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Chapter 15

Innate Humoral Defense Factors

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INTRODUCTION

Until the late 1990s, the innate immune response was considered to be a nonspecific and temporary expedient to "buy time" until the activation of adaptive immunity. We now know that germ-line-encoded receptors or other molecules with pattern-recognition capabilities can detect and respond to generally distinct microbial structures, which are conserved and shared by related groups of microorganisms. Moreover, innate immune recognition mechanisms act as sophisticated mediators between detection of infection and induction of the appropriate adaptive immune response (Fearon, 1997; Medzhitov, 2007). However, innate defense mechanisms against infectious agents have a much older history, and the exocrine secretions of the body contain a great number of distinct soluble factors that protect the body from the majority of potential pathogens that enter along with food and air or by intimate contact between individuals. These constitute the principal subject of this chapter.

The contribution of innate defense factors to the protection of mucosal surfaces against microbial colonization and aggression is probably greatly underestimated. Evidence for this is seen in IgA-deficient subjects who lack secretory IgA antibodies in their secretions and yet are not severely compromised, even though the deficiency may be partially compensated for by secretory IgM or small amounts of IgG in secretions (see Chapter 105). Furthermore, innate defense mechanisms help to define the minimal requirements for successful colonization by commensal and pathogenic organisms: those that cannot adapt to these conditions will be incapable of maintaining themselves within the host. Much of the earlier work on innate defense systems in mucosal secretions was performed in saliva or milk, which are readily accessible in large quantities. However, it has become apparent that similar mechanisms operate in most secretions, although with significant differences of detail. Increased attention to the antimicrobial factors present in the genital tract occasioned by the AIDS epidemic, for example, has revealed that this tract has multiple non-immunoglobulin-based defense mechanisms (Quayle, 2002).

It is important to note that the mucosal surfaces first present a mechanical barrier, consisting of the epithelium itself, which varies from a single columnar cell layer in the gastrointestinal and respiratory tracts to a stratified and sometimes keratinized epithelium in the mouth and lower female genital tract (vagina). Most such surfaces are reinforced by the copious secretion of mucus, which is propelled by peristaltic or ciliary action and is thought to physically entrap microparticles, including microorganisms; however, specific

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interactions between mucins and bacterial receptors may also be involved (Lichtman et al., 1996; see also Chapter 14). Furthermore, mucosal surfaces desquamate, and there is a considerable turnover of epithelial cells (estimated at 10^{11}/day from the human small intestine alone; Potten and Morris, 1988) which, on shedding, carry with them a burden of attached microorganisms.

Sensitive immunochemical assays can usually reveal the presence of complement proteins, especially the major components such as C3, in various secretions. Furthermore, epithelial cells have been found to synthesize complement components C3, C4, and factor B (Strunk et al., 1988). However, the concentrations of complement components present are usually well below those found in serum, and consequently it is uncertain whether complement operates as a fully functional system in secretions, especially in the absence of overt inflammation in the corresponding mucosal tissue. On the other hand, high concentrations of biologically active complement components are detected in the gingival crevicular fluid, which bathes the space (crevice) between the teeth and the free gingiva (Hajishengallis, 2010).

Phagocytes represent a major component of innate defense at the cellular level, and all classes of phagocytes, including macrophages, neutrophils, eosinophils, and mast cells, occur within mucosal tissues. Some of them develop special characteristics in accordance with their location, for example, mucosal mast cells and lamina propria macrophages. Although the activities of phagocytes may be largely confined to the tissues themselves, at least under normal healthy circumstances, rather than taking place within the lumen, the microenvironment close to the mucosal surface may permit phagocytic activity, as in the case of alveolar macrophages. As most secretions are hypotonic, phagocytes probably do not survive with functional activity for long in the bulk fluid phase. However, their contents may be released upon lysis or through induced degranulation and thereby contribute to the soluble antimicrobial factors in the secretions. Part of the lactoferrin (Lf) and lysozyme, as well as myeloperoxidase (as distinct from secretory peroxidase), found in whole saliva or milk, for example, originates from neutrophils emigrating into the gingival crevice or the lactating mammary gland (Moldoveanu et al., 1982). Milk, however, is isotonic and contains variable numbers of both macrophages and neutrophils, depending upon the stage of lactation and both exogenous and endogenous stimuli. Huge numbers of neutrophils accumulate rapidly in milk in response to infection (mastitis), but their phagocytic activities are compromised by constituents of milk, particularly casein, which they ingest in competition with the microorganisms. This results in premature activation, diversion, and attenuation of the neutrophil intracellular killing mechanisms (Grazioso and Buescher, 1996; Russell and Reiter, 1975; Paape et al., 2003).

The humoral arm of innate immunity also contains a heterogeneous group of pattern-recognition molecules, which act as functional predecessors of antibodies ("ante-antibodies"). These molecules together with the classical soluble innate defense factors form an integrated system with complementary specificity, action, and tissue distribution, and they communicate with the cellular arm of innate immunity (phagocytes) to coordinate or invigorate the innate host defenses to mucosal pathogens.

PATTERN-RECOGNITION MOLECULES

The humoral arm of innate immunity includes a heterogeneous group of pattern-recognition molecules (PRMs) such as pentraxins, collectins, ficolins, and certain complement components such as C1q (Bottazzi et al., 2010; Holmskov et al., 2003). Soluble PRMs can be released either locally by stimulated inflammatory cells or systemically by certain epithelial tissues (e.g., the liver). For local release, some soluble PRMs are stored in the granules of neutrophils, whereas other PRMs are produced de novo as early gene products in activated mononuclear phagocytes and dendritic cells. Despite their structural heterogeneity, these molecules share several basic, evolutionarily conserved functions, such as complement activation and regulation, agglutination and neutralization, opsonization, and regulation of inflammation. Through these activities, humoral PRMs—produced in response to microbes or tissue damage—interact with and regulate the effectors of the cellular arm of innate immunity (Bottazzi et al., 2010). Certain cell-associated pattern-recognition receptors (e.g., Toll-like receptors (TLRs) and CD14) may naturally be found in the fluid phase after their release from the cell surface and, therefore, can act as humoral PRMs. These molecules can regulate cellular activation positively or negatively, depending on their concentration and environmental context (Kitchens and Thompson, 2005).

Pentraxins

Pentraxins constitute a conserved family of PRMs characterized by a radial pentameric structure. They are divided into short and long pentraxins: C-reactive protein (CRP) and serum amyloid P (SAP) are prototypical short pentraxins, whereas pentraxin 3 (PTX3) is the prototypical member of the long pentraxin subfamily (Inforzato et al., 2012). Short and long pentraxins share a 200-amino-acid-long C-terminal domain that contains a motif known as the pentraxin signature (HxCxS/TWxS). In addition, long pentraxins contain an unrelated N-terminal domain.

