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Article *in* Oral Diseases · February 2002

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Saliva – the defender of the oral cavity

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Introduction

It is generally accepted that saliva is of paramount importance for the maintenance of oral health. This is based on the numerous studies reporting subjective and objective functional losses that occur in persons who lack the ability to produce adequate volumes of saliva. These include dry mouth feeling (xerostomia), difficulty with swallowing food, and an increased susceptibility for opportunistic infections. The last issue points to an active protective role of saliva in maintaining oral health under normal conditions. The mild climate present in the oral cavity, i.e. an elevated temperature, a high humidity and regular supply of foodstuffs, fosters the growth of a myriad of different aerobic and anaerobic microorganisms, which together form a complex and stable ecosystem. For example, the oral mucosal surfaces of the newborn infant are the portal of entry for the majority of pathogenic microorganisms from the first day of life (Seidel *et al.*, 2001). Saliva plays a key role in maintaining the steady-state of this system, as becomes clear when the salivary clearing is blocked, for instance in sedated patients in intensive care. In the majority of these patients within 2 weeks a shift in the oral microflora occurs to Gram-negative species, which subsequently spread into the respiratory tract causing pulmonary afflictions. This is one example of the crucial role played by saliva in the maintenance of general health, but similar observations can be made in other patients suffering from an impaired saliva secretion. It is recognized for years that saliva contains many components that, in one way or another interact with microorganisms, in this way controlling the composition of the oral microflora. In the seventies and eighties of the previous century, the main proteins and peptides in

human saliva have been identified and characterized (see Figure 1). Still for a lot of proteins the precise biological role remained elusive as translation of biochemical properties to biological functions proved to be difficult or resulted in erroneous concepts. In the seventies, research focused on elucidation of the role played by saliva in the protection of dental enamel and identified a large number of proteins that *in vitro* were involved in the formation of pellicles on hydroxyapatite. Henceforth they were attributed a role in the protection of tooth surfaces. The insight that a lot of so-called saliva-specific or pellicle-specific proteins also were present in other parts of the human body has stimulated further investigation to the biological role in the innate protection of mucous oral epithelia (Schenkels, Veerman and Nieuw Amerongen, 1995b). As a consequence, for some salivary proteins the existing concepts were refined, while for others a completely different role was found, e.g. as microbicidal agents, or as physiological inhibitors of proteinases.

In earlier days the immunoglobulins in saliva have received much attention in relation to their specific protective function to a single type of microorganism. However, nowadays it has become more clear that, in addition to this acquired immune system, also an innate immune system has been secreted into saliva. In the last years more light has been shed on the protective functions of the peptides and of the (glyco)proteins of the innate immune system, contributing to the first line of oral defence (Frohm Nilsson *et al.*, 1999). In addition, recent research on bacteriostatic (glyco)proteins revealed that they have hidden domains possessing microbicidal properties that come available after proteolysis. Although it appears at first glance on Figure 1 that a redundancy to defence mechanisms in saliva is present (Rudney, Hickey and Ji, 1999), this has been suggested only by *in vitro* studies. *In vivo* it is clear that the inhibiting and killing effects are regulated precisely so that an ecology exists in an equilibrium system in the oral cavity.

This mini-review, primarily dealing with the defensive systems in saliva, will in particular focus on the more

