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Alpha-Lactalbumin in Human Milk Alters the Proteolytic Degradation of Soluble CD14 by Forming a Complex

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ABSTRACT: Mother's milk represents a foundational step in the proper development of newborn immunity. This is achieved, in part, through the action of numerous regulatory proteins such as soluble cluster of differentiation 14 (sCD14) found in significant quantities in human milk (~25–50 μg/mL). In adults, CD14 stimulates cytokine production in response to lipopolysaccharide (LPS), the major lipid component found in the outer membrane of Gram-negative bacteria. However, the fate and function of sCD14 in the neonatal gastrointestinal (GI) tract are unknown and may function differently from adults. Therefore, we administered human sCD14 to experimental animals and observed that it persisted in the upper GI tract after feeding. In our search for potential proteolytic protectants, immunoprecipitation of sCD14 from human milk revealed a 15-kD novel protein that copurified with sCD14. Mass spectrometry analysis of the protein identified alpha-lactalbumin. CD14 was also identified by immunoblot after immunoprecipitation of alpha-lactalbumin from milk. *In vitro* digestion assays revealed that purified alpha-lactalbumin decreases the proteolytic degradation of human milk derived sCD14 *in vitro*, suggesting a mechanism by which this key LPS receptor may remain functional in the neonate gut. **(*Pediatr Res* 68: 490–493, 2010)**

As the immunological analysis of human milk enters its sixth decade (1), considerable evidence supports a role for human milk, with its estimated 100,000 components, in regulating the early phase of gut colonization by microorganisms and in the development of the infant's innate immunity (2). It has been demonstrated that breast-fed newborns experience a lower incidence of gastrointestinal (GI) infections including inflammatory, respiratory, and allergic disease in comparison with formula-fed infants (3–6). Protection by milk has been attributed to maternal immunocompetent cells, immunoglobulins, immune reactive peptides, anti-infectious oligosaccharides, growth factors, lactoferrin, and complement components (7).

The bacterial pattern recognition receptor, cluster of differentiation 14 (CD14), plays a pivotal role in the immune recognition and reactivity to microbial cell wall components from Gram-negative and Gram-positive bacteria (8,9). Two soluble forms of CD14, 48 kD and 52 kD, are found in normal human plasma at a concentration of 2–3 μg/mL (9,10). Soluble CD14 (sCD14) binds to whole bacteria and bacterial cell wall components and mediates bacterial-induced proinflammatory cytokine production from cells that typically do not express membrane-bound CD14 (11). The biology of CD14 in the breast, in milk, and in the gut is emerging (7). The average concentration of sCD14 in human milk is 25–50 μg/mL, and consumption of ~1 liter of milk per day by breast feeding infants suggests the GI tract is exposed to milligram quantities of this potent immunostimulatory protein. CD14 protein concentrations in bovine milk are less than that in human milk, only 5–11 μg/mL, but increase in response to bacterially induced mastitis (12,13). Recombinant CD14 also reduces intramammary infection by *Escherichia coli* (*E. coli*) in cows (14,15), suggesting that CD14 in bovine milk plays an important immunological role during mastitis. Conversely, mice transgenically altered to express concentrations of CD14 in milk similar to that in humans, 31–316 μg/mL, show no change in susceptibility to *E. coli* infection but show a considerable decrease in infection-related edema (16). This suggests sCD14 may reduce the inflammatory response to pathogenic bacteria.

The importance of sCD14 in the health and nutrition of breast-feeding infants including its role in the early colonization of microbes is poorly understood. We tested whether sCD14 forms a complex with other proteins present in human milk, and how this might influence CD14 proteolysis by GI tract proteases.

METHODS

Chemicals and reagents. For immunoprecipitation, mouse anti-human CD14 IgG (Clone UCHM-1; Fisher Thermo Scientific, Ottawa, ON), goat anti-human alpha-lactalbumin IgG (Santa Cruz Biotechnology, Santa Cruz, CA), mouse and goat preimmune IgG (Sigma Chemical Co. Aldrich, St. Louis, MO), and Protein A agarose (Sigma Chemical Co. Aldrich) were purchased. For the proteolytic sensitivity assay, alpha-lactalbumin (Sigma Chemical Co. Aldrich), lysozyme (Sigma Chemical Co. Aldrich), and trypsin (Sigma Chemical Co. Aldrich) were also purchased. [^{14}C]-Methyl iodide 250 μCi (10 mCi/mmol; Sigma Chemical Co. Aldrich) was used to label BSA (Sigma Chemical Co. Aldrich), and [^{14}C]-methyl iodide 2 mCi (52.9 mCi/mmol; Perkin Elmer, Waltham, MA) was used to label recombinant human CD14 (rhCD14; R&D Systems, Minneapolis, MN). Other reagents purchased include hydrogen peroxide 30% (wt/vol) solution in water (Sigma Chemical Co. Aldrich), perchloric acid 70% (Sigma Chemical Co. Aldrich), Solvable (Perkin Elmer), and ScintiSafe Plus 50% LSC-Cocktail (Fisher Thermo Scientific).