CRP is a 25-kDa acute-phase protein that is produced by hepatocytes in response to interleukin (IL)-6 (Bottazzi et al., 2010). It was originally identified for its ability to bind the C-polysaccharide of *Streptococcus pneumoniae* in a calcium-dependent manner. CRP activates complement

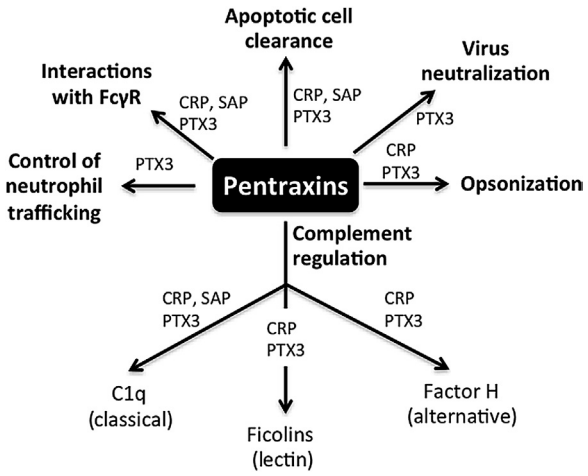

FIGURE 1 Versatile functions of the pentraxin family acting as a bridge between the soluble and the cellular arms of innate immunity. Pentraxins (CRP, SAP, PTX3) mediate diverse activities, ranging from pathogen control to regulation of inflammation. Their pattern-recognition capacity allows them to bind and neutralize viruses or opsonize microbes to enhance their uptake and killing by phagocytes. Pentraxins regulate complement by interacting with all three initiation pathways: with C1q of the classical pathway (CRP, SAP, PTX3), with ficolins that initiate the lectin pathway (CRP, PTX3), and with factor H, a major regulator of the alternative pathway (CRP, PTX3).

C1q in the presence of phosphorylcholine, which is present on apoptotic cells and is also a constituent of the capsule of certain bacteria. CRP can also bind phosphoethanolamine, a constituent of the cell membrane of *Salmonella enterica*. These activities enable CRP to confer protection against a number of microbial pathogens through opsonization, including *S. pneumoniae* (Szalai et al., 1995), *Haemophilus influenzae* (Lysenko et al., 2000), and *Sa. enterica* (Szalai et al., 2000), as demonstrated in mouse models of infection. Moreover, CRP can opsonize apoptotic cells and promote their clearance (Inforzato et al., 2012) (Figure 1).

SAP has a molecular mass similar to that of CRP and 51% identity with CRP and is also produced by hepatocytes in response to IL-6 (Bottazzi et al., 2010). SAP can bind to phosphorylcholine and, similar to CRP, promote apoptotic cell clearance. SAP can also bind to microbial surface structures such as lipopolysaccharide (LPS) and terminal mannose or galactose residues. Important pathogens detected by SAP include *Streptococcus pyogenes* and *Neisseria meningitidis*. In vivo studies to determine the role of SAP in infection have produced mixed results, as well as data that cannot be readily interpreted. For instance, SAP was shown to protect against infection with pathogens that it cannot bind (*Listeria monocytogenes*) (Singh et al., 1986), whereas it enhanced the virulence of some of the pathogens it can interact with (*S. pyogenes* and rough strains of *Escherichia coli*) (Noursadeghi et al., 2000). The latter effect might be attributed to an anti-opsonic effect of SAP bound to the pathogen, thereby reducing its opsonophagocytic killing. This suggests that certain SAP-coated pathogens may evade immunity rather than being tagged for opsonophagocytosis. Whereas CRP can be used as a diagnostic marker of inflammation (within 24 h, its plasma concentration rises by as much as 1000-fold over the baseline, ≤3 mg/L), the levels of SAP are virtually invariant (30–50 mg/L) (Bottazzi et al., 2010).

PTX3 is a versatile soluble PRM that acts as a bridge between the soluble and the cellular arms of innate immunity. In addition to a C-terminal pentraxin-like domain that is homologous to that of CRP and SAP, PTX3 contains a unique N-terminal region, which mediates some of its binding activities (e.g., conidia of *Aspergillus fumigatus*) (Bottazzi et al., 2010). PTX3 is rapidly induced in a variety of cell types, including myeloid dendritic cells and phagocytes, in response to TLR activation. In neutrophils, PTX3 is constitutively stored in the specific granules and is released by several stimuli, including TLR agonists (Maina et al., 2009).

PTX3 binds selected fungi, bacteria, and viruses (e.g., *A. fumigatus* conidia, *Paracoccidioides brasiliensis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, cytomegalovirus, and H3N2 influenza virus); several complement components (e.g., C1q and factor H); apoptotic cells; and cell-adhesion molecules (P-selectin). These versatile binding activities allow PTX3 to perform distinct functions, including promotion of innate defenses against infection (opsonization, virus neutralization), clearance of apoptotic cells, control of neutrophil trafficking, and regulation of complement activation (Inforzato et al., 2012) (Figure 1). In fact, all pentraxins are involved in complement regulation through interactions with C1q (CRP, SAP, PTX3), ficolins (CRP, PTX3), and factor H (CRP, PTX) (Figure 1).

The role of PTX3 in protective immunity was established by observations that PTX3-deficient mice are more susceptible than normal mice to infection caused by pathogens recognized by PTX3, such as *A. fumigatus* and cytomegalovirus (Inforzato et al., 2012). Mechanistically, PTX3 acts as an opsonin, which not only enhances phagocytosis and killing of pathogens but also promotes dendritic cell maturation and polarization, thereby contributing to the activation of the adaptive immune response (Bottazzi et al., 2010). Intriguingly, polymorphisms in the PTX3 gene are associated with risk for pulmonary tuberculosis and *P. aeruginosa* infections in cystic fibrosis (Olesen et al., 2007; Chiarini et al., 2010). Furthermore, PTX3 mediates a variety of antiviral activities against influenza viruses, including inhibition of virus-induced hemagglutination and viral neuramidase activity, as well as neutralization of virus infectivity in vitro (Reading et al., 2008).

Regarding its complement connection, PTX3 can bind C1q. The interaction of PTX3 with C1q bound to plastic (mimicking a microbial surface) leads to activation of the classical complement cascade. In contrast, fluid-phase PTX3 sequesters C1q and inhibits C1q-dependent

complement activation. These data were interpreted to mean that PTX3 can promote the clearance of pathogens tagged by PTX3, whereas in the fluid phase PTX3 may protect against unwarranted complement activation (Bottazzi et al., 2010). PTX3 also interacts with factor H, a major negative regulator of the alternative pathway. This interaction promotes the deposition of functionally active factor H and iC3b on apoptotic cells and prevents excessive activation of the alternative complement cascade, thereby limiting tissue damage under inflammatory conditions (Deban et al., 2008).

PTX3 can also bind P-selectin and thereby inhibit the rolling of leukocytes on the endothelium. In this way, neutrophil-released PTX3 can regulate local inflammation by suppressing P-selectin-dependent recruitment of neutrophils to peripheral tissues, as shown in a model of acute lung injury (Deban et al., 2010). This anti-inflammatory activity is dependent on the *N*-linked glycosidic moiety of PTX3, which is reminiscent of a similar regulatory activity of IgG mediated by its Fc core polysaccharide (Kaneko et al., 2006). In fact, PTX3 is just one of two known secreted molecules that can directly block the leukocyte adhesion cascade and limit neutrophil extravasation at mucosal sites, the other one being the endothelial glycoprotein Del-1, an antagonist of the LFA-1 integrin (Eskan et al., 2012).

The ability of PTX3 to bind and opsonize microbial structures, regulate complement activation, and exert polysaccharide-dependent anti-inflammatory activity suggests that it acts as a functional predecessor of antibodies (an ante-antibody) (Bottazzi et al., 2010). In this context, it is worth noting that PTX3 (as well as CRP and SAP) interacts with FcγR, in particular FcγRIII/CD16 and FcγRII/CD32, and activates phagocytosis and cytokine secretion (Lu et al., 2008).

