Human milk lactoferrin inactivates two putative colonization factors expressed by Haemophilus influenzae

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ABSTRACT Haemophilus influenzae is a major cause of otitis media and other respiratory tract disease in children. The pathogenesis of disease begins with colonization of the upper respiratory mucosa, a process that involves evasion of local immune mechanisms and adherence to epithelial cells. Several studies have demonstrated that human milk is protective against H. influenzae colonization and disease. In the present study, we examined the effect of human milk on the H. influenzae IgA1 protease and Hap adhesin, two autotransported proteins that are presumed to facilitate colonization. Our results demonstrated that human milk lactoferrin efficiently extracted the IgA1 protease preprotein from the bacterial outer membrane. In addition, lactoferrin specifically degraded the Hap adhesin and abolished Hap-mediated adherence. Extraction of IgA1 protease and degradation of Hap were localized to the N-lobe of the bivalent lactoferrin molecule and were inhibited by serine protease inhibitors, suggesting that the lactoferrin N-lobe may contain serine protease activity. Additional experiments revealed no effect of lactoferrin on the H. influenzae P2, P5, and P6 outer-membrane proteins, which are distinguished from IgA1 protease and Hap by the lack of an N-terminal passenger domain or an extracellular linker region. These results suggest that human milk lactoferrin may attenuate the pathogenic potential of H. influenzae by selectively inactivating IgA1 protease and Hap, thereby interfering with colonization. Future studies should examine the therapeutic potential of lactoferrin, perhaps as a supplement in infant formulas.

Acute otitis media is a suppurrative infection of the middle ear and is especially common during early childhood (1). By 3 years of age, 80% of children have suffered from acute otitis media and 40–50% have experienced at least three episodes (2). Otitis media accounts for over one-third of pediatric office visits in the United States and is the most common reason for prescription of oral antibiotics (3). After each episode, fluid persists in the middle ear for weeks to months, causing hearing impairment (4) that sometimes results in deficiencies in language acquisition, speech development, and cognitive achievement (5).

Most cases of otitis media are caused by infection with Streptococcus pneumoniae, Haemophilus influenzae, or Moraxella catarrhalis (6, 7). Infection begins with colonization of the nasopharynx, followed by contiguous spread through the eustachian tube to the middle ear. Colonization is a complex process and involves the interplay of bacterial and host factors. Successful colonization requires that an organism evade local immune mechanisms and overcome the mucociliary escalator. Both S. pneumoniae and H. influenzae secrete an IgA1 protease, which specifically cleaves and inactivates human IgA1, the predominant secretory antibody in the upper respiratory tract (8, 9). All three of these respiratory pathogens also elaborate adhesins, which promote attachment to host epithelium and are critical in preventing physical removal from the mucosal surface.

IgA1 proteases are the prototypes of a family of Gram-negative bacterial proteins that undergo a process known as autosecretion (10). The H. influenzae strain Rd protease is synthesized as a 185-kDa protein with four domains, including an N-terminal signal sequence, an internal serine protease domain (IgAα), a highly basic and helical alpha domain (IgAγ), and a C-terminal beta or helper domain (IgAδ) (11, 12). The signal sequence directs export across the bacterial inner membrane and is then cleaved. Subsequently, the remainder of the protein (hereafter called the preprotein) inserts into the outer membrane by way of the beta domain, which is predicted to form a β-barrel structure with a hydrophilic channel, thus allowing for translocation of the protease and the alpha domain to the extracellular space. Ultimately, the protease domain gains catalytic activity and cleaves within the alpha domain to release itself from the surface of the organism.

