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REVIEW / SYNTHÈSE

Lactoferrin: an alternative view of its role in human biological fluids[1]

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Abstract: Lactoferrin is a major component of biologically important mucosal fluids and of the specific granules of neutrophils. Understanding its biological function is essential for understanding neutrophil- and mucosal-mediated immunity. In this review, we reevaluate the in vivo functions of human lactoferrin (hLF) emphasizing in vivo studies and in vitro studies performed in biologically relevant fluids. We discuss the evidence in the literature that supports (or does not support) proposed roles for hLF in mucosal immunity and in neutrophil function. We argue that the current literature supports a microbiostatic role, but not a microbicidal role, for hLF in vivo. The literature also supports a role for hLF in inhibiting colonization and infection of epithelial surfaces by microorganisms and in protecting tissues from neutrophil-mediated damage. Using this information, we briefly discuss hLF in the context of the complex biological fluids in which it is found.

Key words: lactoferrin, host defense proteins and peptides, antimicrobial.

Résumé : La lactoferrine est une composante majeure des liquides d'importance biologique des muqueuses et des granules spécifiques des neutrophiles. Il est essentiel de comprendre ses fonctions biologiques afin de comprendre l'immunité dépendante des muqueuses et des neutrophiles. Dans cet article de revue, nous réévaluons les fonctions in vivo de la lactoferrine humaine (hLF) en mettant l'emphase sur des études réalisées in vivo et in vitro sur des liquides d'intérêt biologique. Nous discutons des preuves présentées dans la littérature qui appuient ou non les rôles proposés de la hLF dans l'immunité muqueuse et dans la fonction des neutrophiles. Nous soutenons que la littérature actuelle appuie le rôle microbiostatique de la hLF, mais pas son rôle microbicide in vivo. La littérature appuie aussi le rôle de la hLF dans l'inhibition de la colonisation et de l'infection des surfaces épithéliales par les microorganismes, et dans la protection des tissus du dommage causé par les neutrophiles. À partir de cette information, nous discutons brièvement de la hLF dans le contexte de liquides biologiques complexes dans lesquels elle se trouve.

Mots-clés : lactoferrine, protéines et peptides de défense de l'hôte, antimicrobiens.

[Traduit par la Rédaction]

Introduction

Human lactoferrin (hLF) is an 80 kDa iron-binding glycoprotein (Baker and Baker 2004). It is present at moderate to high levels in human milk, tear film, upper airway fluids, seminal plasma, and the cervical mucus plug (Table 1); it is present at low to very low levels in ear wax, saliva, small intestine, vaginal fluid, and amniotic fluid (Table 1); it is absent from the lung alveoli (Masson et al. 1966) and the skin (Mason and Taylor 1978); and there are no reports of hLF being present in the colon or the urinary tract. hLF is one of a multitude of host defense proteins and peptides (HDPPs) (Wang et al. 2009) that constitute a vital first line defense against invading microorganisms. The principle HDPPs found in the mucosal fluids that contain hLF include the α- and β-defensins, cathelicidin 18 (also know as cationic anti-

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Table 1. Reported concentrations (μg/mL) of HDPPs in various tear and mucosal compartments.

Tissue	HNP-1,2,3	HD-5,6 (HNP-5,6)	HBD-1	HBD-2	HBD-3
Human colostrum					
Human milk					
Open eye tear fluid	0.2–1			0.33	
Closed eye tear fluid					
Bronchoalveolar lavage fluid[a]	0.014–0.143		0.000051–0.002000	0–0.0000004	
Respiratory epithelial lining fluid	10		1 (inflamed)	1 (inflamed)	
Nasal	9.2		1 (inflamed)	0.0679–4	
Saliva	2.7–8.6		0.15	0.15	0.31
Intestinal lavage[a]					
Duodenojejunal aspirate					
Ileal effluent					
Small intestine crypts		50–250			
Small intestine crypts after exocytosis		up to 100000[e]			
Seminal plasma					
Vaginal lavage fluid[a,b]	2–5	0.007–0.025	0.015–0.035	0.005–0.040	
Vaginal fluid (undiluted)[b]	0.35		0.04	0.57	
Cervical mucus (nonpregnant)[b]					
Cervical mucus plugs (pregnant)	12 μg/g	0	1 μg/g	0	
Amniotic fluid[c]	0.00280–0.00556			0.003–0.004	0.003
Ear wax[d]	11.85 (μg/g protein)		0.14 (μg/g protein)	0.31 (μg/g protein)	0.95 (μg/g protein)

Note: HDPPs, host defense proteins and peptides; BPI, bactericidal/permeability increasing protein; SLPI, secretory leukoprotease inhibitor; LF, lactoferrin;
[a]Lavage is estimated to dilute the concentration of target HDPPs by 100-fold or more.
[b]In addition to individual variation, levels can change depending on the phase of the menstrual cycle.
[c]In addition to individual variation, levels can change depending on the period of the pregnancy.
[d]Up to 60% of a cerumen plug consists of cellular components; LL-37, SPLI, and BPI are mosty cell bound.
[e]This value is derived from mouse studies.
[f]Samples are from celiac patients. These patients had higher than normal immunohistochemical staining for hLF in their intestines. Ten of seventeen
[g]hLF levels increase from 1–2 μg/mL to 5–30 μg/mL after 20–30 weeks of pregnency. The source of this hLF, whether it is from maternal uterine secre-
[h]Level of lactoperoxidase found in the whey fraction of human milk.

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microbial protein 18 or CAP18), bactericidal/permeability increasing protein (BPI), lactoperoxidase, peptidoglycan recognition protein (PGLYRP) family members, the histatins, lysozyme, secretory phospholipase A2 (sPLA2), secretory leukoprotease inhibitor (SLPI), elafin, surfactant proteins A and D (SP-A and SP-D), IgA, calprotectin, lipocalin 1 and 2, REG3, cystatins, prolactin-inducible protein, salivary gp-340, and PLUNC/SPLUNC family members. These HDPPs, however, are by no means the only HDPPs present in mucosal fluids; for example, Gorr and Abdolhosseini (2011) list over 50 HDPPs found in the oral cavity alone.

Most HDPPs are multifunctional with antiviral, antibacterial, antifungal, wound healing, antiinflammatory, and immunomodulatory properties (see reviews by Ganz 2004, Bowdish et al. 2006, Wiesner and Vilcinskas 2010, and Steinstraesser et al. 2011). HDPPs are expressed by prokaryotes, protozoa, plants, invertebrates, and vertebrates; short cationic amphiphilic peptides are particularly widespread (Zasloff 2002; Bulet et al. 2004). HDPPs are always present as cocktails (Wiesner and Vilcinskas 2010). This allows for a broad range of protection, and, in addition, HDPPs display synergism with other HDPPs (for a few examples of synergism see Levy et al. 1994, Weinrauch et al. 1995, Yan and Hancock 2001, and Cole et al. 2002).

Each tissue is exposed to a different set of microorganisms and each tissue has its own HDPP profile (see Table 1; Wiesner and Vilcinskas 2010). During an infection, the generation of HDPPs is upregulated, expression of genes coding for HDPPs can be activated, mature HDPPs can be generated by proteolytic digestion of inactive propeptides, and HDPPs can be deposited at sites of infection by neutrophils (Devine 2003; Levy 2004; Auvynet and Rosenstein 2009; Diamond et al. 2009). Virtually all HDPPs are reported to have multiple antimicrobial activities. HDPPs exert their antimicrobial activities by permeabilizing microorganism membranes, interfering with essential metabolic pathways, digesting microbial structures, interfering with the function of virulence factors, blocking attachment of microbes to the epithelia, and sequestering essential elements away from invading microbes (Ganz 2004; Levy 2004; Diamond et al. 2008; Nevalainen et al. 2008; Gutsmann and Seydel 2010; Wiesner and Vilcinskas 2010). A cocktail of HDPPs not only gives broader activity against microorganisms, but evolving resistance to an HDPP cocktail is much less likely to occur than evolving resistance

CAP18/LL-37	BPI	Histatin 1, 3, 5	Cystatins	Refs.
	0.0278	0 (by PCR)	4	Sathe et al. 1998; Zhou et al. 2004; Peuravuori et al. 2006; Kolar and McDermott 2011
			19.7	Sathe et al. 1998
				Schnapp and Harris 1998; Singh et al. 1998; Ashitani et al. 2001
				Ganz 2002, 2004
				Cole et al. 1999, 2002
1.6	0.078	10–65	up to approximately 40–100	Jensen et al. 1994; Campese et al. 2009; White et al. 2009; Gorr and Abdolhosseini 2011
				Muller et al. 2005; Ouellette 2005; Ouellette 2011
				Ayabe et al. 2000; Ouellette and Bevins 2001; Ouellette 2005
48–86				Malm et al. 2000; Andersson et al. 2002
				Valore et al. 2006
			32	Valore et al. 2002
				Hein et al. 2002
	0.000–0.002			Espinoza et al. 2003; Soto et al. 2007
0.28 (μg/g protein)	0.20 (μg/g protein)			Schwaab et al. 2011

sPLA2, secretory phospholipase A2; hLF, and human lactoferrin.

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patients had no measurable hLF in their duodenojejunal aspirate, and 7 patients had values ranging from 0.22–0.70 μg/mL in their duodenojejunal aspirate. tions, maternal uterine neutrophils, or from the developing embryo, is not known.

to a single potent antimicrobial agent. That these cocktails are effective is shown by the fact that they are present throughout the plant and animal kingdoms. Moreover, while humans (and all other living organisms) are exposed to a multitude of microorganisms daily, infections are rare events.

This review reevaluates the in vivo function of hLF based on in vivo studies and in vitro studies performed in biologically relevant fluids. However, the first section of this review is devoted to a question that is seldom explored in reviews of lactoferrin (LF); what is the evidence that hLF is actually important in human physiology?