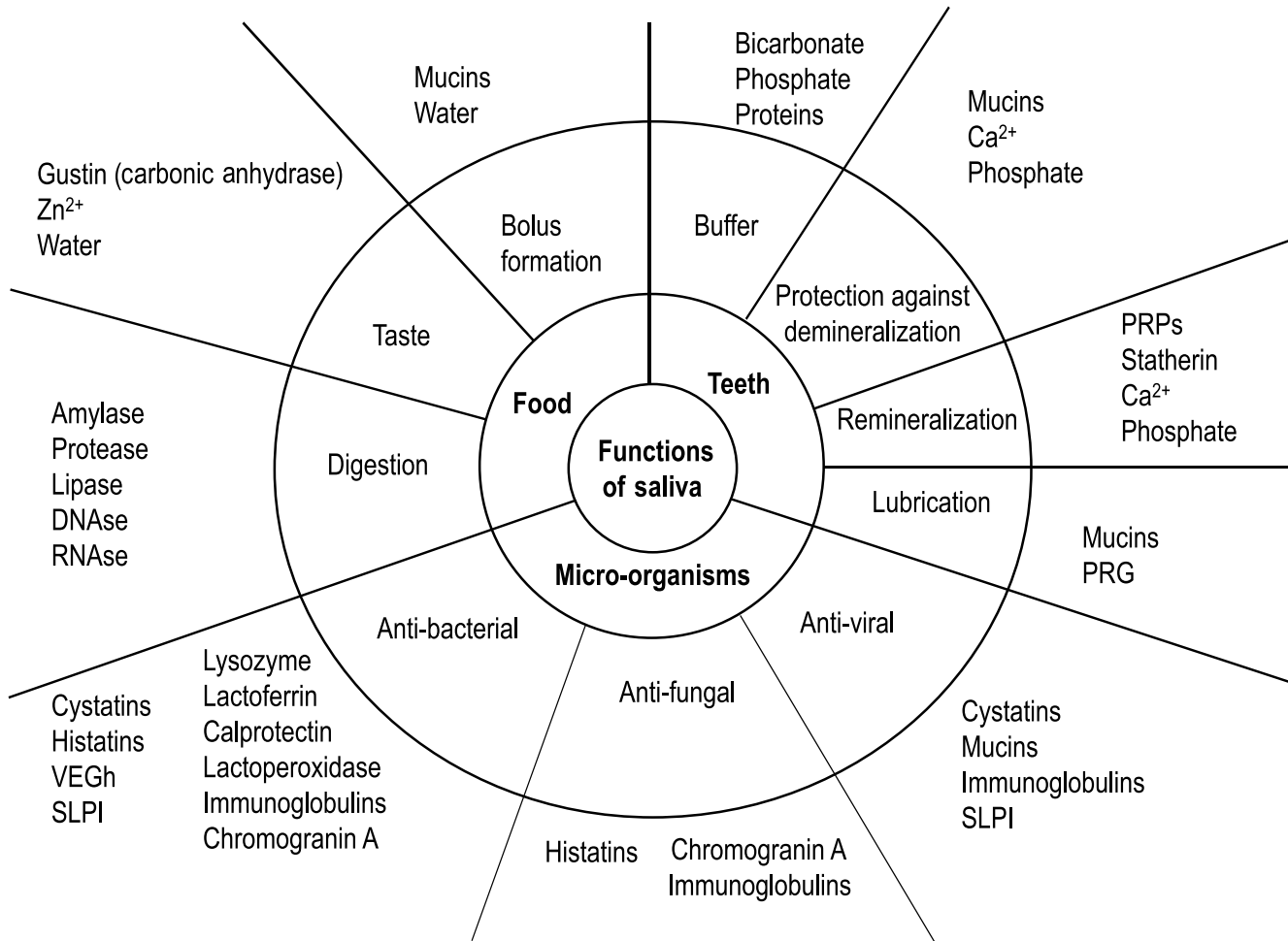


Figure 1 Schematic presentation of the main functions of saliva in relation to its constituents

recently obtained data, aiming to (re)interpret earlier observations on the basis of our present knowledge. In this respect it is intended to give an update of the current knowledge and insights in the protective role of salivary components.

Blood group active glycoproteins: mucins and salivary agglutinin

Salivary mucins

The earliest studies on salivary mucins concluded that these glycoproteins occurred as a single high molecular weight species containing blood group activity (Milne and Dawes, 1973). Later studies, using more stringent isolation and analytical techniques demonstrated that human saliva contains two genetically distinct mucin types, designated MG1 and MG2 (Levine *et al*, 1987; Nieuw Amerongen, Bolscher and Veerman, 1995; Tabak 1995), originating from the MUC5B and MUC7 gene, respectively (Bobek *et al*, 1993; Desseyne *et al*, 1997). MUC7 (MG2) has been found to exist as at least two glycoforms, MG2a and MG2b, respectively (Biesbrock, Bobek and Levine, 1997). In addition, genetic polymorphism has been shown for

MUC7, which is associated with asthma (Kirkbride *et al*, 2001). Salivary MUC5B (MG1), which displays blood group activity, exists in at least three different glycoforms, differing in sialic acid and sulphate content, depending on the glandular source (Veerman *et al*, 1992; Bolscher *et al*, 1995). Even within one glandular secretion, different MUC5B glycoforms could be distinguished (Veerman *et al*, 1992, 1996; Bolscher *et al*, 1995; Thornton *et al*, 1999), highlighting the extreme inter- and intramolecular heterogeneity of this class of salivary glycoproteins. MUC5B functionally and structurally belongs to the classical mucins, which are the main constituents of the slime layers that cover the mucous epithelia throughout the body, e.g. in the gastrointestinal tract, the urogenitary tracts, and the respiratory tracts. These secretory mucins, although encompassing a genetically heterogeneous family, have a comparable architecture: they are composed of disulphide linked monomers that contain heavily glycosylated domains, interspersed with less glycosylated peptide domains ('naked' peptides). Because of their high carbohydrate content (>80%), their large dimensions (>1 µm), and their extended thread-like structure, the classical secretory

mucins already at low concentrations form hydrophilic viscoelastic gels. These gels function as barriers, protecting the underlying epithelium against mechanical damage and preventing direct entrance of noxious agents, including bacteria and viruses, into the underlying vulnerable epithelium. In the oral cavity, MUC5B is present in the protein films (pellicles) covering the enamel and epithelial surfaces, and in this quality protect against acidic attacks (Nieuw Amerongen, Oderkerk and Driessen, 1987) and modulate the microbial colonization of these surfaces (Table 1).