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Abbreviations: CD14, cluster of differentiation 14; **sCD14,** soluble CD14

14C-Labeling of proteins. A protein solution (BSA or rhCD14 at 1 mg/mL and 0.01 mg/mL) was adjusted to pH 10.5 and lyophilized. *In vacuo* methylation was performed with ^{14}C-methyl iodide (10 mCi/mmol) as previously described (17) with the following modifications. The *in vacuo* reaction was performed in a sealed glass test tube and incubated at 85°C for 48 h. Lyophilized and labeled proteins were recovered by resuspension in 500 μL of a 1% solution of NH_4HCO_3 and incubated at 37°C for 2 h to remove ^{14}C-methyl esters formed with carboxyl groups on the protein. The sample was relyophilized to volatilize and remove ^{14}C-methanol that may have formed during the reaction.

Animals. The study was approved by the University of Ottawa's Animal Care Committee and performed in accordance with the University of Ottawa's Animal Care and Veterinary Services. Sprague Dawley rat pups (10 d old) weighing from 19 to 26 g were separated from their mother and housed in an incubator at 37°C, while being fasted for 2 h. The pups were returned to their mother and gavage fed a 250-μL bolus of either 25 μg/mL ^{14}C-BSA (34,400 dpm/μg) or ^{14}C-CD14 (163,000 dpm/μg). Animals were anesthetized at 20 min and 8 h postgavage, euthanized by cardiac puncture, and had organs harvested. Organs were stored at −70°C until needed. For determining the amount of degraded ^{14}C-BSA or ^{14}C-CD14 in the rat pups, stomach contents, normalized for volume, were resuspended in 500 μL of PBS, centrifuged, and the supernatant recovered. Supernatants were clarified using a 0.22-μm spin filter. After clarification, samples were applied to an Amicon (Thermo Scientific) centrifugal filter with a 30,000 molecular weight cutoff that captured degraded protein in the flow through and retained full-length protein in the retentate. Flow through and retentate were resuspended in 2-mL liquid scintillation cocktail and counted using a Tri-Carb liquid scintillation counter (Perkin Elmer).

Milk samples: Preparation and analysis. This study was approved by the Research Ethics Board of the Children's Hospital of Eastern Ontario (Ottawa, Ontario) and the University of Ottawa Research Ethics Board. Human milk samples used in this study were collected from healthy nursing mothers and stored at −80°C. Before use, human milk samples were briefly centrifuged at 5000×g to separate milk fat and whole cells from milk plasma.

Immunoprecipitation of human milk. Defatted human milk plasma was depleted of existing immunoglobulins with Protein A agarose beads (Sigma Chemical Co. Aldrich) as previously described (18). Immunodepleted milk plasma was incubated with either 10 μL (10 μg) of rabbit anti-mouse IgG for sCD14 immunoprecipitations or 10 μL (10 μg) of goat anti-mouse IgG for alpha-lactalbumin immunprecipitations and removed by treatment with Protein A agarose. Milk was then treated with either 30 μL (3 μg) of anti-sCD14 IgG or 10 μL (5 μg) of anti-alpha-lactalbumin IgG for 1 h at 4°C. After antibody incubation, 70 μL of Protein A agarose was added and incubated for an additional hour at 4°C. Protein A agarose beads were washed five times with 750 μL of PBS containing 0.1% Triton-×100 to remove any nonspecifically bound proteins. Proteins were eluted from agarose beads by treatment with 300-μL (pH 1.9) elution buffer with a formic acid:acetic acid:water ratio of 25:75:900 (vol/vol/v) for 1 h at 4°C. Samples were microcentrifuged to pellet agarose, and eluates were concentrated with an Amicon Ultra Filtration column with a molecular weight cutoff of 5 kD (Fisher Thermo Scientific). Columns were spun at 4200 rpm in a Beckman J-6B swinging bucket rotor for 25 min and repeated. After the second spin, filter membranes were washed with 500-μL distilled water, centrifuged, resuspended in 300 μL of neutralization buffer [100 mM Tris HCl pH 9.0], and centrifuged again. Samples were recovered in a final volume of 40 μL for SDS-PAGE and immunoblot analysis.