The importance of hLF

Evidence from animal models

The importance of hLF may appear to be a rather foolish topic to explore, but it is more relevant than it first appears. LF knockout mice have been generated and characterized; these animals develop normally, are healthy and fertile, and respond normally to bacterial challenge (Ward et al. 2003, 2008). This is in stark contrast to knockdown or knockout of the activity of several other HDPPs. Mice deficient in the protease matrilysin that cleaves and activates α-defensins in the mouse intestine do not produce active intestinal α-defensins and are significantly more susceptible to *Escherichia coli* and to *Salmonella typhimurium* challenge than their wild-type counter parts (Wilson et al. 1999). Knockout of mouse β-defensin-1 (mBD-1) increases colonization of the bladder by *Staphylococcus* species (Morrison et al. 2002) and results in delayed clearance of *Haemophilus influenzae* from the lung (Moser et al. 2002). Decreased expression of mBD-1, mBD-2, or mBD-3 by application of small interfering RNA (siRNA) to the eyes of mice results in increased bacterial infection of the cornea (Wu et al. 2009*a*, 2009*b*; Augustin et al. 2011). siRNA knockdown of mouse cationic antimicrobial protein 18 (CRAMP) in the eyes of mice also results in increased bacterial infection of the cornea (Augustin et al. 2011). CRAMP knockout mice have significantly greater susceptibility to bacterial infection of the eye (Huang et al. 2007), intestine (Iimura et al. 2005), and bladder (Chromek et al. 2006). Sheep treated with dapsone to inhibit airway lactoperoxidase had significantly greater susceptibility to *Pasteurella haemolytica* challenge (Gerson et al. 2000). Knockout of lysozyme M in mice results in colonization of the normally sterile lower airways by lactobacilli (Markart et al. 2004), increased sensitivity to intratracheal

Table 1. *(concluded).*

Tissue	Elafin	SLPI	IgA	Lysozyme	LF
Human colostrum			30000	370	3000–8000
Human milk			1000	890	1000– 4800
Open eye tear fluid	0.1–2	8.1	2600	1620–2000	630–2900
Closed eye tear fluid	1–2	52	10000	1800	1800
Bronchoalveolar lavage fluid[a]				6.75	11.83
Respiratory epithelial lining fluid		10–100		100–1000	100–1000
Nasal		10–90		250–500	80–200
Saliva		2.9		40	4–20
Intestinal lavage[a]		0.000053			
Duodenojejunal aspirate					0–0.7[f]
Ileal effluent					0.024–0.052
Small intestine crypts					
Small intestine crypts after exocytosis					
Seminal plasma					112
Vaginal lavage fluid[a,b]		0.05–0.020		0.5–3	
Vaginal fluid (undiluted)[b]		0.7		13	0.9
Cervical mucus (nonpregnant)[b]		0.035–0.042			
Cervical mucus plugs (pregnant)		750 μg/g		660 μg/g	100 μg/g
Amniotic fluid[c]					1–2 to 5–30[g]
Ear wax[d]		0.70 (μg/protein)			6.36 (μg/g protein)

Note: HDPPs. host defense proteins and peptides; BPI, bactericidal/permeability increasing protein; SLPI, secretory leukoprotease inhibitor; LF, lactoferrin;
[a]Lavage is estimated to dilute the concentration of target HDPPs by 100-fold or more.
[b]In addition to individual variation, levels can change depending on the phase of the menstrual cycle.
[c]In addition to individual variation, levels can change depending on the period of the pregnancy.
[d]Up to 60% of a cerumen plug consists of cellular components; LL-37, SPLI, and BPI are mosty cell bound.
[e]This value is derived from mouse studies.
[f]Samples are from celiac patients. These patients had higher than normal immunohistochemical staining for hLF in their intestines. Ten of seventeen
[g]hLF levels increase from 1–2 μg/mL to 5–30 μg/mL after 20–30 weeks of pregnency. The source of this hLF, whether it is from maternal uterine secre-
[h]Level of lactoperoxidase found in the whey fraction of human milk.

challenge with *Klebsiella pneumoniae* (Markart et al. 2004), increased susceptibility to middle ear challenge with *Streptococcus pneumoniae* 6B (Shimada et al. 2008), and impaired resolution of inflammation in response to challenge with *Micrococcus luteus* (Ganz et al. 2003). SP-A knockout mice have increased susceptibility to infection by group B *Streptococcus* (LeVine et al. 1997), *Pseudomonas aeruginosa* (LeVine et al. 1998), *Pneumocystis carinii* (Linke et al. 2001), and *Pneumocystis murina* (Linke et al. 2005) infection. SP-D knockout mice have increased susceptibility to *S. pneumoniae* infection (Jounblat et al. 2005) and have impaired resolution of lipopolysaccharide (LPS) -induced inflammation (Ikegami et al. 2007). Mannose-binding lectin knockout mice have increased sensitivity to *Staphylococcus aureus* infection (Shi et al. 2004). Thus, while knockdown or knockout of many HDPPs impairs the host response to invading microbes, there is no evidence from animal models that LF is important in mucosal immunity.

The LF knockout mice described by Ward et al. (2008) do, however, have a mild phenotype: (*i*) their neutrophils have a defective oxidative burst response when stimulated with phorbol myristate-13-acetate (PMA) in vitro; (*ii*) their total white blood cell counts are slightly decreased; and (*iii*) neutrophil and eosinophil percents in their peripheral blood are elevated. The impaired response to PMA in vitro is often cited as proof that LF is involved in the neutrophil oxidative burst in vivo. It must be remembered, however, that this is an extremely artificial system in which neutrophils are exposed to a single agent, the protein kinase C (PKC) activator PMA, in vitro. Activation of PKC by PMA is sufficient to cause assembly of the NADPH oxidase complex at the membrane (Sheppard et al. 2005), but this complex is inactive. Activa-

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Lactoperoxidase	Lipocallin-1 (tear lipocalin)	Calprotectin (S100A8 + S100A9)	sPLA2	
				Hanson and Winberg 1972; Mickleson and Moriarty 1982; Levay and Viljoen 1995; Montagne et al. 2001; Roseanu and Brock 2006; Weinberg 2009
770[a]				Hanson and Winberg 1972; Mickleson and Moriarty 1982; Levay and Viljoen 1995; Montagne et al. 2001; Shin et al. 2001; Roseanu and Brock 2006; Weinberg 2009
	2200		32–37	Kijlstra et al. 1983; Levay and Viljoen 1995; Qu and Lehrer 1998; Sathe et al. 1998; Sack et al. 2001; Flanagan and Willcox 2009; Weinberg 2009
	3600			Sathe et al. 1998; Sack et al. 2001
				Thompson et al. 1990
3–12				Vogelmeier et al. 1991; Ganz 2002, 2004; Wijkstrom-Frei et al. 2003
				Cole et al. 1999; Cole et al. 2002
1.9		1.93		Tenovuo et al. 1987; Weinberg 2009; Gorr and Abdolhosseini 2011
				Si-Tahar et al. 2000
				Tedeschi et al. 1987
				Troost et al. 2002
				Buckett et al. 1997
		5–14		Valore et al. 2006
		34		Valore et al. 2002; Venkataraman et al. 2005
				Moriyama et al. 1999
		38 μg/g		Hein et al. 2002
		1.5–11.9		Heller et al. 1995; Levay and Viljoen 1995; Espinoza et al. 2003
				Schwaab et al. 2011

sPLA2, secretory phospholipase A2; hLF, and human lactoferrin.

patients had no measurable hLF in their duodenojejunal aspirate, and 7 patients had values ranging from 0.22–0.70 μg/mL in their duodenojejunal aspirate.
tions, maternal uterine neutrophils, or from the developing embryo, is not known.

tion of the NADPH oxidase and generation of an oxidative burst requires a second event, association of Rac with the NADPH oxidase complex (Sheppard et al. 2005; Nordenfelt and Tapper 2011). This second event can be actuated by toll-like receptor 4 (TLR-4) signaling. It is well known that LPS, which signals via TLR-4, primes the neutrophil oxidative burst (DeLeo et al. 1998), indicating that TLR-4 signaling can have an activating role in the assembly of an active NADPH oxidase complex; also, TLR-4 signaling can activate Rac in fibroblasts (Nishida et al. 2010). Since LF binds to TLR-4 and induces TLR-4 signaling (Ando et al. 2010), LF is able to provide the second signal required for full activation of neutrophils in response to PMA in vitro. In the presence of microorganisms, on the other hand, microbial antigens provide the required secondary signals; consequently, as shown by Ward et al. (2008), LF-knockout mouse and wild-type mouse neutrophils respond identically to bacterial challenge both in vitro and in vivo (see Figs. 4, 5, 6, 7, 8, and 9 in Ward et al. 2008).

The effects of LF deficiency on leukocytes (total white blood cell counts are slightly decreased and neutrophil and eosinophil percents in the peripheral blood are elevated in Lf-knockout mice) show that LF does have an effect in vivo. To date, there are 2 signaling receptors that have been proven to transduce LF signals into the cell, TLR-4 (Ando et al. 2010) and low-density lipoprotein receptor-related protein 1 (LRP1) (Grey et al. 2004). A possible mechanism by which LF could affect lymphocyte development is the following: LRP1 interacts with Frizzled and downregulates Wnt signaling (Zilberberg et al. 2004). Since Wnt signaling is important for lymphocyte development (Staal and Sen 2008), LRP1 would depress lymphocyte development. LF binding to

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LRP1 and induction of LRP1 internalization could interfere with LRP1 interaction with Frizzled and upregulate Wnt signaling. Therefore, LF would enhance lymphocyte development, and in LF-knockout mice, lymphocyte development would be somewhat depressed.

A possible mechanism by which LF could affect the peripheral blood levels of neutrophils and eosinophils is via TLR-4 signaling. Stimulation of TLR-4 signaling enhances activation of neutrophils and migration out of the blood and infiltration into the tissues (Sabroe et al. 2005). In the absence of serum LF, the basal level of TLR-4 signaling is lower than in wild-type mice, and, consequently, activation of neutrophils and migration out of the blood would be lower in the knockout animals. This would result in higher numbers of neutrophils remaining in the blood. The effect on eosinophils is a little different. TLR-4 signaling is reported to have little effect on activation of eosinophils and infiltration into the tissues (Nagase et al. 2003). Instead, eosinophils respond to cytokines released by TLR-4-stimulated monocytes and leave the blood and infiltrate into the tissues (Kobayashi et al. 2009). Therefore, similarly to the effect on neutrophils, decreased TLR-4 signaling would result in fewer tissue infiltrating eosinophils and more circulating eosinophils. Direct experimental evidence that refutes or supports these hypotheses is lacking, but the results of LF-knockout in mice does demonstrate that LF does have a measurable in vivo effect.

Perhaps the most salient observation made of the LF-knockout mice was a nonsignificant increase in spontaneous abscess formation (Ward et al. 2008). Importantly, the LF mice were housed in barrier-controlled specific pathogen-free (SPF) conditions, and, consequently, opportunistic infection of these mice was limited. If LF acts to protect against opportunistic infection, the importance of this activity would be more likely to be observed in mice raised in barrier-free conditions than in SPF conditions. Unfortunately, the effect of raising LF-knockout mice in barrier-free conditions have not yet been reported.

Evidence from humans

There are several reports of health benefits conferred by HDPPs. For example, Pütsep et al. (2002), Boman (2003), and Carlsson et al. (2006) describe Kostmann syndrome (also known as severe congenital neutropenia). Kostmann syndrome is associated with severe periodontitis. The saliva, plasma, and neutrophils from Kostmann patients are deficient in LL-37 and patients also have a >50% decrease in neutrophil α-defensins. The neutrophils themselves have normal levels of LF and a normal oxidative burst. Treatment with granulocyte-colony-stimulating factor restores the number of neutrophils to normal but patients continue to lack LL-37 and exhibit periodontal disease. A bone marrow transplant in a single patient restored both neutrophil numbers and the levels of LL-37 and α-defensins and no further dental problems were noted. This data strongly support the idea that CAP18 and (or) α-defensins are critical for human health. Another HDPP that was found to be associated with human health is elafin. Analysis of the genital secretions of HIV-resistant Kenyan sex-workers found that elevated levels of elafin were associated with resistance to HIV infection (Iqbal et al. 2009). A third example is a study that found that patients with SP-D deficiency have more frequent pneumonias and their long-term outcome is worse than that of the patients with detectable SP-D (Griese et al. 2008). A fourth study reports that neutrophils in human newborn blood display an apparently selective deficiency of BPI compared with other granule proteins, and this deficiency appears to account for the reduced antibacterial activity of extracts of newborn neutrophils toward BPI-sensitive Gram-negative bacteria (Levy et al. 1999).