Besides in the mucous acini of the (sero)mucous salivary glands (submandibular, sublingual, palatal and labial glands), MUC5B is also expressed in other body tissues, including the submucosal tracheobronchial glands, the gall bladder and the endocervix (Gipson *et al.*, 1997; Sharma *et al.*, 1998; Van Klinken *et al.*, 1998). In contrast, the expression of MUC7 is confined largely to the serous cells of the (sero)mucous salivary glands and of the respiratory tracts (Biesbrock *et al.*, 1997; Nielsen *et al.*, 1997; Sharma *et al.*, 1998; Bolscher *et al.*, 1999). MUC7 and MUC5B are structurally and functionally completely different molecules. MUC7 is a relatively small (Mr 125 kDa) monomeric species, having low viscoelastic properties. The carbohydrate side chains of MUC7 are predominantly sialylated di- and trisaccharides, while those in MUC5B are much more heterogeneous, with sizes that may vary between two and >20 residues. Furthermore, in contrast to MUC5B, MUC7 lacks blood group active carbohydrates of the ABO-system, a feature that was considered a characteristic trait of mucins (Prakobphol *et al.*, 1998).

When *in vitro* tested, preparations containing high molecular weight blood group reactive glycoproteins cause a wide variety of oral microorganisms to agglutinate, highlighting binding of these components with bacterial receptors. Extrapolated to *in vivo* situations, it is tempting to speculate that this is a physiological mechanism for clearing, to diminish overload of bac-

teria. It has become clear now that MUC7 and the salivary agglutinin (see below) are among the major bacteria-agglutinating factors in saliva. Several studies have reported binding of MUC7 to a variety of microorganisms, e.g. *Streptococcus sanguis*, *S. mitis*, *S. gordonii*, *Actinobacillus actinomycetemcomitans*, *Pseudomonas aeruginosa* and *Escherichia coli* (Murray *et al.*, 1992; Reddy and Levine, 1993; Groenink *et al.*, 1996; Moshier, Reddy and Scannapieco, 1996). In addition to carbohydrate residues, e.g. sialic acid (Reddy *et al.*, 1993; Groenink *et al.*, 1996), unglycosylated peptide domains in MUC7 are involved in the interaction with bacteria (Moshier *et al.*, 1996; Liu *et al.*, 1999; Groenink *et al.*, 1999b). Interestingly, the histidin-rich N-terminal peptide domain of MUC7, which encompasses residues 23–37, by itself has bactericidal properties, probably because it is able to bind and disturb the bacterial membranes (Liu *et al.*, 2000; Situ and Bobek, 2000).

Studies aimed at identification of species specifically binding to (isolated) MUC5B preparations have demonstrated only few species capable of binding to this mucin, including *Haemophilus parainfluenzae* and *Helicobacter pylori* (Veerman *et al.*, 1995, 1997; Bosch *et al.*, 2000), whereas other species, including *Streptococci* do not bind (Murray *et al.*, 1992).

Agglutinin

Salivary agglutinin is a highly glycosylated protein, with a molecular mass of approximately 340 kDa, that carries blood group active antigens. Except for the presence of blood group antigens, salivary agglutinin, which is identical to gp-340 expressed in lung (Holmskov *et al.*, 1999; Prakobphol *et al.*, 2000; Ligtenberg, Veerman and Nieuw Amerongen, 2001) shares a number of features with MUC7: both are monomeric, heavily glycosylated proteins, with extremely sticky properties. As a consequence, under native conditions these proteins occur associated with a variety of

Table 1 Characteristics of salivary defence systems

Defence system	Molecular mass (kDa)	pI	Cellular origin	Salivary glands	Antimicrobial activity
S-IgA	400		B-lymphocytes	All	Spec. immune reaction
Histatins	1–4	>9	Acini	(Sero)mucous	Killing microorganisms
Defensin-2			Epithelium		Killing microorganisms
Cystatins	14	4.5–9	Serous acini, SD	SM, SL, Lip	Inhibitor of cysteine proteinases
VEGh	20		Acini	Von Ebner's gland	Inhibitor of cysteine proteinases
SLPI	11	>9			Inhibitor of serine proteinases
TIMP-1	28			Par, SM	Inhibitor of metallo-proteinases
EP-GP	18	4.5	Serous, mucous acini	All, excepted Par	Binds to CD4
Lactoferrin	76		ID, demilune	Par, SM	Bacteriostatic, microbicidic
Lactoperoxidase	78		Serous acini	Par, SM	Bacteriostatic
Lysozyme	14	>10	ID	Par, SM	Kills Gram-positive bacteria
Chitinase	40	4.5		All	Kills fungi
Agglutinin	340		Duct, demilune	Par, SM, SL	Aggregation
Mucins					
MUC5B	>1000	<4	Mucous acini	All seromucous	Aggregation, encapsulation
MUC7	200	<4	Serous acini		
Calprotectin			Monocytes	Epithelium, Par	Antimicrobial
Chromogranin A	48	Acidic	Duct	SM	Antibacterial, antifungal