Collectins

The collectins constitute a family of lectins that possess triple-helical collagen-like domains as well as C-terminal Ca^{2-}-dependent lectin domains (Holmskov et al., 2003). The best known is the mannose-binding lectin (MBL), which recognizes carbohydrate patterns on the surface of numerous bacteria, viruses, and fungi, resulting in activation of the lectin pathway of the complement system (Ricklin et al., 2010). MBL can also initiate complement activation by binding to IgA (Roos et al., 2001). Another well-known collectin is bovine conglutinin, which interacts with the complement breakdown product iC3b and is able to neutralize influenza A virus (Hartshorn et al., 1993). Surfactant protein (SP)-A and SP-D (which is closely related to conglutinin) occur in the lung surfactant, and are synthesized by type II alveolar cells (Hoppe and Reid, 1994). SP-A and possibly SP-D can bind to bacteria and viruses via carbohydrate groups and promote their phagocytosis by alveolar macrophages, possibly through interaction with the C1q or related receptors (Nepomuceno et al., 1997). Moreover, SP-A inhibits TLR2-dependent *St. aureus* peptidoglycan-induced tumor necrosis factor (TNF) release in human monocytic cells and rat alveolar macrophages by binding directly to TLR2 (Murakami et al., 2002a). SP-A can regulate the innate immune response also by binding CD14 and thereby preventing cellular activation in response to certain serotypes of LPS (Sano et al., 1999).

Ficolins

Ficolins are structurally and functionally related to collectins and, similarly, can activate the lectin pathway of the complement cascade by binding to carbohydrate patterns on the surface of microbes (Holmskov et al., 2003; Liu et al., 2005). They are produced in the liver by hepatocytes and in the lung by type II alveolar cells, as well as by neutrophils and monocytes. Three ficolins have been identified in humans: L-ficolin (ficolin-2), H-ficolin (ficolin-3), and M-ficolin (ficolin-1) (Bottazzi et al., 2010). Ficolins show specificity for *N*-acetylglucosamine residues in complex oligosaccharides, but not for mannose or high-mannose-type oligosaccharides. Individual members display additional specificities, e.g., H-ficolin binds to *N*-acetyl-D-galactosamine and D-fucose, M-ficolin binds to sialic acid, and L-ficolin recognizes lipoteichoic acid and 1,3-β-D-glucan, the major component of yeast and fungal cell walls.

The structural motifs recognized by ficolins are complementary to those detected by MBL and thus appear to target distinct (though occasionally overlapping) microbial species, thereby covering a wide range of pathogen targets. In addition to initiating the lectin pathway, ficolins promote opsonophagocytosis of a number of microbes, including *E. coli* (M-ficolin), *St. aureus* (ficolin B; mouse homolog of M-ficolin), *Salmonella typhimurium* (L-ficolin), and serotype III *Streptococcus agalactiae* (L-ficolin in complex with MBL) (Teh et al., 2000; Liu et al., 2005; Aoyagi et al., 2005). Moreover, L- and H-ficolin can bind apoptotic cells, leading to C3 and C4 deposition, thereby promoting their clearance via the lectin pathway (Kuraya et al., 2005; Honore et al., 2007).

Galectins

Galectins comprise a family of β-galactoside-binding lectins, which have at least one carbohydrate-recognizing domain and can also be found in soluble form. They interact with host carbohydrate ligands in embryogenesis and development and with glycans on microbial surfaces (viruses, bacteria, protists, and fungi). This allows galectins to function as immunomodulatory PRMs in innate immunity. However, galectin-mediated pathogen recognition may not necessarily contribute to innate immune defenses, as in

many cases it is exploited by the pathogens to promote their virulence (Vasta, 2012).

Soluble CD14 and TLRs

At least some pattern-recognition receptors are naturally found in soluble form, which may have a contributory or regulatory effect on the function of the cell-associated version. A classic example is CD14, a 55-kDa glycoprotein that can be attached to the cell membrane by means of a glycosylphosphatidylinositol (GPI) anchor and is therefore devoid of a cytoplasmic domain (Haziot et al., 1988). In addition to the membrane form of CD14 (mCD14), the molecule can also be found in two soluble forms (sCD14): one that is proteolytically liberated from its GPI anchor (48-kDa sCD14) and a second that escapes from the cell membrane (55-kDa sCD14) (Bufler et al., 1995; Haziot et al., 1988). SCD14 can be found in serum at 2–3 μg/mL and in other biological fluids such as milk, cerebrospinal fluid, and urine (Bussolati et al., 2002; Labéta et al., 2000; Landmann et al., 2000).

Like mCD14, sCD14 also binds LPS and the resulting complexes can activate mucosal epithelial and other cells that do not express mCD14 (Pugin et al., 1993), and the activating signals are transduced by TLRs (Akira, 2001). Milk-derived sCD14 may facilitate neonatal intestinal epithelial cell responses to LPS (Labéta et al., 2000), but conversely, injury may be induced by LPS in renal tubular epithelial cells, leading to proteinuria (Bussolati et al., 2002). In contrast to the agonistic activity of sCD14 in mCD14-negative cells, high levels of sCD14 may inhibit LPS-induced TNF release in monocytes/macrophages (Haziot et al., 1994). Mechanistically, sCD14 can compete with mCD14 for LPS thereby promoting LPS efflux from mCD14 and transfer to plasma lipoproteins (Kitchens and Thompson, 2005). In vivo studies suggest that sCD14 may exert protective local proinflammatory effects in tissues, whereas, conversely, it may exert systemic anti-inflammatory effects (Kitchens and Thompson, 2005). In this regard, sCD14 levels are upregulated in sepsis (Landmann et al., 2000), possibly mitigating the severity of disease, because LPS lethality in mice was diminished by recombinant sCD14 (Haziot et al., 1995).

The LPS-binding protein (LBP), which is an acute-phase protein, transfers LPS monomers from micelles to CD14, resulting in cell activation, or from aggregates or sCD14–LPS complexes to high-density lipoprotein, resulting in LPS neutralization (Akira, 2001). Thus low LBP concentrations may enhance the biological activity of LPS, whereas during the acute phase high concentrations may be inhibitory, as suggested by the protective effect of LBP against endotoxin shock in mice (Lamping et al., 1998). Thus, similar to sCD14, LBP also has dual stimulatory and inhibitory effects that are believed to promote protective inflammation against gram-negative bacteria in local sites while preventing potentially destructive inflammation in response to LPS.

Soluble forms of TLRs may act as decoy receptors with regulatory function. The original observation involved an alternatively spliced murine TLR4 mRNA, which results in the expression of a secretory form of TLR4 (sTLR4) devoid of transmembrane and intracellular domains (Iwami et al., 2000). Functionally, sTLR4 inhibits LPS-induced NF-κB activation and TNF release. Since that observation, soluble forms of several TLRs (e.g., TLR1, TLR2, TLR4, TLR6) have been detected in human plasma, milk, parotid saliva, or amniotic fluid (Kacerovsky et al., 2012; Kuroishi et al., 2007; LeBouder et al., 2003). In terms of function, human sTLRs act as negative regulators of TLR-mediated cellular activation. For instance, sTLR2 in parotid saliva can bind TLR2 agonists in the fluid phase and thereby inhibit TLR2-dependent cytokine induction (Kuroishi et al., 2007). More recently, a soluble form of TLR9 was shown to be generated by a specific proteolytic event in the endolysosomal compartment and to inhibit TLR9-dependent signaling (Chockalingam et al., 2001).

The property of soluble PRMs to mediate agonistic or antagonistic effects renders them attractive tools for manipulating the innate immune system, either for promoting protective immunity or for inhibiting unwarranted inflammation in infectious or autoimmune diseases.

LYSOZYME AND HAMLET

Since the initial discovery of lysozyme in 1922 in tears and nasal secretions by Alexander Fleming, a huge literature has accumulated on its structure, function, genetics, biosynthesis, regulation, enzyme activity, and properties, and the reader is referred to a definitive monograph, which summarizes much of this information (Jollès, 1996). Enzymatically, lysozyme (muramidase; EC 3.2.1.17) hydrolyzes the β(1–4) glycosidic bond between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine in bacterial peptidoglycan (Chipman and Sharon, 1969).