There are currently no known human diseases related to LF deficiency. Cystic fibrosis (CF) is sometimes cited as proof that hLF is essential to human health. CF patients become colonized by *P. aeruginosa* (Lyczak et al. 2002; Cystic Fibrosis Foundation 2010), and their airway fluids contain proteases that degrade hLF (Britigan et al. 1993). However, CF is caused by mutations that result in inactivation of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel (Rogan et al. 2011). Inactivation of this channel impairs transport of Cl^-, HCO_3^-, and SCN^- into the airway fluid (Riordan 2008; Itani et al. 2011). Defective transport of these ions has 2 consequences: (*i*) a decrease in airway fluid resulting in a highly viscous fluid that impairs mucociliary clearance (Donaldson et al. 2006) and (*ii*) a dysfunctional lactoperoxidase system (Conner et al. 2007). These 2 defects, not the late-stage proteolytic degradation of hLF, allow the colonization of the normally sterile lower airways by invading microorganisms.

Another condition that is sometimes cited as an example of the importance of hLF as an HDPP is specific granule deficiency (SGD). This disease is caused by a mutation in the gene encoding the transcription factor CCAAT/enhancer binding protein-ε (C/EBPε) (Lekstrom-Himes et al. 1999; Gombart et al. 2001). This results in defective formation of neutrophil granules and severe decreases in neutrophil collagenase, gelatinase, DEFAs 1–3, CAP18, BPI, and hLF (Shiohara et al. 2004). SGD neutrophils are defective in chemotaxis and microbicidal activity, and SGD patients suffer from recurrent infections (Dinauer et al. 2000). C/EBPε-knockout mice (Yamanaka et al. 1997; Dinauer et al. 2000; Shiohara et al. 2004), but not LF-knockout mice (Ward et al. 2008), have a phenotype very similar to SGD patients. Therefore, lack of hLF does not by itself cause the pathological features of SGD. Furthermore, SGD patients produce normal amounts of mucosal LF (Lomax et al. 1989; Raphael et al. 1989). Finally, neutrophils in LF-knockout mice have no observed in vivo abnormalities (Ward et al. 2008). Together, these data do not support a role for hLF deficiency in the pathological features of SGD.

There are reports of LF single nucleotide polymorphisms (SNPs) that are associated with disease.[1] One SNP is K29R

[1]The interested reader will find that the SNP-amino acid number noted in some of the cited references differs from the SNP-amino acid number in the NIH SNP database. This discrepancy is due to the fact that the reference sequence used at the time of this writing to identify hLF SNPs was NM_002343.3. This RefSeq translates the N-terminal of hLF as MKLVFLVLLFLGALGLCLAG**RRRR**SV rather than the previously published MKLVFLVLLFLGALGLCLAG**RRRR**SV. Therefore, the amino acids affected by the SNP have location numbers in the current SNP database of one less than in previously published hLF protein sequences.)

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(K28R refSNP No. rs1126478; position 47 in the unprocessed protein). In a study with 17 healthy control subjects and 9 patients with localized juvenile periodontitis, the Lys variant was strongly associated with disease (Velliyagounder et al. 2003). The basis for the increased resistance of the Arg variant to localized juvenile periodontitis is unknown. The 2 variants had equivalent iron-binding and iron-releasing activities, and the Lys variant had equivalent antimicrobial activity against Gram-negative bacteria and significantly greater activity against Gram-positive bacteria. The Lys variant also induced expression of tracheal antimicrobial peptide (Diamond et al. 1991) to a much greater extent than the Arg variant. A follow up study in a larger population was performed, but the results of the association between the Arg and Lys variants and disease were not published. A report from Taiwan of a population consisting of 65 subjects with aggressive periodontitis, 278 subjects with chronic periodontitis, and 88 healthy subjects found a mild but statistically significant association between the Arg variant and aggressive periodontitis (Wu et al. 2009*c*); a result in direct contrast with the result of Velliyagounder et al. (2003). Wu et al. (2009*c*) conclude that the Lys/Arg LF SNP might be associated with aggressive periodontitis. Another report on the association between LF and periodontitis (Jordan et al. 2005) found an association between the T11A SNP (A10T refSNP No. rs1126477; position 29 in the unprocessed protein) and aggressive periodontitis. The study population consisted of 77 Caucasian patients and 131 healthy Caucasian controls and 46 African-American patients and 78 healthy African-American controls. There was no association between the T11A polymorphism and disease in the Caucasian population, but the Thr variant was associated with disease in the African-American population. This report provided no information concerning the K28R SNP and periodontitis. Another LF SNP possibly associated with disease is E561D (E560D refSNP No. rs2073495; position 579 in the unprocessed protein). A study of a group of 105 HSV keratitis patients and 145 control subjects found that the Glu variant was associated with disease (Keijser et al. 2008). The study also reports that neither the K29R nor the A11T SNPs were associated with HSV keratitis. Finally, a study of 762 students found a modest association between the L632L SNP (refSNP No. rs7645243; rs7645243 is now merged into rs9110) and traveler's diarrhea in female students (Mohamed et al. 2007). There was no association of this SNP with disease in male students. How this cds-synonymous SNP could alter the activity of hLF and why the association is restricted to women is unknown. The association of SNPs L632M and L632V with disease was not reported. The study found no association between 8 other SNPs (refSNP Nos. rs34278344, rs2073495 (E561D), rs34827868, rs1126478 (K29R), rs4637321, rs4683233 (T11A) (merged into rs1126477), rs2239692, and rs2239692) and disease.

Finally, a single individual (now deceased) with a LF deficiency has been described (Lin et al. 2001). This patient did not have any detectable hLF in his plasma, serum, or saliva; other mucosal secretions were not available for hLF assay. The patient did not have a history of recurrent infections nor was he predisposed to infections.

Postulations based on the reported data

LF is widely expressed by placental mammals, even those that do not secrete LF into their milk. This indicates that LF has an important function in mammalian physiology. However, direct experimental evidence that the endogenous LF present in mammalian milk, mucosal secretions, and neutrophils is important is lacking. Is it possible to reconcile the wide occurrence of LF in placental mammals with the lack of effect when LF is removed from mammalian secretions? One obvious point is that LF is present in a cocktail of HDPPs, many of which have similar antimicrobial and immune-modulating activities commonly ascribed to LF. Therefore, removal of the LF component would be expected to have a minor impact on the overall activity of LF-containing fluids toward most microorganisms. Consequently, multiple knockouts (which have not yet been reported) may be required to ascertain the true importance of LF.

Another factor in the mild phenotype of LF-knockout mice has to do with the distribution pattern of LF. LF is present at high levels in tears and airway fluids, mucosal fluids that come into extensive contact with external microorganisms. This suggests that LF acts as a barrier to infection by external microorganisms. A barrier-controlled SPF environment also acts as a barrier to infection by external microorganisms, and, importantly, the LF-knockout mice described by Ward et al. (2003, 2008) were raised in a barrier-controlled SPF environment. Consequently, the importance of LF function in these animals was lessened. It is possible that LF-knockout mice would be more susceptible than their wild-type counter parts to infection if they were raised in barrier-free conditions. Unfortunately, the results of such an experiment have not yet been reported.

LF is also widely present in the neutrophils of placental mammals; however, as described by Ward et al. (2008), neutrophils from control and LF-knockout animals have identical activity toward bacteria both in vitro and in vivo. One possibility is that LF plays a role in microorganism killing by neutrophils that has yet to be identified. Given the high levels of other HDPPs in the neutrophil phagolysosome (Segal 2005; Borregaard et al. 2007; Nordenfelt and Tapper 2011), the importance of LF in the killing of microorganisms by neutrophils is not certain and organisms killed by LF-positive neutrophils and resistant to LF-negative neutrophils have not been identified. Another possibility is suggested by the fact that while neutrophil phagolysosomes contain both myeloperoxidase and LF, macrophage phagolysosomes contain neither myeloperoxidase nor LF. Myeloperoxidase is an iron-binding protein that catalyzes the oxidation of chloride ions to generate the antimicrobial agent hypochlorous acid (Klebanoff 2005). Hypochlorous acid is extremely reactive and destruction

$$[1] \quad H_2O_2 + Cl^- \xrightarrow{MPO} OCl^- + H_2O$$

of the iron-binding myeloperoxidase could result in release of iron and uncontrollable iron-catalyzed generation of oxygen radicals and toxic metabolites (Halliwell and Gutteridge 1984). Iron bound by LF is not available for production of oxygen radicals (Britigan et al. 1987); therefore, the presence of LF in the phagolysosome would help to prevent uncontrollable free-iron catalyzed reactions and maintain the controlled enzymatic generation of hypochlorous acid. In addition, hLF can be induced to high levels in inflamed tissues because of neutrophil activity; for example, it can be present at up to

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600 μg/mL in inflamed gingival crevicular fluid (Gorr and Abdolhosseini 2011). Binding free iron would also decrease extracellular oxygen radical production and decrease tissue damage at sites of high neutrophil activity. While these speculations remain to be tested, it is possible that neutrophil LF needs to be examined in the context of inflammation, both acute and chronic, rather than simple microbial killing.

The lack of any diseases associated with LF coupled with the mild phenotype of the LF-knockout mouse suggests that LF deficiency is either asymptomatic or lethal in humans but not in mice. We argue that the second possibility is the correct one. As discussed above, it is a reasonable conjecture that if the LF-knockout mice described by Ward et al. (2003, 2008) were raised in a barrier-free facility, the incidence of opportunistic infection would increase. Also, it is possible that there is an association between some hLF SNPs and disease. Consequently, it is plausible that a defect in LF would not be asymptomatic. On the other hand, what is the evidence that LF is essential in humans and not in mice? It is very unlikely that the host defense function of hLF is essential, at least early in life; the fetus develops in a sterile environment and is commonly delivered in an aseptic facility. In humans, however, the LF gene encodes 2 variants, LF and Δ-LF. Δ-LF is a transcription factor involved in regulation of the cell cycle and apoptosis (reviewed by Mariller et al. in this issue of Biochemistry and Cell Biology). Thus, Δ-LF function could very well be essential during human embryogenesis, and this could account for the lack of hLF deficiency in humans. The fact that the single patient deficient in hLF described in the literature had severe neurological dysfunctions (Lin et al. 2001) agrees with this hypothesis.