salivary proteins, including S-IgA (Biesbrock, Reddy and Levine, 1991; Mehrotra, Thornton and Sheehan, 1998). Both MUC7 and salivary agglutinin are expressed in the serous cells of the submandibular, sublingual and labial glands (Sharma *et al*, 1998; Bikker *et al*, 2002). However, agglutinin, in contrast to MUC7, is also synthesized in the serous parotid gland. In the parotid gland agglutinin was localized only in the ductal cells, whereas in the submandibular gland agglutinin was detected in both serous acinar cells and serous demilune cells capping the mucous acini (Bikker *et al*, 2002). Agglutinin, initially identified as the protein responsible for the *S. mutans* aggregating properties of parotid saliva (Ericson and Rundegren, 1983), was later found to mediate also the binding between *S. mutans* and *S. sanguis* (Lamont *et al*, 1991). This salivary glycoprotein, which very likely is identical to the Eikenella aggregating factor (EcAF) isolated by Ebisu, Fukuhara and Okada (1988), is also detectable as a component of the salivary pellicle on the tooth surface (Carlén and Olsson, 1995). It now has become clear that agglutinin, like MUC7, binds to a wide variety of microorganisms, including *S. mutans*, *S. salivarius* and *S. sanguis* (Ligtenberg *et al*, 2000). Evidence has been produced that salivary agglutinin is genetically very similar, if not identical, to gp-340, a glycoprotein present in lung washings (Prakobphol *et al*, 2000; Ligtenberg *et al*, 2001). Gp-340 binds to surfactant protein-D, resulting in enhanced phagocytosis and killing of microorganisms by neutrophils and macrophages (Holmskov *et al*, 1999). Gp-340 is also identical to DMBT-1 (protein Deleted in Malignant Brain Tumours), a member of the scavenger receptor family, a group of proteins that, because of their capacity to bind to a broad variety of ligands, has been implicated in the innate immune system (Mollenhauer *et al*, 2000).

Cystatins: cysteine proteinase inhibitors

In the eighties it became apparent that a group of human salivary proteins, which initially were implicated in the control of mineralization, possesses cysteine proteinase inhibitory activity (Shomers *et al*, 1982). On basis of sequence homology these proteins have been classified as members of family 2 cystatins. In saliva at least nine different cystatin isoforms are secreted, including the neutral cystatin SN, three moderately anionic isoforms of cystatin SA, three or four isoforms of the more anionic cystatin S and cystatin C, a cationic cystatin (Henskens, Veerman and Nieuw Amerongen, 1996; Tseng *et al*, 2000). More recently the presence of cystatin D has also been demonstrated in human saliva (Hall *et al*, 1998). Because of their proteinase inhibiting properties, cystatins have been suggested to play a role in controlling proteolytic activity, either from the host (released during inflammatory processes) or from microorganisms (Blankenvoorde *et al*, 1996). However, there is experimental evidence suggesting that cystatin C, although a minor constituent of the total population of oral cystatins, contributes most to the cysteine protein-

ase inhibiting activity of saliva (Henskens *et al*, 1996). This is corroborated by kinetic studies showing that of all salivary cystatins, cystatin C displays the most avid inhibition of model cysteine proteinases, e.g. cathepsins and papain. Both egg-white cystatin and cystatin S can inhibit partially proteolytic enzymes released in *P. gingivalis* culture medium (Blankenvoorde *et al*, 1996). In addition, these proteins inhibit growth of specifically *P. gingivalis*, but this activity appeared not to be linked to their enzyme-inhibiting properties (Blankenvoorde *et al*, 1998).

In rodents, cystatins are absent from saliva under normal conditions but become induced after various treatments, including administration of the β -adrenergic agonist isoproterenol, feeding with tannin-rich food (sorghum), application of papain and turpentin on the oral tissues, or cutting the incisors (Bedi 1991; Chaparro, Yu and Shaw, 1998). Salivary cystatins are constitutively secreted in humans, but evidence has been produced that in particular cystatin C levels are increased following severe inflammation in periodontal diseases (Henskens *et al*, 1994). A putative role of cystatins in mediating inflammatory responses is suggested by data demonstrating that *in vitro* family 2 cystatins can up-regulate cytokine production in gingival fibroblasts, a process which probably does not involve its proteinase inhibitory site (Kato *et al*, 2000).

Von Ebner glands protein (VEGh) and secretory leucocyte proteinase inhibitor (SLPI)

VEGh, as a cysteine proteinase inhibitor

VEGh is a salivary protein secreted by the Von Ebner glands located around the circumvallate and foliate papillae of the tongue. It is also known as tear-specific prealbumin (TSPA). VEGh belongs to the lipocalin superfamily, the members of which possess very similar structural features, despite a nearly complete absence of sequence homology. Originally it has been assumed that VEGh was involved in the perception of bitter taste by binding lipophilic bitter compounds and transporting them to the taste buds. However, later it was demonstrated that VEGh can act as inhibitor of cysteine proteinases (Van't Hof *et al*, 1997). This activity has been attributed to the presence of an inhibitory domain in its N-terminus, structurally resembling a motif in cystatins that is required for inhibitory activity. Evidence has also been produced that VEGh can act as an oxidative-stress induced scavenger of peroxidation products (Redl *et al*, 1999; Lechner, Wojnar and Redl, 2001). The recent finding that tear lipocalin, which is identical to VEGh, possesses endonuclease activity, points to still another function for this protein, as nucleases may act as antiviral agents and inhibit the replication of both DNA and RNA viruses (Yusifov *et al*, 2000). The ubiquitous presence of VEGh in various secretions, including saliva, tears and semen, would indeed favour a more general role, e.g. as antiviral agent, over tissue-specific functions such as a carrier for bitter compounds in

saliva, a retinol-binding protein in tear and an androgen-binding protein in semen (Abduragimov *et al*, 2000). It can be speculated that the proteinase-inhibitory activity of VEGh protects the whole molecule against proteolytic inactivation, but is not linked with its main physiological function.