Conventional or type *c* lysozyme has been identified widely in mammals, birds, and reptiles, and so-called goose-type lysozyme *g* is found in birds, mammals, and fish. Lysozyme *c* also occurs in insects, whereas another form, lysozyme *i*, has been identified in invertebrates (see Chapter 9), and there are other lysozymes in bacteria and phages, fungi, and plants. Thus lysozyme is one of the most phylogenetically ancient and widespread of all defense factors.

Some forms of lysozyme *c* bind calcium. Lysozyme *c* was the first enzyme to be sequenced and resolved by X-ray crystallography and to have its mechanism of action proposed. Identical forms of lysozyme *c* occur in various human body fluids and tissues, and it is abundant in the specific granules of neutrophils. The concentrations of lysozyme in different secretions vary widely (Table 1). Concentrations can also differ markedly between species: for example,

TABLE 1 Concentrations of Some Major Innate Humoral Factors in Human Secretions

Factor	Salivary[a,b]	Lactational	Lacrimal	Intestinal	Genital	Respiratory
Lysozyme	10–80 μg/mL (unst. parotid) 10–200 μg/mL (unst. whole)	55–75 μg/mL	1.2–1.3 mg/mL	43–106 μg/mL	13 μg/mL (vaginal fluid) 1 mg/mL (cervical mucus plug)	250–500 μg/mL (nasal secretions)
Lactoferrin	7–20 μg/mL (unst. parotid) 8.5–24 μg/mL (unst. whole)	1–3 mg/mL 4–15 mg/mL (colostrum)	1.7 mg/mL	1–26 μg/mL (pancreatic juice)	1 μg/mL (vaginal fluid) 0.1 mg/mL (cervical mucus plug) 1.2 mg/mL (semen)	80–200 μg/mL (nasal secretions) 50–150 μg/mL (BAL)
Peroxidase	2–13 μg/ml (unst. parotid 1–7 μg/mL (unst. whole)	10–15 μg/mL (colostrum) less in late milk	30–40 μg/mL			

[a]Other factors in saliva (whole): histatins, 14–47 μg/mL; cystatin S, 7.3–8.2 μM; cystatin SN, 2.8 μM; secretory leukocyte protease inhibitor, 1–10 μg/mL.
[b]Unst., unstimulated; BAL, bronchoalveolar lavage.

FIGURE 2 Ribbon diagram showing the molecular structure of human lysozyme (Protein Data Bank ID#1JWR; Higo and Nakasako, 2002).

human milk contains approximately 3000 times higher levels of lysozyme than does cow's milk (Chandan et al., 1964). Lysozyme within a given fluid may originate from various sources: for example, in human saliva lysozyme is produced by the salivary glands and by oral phagocytes derived from the gingival crevices (Korsrud and Brandtzaeg, 1982; Moro et al., 1984).

Lysozyme consists of a single polypeptide chain of 119–130 amino acid residues; the human enzyme contains 129 residues, M_r 14,600, p*I* 10.5. There are four disulfide bridges that stabilize the molecule in a compact ellipsoidal shape (for review, see Jollès and Jollès, 1984). The crystallographic structure (Figure 2) reveals two domains: one α-helical domain comprising the N- and C-terminal segments and a smaller β-sheet domain (for review, see Strynadka and James, 1996). Glu35 and Asp52 are highly conserved in the active site. Genetically, lysozyme *c* is encoded by a relatively small 10-kb gene of four exons with three introns (Irwin et al., 1996).

Although lysozymes isolated from different species or even from different tissues of the same species show similar biological activity, they differ biochemically and in specific activity. Human lysozyme has a higher specific activity and more potent antibacterial effect against oral microorganisms than hen egg-white lysozyme (Iacono et al., 1980). However, few species of bacteria are directly lysed by lysozyme, and other modes of action independent of its enzymatic activity include the activation of bacterial autolysins, bacterial aggregation, blocking of bacterial adherence, and inhibition of acid production by oral microorganisms (Iacono et al., 1985; Laible and Germaine, 1985; Twetman et al., 1986; Wang and Germaine, 1991). Moreover, cell walls of oral streptococci weakened by cleavage of peptidoglycan (Cho et al., 1982) become susceptible to lysis by the addition of detergents or monovalent anions such as bicarbonate, fluoride, thiocyanate, and chloride, which predominate in saliva (Pollock et al., 1987). Its cationic property (p*I* 10.5) may allow lysozyme to exert bactericidal activity analogous to other cationic proteins, and although binding to oral bacteria is strongly dependent on pH and ionic strength, bacteriolysis caused by lysozyme may be physiologically significant in the oral environment, where these parameters can fluctuate markedly. However, there is no clear evidence that salivary levels of lysozyme are related to the occurrence of dental caries or periodontal disease (Tenovuo, 1989). Remarkably,

lysozyme is reported to display inhibitory activity against HIV-1 (Lee-Huang et al., 1999).

Several synergistic effects between lysozyme and other nonimmunoglobulin or immunoglobulin defense factors have been reported. These include a bactericidal effect on *Streptococcus mutans* exerted by lysozyme and iron-depleted Lf (Soukka et al., 1991) and the inhibition of glucose uptake in oral streptococci by lysozyme and components of the salivary peroxidase/H_2O_2/SCN^- system, including the oxidation product hypothiocyanite (Lenander-Lumikari et al., 1992). Possibly the membrane damage caused by the cationic nature of lysozyme facilitates the diffusion of thiocyanate oxidation products into the cell.

Lysozyme displays sequence homology with α-lactalbumin, an abundant protein in milk that together with galactosyltransferase forms lactose synthase. It is thought that α-lactalbumin evolved from calcium-binding lysozyme *c* after the divergence of birds and mammals, as it retains the conserved calcium-binding residues found in lysozyme *c* (McKenzie and White, 1991). Although normally devoid of muramidase or antibacterial activity, a variant form of α-lactalbumin was found to induce apoptosis in tumor cells (Svensson et al., 1999). This variant, designated HAMLET (human α-lactalbumin made lethal to tumor cells), assumes an oligomeric "molten globule" conformation induced by the release of calcium at low pH in the presence of oleic acid (Svensson et al., 2000). As these conditions occur in the nursing infant stomach, it was speculated that HAMLET might serve to protect the infant gut. HAMLET is bactericidal against *S. pneumoniae* (Håkansson et al., 2000; Hakansson et al., 2011) by a mechanism akin to starvation and involving sodium-dependent calcium influx (Clementi et al., 2012). At sublethal concentrations HAMLET augments the effects of common antibiotics against the same pathogen (Marks et al., 2012).

LACTOFERRIN

Lf is a member of the transferrin family of iron-binding proteins. Like plasma transferrin, Lf can reversibly bind two ferric ions, but, unlike transferrin, it is primarily a protein of exocrine secretions (Table 1) and neutrophil granules, and it does not play a major role in iron transport. Lf-knockout mice show no abnormalities of iron status (Ward et al., 2003). Although many functions have been proposed for Lf relating to antimicrobial, anti-inflammatory, and immunomodulatory activities, it is uncertain which of these are relevant in vivo. Lf is synthesized by a variety of mucosal tissues, major sites being the mammary gland and the genital tract (reviewed by Teng, 2002), although some mammals (e.g., rats and rabbits) contain no Lf in their milk (Masson and Heremans, 1971). Lf is found in the secondary granules of neutrophils and is synthesized by myeloid precursors, not mature neutrophils (Rado et al., 1987).