The H. influenzae Hap protein is a nonpilus protein that promotes intimate interaction with human epithelium (13). It was originally identified on the basis of its ability to confer a capacity for in vitro attachment and invasion when expressed in a nonadherent, noninvasive laboratory strain of H. influenzae (13). Hap shares significant sequence homology (30–35% identity and 51–55% similarity) with the H. influenzae and Neisseria gonorrhoeae IgA1 proteases and undergoes autosecretion by means of an analogous pathway. Hap is produced as a 155-kDa protein with three functional domains, including an N-terminal signal sequence, a surface-localized serine protease domain (Hapγ), and a C-terminal outer membrane domain (Hapδ) (14). To date, there is no evidence for an alpha domain. Ultimately, the Hapδ domain mediates an autoproteolytic cleavage event, releasing itself from Hapδ and from the surface of the organism. Recent evidence indicates that attachment to host cells is a function of the preprotein (Hap, linked to Hapδ), before autoproteolytic cleavage (D.R.H. and J.W.S.T.G., unpublished work).

A number of studies have demonstrated that breast-fed infants have fewer episodes of otitis media and a lower incidence of nasopharyngeal colonization with S. pneumoniae, H. influenzae, and M. catarrhalis than do formula-fed infants (2, 16–20). Human breast milk contains secretory IgA and...
several nonantibody proteins with antibacterial properties, including lactoferrin, lysozyme, and peroxidase. The precise mechanism by which these factors influence bacterial colonization of the nasopharynx and spread to the middle ear has not been established.

In the present study, we examined interactions between human milk and H. influenzae and observed that lactoferrin efficiently extracted the IgA1 protease preprotein from the bacterial outer membrane. We also found that lactoferrin specifically degraded and inactivated the Hap adhesin. Both extraction and degradation were inhibited by pretreatment of lactoferrin with broad serine protease inhibitors, suggesting that lactoferrin may have protease activity. Experiments with three additional H. influenzae outer-membrane proteins revealed no effect, suggesting that this newly recognized activity may be specific for certain autotransported proteins.

MATERIALS AND METHODS

Bacterial Strains and Plasmids and Growth Conditions. H. influenzae strain Rd is a capsule-deficient serotype d strain that secretes IgA1 protease but contains a nonfunctional hap gene because of a spontaneous nonsense mutation at codon 710 (13, 21). H. influenzae strain Rd3–13 is a derivative of Rd with a mutant IgA1 protease that lacks protease activity and is locked in the full-length state; it was constructed by Y. Fishman and A. Wright (Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, MA) and contains a valine residue in place of the active site serine. H. influenzae strain DB117 is a derivative of Rd with a mutation in the rec-1 gene and is deficient in recombination (22). H. influenzae strain 1479 is a clinical isolate that was originally recovered from the sputum of a patient with chronic bronchitis and is serologically nontypeable (23).

The plasmid pJS106 contains the wild-type hap gene in the vector pGJB103 and was described previously (13). pHapS243A encodes a Hap derivative that contains an alanine residue in place of the active site serine at position 243 (14); the mutant protein is locked in the full-length form and remains cell-associated (ref. 14; D.R.H. and J.W.St.G., unpublished work). pHapD encodes a Hap derivative composed of the Hap signal sequence fused directly to the Hap C-terminal domain (Hapβ), thus lacking the central Hap domain (D.R.H. and J.W.St.G., unpublished work).

H. influenzae strains were grown as described previously (24) and were stored at −80°C in brain–heart infusion broth with 20% glycerol.

Preparation of Human Milk Samples. After obtaining informed consent, human milk was collected between 3 days and 4 months postpartum from healthy mothers taking no antibiotics. All samples were collected in sterile beakers and within 3 hr of collection were centrifuged at 10,000 × g for 20 min at 4°C to remove lipids and cells. The resulting milk whey was stored at −80°C and was prepared for use by thawing slowly, without further modifications.

Milk whey proteins were fractionated by first precipitating with 40 vol of cold acetone, then subjecting to anion exchange (DE-52, Whatman) and molecular sieve chromatography (Bio Gel P-200, Bio-Rad). All steps were carried out in neutral buffers at either room temperature or 4°C. Human Milk and Lactoferrin Studies. Recombinant lactoferrin and recombinant lactoferrin N-lobe (residues 1–334) were purified from baby hamster kidney (BHK) cells, as previously described (25, 26). Recombinant lactoferrin purified from Aspergillus awamori was a generous gift from Agen- nix Corporation (Houston, TX) and was deglycosylated according to the method of Baker et al. (27).