SLPI, inhibitor of serine proteinases

Another example of salivary protein with more than one function is the SLPI (Shugars, Watkins and Cowen, 2001). This protein, for the first time isolated from respiratory secretions, is an inhibitor of serine-proteinases (designated a serpin), including neutrophil elastase, chymotrypsin and Cathepsin G. Besides in human airway submucosal glands, SLPI is expressed in various secretory tissues, including the submandibular, sublingual, parotid, and minor salivary glands. In addition to its proteinase inhibitory properties, SLPI has antimicrobial and antiviral properties (McNeely *et al*, 1995). Interestingly, mice knocked out for the gene encoding SLPI show impaired cutaneous wound healing with increased inflammation and elastase activity, pointing to a pivotal role for SLPI in wound healing.

TIMPS: tissue inhibitors of metalloproteinases

In parotid and submandibular secretions TIMP-1 has been identified, a member of the family of tissue inhibitors of matrix metalloproteinases (MMPs) (Hayakawa *et al*, 1994). Considering their potent inhibitory action against MMPs, including collagenase, gelatinase and stromelysin, TIMPs are thought to play an important role in turnover and remodelling of the extracellular matrix. In saliva the main type of collagenase derives from the host leucocytes, suggesting a role of salivary TIMP-1 in control of inflammatory diseases. Such a function would also be suggested by the finding that TIMP levels in saliva of periodontitis patients, which have elevated collagenase activities, are decreased compared with those in healthy controls. However, other unique functions of TIMPs have been reported, including erythroid potentiating activity, cell growth-promoting activity, and stimulation of osteoclastic bone resorption (Murate and Hayakawa 1999). Interestingly, the latter activity could not be ascribed to merely their inhibitory effect on matrix proteolysis, indicating that TIMPs, like VEGh and SLPI, are multifunctional proteins.

Extra-parotid glycoprotein (EP-GP)

EP-GP is another example of a protein that originally had been implicated in the formation of the dental pellicle, because of its high affinity for hydroxyapatite (Rathman *et al*, 1989). Later studies demonstrated that EP-GP protein is present in several excretory body fluids, including sweat, semen and tears, and is identical to secretory actin binding protein (SABP) and Gross Cystic Disease Fluid Protein (GCDFP-15) (Schenkels *et al*, 1994, Schenkels, Veerman and Nieuw Amerongen, 1995a, 1995b; Caputo *et al*, 1999). The ubiquitous presence in mucosal secretions pointed to a

more general protective role for EP-GP, a concept that was supported by the observation that this protein binds to microorganisms (Schenkels *et al*, 1997) as well as to the CD4 receptor on monocytes (Autiero *et al*, 1997; Gaubin *et al*, 1999). New light was shed on its function when evidence was produced that GCDFP-15 is an aspartyl proteinase with a very narrow substrate specificity: *in vitro* studies showed that GCDFP-15 hydrolyses gelatine and fibronectin, but not bovine serum albumin (BSA), laminin, casein, vitronectin and CD4 (Caputo *et al*, 2000). GCDFP-15, which under non-pathological conditions is absent from breast tissues, is used as a marker for apocrine carcinoma of the breast and the skin (Caputo *et al*, 2000). In view of the expression in malignant tissues, it is speculated that the fibronectin-degrading activity of GCDFP-15 facilitates cell-invasion by cleaving the extracellular matrix scaffold between cells, thus detaching cell membranes from adhesion sites. Under physiological conditions EP-GP might be involved in the turnover of mucous tissues by facilitating the detachment of epithelial cells, but experimental evidence in support of this function is still lacking.

Lactoferrin

The presence of lactoferrin in secretions such as tears, milk and saliva is usually linked with its iron-binding properties. There is general consensus that the long-known bacteriostatic effects of lactoferrin are the result of its iron-depriving effects. In addition, in the nineties it was shown that both bovine and human lactoferrin contain a hidden antimicrobial domain that is liberated from the molecule after proteolytic digestion by pepsin. Within this domain, comprising the 1–47 N-terminal amino acids of human lactoferrin (designated lactoferricin-H), two bacteriocidal fragments can be mapped, residues 1–11 and residues 18–31, respectively (Bellamy *et al*, 1992; Groenink *et al*, 1999a; Lupetti *et al*, 2000). Despite a high sequence homology between the human and the bovine lactoferrin, the antimicrobial properties of peptides derived from the latter are considerably stronger (Bellamy *et al*, 1993; Groenink *et al*, 1999a). One of the target microorganisms of lactoferrin is the periopathogenic bacterium *A. actinomycetemcomitans*. It has been reported that in periodontitis patients, carrying *A. actinomycetemcomitans*, a negative correlation exists between the number of subgingival *A. actinomycetemcomitans* and the lactoferrin concentration in saliva (Groenink *et al*, 1999b). In addition, it inhibits also the growth of *Helicobacter pylori* in an *in vitro* culture system and *H. felis* in an *in vivo* mouse model (Dial *et al*, 2000).