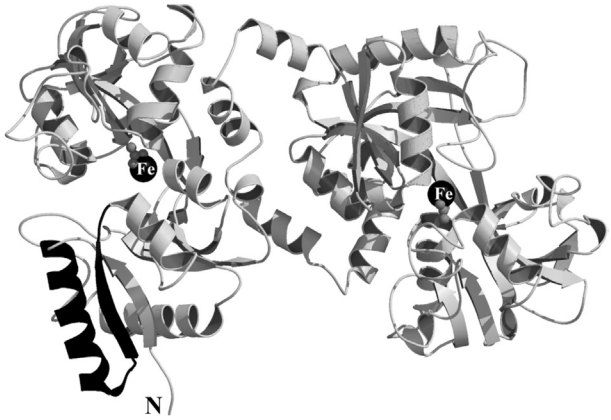

FIGURE 3 Ribbon diagram showing the structural organization of the lactoferrin molecule. The N-lobe is on the left, the C-lobe on the right. In each lobe the bound iron atom is shown as a black sphere in the center, with its associated carbon atom. The antibacterial domain on the surface of the N-lobe is highlighted in black, and the N-terminus, where a cluster of positively charged residues is found, is labeled N. *Figure courtesy of Heather Baker.*

For a comprehensive review of all aspects of Lf structure and function see Schryvers and Vogel (2002).

Lf from a number of species is a single-chain glycoprotein with a molecular weight of about 80 kDa and shows ~60% sequence homology with serum transferrin (Baker, 1994). The polypeptide chain is folded into two lobes, which show ~40% sequence homology, indicative of an ancestral gene duplication (Figure 3). Each lobe is in turn folded into two domains, separated by a cleft in which each iron-binding site is located. In all cases Fe^{3+} is liganded to an aspartic acid, two tyrosines, and a histidine, together with two oxygen atoms from a synergistically bound carbonate ion (Baker, 1994). Iron binding and release are associated with conformational changes in the protein (Anderson et al., 1990). The open "apo" form is more susceptible to denaturation and proteolytic digestion than the Fe-bound ("holo") form. Iron is released at pH <3, whereas in transferrin iron release occurs at pH ~5.5 (Mazurier and Spik, 1980). This probably explains why transferrin, but not Lf, can donate iron to cells via receptor-mediated endocytosis.

Lf is a highly basic protein (p*I* ~9) (Moguilevsky et al., 1985), which makes it an extremely "sticky" protein that binds readily to other macromolecules (Lampreave et al., 1990) or to mucosal surfaces. This property has also hampered attempts to characterize true Lf receptors, and only the enterocyte brush border receptor, which interacts with the basic N-terminal region of Lf (El Yazidi-Belkoura et al., 2001), has been cloned and functionally expressed (Suzuki et al., 2001). Lf is taken up and catabolized by hepatocytes (McAbee et al., 1993). Internalization by T lymphocytes but not monocytes has also been reported (Bi et al., 1996; Ismail and Brock, 1993), resulting in mitogen-activated protein kinase activation (Dennin-Duthille et al., 2000).

Lf has been reported to inhibit the growth or reduce the infectivity of numerous infectious agents, including bacteria, viruses, and parasites. Apo-Lf is bacteriostatic, as it inhibits the growth of various bacteria in vitro, and addition of iron abolishes its activity, indicating that inhibition of iron uptake is involved. Many microorganisms are susceptible to iron deprivation, including mucosal pathogens such as enteropathogenic *E. coli*, *Salmonella* spp., and *Vibrio cholerae* (Weinberg, 2001). However, many bacteria can overcome this effect by secreting low-molecular-weight high-affinity iron chelators (siderophores) that can remove iron from Lf, or by scavenging iron from other sources (Brock et al., 1991). Others, notably *Haemophilus* and *Neisseria*, express specific Lf receptors, regulated by iron levels, which allow them to acquire Lf-bound iron (Gray-Owen and Schryvers, 1996). Lack of iron in *P. aeruginosa* prevents it from forming a biofilm, thereby making it more susceptible to other host defense mechanisms (Singh et al., 2002). In contrast, Fe–Lf inhibits binding of *Actinobacillus actinomycetemcomitans* to buccal epithelial cells (Fine and Furgang, 2002), suggesting that low iron levels might aid colonization.

Bactericidal activity, independent of iron binding (Arnold et al., 1977), probably involves increased membrane permeability (Ellison and Giehl, 1991), mediated by the basic N-terminal region of Lf, as the isolated lactoferricin peptides are more active (Bellamy et al., 1992). Lf binds to various bacterial surface molecules, e.g., the cell wall teichoic acid of *St. aureus* (Vorland et al., 1999), *E. coli* LPS and porins (Vorland et al., 1999; Sallmann et al., 2000), pneumococcal surface protein A (Hammerschmidt et al., 1999), and a 120-kDa surface protein in *Gardnerella vaginalis* (Jarosik and Land, 2000).

In a novel antibacterial mechanism, Lf inactivates two colonization factors, IgA1 protease and Hap adhesin, of *H. influenzae*, owing to an intrinsic serine protease activity (Hendrixson et al., 2003), implying that Lf is bifunctional. Similarly Lf binds to and cleaves the hemoglobin receptor of *Porphyromonas gingivalis*, preventing it from obtaining hemoglobin-bound iron (Shi et al., 2000); however, the lactoferricin peptide was also effective, although it does not contain the putative proteolytic region (Hendrixson et al., 2003), suggesting that proteolytic activity from the organism contributed.

Antibacterial activity of Lf has been amply confirmed in vitro, but evidence for its activity in vivo is less convincing. Lf occurs at high concentration (~1 mg/mL) in human milk, and breast-fed babies are less susceptible than bottle-fed babies to gastrointestinal infection, suggesting that Lf might be an important factor in breast milk. However, attempts to modify the gastrointestinal microbiota of bottle-fed infants by supplementing formula milks with Lf have failed to confirm this (Roberts et al., 1992). Nevertheless, oral administration of Lf can protect against bacterial infection in animal models, for example, amelioration of *Helicobacter* infection possibly by glycan-mediated interference with bacterial adherence (Wang et al., 2001), reduction of the overgrowth and translocation of enterobacteria in mice fed bovine milk (Teraguchi et al., 1995), reduced severity of systemic *E. coli* infection in neonatal rats infected orally (Edde et al., 2001), and protection of rabbits against *Shigella flexneri* infection, through inhibition of bacterial uptake by host cells (Gomez et al., 2003). An antibacterial effect of Lf dependent upon iron binding was revealed when β_2-microglobulin-knockout mice, which suffer from an iron overload condition similar to human hemochromatosis, became less susceptible to tuberculosis after treatment with Lf (Schaible et al., 2002). These studies suggest that Lf might have therapeutic benefit in certain bacterial infections in humans.

Lf can prevent infection by a variety of DNA and RNA viruses, including cytomegalovirus, HIV, herpesvirus, hepatitis B and C viruses, rotavirus, respiratory syncytial virus, and enterovirus 71 (van der Strate et al., 2001). Lf appears to be most effective at the early stages of infection, acting either by blocking viral receptors on host cells or by binding directly to the virus. Lf blocks entry of rotavirus into enterocytes by a mechanism that is independent of iron saturation, but enhanced by desialylation, and mimicked by two tryptic peptides distinct from the lactoferricin region (Superti et al., 2001). A C-terminal region peptide of Lf binds to hepatitis C virus E2 protein and prevents interaction with the host cell (Nozaki et al., 2003). In contrast, inhibition of hepatitis B virus infection in vitro depends upon the interaction of Lf with hepatocytes rather than the virus (Hara et al., 2002). The lactoferricin region of Lf is partly responsible for its ability to inhibit binding of HIV to the CXCR4 or the CCR5 chemokine receptor (Berkhout et al., 2002).