The various reported associations of hLF SNPs with disease are suggestive, but apparent contradictions need to be resolved. For example, for the association between K29R and periodontitis, why were the results of the larger follow up study testing the association between this SNP and localized juvenile periodontitis found by Velliyagounder et al. (2003) not published? Why were the results of the study by Wu et al. (2009*c*), which found an association between the K29R SNP and aggressive periodontitis, in direct contradiction to the study by Velliyagounder et al. (2003)? Why did the study by Jordan et al. (2005), which found an association between the T11A SNP and aggressive periodontitis, not mention the K29R SNP? Possibly, differences in the type of periodontitis and in the make up of the HDPP cocktail in the saliva and neutrophil compartments of different populations could account for these seeming discrepancies, but this speculation remains to be tested. Another seeming inconsistency is the association between the cds-synonymous SNP L632L and traveler's diarrhea found by Mohamed et al. (2007). The authors speculate that the SNP may affect hLF expression; however, this needs to be tested and reported. Also the frequencies of the missense SNPs L632M and L632V need to be reported. The association between the E561D SNP and HSV keratitis reported by Keijser et el. (2008) may be the best proof that hLF does have important antimicrobial activity in humans. Further studies verifying this association and examining why the Asp variant is more effective than the Glu variant in preventing HSV infection of the cornea are warranted.

Antimicrobial activities of LF

LF is microbiostatic

Initial studies on the effect of LF on the viability of microorganisms concluded that LF was microbiostatic and that this microbiostatic activity was the result of sequestering iron away from the test microorganism (Oram and Reiter 1968). Later it was noted that in addition to its iron-sequestering activity, LF exerted an iron-sensitive antimicrobial activity toward some microorganisms that was not reversed or prevented by the addition of iron (Arnold et al. 1981, 1982). Consequently, it was conjectured that, in addition to sequestering iron, apo-LF (iron-free LF) had iron-independent antimicrobial activity and that due to conformational changes that occurred upon binding iron, holo-LF (iron-bound LF) lacked this activity. In addition, early assays in low ionic strength fluids without divalent cations also indicated that hLF was microbicidal. Here, we argue that when additional, more recent studies are evaluated, apo-hLF in biologically relevant fluids is microbiostatic, and even the highest levels of hLF found in vivo are not microbicidal.

Like the other cationic HDPPs, hLF has a broad spectrum of microbicidal activity in low ionic strength fluids lacking Ca^{2+} and Mg^{2+}. However, the ionic environment has an enormous effect on the microbicidal activity of cationic HDPPs. First, attraction of cationic HDPPs, such as LF, for negatively charged target membranes is highly dependent upon the ionic strength of the fluid in which the microbe and the HDPP are present; the interaction between a pentavalent cationic HDPP and a pentavalent anionic section of membrane is predicted to decrease 2000-fold if the ionic environment is changed from a 10 mmol/L HEPES-Na buffer (pH 7.5) to a buffer containing 100 mmol/L NaCl (Nikaido 2003). Therefore, in fluids with physiological concentrations of salts, much higher levels of cationic HDPPs are required to target negatively charged bacterial membranes than are required in fluids with low salt concentrations. Bacterial membranes are structurally different from mammalian membranes, one important difference is that the outer leaflets of mammalian membranes are composed of zwitterionic phospholipids and are consequently neutral whereas the membranes of Gram-negative and Gram-positive bacteria are negatively charged (see Matsuzaki (1999) and Yeaman and Yount (2003) for a discussion of the differences between bacterial and mammalian membranes and how these differences affect the targeting of these membranes by cationic HDPPs).

A second critical effect of the ionic environment is that divalent cations stabilize the outer membrane of Gram-negative bacteria and help shield the lipid A moiety of LPS from interaction with large cationic proteins such as LF (Nikaido 2003; Schneck et al. 2009; Oliveira et al. 2010; for the interested reader, a very brief account of the effects of divalent cations on the outer membrane of Gram-negative bacteria is presented in Supplementary data Chapter 1).[2] Therefore, in fluids with physiological concentrations of calcium and magnesium, Gram-negative bacteria are highly resistant to LF.

[2]Supplementary data are available with the article through the journal Web site (www.nrcresearchpress.com/doi/suppl/10.1139/o2012-013).

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Another factor is the ability of hLF to sequester Ca^{2+} away from the target microorganism. As described by Rossi et al. (2002): (*i*) bovine lactoferrin (bLF) is able to bind Ca^{2+} with a K_d of about 6 μmol/L; (*ii*) the bLF protein itself does not possess Ca^{2+}-binding activity, rather it is the sialic acid residues present on the glycan chains of bLF that bind Ca^{2+}; (*iii*) even when separated by a dialysis membrane, EDTA, apo-bLF, and holo-bLF each caused release of LPS from the outer membrane of *Pseudomonas aeruginosa* PAO1, and this effect was abolished by addition of Ca^{2+} or Mg^{2+} to the assay buffer. Therefore, in fluids with low concentrations of calcium and magnesium, the ability of bLF to bind and sequester Ca^{2+} away from the target microorganism could significantly impact the viability of the test microorganism. In biological fluids, on the other hand, the Ca^{2+}-binding sites of bLF are saturated, and bLF does not interfere with acquisition of Ca^{2+} (Rossi et al. 2002). Since bLF and hLF have similar sialic acid residues present in their glycans chains (Spik et al. 1988), it is reasonable to suppose that hLF should also be able to chelate Ca^{2+}.

Importantly, tears and mucosal fluids contain NaCl in concentrations of 100 mmol/L or more and divalent cations between 1 and 2 mmol/L. In a study comparing the effects of different methods for assessing the bactericidal and membrane permeabilizing effects of small cationic peptides derived from lactoferricin, Sánchez-Gómez et al. (2008) report that (*i*) testing in low ionic strength solvents resulted in a vast overestimation of bactericidal activity and (*ii*) addition of 1 mmol/L Mg^{2+} and Ca^{2+} to a low ionic strength buffer abolished the bactericidal activity of many of the peptides tested.

Unfortunately, there are few studies of the microbicidal activity of hLF in physiologically relevant fluids. Currently, the data reported in the literature does not support a microbicidal role for hLF in tear or mucosal fluids. Table 2 lists the results of studies of hLF in which salts or divalent cations were present in the assay buffer. In these studies, when holo-hLF was tested, it was consistently found to lack microbicidal activity. As can be seen in Table 2, in 3 studies (Arnold et al. 1980, 1981; Kalmar and Arnold 1988) the microbicidal effect of hLF was salt or cation insensitive. Importantly, in these studies the assay solution was not buffered. When the solution was buffered (Arnold et al. 1981) or the pH was raised (Arnold et al. 1981; Kalmar and Arnold 1988), the microbicidal activity of hLF was abolished. In buffered solutions with higher levels of salts or divalent cations, hLF either had no effect on the target microorganism or was microbiostatic. There are no reports of hLF killing target microorganisms in fluids with relevant levels of buffering ions, salts, and divalent cations.

Not surprisingly, LF-mediated membrane damage is also inhibited by the conditions that inhibit its microbicidal activity. Ellison et al. (1988, 1990) report that in Hanks balanced salt solution without Ca^{2+} or Mg^{2+}, apo-hLF caused release of LPS from *Escherichia coli* CL99-2, *Salmonella typhimurium* SH4247, and *Salmonella typhimurium* SL696. When Ca^{2+} or Mg^{2+} was added back, apo-hLF did not cause release of LPS from these bacteria. Holo-hLF did not cause release of LPS under any of the test conditions.

An interesting finding was reported in the study by Arnold et al. (1981). Stationary phase bacteria were resistant to hLF. The HDPPs known as peptidoglycan recognition proteins (PGLYRPs) also affect growing but not stationary cells. Dziarski and colleagues have described PGLYRPs (Dziarski and Gupta 2006; Lu et al. 2006; Wang et al. 2007*a*; Kashyap et al. 2011) and have shown that these HDPPs interact with target cells at the site of daughter cell separation during cell division and activate a stress-response system that detects and disposes of misfolded proteins. Inappropriate activation of this system can result in inhibition of cell growth or cell death. They speculate that the mechanism by which PGLYRP mediates its antimicrobial activity may be shared by other HDPPs. Currently, however, there are no reports regarding LF and this possible mechanism of action.

hLF has been shown to remove the hemoglobin receptor protein (HbR) from the cell surface of *Porphyromonas gingivalis* (Shi et al. 2000). Significant reduction of cell surface HbR occurred at 13.6 μmol/L (~1000 μg/mL) hLF. Removal was inhibited by cysteine protease inhibitors, suggesting the possibility that the protease activity of hLF is required for removal of HbR (hLF protease activity is discussed below). However, removal of HbR is also accomplished by 24.8 μmol/L bovine lactoferricin (bLFcin), which has no proteolytic activity. The effect of protease inactive hLF on HbR removal from *P. gingivalis* cell surfaces was not assessed in these studies. Unfortunately, the assay was performed in PBS without Ca^{2+} or Mg^{2+}. Given the critical importance of divalent cations on the stability of the outer membrane of Gram-negative bacteria, it is essential that the effect of physiological levels of Ca^{2+} and Mg^{2+} on hLF activity be assessed before any conclusions on hLF activity can be reached. Also, since in healthy individuals hemoglobin is not an important source of iron in mucosal fluids, the relevance of this activity in vivo is uncertain. On the other hand, if hLF does have this activity in mucosal fluids, in patients with periodontal disease, inhibition of hemoglobin as a source of iron may be physiologically relevant.

Iron binding by hLF is its best characterized property (Baker and Baker 2005). Although some microorganisms express siderophores that have a higher affinity for iron than hLF and are therefore able to obtain iron in the presence of hLF (Chu et al. 2010) and some microorganisms express hLF receptors and use iron bound hLF as an iron source (León-Sicairos et al. 2005; Beddek and Schryvers 2010), hLF is able to sequester iron away from most microorganisms, thereby inhibiting their growth (Masson et al. 1966; Arnold et al. 1980; Aguila et al. 2001; Weinberg 2009). To date, sequestering iron away from microorganisms is the only verified method by which hLF inhibits the growth of invading microbes. In addition to inhibiting the growth of microorganisms, the iron-sequestering function of LF inhibits biofilm formation by *P. aerugionsa* (Singh et al. 2002; Singh 2004), *Burkholderia* species (Caraher et al. 2007), *P. gingivalis*, and *Prevotella intermedia* (Wakabayashi et al. 2009).

Taken together, the results from the literature discussed here indicate that the hLF protein is microbiostatic rather than microbicidal, and hLF does not destabilize membranes in vivo. hLF may be able to exert its microbiostatic activity by mechanisms other than sequestering iron, but such mechanisms have not been verified in biologically relevant fluids.

Table 2. The antimicrobial effect of hLF in studies in which salt or divalent cations were present in the assay solution.