It is conceivable that antimicrobial domains will be released during digestion of lactoferrin in the gastrointestinal tract. This would support the idea that salivary proteins besides the mouth, also can be involved in protection downstream along the gastrointestinal tract. It is now clear that lactoferrin is a multifunctional protein having bacteriostatic, bacteriocidal, fungicidal, antiviral and anti-inflammatory and immunomodulatory

properties (Soukka, Tenovou and Lenander-Lumikari, 1992; Nikawa *et al.*, 1993).

Antimicrobial enzymes: lactoperoxidase, lysozyme and chitinase

Peroxidase activity in saliva is derived from two sources: human salivary lactoperoxidase (HS-LPO) is synthesized and secreted by the salivary glands, whereas myeloperoxidase (MPO) is found in polymorphonuclear (PMN) leucocytes, which migrate into the oral cavity at gingival crevices. Reportedly, the contribution of MPO to the total salivary peroxidase can vary between 30 and 75%, possibly depending on the oral health of the subjects participating (Thomas *et al.*, 1994; Ortiz *et al.*, 1997). Salivary peroxidases catalyse the formation of bactericidal compounds, e.g. hypothiocyanate, by peroxidation of thiocyanate. Because of the antimicrobial effects of the lactoperoxidase system, dentifrices and mouthrinses have been marketed which enhance the endogenous activity of salivary peroxidase, by supplementing H₂O₂-generating enzyme systems.

Lysozyme is ubiquitously present in body fluids, including saliva, tears, bronchial mucus and sweat (Schenkels *et al.*, 1995b). The well-known antimicrobial activity of lysozyme is generally linked with its lytic action on bacteria by catalysing the hydrolysis of cell wall polysaccharides. In addition, non-enzymatic bactericidal activity has been documented for lysozyme as well, which has been attributed to activation of bacterial autolysins (Laible and Germaine, 1985).

Recently, evidence has been produced that chitinase, which catalyses the hydrolytic cleavage of chitin (–GlcNAcβ(1–4)GlcNAc–) is not only present in serum, but also in saliva, where it is derived from the parotid, submandibular, sublingual and palatine glands (Van Steijn *et al.*, 1999). As chitin is a constituent of the yeast cell walls, chitinase activity may play a role in the protection against colonization of oral epithelial cells by yeast. Interestingly, in saliva of periodontal patients, chitinase levels are significantly elevated compared with those in healthy controls (Van Steijn *et al.*, 2002). After periodontal treatment, a decrease in activity of chitinase was found, suggesting that its expression is regulated by inflammatory mediators.

Antimicrobial peptides: histatins and defensins

In the past decennia much attention has been paid to the salivary histatins, a family of structurally related peptides that are enriched in arginine, histidine and lysine residues. In human saliva at least 12 histatin-like peptides have been identified, the majority of which are degradation fragments of two parent molecules, histatin 1 and histatin 3 (Troxler *et al.*, 1990). Of all histatins, histatin 1, histatin 3 and histatin 5 (which is derived from histatin 3), are most abundant in parotid saliva. Presumably because of its relatively potent fungicidal properties (Oppenheim *et al.*, 1988), histatin 5 has been subject to numerous studies. Besides killing of bacteria, such as *S. mutans* (Mackay *et al.*, 1984) and yeasts, histatin 5 has been implicated in a variety of processes, including pellicle formation (Jensen,

Lamkin and Oppenheim, 1992), neutralization of potentially noxious substances, e.g. polyphenols (Yan and Bennick, 1995), chelation of metal ions (Melino *et al.*, 1999; Gusman *et al.*, 2001a), inhibition of inflammatory cytokine induction (Imatani *et al.*, 2000), and inhibition of host and bacterial proteinases, including metalloproteinases and cysteine proteinases (Gusman *et al.*, 2001a, 2001b). The affinity of histatins for such a large repertoire of chemically and structurally different ligands obviously is linked to the unique chemical and structural feature of this molecule. For example, histatin 5 molecules have a flexible structure: in water they have a random-coil structure, while in apolar media they can adopt an α-helical structure. The absence of a strict secondary structure, in addition to the presence of both charged and hydrophobic domains, is probably the key to the multibinding features of histatins, as it will favour binding to structurally and chemically widely different molecules. Nevertheless it is still unclear what the main oral function of histatins would be and whether *in vivo* these are truly multifunctional compounds. Their antimicrobial properties are most appealing and therefore have attracted a lot of interest (Edgerton *et al.*, 1998; Helmerhorst *et al.*, 1999a, 1999b, 2001). Although it was hypothesized that the mechanism of killing activity of cationic peptides was by pore formation in the cellular membrane (Hancock 1997), there is much evidence that the various cationic peptides may have different types of killing mechanisms (Ruisen *et al.*, 2001; Van't Hof *et al.*, 2001). Still it has to be noted that histatin 5, compared with antimicrobial peptides in other parts of the human body, e.g. defensins, *in vitro* display relatively weak microbicidal properties that are readily abolished at moderately elevated ionic strength.