The purity of recombinant protein preparations was confirmed by mass spectroscopy using a linear matrix-assisted laser desorption ionization-time-of-flight instrument (Voyag-
Active fractions contained no IgA, indicating that extraction was independent of milk antibody.

To confirm that lactoferrin alone was capable of extraction, assays were performed with preparations of recombinant human lactoferrin produced in either BHK cells or A. awamori. In addition, recombinant lactoferrin N-lobe was examined, again after purification from BHK cells. Recombinant protein was used in a concentration of 1 mg/ml (13 μM), approximating the value reported for lactoferrin in human milk (34). As shown in Fig. 1C, lactoferrin purified from BHK cells removed the IgA protease preprotein from strain Rd3-13 and then degraded the extracted protein. Deglycosylated recombinant lactoferrin from A. awamori and recombinant lactoferrin N-lobe from BHK cells had an identical effect (not shown). To ensure that no other proteins were present in the preparations of recombinant protein, molecular mass measurements were performed by using mass spectroscopy. Intact glycosylated lactoferrin from BHK cells was 79,338 Da, and glycosylated lactoferrin N-lobe was 36,890 Da, in both cases very close to predicted values. Additional analysis demonstrated that lactoferrin iron content had no influence on either extraction or degradation (not shown).

**Human Lactoferrin Degrades and Inactivates the H. influenzae Hap Adhesin.** To address whether lactoferrin also extracts other autotransported proteins from H. influenzae, we examined the effect of 13 μM human milk lactoferrin on the Hap adhesin, which bears structural similarity to IgA1 protease. Interestingly, lactoferrin treatment of strain DB117 expressing wild-type Hap resulted in specific proteolysis of Hap. As shown in Fig. 2A, Western blot analysis of whole cells revealed loss of the preprotein and appearance of a C-terminal fragment slightly smaller than HapB (39 kDa vs. 45 kDa). To confirm these results and to assess whether proteolysis depends on Hap serine protease activity, we examined the effect of lactoferrin on DB117 expressing Hap with a mutated active site serine (HapS243A). This protein lacks autoproteolytic activity and remains in the outer membrane in the preprotein form (14, 15). Western blot analysis of whole cells again revealed loss of the Hap preprotein and generation of a Hap C-terminal fragment (Fig. 2A, lanes 3 and 7). Treatment of DB117 expressing a Hap derivative containing the Hap signal sequence fused to HapB also resulted in generation of the cell-associated 39-kDa C-terminal fragment (Fig. 2A, lanes 4 and 8).

To establish that these results were because of lactoferrin and not some other component of milk whey, we examined the effect of recombinant human lactoferrin. As shown in Fig. 2B, 13 μM deglycosylated recombinant human lactoferrin prepared from A. awamori generated two products, including the same 39-kDa C-terminal fragment observed with milk-derived lactoferrin and a slightly smaller C-terminal fragment. Additional analysis revealed that HapB or a related fragment of the Hap preprotein was liberated into the supernatant (Fig. 2C). Experiments comparing the proteolysis of Hap by 87 nM, 217
Given that Hap promotes adherence to human epithelial cells, we wondered whether lactoferrin treatment would inhibit Hap-mediated attachment. To address this question, strain DB117 expressing HapS243A was incubated for 1 hr in either PBS alone or PBS with 13 μM lactoferrin, then washed twice and inoculated onto a monolayer of Chang epithelial cells. After incubation for 30 min, adherence was quantitated. [DB117 expressing HapS243A demonstrates augmented in vitro adherence compared with DB117 expressing wild-type Hap, reflecting the fact that attachment is mediated by the preprotein form of Hap (D.R.H. and J.W.St.G., unpublished work), which remains intact and cell-associated when the active site serine is mutated]. As shown in Fig. 3, treatment of DB117/HapS243A with either milk-derived or recombinant lactoferrin resulted in an 85–97% decrease in Hap-mediated adherence. DB117/vector served as a negative control and was nonadherent regardless of lactoferrin treatment. Viability studies demonstrated that lactoferrin treatment had no effect on the survival of either DB117/HapS243A or DB117/pGJB103.