Assay solution	Test organism	Organism	apo-hLF concentration	Condition				Reference
				Low salt	Low salt + divalent cations	High salt	High salt + divalent cations	
Unbuffered saline	*Streptococcus mutans* AHT	(+)	333 μg/mL			Microbicidal		Arnold et al. 1977
Unbuffered saline	*Vibrio cholerae* 569B	(–)	333 μg/mL			Microbicidal		Arnold et al. 1977
Unbuffered saline	*Escherichia coli* 0126	(–)	333 μg/mL			No effect		Arnold et al. 1977
Unbuffered saline	*Streptococcus mutans* AHT	(+)	4.2 μmol/L			Microbicidal		Arnold et al. 1980
Unbuffered saline	*Streptococcus mutans* BHT	(+)	4.2 μmol/L			Microbicidal		Arnold et al. 1980
Unbuffered saline	*Streptococcus mutans* 10449	(+)	4.2 μmol/L			Microbicidal		Arnold et al. 1980
Unbuffered saline	*Streptococcus mutans* 6715	(+)	4.2 μmol/L			Microbicidal		Arnold et al. 1980
Unbuffered saline	*Streptococcus mutans* LM-7	(+)	4.2 μmol/L			Microbicidal		Arnold et al. 1980
Unbuffered saline	*Streptococcus salivarius*	(+)	4.2 μmol/L			Microbicidal		Arnold et al. 1980
Unbuffered saline	*Streptococcus mitior*	(+)	4.2 μmol/L			Microbicidal		Arnold et al. 1980
Unbuffered saline	*Streptococcus pneumoniae* ATCC 6303	(+)	4.2 μmol/L			Microbicidal		Arnold et al. 1980
Unbuffered saline	*E. coli* (nonenteropathogen)	(–)	4.2 μmol/L			No effect		Arnold et al. 1980
Unbuffered saline	*E. coli* (nonenteropathogen)	(–)	42.0 μmol/L			Microbicidal		Arnold et al. 1980
Unbuffered saline	*V. cholerae* 569B	(–)	4.2 μmol/L			Microbicidal		Arnold et al. 1980
Unbuffered saline	*Pseudomonas aeruginosa*	(–)	4.2 μmol/L			Microbicidal		Arnold et al. 1980
Unbuffered saline	*Candida albicans*	Yeast	4.2 μmol/L			Microbicidal		Arnold et al. 1980
Unbuffered saline	*Streptococcus pyogenes*	(+)	83 μmol/L			No effect		Arnold et al. 1980
Unbuffered saline	*Streptococcus lactis* 11454	(+)	83 μmol/L			No effect		Arnold et al. 1980
Unbuffered saline	*Lactobacillus casei*	(+)	83 μmol/L			No effect		Arnold et al. 1980
Unbuffered saline	*Staphylococcus aureus*	(+)	83 μmol/L			No effect		Arnold et al. 1980
Unbuffered saline	*Staphylococcus epidermidis*	(+)	83 μmol/L			Microbiostatic		Arnold et al. 1980
Unbuffered saline	*Escherichia coli* O126:B16	(–)	83 μmol/L			No effect		Arnold et al. 1980
Unbuffered saline	*Escherichia coli* O111	(–)	83 μmol/L			No effect		Arnold et al. 1980
Unbuffered saline	*Enterobacter cloacae*	(–)	83 μmol/L			No effect		Arnold et al. 1980
Unbuffered saline	*Salmonella newport*	(–)	83 μmol/L			No effect		Arnold et al. 1980
Unbuffered saline	*Shigella sonnei*	(–)	83 μmol/L			No effect		Arnold et al. 1980
Unbuffered	*Legionella pneumophila*	(–)	260 μg/mL	Microbicidal	No effect[c]			Bortner et al. 1986
Unbuffered	*S. mutans* 10449	(+)	1.2 μmol/L	Microbicidal		Microbicidal	Microbicidal	Arnold et al. 1981
Unbuffered + iron	*S. mutans* 10449	(+)	1.2 μmol/L	Microbicidal				Arnold et al. 1981
Unbuffered	*S. mutans* 10449	(+)	0.12 μmol/L	Microbicidal				Arnold et al. 1981
Unbuffered pH 8.0	*S. mutans* 10449	(+)	0.12 μmol/L	No effect				Arnold et al. 1981
50 mmol/L K-phosphate (low pH)	*S. mutans* 10449	(+)	0.12 μmol/L	No effect				Arnold et al. 1981
50 mmol/L K-phosphate (high pH)	*S. mutans* 10449	(+)	0.12 μmol/L	No effect				Arnold et al. 1981
HEPES (low pH)	*S. mutans* 10449	(+)	0.12 μmol/L	No effect				Arnold et al. 1981
HEPES (high pH)	*S. mutans* 10449	(+)	0.12 μmol/L	No effect				Arnold et al. 1981
Unbuffered pH 5.5	*Actinobacillus actinomycetemcomitans*	(–)	1.3 μmol/L			Microbicidal	Microbicidal	Kalmar and Arnold 1988

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Table 2 (*continued*).

Assay solution	Test organism	Organism	apo-hLF concentration	Condition				Reference
				Low salt	Low salt + divalent cations	High salt	High salt + divalent cations	
Unbuffered pH 6.0	*A. actinomycetemcomitans*	(–)	1.3 μmol/L			No effect		Kalmar and Arnold 1988
WMS broth[a]	*Escherichia coli* CL99-2		2000 μg/mL		Microbio-static			Ellison et al. 1988
Todd Hewitt broth	*Streptococcus mutans* ATCC 6715-13 ser. g	(+)	312–2500 μg/mL	Microbiostatic				Visca et al. 1989
M9 minimal medium[b]	*Escherichia coli* 803	(–)	500 μg/mL		Microbio-static			Visca et al. 1990
M9 minimal medium[b]	*Escherichia coli* CSH26	(–)	500 μg/mL		Microbio-static			Visca et al. 1990
M9 minimal medium[b]	*Escherichia coli* C600	(–)	500 μg/mL		Microbio-static			Visca et al. 1990
M9 minimal medium[b]	*Escherichia coli* AN263	(–)	500 μg/mL		Microbio-static			Visca et al. 1990
M9 minimal medium[b]	*Escherichia coli* HN13	(–)	500 μg/mL		Microbio-static			Visca et al. 1990
M9 minimal medium[b]	*Escherichia coli* K311	(–)	500 μg/mL		No effect			Visca et al. 1990
M9 minimal medium[b]	*Escherichia coli* EC20	(–)	500 μg/mL		No effect			Visca et al. 1990
M9 minimal medium[b]	*Escherichia coli* EC30	(–)	500 μg/mL		No effect			Visca et al. 1990
M9 minimal medium[b]	*Escherichia coli* EC33	(–)	500 μg/mL		No effect			Visca et al. 1990
PBS	*Porphyromonas gingivalis*	(–)	2000 μg/mL			Microbiostatic		Aguilera et al. 1998
PBS	*Prevotella intermedia*	(–)	2000 μg/mL			No effect		Aguilera et al. 1998
PBS	*Prevotella nigrescens*	(–)	2000 μg/mL			No effect		Aguilera et al. 1998
10 mmol/L K-phosphate wth 1% LB	*E. coli* (clinical strain from urinary tract)	(–)	230 μg/mL	Microbicidal	No effect	No effect		Travis et al. 1999
LB± Na-phosphate	*Shigella flexneri* 5 M90T-A2	(–)	10000 μg/mL			No effect		Gomez et al. 2003
DMEM–HEPES	Enteropathic *Escherichia coli* O127:H6 E2348/69,	(–)	10000 μg/mL			No effect		Ochoa et al. 2003
PBS	*P. gingivalis*	(–)	8000 μg/mL			Microbiostatic		Wakabayashi et al. 2009
PBS	*P. intermedia*	(–)	8000 μg/mL			Microbiostatic		Wakabayashi et al. 2009
5 mmol/L K-phosphate	*C. albicans*	Yeast	5 μmol/L up to 30 μmol/L	Microbicidal		No effect		Viejo-Díaz et al. 2004
5 mmol/L Na-phosphate	*C. albicans*	Yeast	5 μmol/L up to 30 μmol/L	Microbicidal		No effect		Viejo-Díaz et al. 2004
5 mmol/L Tris buffer	*C. albicans*	Yeast	5 μmol/L up to 30 μmol/L	Microbicidal	No effect[d]	No effect		Viejo-Díaz et al. 2004
RPMI/HEPES	*Aspergillus fumigatus*	Fungus	10 μg/mL				Microbiostatic	Zarember et al. 2007

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Table 2 (*concluded*).

Assay solution	Test organism	Organism	apo-hLF concentration	Condition				Reference
				Low salt	Low salt + divalent cations	High salt	High salt + divalent cations	
Isotonic HEPES-sucrose	*Giardia lamblia*	Protozoa	2500	Microbicidal	No effect			Turchany et al. 1995
BI-S-33 media	*Entamoeba histolytica*	Protozoa	31.25 μmol/L	Microbicidal	No effect[e]			León-Sicairos et al. 2006

Note: hLF, human lactoferrin; WMS, ; LB, Luria–Bertani broth; PBS, phosphate-buffered saline; RPMI, 2.5% fetal bovine serum, HEPES, L-glutamine, and $NaHCO_3$; (+), Gram-positive bacteria; (–): Gram-negative bacteria; and apo-hLF, iron-free human lactoferrin.

[a]WMS broth, low ionic strength with 1 mmol/L Mg^{2+}.

[b]M9 minimal medium, low ionic strength, 2 mmol/L Mg^{2+}.

[c]8 mmol/L Mg^{2+} was used in this assay.

[d]4 mmol/L Ca^{2+} was required to abolish the candidacidal activity of hLF.

[e]5 mmol/L Ca^{2+} or Mg^{2+} was required to abolish the amoebacidal activity of apo-hLF.

Lactoferricin

Human lactoferricin (hLFcin) consists of the N-terminal 49 amino acids of mature hLF and is generated by pepsin digestion of the parent protein (Hunter et al. 2005). Comparison of bLFcin and hLFcin reveals important information about the activity of hLFcin. Both hLFcin and bLFcin are small, salt-sensitive, cationic, antimicrobial peptides, but bLFcin is much more potent than hLFcin. For example, bLFcin exhibits more than 15-fold higher bactericidal effect against *E. coli* compared with hLFcin (Bellamy et al. 1992). The structure of these 2 peptides in aqueous solution discloses a key physical feature that allows bLFcin to interact more readily with target membranes than hLFcin. In aqueous solution, bLFcin adopts a β-sheet conformation with several hydrophobic residues clustered on one side of the sheet; therefore, once bLFcin's positively charged residues bring it into contact with the target cell, hydrophobic residues interact with the membrane (Hwang et al. 1998; Farnaud et al. 2004; Gifford et al. 2005; Hunter et al. 2005; Daidone et al. 2011). hLFcin, on the other hand, adopts a coiled structure in aqueous solution without an ordered alignment of hydrophobic residues making its initial interactions with target membranes much weaker than that of bLF (Hunter et al. 2005). The greater microbicidal activity of bLFcin compared with hLFcin could be related to the necessity of maintaining Δ-LF function in humans, as noted above.