Mass spectrometry: Liquid chromatography-coupled mass spectrometry (LC-MS/MS). Protein bands were excised from SDS PAGE gels and subjected to in-gel tryptic digestion as previously described (19,20). Tryptic digestions were performed for 8 h at 37°C with sequencing grade trypsin (Promega, Madison, WI). Peptides from each gel band were extracted, brought to a final volume of 10 μL with 5% formic acid, and analyzed by LC-MS/MS. Using an Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA), samples were loaded at 2 μL/min onto a 75 μm × 50 mm precolumn packed with 5 μm YMC ODS-A C18 beads (Waters, Milford, MA). After sample desalting, the flow was split, and peptides were eluted through a second 75 μm × 50 mm column packed with the same beads at ~200 nL/min using a 5–80% gradient of acetonitrile with 0.1% formic acid for 1 h. The liquid chromatography effluent was electrosprayed into the sampling orifice of a LTQ linear ion trap mass spectrometer (ThermoFisher Scientific, Waltham, MA). MS/MS data were analyzed and matched to human protein sequences in the International Protein Index database using the Mascot search engine. Peptide and MS/MS mass tolerances were set at 2 Da and 0.8 Da, respectively (21).

Proteolysis assay. Human milk CD14 was enriched by step gradient precipitation of milk plasma using ammonium sulfate (45–60%) (22). CD14 immunodot blots showed that CD14 was preferentially enriched at 60% ammonium sulfate concentration. After centrifugation the pellet was resolubilized in 10 mL of ddH_2O overnight at 4°C. Buffer exchange and concentration were performed using an Amicon Filtron concentrator column to a final volume of 1.5 mL. SDS-PAGE separation of ammonium sulfate-enriched CD14 in combination with immunoblots and scanning densitometry (Sigma Plot, Systat Software, Inc. San Jose, CA) estimated a CD14 concentration of ~450 ng/mL. For *in vitro* protease digestion assays, 10 μg of ammonium sulfate-precipitated CD14 in combination with either lysozyme or alpha-lactalbumin was digested at 37°C for either 60 min (trypsin) or 15, 30, 45, or 60 min (trypsin, chymotrypsin, and pancreatin multidigest).

RESULTS

Recombinant CD14 persists in the upper GI tract. Ten-day-old rat pups were gavage fed a 25-μg/mL bolus of either ^{14}C-labeled recombinant-CD14 or ^{14}C-labeled BSA as a control. Molecular weight separation of intact and degraded radiolabeled proteins from stomach contents indicated that ~90% of the BSA was not digested within the first 20 min after gavage feeding (Fig. 1) and was minimally detected in the stomach by 8 h postgavage. Conversely, ~80% of sCD14 was degraded within 20 min of gavage feeding and ~20% persisted in the stomach after 8 h. The findings suggest a pool of CD14 is sequestered in the stomach that does not undergo proteolytic degradation.

Alpha-lactalbumin and sCD14 form a complex in human milk. CD14 and associated proteins were immunoprecipitated from human milk samples from seven different mothers using a mouse monoclonal human-CD14 antiserum bound to a column. Of the seven samples, three novel bands on PAGE gels were identified at ~80 kD, 27 kD, and 15 kD in comparison with the control IgG lane (Fig. 2*A*). The 80-kD band was similar in molecular mass to human lactoferrin (77 kD), an antimicrobial protein that has already been shown to interact with CD14 (23). We also identified a 27-kD protein that is under further investigation. Not all human milk samples tested were positive for these proteins, raising interesting questions about the heterogeneity in human milk and its putative impacts on infant nutrition and health.

Mass spectrometric analysis of the 15-kD band identified alpha-lactalbumin, a major milk protein involved in lactose

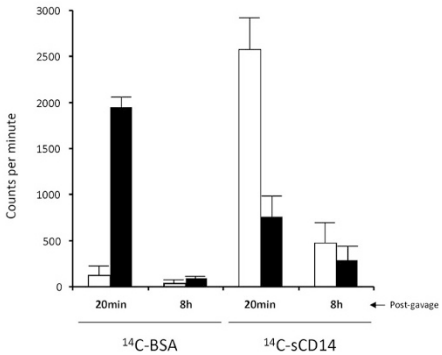

Figure 1. Fate of radio-labeled proteins in the neonate stomach. Ten-day-old Sprague Dawley rat pups (n = 12) were fed with a 250-μL bolus of 25 μg/mL ^{14}C-CD14 or ^{14}C-BSA. Animals were euthanized, and stomachs were collected at 20 min and 8 h postgavage. Stomach contents were separated using a 30,000 molecular weight cutoff membrane to fractionate intact (■) proteins from ^{14}C-labeled, degraded (□) proteins.