**Lactoferrin-Associated Degradation of Hap Is Inhibited by Phenylmethylsulfonyl Fluoride (PMSF).** Our findings with DB117 expressing Hap, HapS243A, or Hapβ suggested that lactoferrin has protease activity. With this idea in mind, we examined the lactoferrin crystallographic structure (35) and primary amino acid sequence for features of protease active.

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**Fig. 2.** Western immunoblots demonstrating that treatment of *H. influenzae* strain DB117 with human milk lactoferrin or deglycosylated *A. awamori* recombinant human lactoferrin results in degradation of the Hap preprotein and Hapβ, with release of Hap into the culture supernatant. (A) Western immunoblot of whole-cell lysates of *H. influenzae* strain DB117 derivatives preincubated with either PBS alone (lanes 1–4) or PBS with 13 μM human milk lactoferrin (lanes 5–8). (B) Western immunoblot of whole-cell lysates of *H. influenzae* strain DB117 derivatives preincubated with either PBS alone (lanes 1–4) or PBS with 13 μM deglycosylated *A. awamori* recombinant human lactoferrin (lanes 5–8). (C) Western analysis of culture supernatants of *H. influenzae* strain DB117 derivatives preincubated with either PBS alone (lanes 1–4) or PBS with 13 μM deglycosylated *A. awamori* recombinant human lactoferrin (lanes 5–8). Western analysis was performed with antiserum Rab730, which reacts with the Hap preprotein, Hap, and Hapβ. The gels in all panels were loaded as follows: lane 1, PBS-treated DB117/vector; lane 2, PBS-treated DB117/wild-type Hap; lane 3, PBS-treated DB117/HapS243A; lane 4, PBS-treated DB117/Hapβ; lane 5, lactoferrin-treated DB117/vector; lane 6, lactoferrin-treated DB117/wild-type Hap; lane 7, lactoferrin-treated DB117/HapS243A; lane 8, lactoferrin-treated DB117/Hapβ. Arrowheads point to the Hap preprotein and Hapβ, arrows point to Hap degradation products, and asterisks indicate Haps.

**Fig. 3.** Effect of human lactoferrin on Hap-mediated *H. influenzae* adherence to human epithelial cells. (A) Adherence to Chang conjunctival cells by DB117/vector and DB117/HapS243A after incubation in PBS, PBS with 13 μM human milk whey lactoferrin, or PBS with 13 μM deglycosylated *A. awamori* recombinant lactoferrin. Adherence was measured in a 30-min assay as previously described (33) and is reported relative to DB117/HapS243A associated with Chang conjunctival cells samples after staining with Giemsa (×2,250). The sample in B was incubated in PBS, and the sample in C was incubated with 13 μM deglycosylated *A. awamori* recombinant lactoferrin.
sites (36). Among the many serine residues in the N-lobe, several are surface-exposed and would be candidate catalytic sites, although none is surrounded by the sequence typical of serine proteases. Lactoferrin lacks nonferrous metal binding sites or features common to other known protease classes, arguing against another catalytic mechanism. To test the possibility that lactoferrin functions as a serine protease, we examined whether PMSF, a broad inhibitor of serine proteases, can inhibit degradation of Hap. As shown in Fig. 4, the partial proteolysis of Hap produced by 433 nM recombinant lactoferrin was significantly inhibited by 7.5 mM PMSF. Similarly, lactoferrin extraction of the IgA protease preprotein was markedly reduced in the presence of either 10 mM PMSF or 10 mM diisopropyl fluorophosphate, another serine protease inhibitor (not shown).