To date, the presence of antimicrobial levels of hLFcin in human mucosal fluids has not been documented. There is, however, evidence that hLFcin may be microbicidal toward selected microorganisms in vivo. Mirza et al. (2011) report on the mechanism by which apo-hLF kills *S. pneumoniae*. Importantly, the assays were carried out in potassium phosphate buffered saline containing 1 mmol/L Mg^{2+}. They demonstrate that apo-hLF killing requires production of serine proteases by *S. pneumoniae*, that these proteases cleave apo-hLF between amino acid residues 78 and 79, generating a short peptide with a hLFcin moiety, that killing by hLF is abolished by serine protease inhibitors, and that the *S. pneumoniae* EF3030 *prtA* (protease) mutant is resistant to hLF-mediated killing. The concentration of hLF in the airway lining fluid is estimated to be approximately 100 to 1000 μg/mL (Table 1). Therefore, the hLF found in human airways is potentially bactericidal against *S. pneumoniae*. This suggests the possibility that hLFcin is microbicidal to microorganisms that produce proteases that cleave mature hLF and generate hLFcin. However, whether antimicrobial amounts of hLFcin are generated in vivo remains to be experimentally verified.

LF blocks colonization/infection of the epithelia

Hendrixson et al. (2003) demonstrated that hLF has protease activity, and mutation of either Ser259 or Lys73 to Ala abolishes this activity. The orientation of these residues is remote from the iron-binding sites of hLF and iron binding does not affect hLF proteolytic activity (Qiu et al. 1998; Hendrixson et al. 2003).

IgA1 protease enhances colonization of mucosal surfaces by *H. influenzae*. Mucosal microbes are coated with sIgA, and this sIgA interferes with attachment of the microbe to the mucosal surface (van der Waaij et al. 1996; van Egmond et al. 2001). IgA1 protease targets IgA that attaches to the

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surface of *H. influenzae*, thereby enhancing the ability of these bacteria to adhere to the epithelial surface (Weiser et al. 2003; He et al. 2011). (*i*) hLF cleaves IgA1 protease between Arg1019 and Arg1020 and between Arg1020 and Arg1021, releasing it from the cell surface; (*ii*) incubation of *H. influenzae* with 1.3 μmol/L of the N-lobe of hLF (this hLF fragment contains the protease domain and 1.3 μmol/L corresponds to ~100 μg/mL hLF) for 1 h removes IgA1 protease from *H. influenzae*; (*iii*) proteolytic removal of IgA1 protease by hLF prevents degradation of the IgA that is bound to *H. influenzae*, thereby decreasing the ability of *H. influenzae* to adhere to and colonize epithelial surfaces (Qiu et al. 1998; Hendrixson et al. 2003). The effect of protease-inactive hLF on the adherence of *H. influenzae* to target surfaces was not assessed in these studies.

Hap adhesion is another protein expressed by *H. influenzae* that promotes adhesion to and colonization of target surfaces (Hendrixson and St Geme 1998). (*i*) hLF proteolytically degrades Hap adhesion; (*ii*) incubation of *H. influenzae* with 1.3 μmol/L of the N-lobe of hLF (this hLF fragment contains the protease domain and 1.3 μmol/L corresponds to ~100 μg/mL hLF) for 1 h removes Hap adhesion from *H. influenzae*; (*iii*) proteolytic removal of Hap adhesion decreases the ability of *H. influenzae* to adhere to and colonize mucosal surfaces (Qiu et al. 1998; Hendrixson et al. 2003). The effect of protease-inactive hLF on the adherence of *H. influenzae* to target surfaces was not assessed in these studies.

Another target of hLF is the type III secretion system expressed by certain Gram-negative bacteria. The type III secretion system is reviewed by Blocker et al. (2001) and Cornelis (2006). The type III secretion system is a large protein complex often referred to as an injectosome. It consists of a basal complex that is in contact with the bacteria cytoplasm, ring-like structures that anchor the injectosome in the inner and outer membranes, a long needle-like structure ~60 nm in length, and a tip complex located at the tip of the needle that interacts with the host cell membrane. When the tip complex comes into contact with a target membrane, hydrophobic translocator proteins are secreted into the injectosome and come into contact with the target membrane where they form a pore allowing bacteria docked on the cell surface to inject effector proteins into the cell (Blocker et al. 1999). Disruption of the type III secretory systems of infectious bacteria inhibits infection of their target cells (Lu and Walker 2001). hLF disrupts the type III systems of *Shigella flexneri* and enteropathogenic *Escherichia coli* (EPEC) by causing loss of *S. flexneri* translocator proteins IpaB and IpaC and EPEC translocator proteins EspA, EspB, and EspD (Gomez et al. 2003; Ochoa et al. 2003; Ochoa and Clearly 2004). hLF is thought to proteolytically degrade the *S. flexneri* translocator proteins, but this has not been shown directly; hLF has been shown to directly degrade purified EspB (Ochoa and Clearly 2004). hLF does not affect the viability of either *S. flexneri* or EPEC, and, consequently, hLF-mediated inhibition of target cell infection by these bacteria is considered to be the result of hLF-mediated disruption of their type III secretory systems (Gomez et al. 2003; Ochoa et al. 2003). The effect of protease-inactive hLF on the infectivity of *S. flexneri* or EPEC has not been assessed.

hLF also inhibits colonization of target surfaces by mechanisms that are not believed to involve disruption of microbial structures. hLF inhibits the adsorption of *Streptococcus mutans* 6715-13 to hydroxyapatite (Visca et al. 1989). The authors of this study suggest that hLF interferes with ionic interactions between *S. mutans* and hydroxyapatite, but the actual mechanism remains unknown. In a more recent study, Wakabayashi et al. (2009) show that at levels as low as 8 μg/mL apo-hLF inhibits biofilm formation by *P. gingivalis* and at 130 μg/mL it inhibits biofilm formation by *P. intermedia*. In addition, these levels of hLF also disrupt already formed biofilms to some extent. The effect of holo-hLF was not assessed in this study, but both apo-bLF and holo-bLF have activity against these bacteria, suggesting that bLF, and perhaps hLF, has an iron-independent mechanism of biofilm prevention/disruption. The actual mechanism, however, is unknown.

hLF is capable of inhibiting the replication of a wide range of viruses, and most studies indicate that hLF prevents infection of the host cell (van der Strate et al. 2001). For the interested reader, the antiviral activity of hLF is discussed in Supplementary data[2] Chapter 2.

Synergism with other HDPPs

Data from studies using biologically relevant fluids is scarce. As with hLF alone, synergism with other HDPPs is salt and divalent cation sensitive. In low ionic strength media without divalent cations, *E. coli* are killed by 2000 μg/mL hLF + 500 μg/mL lysozyme, but killing is abolished in high ionic strength medias or by the addition of either 1 mmol/L Ca^{2+} or 1 mmol/L Mg^{2+} (Ellison and Giehl 1991). hLF (200 μg/mL) + lysozyme (500 μg/mL) was also inactive toward *P. aeruginosa* in heat inactivated, ionically intact nasal fluid (Cole et al. 1999).

Stephens et al. (1980) reported on the synergistic activity between sIgA and hLF on inhibition of enteropathic *Escherichia coli* strain 0111 in tissue culture medium. At 2000 μg/mL, each of these HDPPs was bacteriostatic. sIgA (250 μg/mL) plus hLF (250 μg/mL) had about the same bacteriostatic effect as 2000 μg/mL of the individual HDPPs. Notably, even when 2000 μg/mL of both of these HDPPs was added to the assay, they were bacteriostatic, not bactericidal, consistent with the data in Table 2, indicating that hLF is not microbicidal in fluids containing biologically relevant levels of salts, divalent cations, and buffering ions. Synergism was lost upon addition of iron to the assay. One possible explanation for the synergism displayed by these 2 HDPPs is that IgA agglutinates the bacteria and hLF sequesters iron. As can be seen in Table 1, the levels of both hLF and IgA are high in tear fluid.

In artificial tear fluid, 1800 μg/mL hLF is able to synergize with 5400 μg/mL lysozyme to inhibit the growth or kill (depending on the strain) the Gram-positive bacteria *Staphylococcus epidermidis* (Leitch and Willcox 1998). This study used high levels of hLF and artificially high levels of lysozyme. The antimicrobial activity of hLF was dramatically reduced or abolished when holo-hLF was substituted for apo-hLF. This effect was repeated in a later study by the same group (Leitch and Willcox 1999). The later study also used 1800 μg/mL hLF and 5400 μg/mL lysozyme, so while these results are suggestive, it is unclear whether hLF is able to synergize with lysozyme in vivo. Notably, hLF and lysozyme are present at moderate to high levels in milk, open

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and closed eye tear fluid, airway fluids, and the cervical mucus plug (Table 1).

hLF is also able to synergize with SLPI in cation-depleted nasal fluid (Cole et al. 2002). In this study, nasal fluids were depleted of cationic proteins and the major nasal fluid proteins, lysozyme, SLPI, and hLF, were added back individually or in combination. Depleted nasal fluid (dNF), dNF with SLPI, and dNF with hLF were ineffective against *P. aeruginosa*. Addition of both LF and SPLI restored the antimicrobial activity of dNF. One possible explanation of the synergistic activity is that the protease inhibitor SLPI protects hLF from degradation by proteases released by *P. aeruginosa* (Britigan et al. 1993), allowing intact LF to sequester iron away from the bacteria, thereby limiting its growth. However, this possible mechanism of restoring the growth inhibitory effect of depleted nasal fluids was not tested in this study. Both hLF and SLPI are present at moderate to high levels in closed eye tear fluid, airway fluids, and the cervical mucus plug (Table 1).

hLF binds to LRP1, TLR4, and LPS

hLF is known to bind to 2 signaling receptors, LRP1 (Grey et al. 2004) and TLR-4 (Ando et al. 2010). Neither of these receptors is specific for hLF. LRP1, reviewed by Lillis et al. (2008), binds numerous ligands and is sometimes referred to as a scavenger receptor, and TLR-4 binds several types of microbial antigens (Medzhitov 2001). The effect of binding to LRP1 is dependent on a multitude of other events; LRP1 recognizes more than 50 ligands and is able to associate with at least 15 cytosolic adaptor proteins. hLF binding to LRP1 promotes osteoblast proliferation and survival (Grey et al. 2004; Naot et al. 2005) and keratinocyte migration (Tang et al. 2010), and bLF binding to LRP1 promotes fibroblast movement and proliferation (Takayama et al. 2003; Grey et al. 2004; Takayama and Takezawa 2006), suggesting that hLF may also stimulate fibroblasts. These studies suggest that hLF could be involved in wound healing in vivo. On the other hand, in tissues with elevated neutrophil activity, the overall effect of high neutrophil activity is tissue damage (Witko-Sarsat et al. 2000); therefore, the overall effect of neutrophil hLF in vivo may be to slow down tissue damage. The association of LF and wound repair could be explored using LF-knockout mice, but such a study has yet to be reported.

hLF binding to TLR-4 stimulates TLR-4 signaling (Ando et al. 2010). TLR-4 is expressed by mucosal epithelial cells and numerous cells of the innate and adaptive immune system (Armant and Fenton 2002). Therefore, by binding to TLR-4, hLF can promote the activation of immune effector cells and modulate the expression of a variety of cytokines. However, hLF signaling via TLR-4 is weak (Ando et al. 2010), and in the presence of microbial-antigen binding to TLRs and other pattern recognition receptors, e.g., at septic sites, there is no evidence that LF has any measurable immune stimulatory activity (see Ward et al. 2008).