Evidence for an antiviral role for Lf in vivo is limited. However, Lf administered to suckling mice improved survival rates in experimental hantavirus infection (Murphy et al., 2001). Protection against cytomegalovirus infection in mice appears to be due to enhanced natural killer cell activity rather than to a direct antiviral effect of Lf (Shimizu et al., 1996). A clinical trial of bovine Lf in hepatitis C virus infection revealed temporary improvement in 6 of 45 patients (Okada et al., 2002).

The role of Lf against mucosal parasites remains uncertain. *Tritrichomonas foetus* (Grab et al., 2001) and *Leishmania chagasi* (Wilson et al., 2002) possess mechanisms for obtaining iron from Lf, which might therefore be expected to enhance rather than inhibit their growth.

Whereas the antimicrobial effects of Lf have been known for decades, it has become clear that Lf can also influence immune and inflammatory responses, so that its antimicrobial activity could even be due to immunomodulatory rather than direct antimicrobial effects.

One mechanism by which Lf can be anti-inflammatory is through sequestration of non-transferrin-bound iron at

inflammatory foci, thereby preventing it from catalyzing potentially harmful free radical reactions (Guillen et al., 2000). LPS binding by Lf impairs LPS recognition by soluble or surface CD14 on monocytes (Baveye et al., 1999, 2000) and the subsequent production of IL-6, TNF, and nitric oxide (Mattsby-Baltzer et al., 1996; Choe and Lee, 1999). In neutrophils Lf inhibits binding of LPS to L-selectin and subsequent production of reactive oxygen species (Baveye et al., 2000). LPS-mediated IL-8 production by endothelial cells is inhibited by Lf through its basic N-terminal region (Elass et al., 2002; Zhang et al., 1999). Conversely, immobilized (not soluble) Lf stimulates eosinophil activation (Thomas et al., 2002), suggesting that Lf bound to airway epithelial cells might exacerbate asthma.

Despite extensive research, much remains to be learned about Lf, especially in vivo, as many of its proposed functions are based on in vitro studies. Knowledge of the structure of Lf and its iron-binding properties has been revealed by crystallographic studies (Baker et al., 1998). Increasing evidence suggests that Lf is multifunctional, with iron binding, release of basic peptides, and even proteolytic activity contributing to different functional scenarios. Pediatric clinical trials of Lf have been encouraging in terms of safety, as well as efficacy in certain cases, such as reducing the incidence of late-onset sepsis in infants, but more studies are warranted to further address its safety and efficacy in the treatment of neonatal sepsis, necrotizing enterocolitis, and enteric and respiratory infections (Ochoa et al., 2012; Pammi and Abrams, 2011).

PEROXIDASES

Peroxidase activity is found in exocrine secretions including milk, tears, and saliva, as well as in vaginal fluid (Table 1), mostly derived from enzymes synthesized in the glands, but some activity derives from polymorphonuclear leukocytes (myeloperoxidase; MPO) or possibly eosinophils (eosinophil peroxidase; EPO).

Human salivary peroxidase (SPO) is an ~75-kDa protein that is secreted by the parotid glands. The gene has been cloned and sequenced (Kiser et al., 1996), and its sequence is similar (except for two nucleotides) to that of human lactoperoxidase (LPO) found in milk, suggesting that these two enzymes are products of the same gene. Bovine LPO consists of a single polypeptide chain with one heme group, which is covalently attached to the protein via two ester linkages. Bovine LPO preparations are heterogeneous (Paul and Ohlsson, 1985): a major fraction consists of a single polypeptide chain of 78.5 kDa, and smaller molecules are derived by loss of carbohydrate groups or deamidation of asparagine or glutamine residues. Human SPO is also heterogeneous, and at least three major forms of 78, 80, and 280 kDa have been reported (Månsson-Rahemtulla et al., 1988). Human milk contains at least two peroxidases (Pruitt et al., 1991), LPO and MPO (derived from neutrophils), the relative amounts of which vary widely from sample to sample and depend on the stage of lactation.

Peroxidases protect mucosal surfaces from microorganisms by catalyzing the peroxidation of halides (Cl^-, Br^-, I^-) and the pseudohalide thiocyanate (SCN^-) to generate reactive products that have potent antimicrobial properties. MPO and EPO catalyze the peroxidation of Cl^-, Br^-, I^-, and SCN^-, but LPO and SPO do not catalyze the peroxidation of Cl^-. In the absence of halides or SCN^-, peroxidases behave as catalases and degrade H_2O_2 to water and oxygen. The catalase and peroxidase activities of these enzymes thereby protect mucosal surfaces by preventing the accumulation of toxic products of oxygen reduction.

The net reactions of the peroxidation of thiocyanate at physiological concentrations (Pruitt et al., 1986) are:

$$SCN^- + H_2O_2 \rightarrow OSCN^- + H_2O$$

$$OSCN^- + H^+ \leftarrow\rightarrow HOSCN \quad (pK_a = 5.3)$$

These same reactions can protect some bacteria from H_2O_2 toxicity (Adamson and Carlsson, 1982). Both hypothiocyanous acid (HOSCN) and hypothiocyanite ion ($OSCN^-$) inhibit the growth and metabolism of many species of bacteria (for review, see Pruitt and Reiter, 1985).

The major limiting factor for SCN^- peroxidation in human saliva is the availability of H_2O_2 (Månsson-Rahemtulla et al., 1983; Tenovuo et al., 1981), but concentrations of SCN^- below 0.6 mM may also be limiting (Pruitt et al., 1982), and in human milk, the concentrations of SCN^- are usually below this level. The low peroxidase concentration in human milk also may be a limiting factor (Pruitt and Kamau, 1991; Pruitt et al., 1991). Thiocyanate ion is secreted by salivary, mammary, lacrimal, and gastric glands and can originate from several sources, but the major source of SCN^- is the detoxification of cyanide (CN^-) primarily in the liver by thiosulfate–cyanide sulfurtransferase, yielding nontoxic SCN^-. Thiocyanate is found in saliva (0.35–1.24 mM, increasing to 1.38–2.74 mM in smokers) (Tenovuo, 1985) and milk (0.021–0.122 mM) (Pruitt and Kamau, 1991).

MPO and EPO catalyze the oxidation of Cl^- by H_2O_2 to form hypochlorite (OCl^-), which not only kills bacteria and even helminthic parasites (Gleich and Adolphson, 1986), but is also extremely destructive to tissues (Slungaard and Mahoney, 1991). However, SCN^- is the preferred substrate for both MPO and EPO despite its low concentration in serum, and HOSCN and $OSCN^-$ are nontoxic to human cells and tissues (Hänström et al., 1983; Tenovuo and Larjava, 1984; Thomas and Fishman, 1986).

The peroxidase–SCN^- system has antibacterial effects against many bacteria, including streptococci and lactobacilli, and some periodontal and other mucosal pathogens

(Pruitt and Reiter, 1985; Tenovuo, 1985; Courtois et al., 1992; Ihalin et al., 1998). In streptococci it inhibits glucose incorporation, glycolysis, and acid production by cariogenic bacteria (Pruitt and Reiter, 1985; Lenander-Lumikari and Loimaranta, 2000); it also affects the cytoplasmic membrane and inhibits various respiratory oxidases and reductases in *E. coli*. Yeasts and many viruses are also sensitive to peroxidase–SCN^- (Pourtois et al., 1990; Mikola et al., 1995).