Recently, β-defensins have also been demonstrated in saliva (Krisanaprakornkit *et al.*, 2000; Dale and Krisanaprakornkit, 2001). These antimicrobial peptides, which are induced in epithelial tissues upon inflammation (Harder *et al.*, 2000), are also expressed in duct cells of salivary glands. These peptides are part of the innate immune system; they have broad spectrum antibacterial and antifungal activity and they have properties that may serve to link innate immunity with the acquired immune system.

Calprotectin

Calprotectin is a calcium- and zinc-binding protein derived mainly from granulocytes, monocytes and macrophages. It appears to inhibit microbial growth through competition for zinc (Sweet, Denbury and Challacombe, 2001). The main sources of salivary calprotectin appear to be the gingival crevicular fluid and the oral surface epithelium. As the calprotectin concentration rises markedly in some inflammatory diseases, this protein has been thought to be a marker of inflammatory disease. In periodontitis, calprotectin appears up-regulated and is detected at higher levels in gingival crevicular fluid and tissue specimens. Furthermore, salivary calprotectin levels are raised in patients

with oral candidiasis, consistent with mucosal transudation of calprotectin from inflamed mucosa (Kleinegger, Stoeckel and Kurago, 2001).

Chromogranin A

Chromogranin A is a major protein in adrenal chromaffin cells and adrenergic neurones. In the submandibular glands of the rat, chromogranin A-like immunoreactivity is stored in the exocrine cells in the granular convoluted tubule, and is secreted into saliva by stimulation with noradrenaline and acetylcholine. In human, a prompt elevation of salivary chromogranin A-like immunoreactivity is found in psychosomatic stresses (Nakane *et al*, 1998). Vasostatin-1, the natural N-terminal 1–76 chromogranin A-derived fragment in bovine sequence, displays antibacterial activity against Gram-positive bacteria at micromolar concentrations and is also able to kill a large variety of filamentous fungi and yeast cells (Lugardon *et al*, 2000).

Multifunctional salivary proteins

In the past decades the genetics and the biochemical properties of the major salivary proteins and peptides have been unravelled. Furthermore, the functions of a number of proteins have been identified unambiguously. For example, there is general consensus that S-IgA, directed against specific microorganisms, plays a role in the immunological defence of body secretions, including saliva. However, under stressful conditions the concentration and the composition of the immunoglobulins can change dramatically (Steerenberg *et al*, 1997; Bosch *et al*, 2001). Similarly, the biological role of other proteins is established with reasonable certainty. Examples are the large salivary mucins (MUC5B), which are primarily responsible for the viscoelastic properties of salivary films, salivary peroxidase, involved in the production of antimicrobial inorganic ions, and amylase, involved in digestion of starch. For a number of other proteins that yet lack a clear (enzymatic) activity the function can only be extrapolated on the basis of ‘characteristic’ properties. Although inevitable, such an approach may lead to erroneous concepts, and history has learned that one should exercise caution when claiming functions on the basis of properties alone. So, when on basis of amino acid sequence no clear biological role for an isolated protein can be identified, an often used approach is to study interaction (binding) to various substrates, microorganisms, and proteins, in the hope to gain clues for a biological role. For instance, EP-GP, originally isolated because of its selective adsorption onto hydroxyapatite, was implicated in bacterial clearance when we found it binding to *Streptococci*, *in situ* as well as *in vitro* (Schenkels *et al*, 1997). Finally it turned out that GCDFP-15 (which is identical to EP-GP) is a proteinase with a very narrow substrate specificity, pointing to a role in remodelling of the extracellular matrix (Caputo *et al*, 2000). Another example is VEGh which, initially was implicated in taste perception, because of its glandular origin (the Von Ebner glands in the tongue) (Garibotti *et al*, 1995).

Later VEGh was shown to inhibit cysteine proteinases, and finally it turned out to have endonuclease activity. Another example is the salivary gustin, which because of its zinc-binding properties was linked with taste perception, but now has been identified as a member of the carbonic anhydrase family (Thatcher *et al*, 1998). In this context the term multifunctionality has been coined to indicate the phenomenon that one salivary protein may exert different functions. However, from the foregoing it will be clear that it is not easy to tell whether a property of a protein is linked with its main physiological function, whether it assists in the main physiological function, or is a pure coincidence. For instance salivary histatin 5, the strongest antimicrobial histatin species, contains a zinc-binding motif (Melino *et al*, 1999) and is able to bind copper ions as well (Gusman *et al*, 2001a, 2001b). The capability to bind metal ions suggests a scavenger role for detoxification of metal ions in the oral cavity, a function not related to its putative antimicrobial function. On the other hand, upon binding to zinc, histatin 5 adapts the α -helix conformation, which is pivotal to its antimicrobial activity. In the latter case zinc might be considered as a cofactor, enhancing the antimicrobial function. Because of their polyphenol binding properties, histatins have also been implicated in the detoxification of dietary tannins, a function already attributed to proline-rich proteins (PRPs). However, a characteristic feature of antimicrobial peptides, including histatins, is the presence of distinct cationic and hydrophobic motifs. The latter will have a natural tendency to bind to apolar compounds via hydrophobic interactions. Thus it remains to be demonstrated whether binding to polyphenols indeed is physiologically relevant in case of the histatins, or is a mere side-effect of the structural characteristics linked with its main (antimicrobial?) function.