Finally, hLF binds tightly to LPS through both ionic interactions with the negatively charged backbone and high affinity interaction with the lipid A moiety (Appelmelk et al. 1994; Brandenburg et al. 2001). This interaction inhibits LPS stimulation of TLR-4 (Ando et al. 2010). This suggests that at septic sites, where TLR signaling via microbial antigens will be far greater than TLR-4 signaling via neutrophil-derived hLF, neutrophil hLF will either have little effect on the immune system or, in the presence of LPS, exert antiinflammatory activity.

As noted above, hLF can be induced to high levels in inflamed tissues, such as inflamed gingival crevicular fluid (Gorr and Abdolhosseini 2011), due to neutrophil activity. High levels of neutrophil hLF will (*i*) inhibit colonization and infection of host tissues, (*ii*) inhibit the growth of microorganisms, and (*iii*) protect tissues from damage by binding iron and making it unavailable for oxygen radical production, by binding LRP1 and promoting tissue repair and by binding LPS and dampening LPS-mediated inflammatory signaling. These activities, however, remain to be experimentally verified in vivo. The complex biology of the response to infection and tissue damage may make it necessary to generate animals models with multiple knockouts or knockdowns to uncover the functions of endogenous LF.

LF-containing mucosal fluids

Mucins, reviewed by Linden et al. (2008), are highly *O*-glycosylated glycoproteins and major constituents of mucosal fluids. Mucins serve to protect mucosal surfaces from mechanical insult, dehydration, and infection. Soluble mucins present in mucosal fluids bind and trap microorganisms, and, consequently, removal of mucosal fluid from the body by mechanisms such as mucociliary transport, swallowing, coughing, and blinking also removes entrapped microbes. Transmembrane mucins are a major constituent of the epithelial glycocalyx in all mucosal tissues. Transmembrane mucins act as a physical barrier to inhibit interaction of infectious microorganisms with the epithelial surface. In addition, the extracellular component of these mucins can be shed into the mucosal fluid. Shedding appears to be important in removing invading microbes from the epithelial surface (McGuckin et al. 2011). Physical removal of microorganisms from the body can require a significant amount of time, during which growth and proliferation of the organism could help it evade removal (Knowles and Boucher 2002). The microbiostatic activity of hLF is well suited to the nonlethal, noninflammatory removal of invading microorganisms from the human body by mucosal clearance.

As shown in Table 1, hLF is present at high levels in milk and tear fluid, and it is present at moderate to high levels in airway fluids, seminal plasma, and the cervical mucus plug. hLF is absent or present at only minute levels in lung alveoli, adult intestine, urinary tract, and the nonpregnant female reproductive tract (in the absence of semen). In these tissues, unless hLF is present in much higher concentrations in specific microniches, it is unlikely to have any physiological effect. Of note regarding the distribution pattern of hLF is that hLF is present in saliva, but at substantially lower levels than in tears or airway fluids.

The eye

In the eye, a layer of transmembrane mucin proteins forms a glycocalyx that acts as a barrier to pathogens, protects the epithelium from physical stresses, and is able to activate intracellular signaling pathways (Gipson 2004; Mantelli and

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Argueso 2008; Govindarajan and Gipson 2010). Ocular epithelial cells and their attached mucin glycocalyx are covered with an aqueous layer containing numerous secreted mucins, which is bounded at the air–aqueous interface by a lipid layer (Gipson 2004, 2007; Linden et al. 2008; Mantelli and Argueso 2008; Guzman-Aranguez and Argueso 2010).

Tear film contains high levels of hLF, IgA, SLPI, lipocalin-1, and lysozyme (Table 1). Tear fluid also contains SP-D (Ni et al. 2005; Mun et al. 2009). SP-D and IgA, like ocular mucins, bind microorganisms and inhibit attachment to the epithelial surface (van der Waaij et al. 1996; van Egmond et al. 2001; McCormack and Whitsett 2002; Hickling et al. 2004). Binding of microorganisms by mucins, SP-D, and IgA enable the physical removal of these microorganisms when tear fluid is flushed out of the eye by the pumping action of the blink. SLPI is a serine protease inhibitor with the obvious function of protecting HDPPs and other targets from proteolysis by proteases secreted by microorganisms (Thompson and Ohlsson 1986; Mun et al. 2009). Lipocalin-1, also known as tear lipocalin, binds a wide variety of microbial siderophores, including all major classes of fungal siderophores (Fluckinger et al. 2004). Siderophores are iron-binding molecules utilized by a wide variety of microorganisms to obtain iron (Chu et al. 2010). Consequently, binding iron–siderophore complexes inhibits iron acquisition by these microorganisms and inhibits their growth (Fluckinger et al. 2004; Chu et al. 2010). Lysozyme digests peptidoglycans of the bacteria cell wall (Ibrahim et al. 2002). Destruction of the cell wall inhibits bacterial growth, but is not immediately bactericidal in isotonic solutions. Because the cell wall is protected by the outer membrane of Gram-negative bacteria, lysozyme inhibits the growth of Gram-positive bacteria but is ineffective against Gram-negative bacteria (Ibrahim et al. 2002). Importantly, digestion of the wall by lysozyme does not generate inflammatory microbial antigens, in fact, digestion of cell wall peptidoglycans is antiinflammatory (Ganz et al. 2003; Nash et al. 2006). As discussed above, hLF has been shown to synergize with SLPI, IgA, and lysozyme in vitro. Overall, the microbiostatic actions of hLF, lipocalin-1, and lysozyme prevent outgrowth of microorganisms trapped in tear fluid by mucins, IgA, and SP-D and enable their nonlethal removal from the eye. This effect is vital to eye function as maintenance of eye clarity necessitates antimicrobial defense of the eye without induction of an inflammatory response (Flanagan and Willcox 2009).

Tear film also contains high levels of secreted phospholipase A2 (sPLA2) (Table 1) (sPLA2 is reviewed by Buckland and Wilton (2000) and Nevalainen et al. (2008)). sPLA2 hydrolyzes the sn-2 bond of phospholipids. At physiological levels in biologically relevant fluids, sPLA2, like lysozyme, is active against Gram-positive bacteria, but ineffective against Gram-negative bacteria (Qu and Lehrer 1998; Buckland and Wilton 2000; Buckland et al. 2000; Beers et al. 2002; Murakami and Kudo 2004; Nevalainen et al. 2008). Importantly, the preferential site of sPLA2 action are membranes associated with cell growth and separation (Foreman-Wykert et al. 1999), suggesting that the primary targets of sPLA2 are Gram-positive bacteria that have escaped the microbiostatic function of tear fluid and, consequently, may evade removal from the eye.

As discussed by Bowdish et al. (2005*a*, 2005*b*), the reported levels of HDPPs in mucosal fluids are often below the levels required for antimicrobial activity in biologically relevant fluids. The discussion by Bowdish et al. (2005*a*, 2005*b*) taken in conjunction with the data discussed in this review and the reported levels of HDPPs in tears (Table 1) suggest that in general tear fluid is microbiostatic rather than microbicidal (see Buckland et al. 2000 and McDermott et al. 2006) and that the overall antimicrobial activity of tear fluid resembles the overall activity of hLF. It is microbiostatic and prevents colonization and infection of the ocular epithelium.

In spite of being exposed to a multitude of microorganisms every day, infection of the eye is a very rare event, suggesting that it is unlikely that the eye does not contain potent microbicidal activity in addition to the microbiostatic activity of the overlying tear fluid. One mechanism of epithelial microbicidal activity is described by Kisich et al. (2007). Exposure of keratinocytes to *S. aureus* resulted in deposition of β-defensins (HBD1, HBD2, and HBD3) and LL-37 onto the bacteria, and the amount of HBD3 deposited was microbicidal. Whether these cationic antimicrobial peptides were stored in the cytoplasm of the cell and exocytosed after contact with the bacteria or were bound to the cell surface, possibly via ionic interaction of positively charged HDPPs (notably, a large number of HDPPs are cationic) with negatively charged cell surface glycoproteins, was not addressed in this study. Importantly, focal deposition of microbicidal amounts of HDPPs onto invading microorganisms would account for the generally insignificant levels of many potentially microbicidal HDPPs in mucosal fluids.

Eyes have 2 states of being, (*i*) open, in which trapped microorganisms are rapidly removed from the eye, and (*ii*) closed, in which trapped microorganisms remain in the tear fluid for several hours. The closed eye state is characterized by neutrophil infiltration, an increase in the levels of complement components, and a very large increase in the amount of IgA (Sack et al. 2001). This would appear to represent a shift from passive physical removal of microorganisms from the open eye to active phagocyte-mediated removal of microorganisms from the closed eye. The antiinflammatory activities of hLF and lysozyme are likely to be important in preventing inflammation in the closed eye.

The airways

In the airways, as in all other mucosal surfaces, there is a protective glycocalyx of transmembrane mucin proteins at the epithelial cell surface (airway mucins are reviewed by Lamblin et al. (2001), Knowles and Boucher (2002), and Fahy and Dickey (2010)). Overlying the epithelium there is a fluid layer (the pericilliary layer), a layer of surfactant, and a top layer of mucus (Grubor et al. 2006). Beating cilia move the overlying surfactant and mucus layers out of the airways; this is commonly referred to as the mucociliary escalator.

The principle HDPPs of the airway are SP-A and SP-D (McCormack and Whitsett 2002; Hickling et al. 2004; Kuroki et al. 2007) and hLF, SLPI, lysozyme, and lactoperoxidase (Table 1). As described by Lamblin et al. (2001), airway mucins present a library of carbohydrate determinants to microorganisms in mucosal fluids and bind to a multitude of these organisms. SP-A and SP-D also bind to a wide variety of microorganisms (McCormack and Whitsett 2002; Kuroki

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et al. 2007). Once bound, trapped microorganisms are removed from the airways by mucociliary clearance. One difference between the nose and the bronchial tree is the residency time of inhaled microorganisms. Transport out of nasal passages is fairly rapid (Isaacs et al. 2011), whereas transport out of the lung can require several hours (Knowles and Boucher 2002).

Lactoperoxidase catalyzes the oxidation of thiocyanate to generate the antimicrobial agent hypothiocyanous acid:

[2] $H_2O_2 + SCN^- \xrightarrow{LPO} OSCN^- + H_2O$

This system activates oxidative stress responses in bacteria and is generally microbiostatic (Lenander-Lumikari et al. 1992; De Spiegeleer et al. 2005). Therefore, during removal of microorganisms from the airways, the microbiostatic activity of hLF, SLPI, lysozyme, and lactoperoxidase keep the growth of the trapped microbes in check and prevent these microbes from evading removal.