OTHER FACTORS

Low-Molecular-Weight Inhibitors

Inorganic and organic acids present in many secretions are known to have antimicrobial properties. For example, neutralization of stomach hydrochloric acid results in a 1000-fold reduction in the infectious dose of *Salmonella typhi* (Mims et al., 1995). Lactic acid (largely produced by lactobacilli) is believed to be important in the maintenance of vaginal health and to inhibit the growth of organisms involved in bacterial vaginosis as well as HIV. Human milk contains a large number of oligosaccharides (apart from lactose), some of which are present in concentrations of up to 1–2 g/L, but as they are excreted intact in infants' urine they are thought not to have significant nutritive value. Several have been found to resemble carbohydrate structures present in bacterial cell walls, for example, those of group B streptococci (Pritchard et al., 1992), and to interfere with, for example, the adherence of *E. coli* to uroepithelial cells (Coppa et al., 1990). Sialyllactose and fucosylated milk oligosaccharides have also been found to inhibit cholera toxin and the heat-stable enterotoxin of *E. coli* (Idota et al., 1995; Newburg et al., 1990), presumably by interfering with their binding to ganglioside receptors.

High-Molecular-Weight Glycoprotein Agglutinins

Salivary mucins are reported to form heterotypic complexes with various other proteins, including lysozyme and α-amylase, as well as secretory IgA, thereby enhancing their binding and agglutinating properties (Biesbrock et al., 1991; Iontcheva et al., 1997) (see also Chapter 14). The salivary agglutinin, a 300- to 400-kDa glycoprotein that occurs in parotid secretion and binds various oral streptococci (Rundegren, 1986), is identical to the scavenger receptor gp340, which is present in bronchoalveolar fluid and also binds to lung surfactant protein D (Ligtenberg et al., 2001). In human saliva, gp340 binds HIV-1 gp120 and inhibits viral infection (Wu et al., 2006). However, gp340 expressed on genital tract epithelial cells binds HIV-1 and promotes infection of target cells by otherwise subinfectious levels of the virus (Stoddard et al., 2007).

Proline-Rich Proteins

The proline-rich proteins (PRPs) are a large family of salivary proteins produced by the parotid and submandibular glands and constitute nearly 70% of the total protein of human saliva. Proline accounts for about 25–40% of the amino acids in PRPs (Bennick, 1982), which are encoded by six genes (Azen and Maeda, 1988). Basic and glycosylated PRPs are encoded by four genes, PRB1–PRB4, and acidic PRPs by two genes, PRH1 and PRH2. They are synthesized as precursor proteins (~150 amino acids), many of which are cleaved before secretion to generate more than 20 PRPs in saliva. Interestingly, the submandibular gland expresses only acidic PRPs.

Functional roles of acidic PRPs include binding to hydroxyapatite (thus becoming a part of the acquired enamel pellicle on teeth) and maintaining tooth integrity (reviewed by Lamkin and Oppenheim, 1993). The carboxyl-terminal domain of acidic PRPs is implicated in bacterial binding, but only when the PRPs are adsorbed to hydroxyapatite, which results in a conformational change that exposes receptors for bacterial attachment—so-called cryptitopes (Gibbons et al., 1990). This provides an efficient means for bacteria such as *Actinomyces viscosus, S. mutans,* and *Streptococcus gordonii* to bind to teeth without being cleared from the oral cavity (reviewed by Lamkin and Oppenheim, 1993). Acidic PRPs on the tooth surface can be degraded into peptides having potential defense properties (Madapallimattan and Bennick, 1990). For example, the antimicrobial pentapeptide Arg-Gly-Arg-Pro-Gln is proteolytically generated from acidic PRPs by commensal *Streptococcus* and *Actinomyces* spp. The synthetic pentapeptide desorbed bound bacteria and counteracted sucrose-induced decrease of dental plaque pH in vitro (Li et al., 2000). Salivary PRPs also inhibit herpes simplex virus 1 replication (Gu et al., 1995) and serve as receptors for *Candida albicans* (O'Sullivan et al., 1997).

Another acidic PRP expressed in human lacrimal glands (Dickinson and Thiesse, 1995) shares 45.5% homology with salivary PRP-1, but its function is unknown, although it is thought to interact with ocular microbes.

Basic PRPs, which are expressed only in parotid saliva, have not been well characterized, but they form insoluble complexes with tannin and tannic acid (Baxter et al., 1997; Lu and Bennick, 1998), suggesting that they play a role in protection against harmful dietary tannins (reviewed by Bennick, 2002). Parotid salivary basic proline-rich proteins have been shown to inhibit HIV-1 infectivity, possibly by binding to gp120 (Robinovitch et al., 2001).

Glycosylated PRPs also bind to microorganisms, and depending on their presence in the tooth pellicle or saliva, they facilitate the adherence of bacteria to oral surfaces or their clearance from the mouth, respectively (reviewed by Bennick, 2002).

Histatins

Histatins are a family of at least 12 small, histidine-rich, cationic peptides secreted into human saliva by salivary glands and having antimicrobial activity, especially against fungi such as *Candida* (Pollock et al., 1984; Oppenheim et al., 1988). Histatins 1, 3, and 5 are the most abundant and they have been credited with most of the anticandidal activity, histatin 5 being the most effective (Xu et al., 1991). Histatins 1 and 3 are encoded by the HIS1 and HIS2 genes, respectively (Sabatini et al., 1993); histatin 5 is a proteolytic product of histatin 3, and the other histatins are probably proteolytic derivatives of histatins 1 and 3. Histatins kill various *Candida* species, including those resistant to the antimycotics fluconazole and amphotericin B, and *A. fumigatus* (Tsai and Bobek, 1997a,b; Helmerhorst et al., 1999a). Histatin 5 and its analogs exhibit synergistic effects with amphotericin B against *Aspergillus, Candida,* and *Cryptococcus* strains (van't Hof et al., 2000).

Histatins act through a multistep mechanism, in which the histatin is internalized by *C. albicans* after binding by cell wall Ssa proteins (Li et al., 2006; Jang et al., 2010) and targeted to energized mitochondria (Helmerhorst et al., 1999b). The killing of *C. albicans* is accompanied by the release of intracellular potassium ions, and the TRK1 potassium channel is critical (Pollock et al., 1984; Xu et al., 1999; Baev et al., 2004). Histatins inhibit respiration and induce the formation of reactive oxygen species in *C. albicans* cells (Helmerhorst et al., 2001) and induce an osmotic stress response (Vylkova et al., 2007).

Inhibitory and bactericidal activities of histatins have also been shown against various bacteria, including *S. mutans* (MacKay et al., 1984), *P. gingivalis* (Murakami et al., 1990, 1991; Nishikata et al., 1991; Gusman et al., 2001), *A. actinomycetemcomitans* (Murakami et al., 2002b), *P. aeruginosa, E. coli,* and *St. aureus* (Sajjan et al., 2001).

As natural antimicrobial peptides histatins show little or no toxicity toward mammalian cells, and have a low tendency to elicit resistance, they show potential as novel antimicrobials, although few in vivo studies have been published. Histatin derivatives have shown some efficacy against experimental gingivitis in beagle dogs (Paquette et al., 1997) and in human clinical trials (Mickels et al., 2001; Van Dyke et al., 2002). Histatin 5 has also been examined for efficacy against *C. albicans* infection in a murine model of vulvovaginal candidiasis and a rat model of oral candidiasis (Intini et al., 2003).

Cystatins

Human salivary cystatins belong to family 2 of the cystatin superfamily, all being derived from a common ancestor (reviewed by Bobek and Levine, 1992). Most are potent inhibitors of cysteine proteinases. Salivary cystatins are encoded by four related genes, CST1, 2, 4, and 5, encoding cystatins SN, SA, S, and D, respectively. Cystatins SN, SA, and S are 121-amino-acid proteins with about 88% identity; cystatin D contains 122 residues and shows about 55% sequence homology with the other cystatins. CST1 and 4 are expressed also in a few other tissues in the body, primarily in exocrine epithelia (Dickinson et al., 2002). Potential functions of type 2 cystatins include direct inhibition of endogenous and exogenous cysteine proteinases, control of mineralization at the tooth surface, antibacterial and antiviral activities, and modulation of the immune system (reviewed by Dickinson, 2002).

