamin in the infant intestine, thereby regulating...
the establishment of normal intestinal microflora (17). In addition, results from animal studies suggest that haptocorrin may facilitate vitamin B-12 absorption in the piglet (37, 38). More recently, we found evidence at the cellular level that haptocorrin mediates vitamin B-12 absorption in breastfed infants during the neonatal period when the intrinsic factor system (the mechanism by which vitamin B-12 is absorbed in adults) may not be functioning to its full capacity (39). However, in order for haptocorrin to exert its biological function in the infant gut, haptocorrin needs to survive the conditions of the upper gastrointestinal tract. In the current study, porcine haptocorrin was used because it is commercially available and has extensive biochemical and immunologic similarities to human haptocorrin (14, 20–25).

FIGURE 4. Growth-inhibitory effect of (A) apo-haptocorrin and (B) holo-haptocorrin on enteropathogenic Escherichia coli (EPEC) over time. Apo-haptocorrin and holo-haptocorrin samples in phosphate-buffered saline (2 μg/mL, ▲; 5 μg/mL, ●; and 10 μg/mL, ▣) after exposure to proteases were incubated in a 96-well plate with ~1 × 10⁷ viable EPEC organisms per well in synthetic broth for 0.5–28 h at 37°C. Test samples were compared with the control, which was phosphate-buffered saline without haptocorrin (0 μg/mL, ■), after exposure to proteases. A reduction in the growth of EPEC (± SD; n = 4) was observed at all concentrations of both apo-haptocorrin and holo-haptocorrin by 10 h and was maintained throughout 28 h. *All haptocorrin concentrations were significantly different from the control (P < 0.001) by one-factor ANOVA and Tukey’s test at a given time point.

In the neonatal stomach, the degree of protein hydrolysis is limited. Although hydrogen-potassium ATPase, the enzyme necessary for the secretion of hydrogen ions by the parietal cells of the stomach, is present in its functional form from 13 wk of gestation, HCl secretion in the neonate is not at the level of that in adults (40). Thus, the pH of the gastric contents in the newborn is ~4.0–4.5 but decreases over time (41, 42). Therefore, in the present in vitro digestion studies, we used a pH of 3.5 rather than the pH of 1–2 that is typically found in adults. Pepsin activity has also been detected in the stomach as early as 16 wk of gestation; however, the secretory mechanism in the neonate is not as developed as that of adults (43, 44). It has been shown previously that pepsin activity in a 1-mo-old infant is only 18% of that of adults (42, 45). In addition, because the pH for maximum pepsin activity is ~2, pepsin may not have been able to act at its full capacity at pH 3.5.

Furthermore, the development of a functional response by the pancreas is not complete at birth (46). In piglets, amounts of gastric and pancreatic proteases are low during the first 3–4 wk of age, but increase markedly thereafter (47). The activity of intestinal enterokinase, the enzyme that converts the proteolytic enzyme trypsinogen to trypsin (which in turn activates other zymogens), is also low at birth but increases with age (48). Enterokinase activity at birth has been shown to be 25% of that of 1-y-old infants (49). Moreover, the transit time through the infant gastrointestinal tract is rapid during the neonatal period, thereby decreasing the time that proteins are exposed to proteases (50). The structural analysis of the haptocorrin sample exposed to proteolytic enzymes by MALDI-TOF MS did not reveal a decrease in molecular weight, suggesting that haptocorrin remained intact. Results from the in vitro digestion of haptocorrin incubated in PBS or in human milk showed intact haptocorrin; therefore, it does not appear that a component of human milk contributed to its resistance against proteolytic degradation. Although in vivo experiments were not performed in this study,
haptocorrin may also be resistant to proteolytic degradation in breastfed infants. Trugo and Newport (22) found that porcine milk haptocorrin, regardless of cobalamin saturation, was resistant to degradation in piglets.

Because haptocorrin survived in vitro exposure to digestive enzymes in both human milk and PBS, it is possible that some biochemical characteristic unique to haptocorrin may be contributing to its stability. Haptocorrin is a heavily glycosylated protein; carbohydrates account for ≈30% of its molecular weight. The carbohydrate moieties may stabilize the conformation, protect haptocorrin from proteolysis, or both. A previous study by Gordon et al (51) showed that the carbohydrate core of intrinsic factor (the vitamin B-12-binding protein involved in the absorption of dietary vitamin B-12 in the ileum of adults) may play a role in protecting the protein from hydrolysis by pancreatic proteases in the intestinal lumen. Further studies are needed to test the extent to which the glycans of haptocorrin are a factor in its resistance against proteolytic degradation.

The potential for haptocorrin to escape proteolytic degradation in vivo suggests a possible physiologic role for haptocorrin in the gastrointestinal tract of the breastfed infant. Although previous studies have proposed a bacteriostatic function for haptocorrin in the breastfed infant, direct measurements of the inhibitory activity of haptocorrin on _E. coli_ growth have not been conducted. Gullberg (17) proposed a bacteriostatic role for haptocorrin on the basis of the high vitamin B-12-binding capacity of human milk; however, no microbiological experiments were conducted. In addition, Ford (18) used whole sow milk to examine the bacteriostatic effects of haptocorrin on _E. coli_ native to bovine. However, because whole sow milk was used, the bacteriostatic effects specific to haptocorrin could not be dissociated from those of other milk proteins with similar antimicrobial activity. Our in vitro results showed a growth-inhibitory effect of both apo-haptocorrin and holo-haptocorrin on EPEC. Furthermore, because differences in the ability to suppress the growth of EPEC were not observed between haptocorrin that had been exposed to proteolytic enzymes and haptocorrin that had not, the results suggest that haptocorrin is functional even after exposure to proteases.

The lack of bacterial colonies formed in the presence of haptocorrin suggests a bacteriostatic effect of haptocorrin on EPEC. The decrease in absorbance at 620 nm for haptocorrin-EPEC mixtures further supports this effect (Figure 4). The exact mechanism by which haptocorrin, regardless of vitamin B-12 saturation, exerts this bacteriostatic activity on EPEC needs further investigation. Because both apo-haptocorrin and holo-haptocorrin are present in milk, we speculate that _J_ haptocorrin itself may possess a unique biochemical property that exerts bacteriostatic activity (a plausible property for apo-haptocorrin and holo-haptocorrin), or _2_ haptocorrin may inhibit EPEC growth by sequestering vitamin B-12, rendering it unavailable for _E. coli_ utilization (a plausible action for apo-haptocorrin—this may explain why in vitro, the decrease in absorbance at 620 nm is not seen until ≈10 h).

It has been suggested previously that haptocorrin in human milk has host-defense activity, but experiments using bacteria pathogenic to humans had never been conducted. The present study shows that in vitro, haptocorrin can withstand proteolytic degradation and exert a bacteriostatic effect on a pathogenic strain of _E. coli_. Therefore, haptocorrin has the potential to influence bacterial growth in the gastrointestinal tract of breastfed infants. Further studies are needed to examine the antimicrobial effect of human milk haptocorrin in vivo.

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