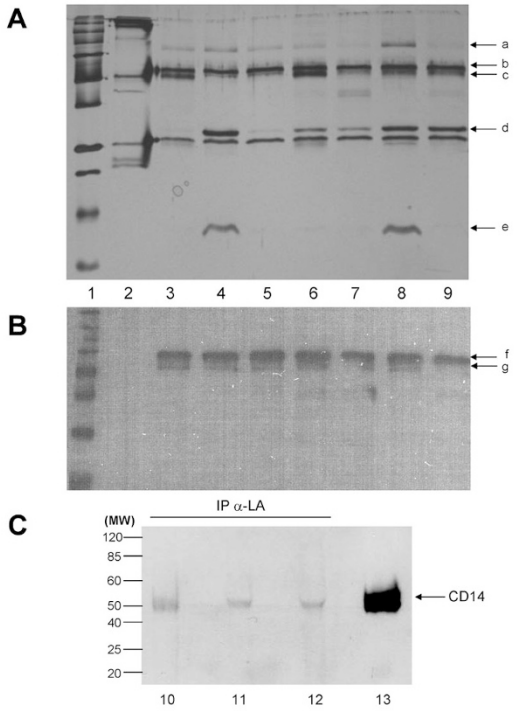

Figure 2. Alpha-lactalbumin and CD14 form a complex in human milk. (*A*) Silver stain of immunoprecipitated CD14 proteins from seven human milk samples (*Lanes 3–9*) resolved by SDS PAGE. *Lane* 1: Molecular weight markers. Novel bands are marked by arrows *a* (~75 kD), *d* (~27 kD), and *e* (~15 kD). Arrows *b* and *c* indicate the position of CD14 isomers. *Lane* 2 shows immunoprecipitation of human milk with a preimmune IgG antisera. (*B*) Western blot of CD14 immunoprecipitated human milk. *Lanes* are similar to (*A*) except that biotinylated molecular weight markers were used (*Lane* 1). CD14 isomers are highlighted by arrows *f* and *g*. (*C*) CD14 immunoblot of alpha-lactalbumin immunoprecipitated from three human milk samples (*Lanes 10–12*) and human milk as a positive control (*Lane 13*).

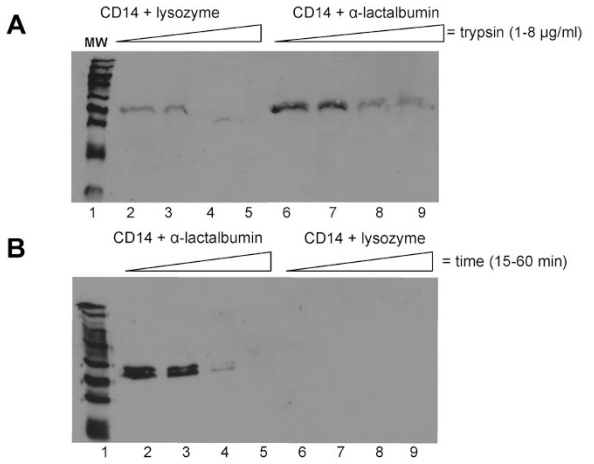

Figure 3. Alpha-lactalbumin delays proteolysis of CD14 *in vitro*. (*A*) Western blot of *in vitro* trypsin digestion of breast milk CD14 performed at neutral pH for 1 h at 37°C with 0, 1, 2, 4, and 8 μg of trypsin in the presence of either alpha-lactalbumin (*right*) or lysozyme (*left*). (*B*) Western blot of *in vitro* digestion using trypsin, chymotrypsin, and pancreatin digest of breast milk CD14. Ten milligrams of CD14 was digested for 15, 30, 45, and 60 min in the presence of either alpha-lactalbumin (*left*) or lysozyme (*right*).

biosynthesis. To further characterize sCD14/alpha-lactalbumin complexes in milk, anti-alpha-lactalbumin IgG was used to reverse-immunoprecipitate sCD14 from human milk (Fig. 2*C*; *Lanes 10–12*). The sCD14 immunoblot analysis demonstrated that sCD14 was coimmunoprecipitated from human milk. Comparison of signal intensity of the immunoprecipitated sCD14 with the human milk control (Fig. 2*C*; *Lane 13*) suggests only a small pool of sCD14 is in the bound state. A unique characteristic of alpha-lactalbumin is that it forms a proteolytically resistant complex with lactose synthetase (24), suggesting that a complex of milk-derived sCD14 with alpha-lactalbumin may be equally resistant to proteolysis.