Making statements about the ‘real’ function of a given salivary protein is further hindered by the fact that saliva harbours myriads of different microorganisms, some of which may be pathogenic, while others are indifferent, or even may be beneficial for the host. All these species successfully have adapted to survive in an environment that basically strives their neutralization and clearance. This makes it difficult to interpret properly data demonstrating binding of salivary proteins to bacteria, as *a priori* one cannot tell whether such an interaction benefits the host or the bacterium. There is little discussion that S-IgA-bacterium binding is an example of an interaction in favour of the host, because of the well-established role of the adaptive immunity in the protection of the body. On the other hand, binding of amylase to certain streptococci (Bergmann and Gulzow 1995) presumably is an example of an interaction that benefits the bacterium, as it endows the bacterium with the capability to digest starch (Scannapieco, Torres and Levine, 1995). Another protein that presumably is abused by microorganisms for their own good is salivary lactoferrin. Because of its iron-depriving effects, lactoferrin is generally considered a bacteriostatic agent. However, a number of pathogens,

including *Neisseria* spp., *S. pneumoniae* and *Haemophilus influenzae*, can utilize iron-loaded human lactoferrin as a sole source of iron.

For a number of other proteins it is simply unknown what binding to bacteria means. For instance, EP-GP binding to bacteria, which also can be demonstrated *in situ*, was first thought to favour microbial clearing. The recent findings that EP-GP specifically can hydrolyse extracellular matrix proteins, leaves the possibility open that it might be used as well by microorganisms to enhance infiltration into the epithelial tissues.

The research in the past decades has produced a wealth of information about the biochemical and functional properties of salivary macromolecules. Figure 1 provides an overview of the functions of saliva, showing that in addition to the same protein having different functions, different proteins can share the same function. In particular there seems to be an overlap in antimicrobial functions of saliva. This may explain why the protective effects of the secretory antimicrobials amply demonstrated *in vitro*, are difficult to demonstrate *in vivo*. Functional overlap will make that no single component is necessary for the overall antimicrobial capacity of the hosts defence system. However, it should be realized that it is difficult to value the significance of defensive and protective systems in saliva. Nowadays in the developed countries there are strict safety-guides for the preparation of foodstuff and drinking water, aimed to minimize the risk of food poisoning and microbial contamination. It can be speculated that this will obscure deficits in the natural protection afforded by innate as well as adaptive immunity, and make it difficult to find significant relationships between the level of a specific compound (e.g. S-IgA) and the susceptibility to diseases. Such relationships might become evident in the more 'natural' situation, when people are daily exposed to microbial threats by ingestion of contaminated foodstuff and drinking water.

Equilibrium in microbial ecology

The oral cavity is colonized by a great number species of microorganisms, varying greatly in quantity. For example, *S. oralis* and *S. sanguis* are abundantly present on the surfaces of the teeth, whereas *S. mutans* forms only a minority of the supragingival plaque (MacPherson, MacFarlane and Stephen, 1991). That an equilibrium system in microbial ecology exists in the oral cavity can be illustrated by the fact that about half of the population carries *Candida albicans* without phenomena of candidiasis. In other words, in these cases candidiasis is an opportunistic disease emerging after lowering of the immune system. The equilibrium in the ecology of the microorganisms in the oral cavity will be maintained not only by the diet but also through the interactive inhibition between the bacterial species themselves. An example of the last mechanism is the discovery of the release of Lantibiotics by *S. salivarius* that can inhibit the growth of *S. pyogenes* (Upton *et al*, 2001).

Back to the future: from purified compounds towards whole saliva

With all the knowledge obtained in the past about the molecular properties of isolated salivary components, it is now time to make a step back and try to understand what their role might be in the complex situation present *in vivo*. In the mouth fluid, a number of salivary proteins are not present as single molecular entities, but in complex with the large salivary mucins. It has been speculated that such complexes could enhance or diminish the multiple biological functions of each member of the complex, but this concept still has to be proven (Iontcheva, Oppenheim and Troxler, 1997). Although it is generally accepted that complexation between salivary macromolecules occurs, and probably influences their biological properties, data about the biochemical and functional properties of these complexes are scarce. One study addressing this issue suggested that binding of MUC7 to *S. aureus* and *P. aeruginosa* may involve a MUC7-S-IgA complex (Biesbrock *et al*, 1991), but this has to be extended and confirmed for other microorganisms as well.

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