In vitro, salivary cystatin SN inhibits human lysosomal cathepsins B, H, and L, and cystatin SA inhibits cathepsin L, which is involved in periodontal tissue destruction (Baron et al., 1999). The results of studies concerning salivary cystatin levels with respect to disease severity in periodontitis are controversial: cystatin levels have been reported to decline, to increase, or not to change (reviewed by Dickinson, 2002), although one study showed that cystatin C (encoded by CST3) increases with disease progression, ostensibly to mitigate further inflammatory tissue damage (Sharma et al., 2012).

Cystatins can interfere with viral replication dependent on host or viral cysteine proteinases. Thus cystatin SN inhibits replication of herpes simplex virus-1 (Gu et al., 1995; Weaver-Hiltke and Bobek, 1998), but not as effectively as cystatin C, and cystatin D inhibits replication of coronavirus (Collins and Grubb, 1998).

Many types of cystatins have a wide range of effects on immune cells (reviewed by Dickinson, 2002). The list now includes SD-type cystatins, SA1 and SA2, which have been found to adhere to human fibroblasts through cell surface molecules, mainly CD58, leading to the expression and release of IL-6 (Kato et al., 2002). Salivary cystatins may thus regulate the cytokine network in gingival connective tissues.

Secretory Leukocyte Protease Inhibitor

Secretory leukocyte protease inhibitor (SLPI), found in the highest concentrations in saliva, but present also in breast milk and genital secretions, is an 11.7-kDa protein that exhibits antimicrobial activities and is thought to play a critical role in mucosal defense. SLPI is a potent inhibitor of serine proteinases (such as human leukocyte elastase) and is also capable of inhibiting HIV-1 infectivity in vitro (McNeely et al., 1995). In the oral cavity SLPI may impede the ability of HIV-1 to infect additional target cells (Wahl et al., 1997). SLPI blocks HIV-1 infection of macrophages and primary T cells at concentrations (1–10 μg/mL) that occur naturally in saliva (Shugars and Wahl, 1998). These findings suggest that SLPI may be partially responsible for the low rate of oral transmission of HIV-1. Although numerous studies

report that SLPI protects cultured mononuclear cells against infection, the inhibition of HIV-1 infection of human macrophages is highly variable (Konopka et al., 1999; Turpin et al., 1996). These discrepancies have been attributed to factors such as variability of macrophage susceptibility to HIV-1 infection and the quality of SLPI preparations (Shine et al., 2002). In addition to SLPI, there are other endogenous inhibitors of HIV-1 in oral secretions, including thrombospondin, Lf, mucins, cystatins, and PRPs. Thrombospondin, which aggregates the virus and blocks virus–CD4 interaction during viral entry, is effective at physiological concentrations (Crombie et al., 1998), whereas the others inhibit HIV only at supraphysiological concentrations.

The production of SLPI (and also lysozyme, but not Lf or total protein) is diminished among healthy older adults, especially males (Shugars et al., 2001), but the impact of this on the increased risk of oral disease with advanced age remains to be determined. In infant saliva SLPI has been shown to play an important role in reducing HIV-1 transmission from mother to child through breast milk of HIV-1-infected mothers (Farquhar et al., 2002).

SLPI, which is found in vaginal secretions, has also been shown to be bactericidal for *Neisseria gonorrhoeae* (Cooper et al., 2012).

Other Antimicrobial Proteins

Several innate antimicrobial proteins have come to attention because they are induced in epithelial cells directly by microbial contact or as a result of activation of Th17 cells and the secretion of IL-17 and IL-22, which promote the synthesis of these proteins (Ouyang and Valdez, 2008). They include angiogenins, RegIIIγ, certain S100 proteins, and lipocalin-2.

Angiogenins are released by the liver into the circulation in the acute-phase response. Angiogenin-4, expressed by intestinal Paneth cells in mice when stimulated by gut commensal bacteria such as *Bacteroides thetaiotaomicron* (Hooper et al., 2003), is a 144-residue protein and a member of the ribonuclease family. It is secreted along with lysozyme from Paneth cell granules giving rise to crypt concentrations exceeding 1 mM. It displays potent selective bactericidal activity against gram-positive bacteria such as *Enterococcus faecalis, L. monocytogenes,* and *S. pneumoniae* and the yeast *C. albicans* at concentrations as low as 1 μM, but not against *B. thetaiotaomicron* or other commensals.

RegIIIβ and RegIIIγ are members of the regenerating protein family of C-type lectins having diverse functions. A human homolog, HIP/PAP (hepatocarcinoma–intestine–pancreas/pancreatitis-associated protein) is found in the small intestine (Cash et al., 2006b). Murine RegIIIγ is highly expressed in the gastrointestinal tract and has potent antimicrobial properties, especially against gram-negative bacteria (Cash et al., 2006a; Zheng et al., 2008), and it is credited with maintaining the intestinal crypts in an essentially sterile condition (Vaishnava et al., 2011). RegIIIγ has been identified in lung epithelial cells and shown to be effective against *St. aureus* (Choi et al., 2013).

S100 proteins are a family of 24 calcium-binding proteins associated with inflammation (Donato et al., 2013). S100-A7, S100-A8, and S100-A9 are involved in antibacterial defense at mucosal surfaces and are upregulated by IL-17 or IL-22 (Liang et al., 2006). S100-A8 (also known as myeloid-related protein-8; MRP8) and S100A9 (MRP14) are abundant cytoplasmic proteins in neutrophils and can form MRP8/14 heterodimers (also known as calprotectin) with antimicrobial activity (Achouiti et al., 2012; Ma et al., 2010). In mice, these antimicrobial proteins protect against lung infection with *Klebsiella pneumonia* (Achouiti et al., 2012), whereas treatments that interfere with IL-17 production diminish the release of MRP8/14 and exacerbate pulmonary mucosal infection with *S. pneumoniae* (Ma et al., 2010). However, S-100 proteins are also endogenous activators of TLR4 and have been implicated in endotoxin-induced shock (Vogl et al., 2007) and in inflammatory bowel disease (Manolakis et al., 2011). They are moreover detected in inflamed knee joints of mice, where they were shown to contribute to pericellular matrix degradation via upregulation of matrix metalloproteinases (van Lent et al., 2008).

Lipocalin-2 binds to iron–enterochelin complexes, which are used by various enterobacteria to acquire iron in the iron-poor setting of the gastrointestinal tract, thereby depriving the bacteria of this essential nutrient and protecting, for instance, against infection with *E. coli* (Flo et al., 2004; Goetz et al., 2002). Lipocalin is also found in neutrophils, where it is stored in specific granules (Goetz et al., 2002), and its expression is greatly enhanced through stimulation of TLR2/1, TLR4, or TLR5 (Flo et al., 2004). It is moreover expressed in the epithelia of the respiratory and alimentary tracts (Cowland and Borregaard, 1997). It has been shown that this antimicrobial protein can also mediate protection at mucosal surfaces. Specifically, it was shown that lipocalin-2 is induced by TLR4 activation in response to *K. pneumoniae* infection and mediates the clearance of this pathogen from the lungs (Chan et al., 2009). Also known as neutrophil gelatinase-associated lipocalin, lipocalin-2 can additionally be induced by the cytokines IL-1β and IL-17 (Chan et al., 2009).

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