Alpha-lactalbumin alters the proteolytic degradation of human milk sCD14. *In vitro* digests using trypsin alone or in combination with chymotrypsin and pancreatin were performed to investigate whether such a complex alters CD14 stability. The sCD14 from human milk is known to be proteolytically resistant to pepsin digestion and partially resistant to pancreatin digestion *in vitro* (25). The present results show that a higher concentration of trypsin is required to completely digest CD14 in the presence of alpha-lactalbumin than the control protein, lysozyme (Fig. 3*A*). Digestion of CD14 with a protease cocktail of trypsin, chymotrypsin, and pancreatin was also delayed in the presence of alpha-lactalbumin compared with lysozyme (Fig. 3*B*). The data demonstrate that CD14 is protected by alpha-lactalbumin and the consequence of this interaction *in vivo* may account for the persistence of CD14 in the upper GI tract, Fig. 1 (25).

DISCUSSION

Paradoxically, both sCD14 and Gram-negative bacteria are found in human milk (7,26) and yet do not seem to trigger inflammation in the GI tract of breast-fed infants or in the mammary gland of the lactating mother. This suggests that the normal physiological response of sCD14 may be altered in these compartments.

Alpha-lactalbumin is a major protein found in human milk (20–25% of total protein) and has several physiological functions (27,28). Partial digestion of alpha-lactalbumin leads to the production of transient peptides having both antimicrobial and immunostimulatory properties to aid against infection (28). A novel folding variant, termed a molten globule state, of multimeric alpha-lactalbumin has been discovered, which has antimicrobial activity (29) and enhances apoptosis of tumors (30). The interaction of alpha-lactalbumin with sCD14 reported here extends the functionality of this dynamic milk protein. The persistence of recombinant CD14 in the neonate stomach (Fig. 1) suggests that sCD14 complexed with alpha-lactalbumin may permit sCD14 to migrate further along the GI tract than would be possible in the absence of this interaction. Therefore, sCD14 and alpha-lactalbumin occupy a specific temporal window during infant development, most notably after birth when the child begins feeding from the breast, and sCD14 levels are at their highest concentration in human milk (25,31,32). This suggests that early colonization of the gut and the presence of sCD14/alpha-lactalbumin complexes may be a coordinated process to ensure population by appropriate commensal microbes. The persistence of sCD14 in the upper GI tract suggests its biological role in limiting the colonization of

microorganisms within this region as the upper GI tract houses seven orders of magnitude fewer bacteria than the colon (33).

Interestingly, sCD14/ alpha-lactalbumin did not occur consistently in all milk samples analyzed (Fig. 2). Milk samples used in this study were obtained from mothers who had been actively breast feeding from weeks to months postpartum, suggesting this protein complex may be temporally restricted, especially given that sCD14 is maximally expressed in colostrum and early milk (32). In human milk, sCD14 exists in a complex with at least three other proteins: a 15-kD protein, which is alpha-lactalbumin; a 77-kD protein, which is likely lactoferrin; and a yet uncharacterized 27-kD protein. Three of the four proteins are known to have antimicrobial activity. Recent work from Ohnishi *et al.* (34) suggests that His-tagged CD14 purified from *Pichia pastoris* seemed to inhibit *E. coli* growth. We have observed that human milk-derived sCD14 also reduces the viability of *E. coli in vitro* (unpublished observations). Together this demonstrates that sCD14 is part of a larger specialized complex whose combined antimicrobial activity would target a wide range of microorganisms in the upper GI tract. This so-called "gatekeeper complex" may serve two functions. The first is surveillance that may function to limit colonization of the upper GI tract. The second is planned obsolescence, whereby components of the complex, such as CD14, are degraded before reaching the colon thereby minimizing CD14-dependent inflammation. Alpha-lactalbumin also presents an interesting structural paradigm because of its ability to form nanotubes (35,36), which in the hydrolytic environment of the GI tract may function as a scaffold to shield sCD14 and other bioactive milk components from degradation.

Previous work from our laboratory has demonstrated that human milk-derived sCD14 is not detectable in the stool of newborn breast-fed infants and is adsorbed, sequestered, or degraded (25). In combination with the present results, sCD14 is still observed to be sensitive to proteolysis; however, the presence of a resistant pool in the stomach does not preclude the possibility of CD14 degradation further along the GI tract. Therefore, a more complete picture of the digestive fate of sCD14 in the GI tract of neonates including how this might influence gut colonization is warranted.

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