To address the specificity of the interaction between lactoferrin and \textit{H. influenzae} surface proteins, we examined whether lactoferrin treatment results in extraction or degradation of the \textit{H. influenzae} major outer-membrane proteins P2, P5, and P6. Like IgA\(_b\) and Hap\(_b\), P2, P5, and P6 are predicted to form \(\beta\)-barrel structures comprised of a series of transmembrane antiparallel amphipathic \(\beta\)-sheets (37–40). However, these outer-membrane proteins lack the large extracellular domains that link IgA\(_b\) and Hap\(_b\) to their N-terminal passenger domains and typify the autotransported proteins. As shown in Fig. 5, treatment with milk-derived lactoferrin under conditions leading to extraction of the IgA1 protease preprotein and proteolysis of Hap had no appreciable effect on P2, P5, or P6. All three of these proteins remained cell-associated and intact.

**DISCUSSION**

Earlier studies showed that lactoferrin, a bilobed protein found in milk, mucosal secretions, and neutrophils, plays an important role in host defense (15, 41). Lactoferrin efficiently binds iron, starving bacteria of this essential nutrient. In addition, lactoferrin is bactericidal for many Gram-negative species, and typifies the autotransported proteins. As shown in Fig. 5, treatment with milk-derived lactoferrin under conditions leading to extraction of the IgA1 protease preprotein and proteolysis of Hap had no appreciable effect on P2, P5, or P6. All three of these proteins remained cell-associated and intact.

**Fig. 4.** Effect of serine protease inhibitor PMSF on lactoferrin-associated proteolysis of \textit{H. influenzae} Hap. DB117/HapS243A was incubated in PBS (lane 1), PBS with 433 nM deglycosylated \textit{A. awamori} recombinant lactoferrin (lane 2), PBS with 433 nM recombinant lactoferrin and 7.5% 2-propanol (lane 3), or PBS with 433 nM recombinant lactoferrin and 7.5 mM PMSF in 2-propanol (lane 4). Whole-cell lysates were prepared and examined by Western analysis with antiseraur Rab 730, which reacts with the Hap preprotein, Hap\(_b\), and Hap\(_a\). The arrowhead points to the Hap preprotein, and arrows point to Hap degradation products.

**Fig. 5.** The \textit{H. influenzae} P2, P5, and P6 outer-membrane proteins are not removed or degraded by exposure to human milk whey. Bacteria were grown to mid-logarithmic phase and then incubated with either buffer (lanes 1 and 2 in all panels) or human milk whey (lanes 3 and 4 in all panels). Whole-cell lysates (lanes 1 and 3 in all panels) and corresponding supernatants (lanes 2 and 4 in all panels) were examined by Western immunoblot analysis. A was probed with antiserum #331 against IgA1 protease, B was probed with mAb 6G3 against P2. C was probed with mAb 2C7 against P5, and D was probed with mAb 7F3 against P6. Strain Rd3–13 was used for the analysis of IgA1 protease, P5, and P6. Strain 1479 was used for the analysis of P2, as antibody 6G3 is specific for the strain 1479 P2 protein. The IgA1 protease preprotein was transferred to the supernatant, while P2, P5, and P6 were unaffected.

The precise mechanism by which lactoferrin extracts the IgA1 protease preprotein without affecting bacterial viability is unknown. However, our experiments with serine protease inhibitors suggest that extraction is related to lactoferrin proteolytic activity, localized to the N-lobe of the protein. Our
observations with Hap provide additional evidence that lactoferrin has serine protease activity, with activity residing in the N-lobe. An alternative explanation for our results is that lactoferrin complexes with an unidentified protease in milk or cell-culture media during the process of purification. Arguing against this possibility, native and recombinant lactoferrin purified from multiple sources and prepared by several schemes had the same effect. Furthermore, molecular mass measurements of active recombinant lactoferrin and lactoferrin N-lobe closely approximated the predicted values for these species.

The results of the present study suggest that lactoferrin may have significant therapeutic potential and may be valuable as a supplement in infant formulas. However, since colonization involves complex host–bacterial interactions, it remains unclear whether inactivation of selected factors will attenuate virulence. Thus future studies should compare rates of \textit{H. influenzae} nasopharyngeal colonization and otitis media in infants fed either standard formula or formula supplemented with native or recombinant lactoferrin.

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