As in the eye, the reported levels of airway HDPPs are generally below the levels required for microbicidal activity in biologically relevant fluids (Table 1; see the discussion by Bowdish et al. 2005*a*, 2005*b*). However, again like the eye, infection of the airways is rare, suggesting that microbicidal activity is present. One possibility is that focal deposition of microbicidal levels of HDPPs onto microorganisms that are able to penetrate to the epithelial cell surface, rather than global deposition into the overlying mucosal fluid, could account for the presence of microbicidal activity in the airways in the presence of low levels of microbicidal HDPPs in airway mucosal fluids. Importantly, a large number of HDPPs are cationic and ionic interaction with negatively charged cell surface glycoproteins could result in low levels of these HDPPs in mucosal fluids and microbicidal levels at the epithelial surface. Unfortunately, there are very few in vitro studies that have examined the levels of endogenous HDPPs at the cell surface or in the exocytotic vesicles of mucosal epithelial cells (for an example of such a study see Kisich et al. 2007), and we are aware of no in vivo studies that have been able to accurately determine the levels of HDPPs in these microdomains. While the study by Kisich et al. (2007) supports the concept of focal deposition of HDPPs onto invading microorganisms as an antimicrobial strategy used by the mucosa epithelium, much more investigation into this area of mucosal immunity is needed.

The alveoli, unlike the upper airways, are sterile. The transition from the microbe-permissive upper airways to the microbicidal lower airways is not well defined. Seromucous glands, which secrete mucus and antimicrobial factors, are found all along the upper respiratory trachea and bronchi; the number and size of the glands declines with progression down the airway, and they are not present in the conducting bronchioles, respiratory bronchioles, or alveoli (Fischer 2009). The exact HDPP content of alveolar fluid has not been reliably determined. Important components of alveoli are alveolar macrophages and SP-A and SP-D that are produced by type II cells (Ng et al. 2004). Aside from alveolar macrophages, there is no verified microbicidal activity in the alveoli. hLF is not present in alveolar fluids (Masson et al. 1966). The lack of hLF in alveoli is reasonable since hLF binds to TLR-4 expressed by macrophages and stimulates TLR-4 signaling. While hLF-induced TLR-4 signaling is weak, continuous signaling could lead to damage of this extremely fragile compartment.

The mouth

The function of hLF in saliva is uncertain. hLF levels are markedly lower than in tears and airway fluids (Table 1). The maximum levels reported are only 20 μg/mL (~0.25 μmol/L). There is, however, one report that 10 μg/mL hLF had a significant microbiostatic effect on the growth of *Aspergillus fumigatus* (Zarember et al. 2007). The inhibitory effect was abolished by the addition of 1 μg/mL ferritin, but the concentration of iron in saliva is ~0.1 μg/mL (Haghighat and al-Hashimi 2003). Therefore, the amount of hLF in saliva would appear to be sufficient to sequester iron away from invading microorganisms. In addition, one study reported that in patients with localized aggressive periodontitis, the overall iron-binding capability of hLF was decreased (Fine et al. 2002). On the other hand, there are no reports that the hLF SNPs possibly associated with disease have decreased iron-binding capability. Moreover, many healthy patients have salivary hLF levels lower than 10 μg/mL (Tenovuo et al. 1987). Finally, there are no reports associating low salivary hLF levels with disease.

Another possible function of hLF is protection of secretory glands, rather than the mouth itself, against infection. hLF levels in glandular acini and ducts may be microbiostatic even when hLF levels in saliva are low. However, while hLF has been shown to be present in salivary glands (Reitamo et al. 1980), the levels present in glandular acini and ducts have not been reported.

A third possible function of hLF in the oral cavity is protection of oral epithelia from microbial colonization and infection. As discussed for the eye and airway epithelium, it is possible that the level of hLF (which is cationic) present at the epithelial cell surface may be significantly higher than in saliva due to ionic interaction with negatively charged cell surface glycoproteins. Consequently, it is possible that epithelial cell surface associated hLF may be present at high enough levels (even in people with low levels of hLF in their saliva) to protect the oral epithelium from microbial colonization and infection. However, there are no reports of the levels of oral epithelium-associated hLF (or the association of any of the other oral HDPPs with the oral epithelium).

The adult intestine

There are no reports of hLF expression in the colon, and the luminal content of hLF in the small intestine is minute, <1 μg/mL (Tedeschi et al. 1987; Troost et al. 2002). hLF present in the lumen of the human intestine is far too low to have any antimicrobial function. However, the distribution of hLF in the upper small intestine is quite interesting. It is present on the tips of villi in the duodenum (Mason and Taylor 1978). The ileum and the large intestine are protected by a covering of dense mucus (Kim and Ho 2010; Johansson et al. 2011). Villi in the duodenum and jejunum, however, project through the protective layer of dense mucus and are exposed to intestinal microorganisms (Johansson et al. 2011). Thus, hLF expression may be associated with exposed villi. One possible protective function of hLF is its binding to intelectin 1 (ITLN1) (Suzuki et al. 2005). ITLN1 is a secreted

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lectin that binds to galactofuranose residues present in bacterial cell walls (Tsuji et al. 2001). In the intestine it is expressed by Paneth cells and goblet cells and is believed to have an antimicrobial function (Wrackmeyer et al. 2006). However, ITLN1 also has a second function in the small intestine; it helps stabilize lipid-raft microdomains present in the brush borders of enterocytes (Wrackmeyer et al. 2006; Danielsen and Hansen 2008). These domains contain high levels of digestive enzymes and are important sites of nutrient uptake, but they are also portals for infection (Danielsen and Hansen 2006). As expected, because of its bacterial cell wall binding ability, the presence of ITLN1 in the cell membrane enhances adsorption of bacteria to the cell surface (Tsuji et al. 2009). Therefore, hLF binding to ITLN1 could reduce microbe binding to enterocytes. However, at this time there is no direct experimental evidence to either support or refute this conjecture.

It has been speculated that one of the functions of hLF in the intestine is iron sequestration from invading microorganisms. The physiology of the intestine, however, does not support this speculation. The first point is that the level of hLF in the adult intestine is extremely low, much too low to have an appreciable effect on the iron content of the intestine. Second, the iron content of the intestine is sufficient to support the enormous microbiota of the colon.

The infant intestine

The resistance of hLF and other HDPPs present in milk to digestion, coupled with the reduced digestive capability of the infant gastrointestinal tract, is believed to result in the presence of significant amounts of these HDPPs in the infant intestine (Spik et al. 1982; Brines and Brock 1983; Lönnerdal 2003; Newburg and Walker 2007). Milk contains high levels of mucins and other complex glycoconjugates that bind microorganisms (Peterson et al. 1998*a*, 1998*b*; Newburg 1999; Newburg and Walker 2007). Milk also contains high levels of hLF, IgA, lysozyme, and lactoperoxidase (Table 1). It also contains ~8 mmol/L Ca^{2+}, which is protective for many microorganisms. Consequently, milk gives the infant intestine a microbiostatic character, similar to that of tears and airway mucosal fluids. That breast milk would be microbiostatic rather than microbicidal is reasonable. Ingestion of large amounts of microbicidal material during the development of the intestinal microbiota would most likely generate a significant and damaging inflammatory response, whereas ingestion of large amounts of microbiostatic material would not.

As noted above, the ileum and colon are protected by a coating of dense mucus, but intestinal villi in the duodenum and jejunum project through this protective layer and are exposed to intestinal microorganisms. In contrast to other mucosal fluids, the mucosa of the intestine does contain microbicidal levels of HDPPs, particularly α-defensins secreted by Paneth cells (Table 1). α-Defensins and other HDPPs secreted by Paneth cells are thought to protect exposed intestinal villi from microbial colonization and infection (Sherman et al. 2005; Fernandez et al. 2008). A study in rats, however, suggests that while Paneth cells are important in protecting the infant intestine, there are significantly fewer Paneth cells in the infant intestine than in the adult intestine (Sherman et al. 2005). Possibly, the lack of function Paneth cells may help to decrease inflammation in the infant intestine. Therefore, similarly to microbiostatic tears and airway mucosal fluids, movement of microbiostatic milk will, with minimal inflammation, remove microorganisms from the upper small intestine with its exposed intestinal villa and deposit them in the ileum and colon where the intestinal epithelium is much better protected.

The high level of hLF in milk also affects iron absorption in the infant intestine as has been shown by reports indicating that hLF sequesters iron away from the infant (Davidsson et al. 1994; Ward and Conneely 2004; Collard 2009). Iron binding by hLF will benefit intestinal microorganisms that can use the iron bound to hLF as an iron source (Beddek and Schryvers 2010; Chu et al. 2010) and microorganisms that do not require iron, such as lactobacilli (Weinberg 2009), compete with microorganisms that require iron but cannot use iron-bound hLF as an iron source. Consequently, ingested hLF will be one of the factors that shape the intestinal microbiota of the infant intestine (Roberts et al. 1992; Harmsen et al. 2000).

Seminal plasma

Moderate levels of hLF are present in seminal plasma (Table 1). In addition, spermatozoa are coated with hLF (Wang et al. 2007*b*). The role of spermatozoa-associated hLF is most likely to prevent attachment of vaginal microflora to the spermatozoa. This function of hLF would have 2 consequences as follows: (*i*) to protect the sterile uterine environment from spermatozoa-introduced microorganisms and (*ii*) to protect the spermatozoa itself from infection. The function of seminal plasma hLF that is not associated with spermatozoa is less obvious. One possibility is that the relatively high levels of hLF in seminal plasma ensure saturation of the cell surface of the spermatozoa with hLF. A second possibility is that hLF protects the vagina from penis-introduced microorganisms. Unfortunately, there are no reports of studies examining associations between seminal plasma hLF and vaginal infections.

The female reproductive tract

Microbiostatic levels of hLF have not been reported to be present in the reproductive tract of women who are not pregnant. One possibility, as mentioned above, is that functional levels of hLF are associated with the epithelial cell surface. Another possibility is that the low levels of hLF detected is the result of low level neutrophil activity that helps prevent infection of the uterus (Press and King 1986) (and possibly the vaginal epithelium) by the vaginal microbiota. Currently, there is no evidence that hLF has any function in the reproductive tract of women who are not pregnant other than that associated with neutrophil activity.

About half way through the third trimester, hLF levels increase in amniotic fluid. The source of this hLF, whether it is from maternal uterine secretions, maternal uterine neutrophils, or from the developing embryo, is unknown. Whatever its source, however, it is extremely unlikely that this hLF has any function within the normally microorganism-free amniotic sac (Kim et al. 2009). The levels determined by Pacora et al. (2000) are low, the highest levels of hLF are <6 μg/mL, too low to exert any antimicrobial activity. The most plausible source of this hLF is maternal neutrophils or the fetus. Currently, there is no evidence that hLF has any protective