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Antimicrobial peptides and proteins, exercise and innate mucosal immunity

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Abstract

This review examines the question of whether exercise can be used as an experimental model to further our understanding of innate antimicrobial peptides and proteins (AMPs), in particular lactoferrin and lysozyme, and their role in susceptibility to infection. AMPs are humoral factors of the innate immune system that form a constituent and inducible component of mucosal surfaces. There is strong evidence to suggest that AMPs, in combination with cellular and physical factors, play an important role in preventing infection. While AMPs act directly on microbes, there is increasing recognition that they also exert their protective effect via immuno-modulatory mechanisms, especially in non-inflammatory conditions. Further studies that manipulate physiologically relevant concentrations of AMPs are required to shed light on the role they play in reducing susceptibility to infection. Evidence shows that various forms of prolonged and/or exhaustive exercise is a potent modulator of the immune system, which can either sharpen or blunt the immune response to pathogens. The intensity and duration of exercise can be readily controlled in experimental settings to manipulate the degree of physical stress experienced by animal or human subjects. This would allow for an investigation into a potential dose-response effect between exercise and AMPs. In addition, the use of controlled exercise could provide an experimental model by which to examine whether changes in the concentration of AMPs alters susceptibility to illness.

1. Introduction

There is a higher risk of infection at epithelial surfaces of the body, such as the respiratory, gastro-intestinal, or uro-genital tract and the skin, that interface with, and separate the host from, the external environment. These epithelial surfaces are protected from invading microbes by the innate mucosal/epithelial defense system, which will be referred to as innate mucosal immunity. While the mucosal immune system does not function independently of the systemic immune system, it is regarded as a distinct entity because it has localized defence factors and is autonomously regulated (Toy and Mayer 1996). In addition to its defence mechanisms, the mucosal immune system also suppresses potentially damaging inflammatory activity. This suppression plays an important role in the prevention of chronic inflammation at mucosal surfaces by preventing infection without the initiation of an immune response. Under inflammatory conditions the suppression of inflammatory activity acts as a measure of control to bring the inflammatory process to a conclusion. Dysfunction in mucosal immunity is associated with increased illness and morbidity (Daele and Zicot 2000), suggesting that immune competence at mucosal surfaces is an important factor for the maintenance of health and well-being.

While effective protection of mucosal surfaces requires both innate and adaptive immune components, this review will address the innate mucosal immune system and in particular antimicrobial peptides and proteins (AMPs) with focus on lactoferrin and lysozyme. Innate mechanisms are primarily responsible for preventing pathogens from entering the body and initiating a rapid response should infection occur. The prophylactic role of the innate immune system has, in recent years, received increased

attention as the search continues for ways to reduce the burden of infectious illness worldwide. There is a diverse range of innate physical (cilia, epithelia and mucus), cellular (neutrophils and macrophages) and humoral factors (AMPs) that function as a barrier to infectious agents. Although the role of physical and cellular factors has been well characterized, humoral factors, such as AMPs, have only recently been acknowledged as important components at mucosal surfaces. AMPs are constituent and inducible factors of secretions at mucosal surfaces that display activity against a broad range of pathogens. Their presence in secretions without the need for prior exposure to infectious agents is indicative of their integral role in the innate mucosal immune system.

While there is extensive evidence from *in-vitro* and animal studies that suggest AMPs have a role in innate mucosal defence, their activity in humans needs to be confirmed by *in-vivo* experiments. Host immune status is recognized as an important factor in susceptibility to infection. Studies that employ experimental models of physical stress to manipulate local immune factors, such as AMPs, may shed further insight into the relationship between immunity, stress and infection. We propose that exercise could be a useful experimental model to study changes in the concentration of AMPs and improve knowledge of their role in reducing susceptibility to illness. Heavy and/or prolonged exercise in humans is known to cause transient perturbations in many cellular and humoral immune factors (Gleeson et al. 1999). Investigations have shown that the serum concentration of lactoferrin increases after moderate and high intensity running (Inoue et al. 2004). To date there have been no published investigations examining the relationship between the concentration of AMPs located in respiratory secretions and exercise. Using exercise as an experimental model to study the relationship between physiologically relevant changes in AMPs and

susceptibility to infection may shed further light on the role of AMPs in mucosal immunity.

2. Antimicrobial Peptides and Proteins

Since Alexander Fleming's discovery in the 1920's that lysozyme kills bacteria, there has been a steady interest in the role of AMPs at mucosal surfaces. The term antimicrobial peptide traditionally refers to small (<100 amino acids) cationic peptides that have antimicrobial activity. The discovery in recent years of a wide range of biological factors, such as cytokines, that display antimicrobial activity has broadened the number of innate antimicrobial factors. Throughout this review we will use the generic abbreviation AMP to refer to both small cationic peptides, polypeptides and proteins, such as lactoferrin and lysozyme. An extensive number of AMPs have been identified in plants and animals. Each mucosal location has a unique profile of AMPs (Tjabringa et al. 2005). This site-specific difference is the result of a number of factors, including the effect of commensal microflora and the presence or absence of microbial challenge. AMPs are classified into groups based on structural features, including size, amino acid structure and physical structure and charge (Reddy et al. 2004). In addition to their antimicrobial properties, AMPs exert substantial immunomodulatory influence locally by inducing the secretion of cytokines and recruiting immune cells to sites of infection, and participate in the remodeling of injured epithelia (Bowdish et al. 2005). AMPs contribute to the health and well being of mucosal surfaces by engaging in a diverse range of activities.

The diverse activities attributed to AMPs relate to the fact that they contain multiple functional domains. The antimicrobial properties of lactoferrin are related to the N-terminal fragment of lactoferrin, known as lactoferricin (Wakabayashi et al. 2003). The ability of lactoferrin to act as a microbistatic agent through its iron

102 binding capability, however, relates to the two homologous lobes at either end of the
103 peptide. Kanyshkova and colleagues note that other enzymatic activities displayed by
104 lactoferrin relate to different subfractions of the peptide(Kanyshkova et al. 2003).
105 Similar observations have been made with other AMPs. Investigations into the human
106 cathelicidin LL-37, an AMP secreted from leukocytes and epithelial cells, have
107 identified several isoforms each of which has a different function (Murakami et al.
108 2002). Many AMPs require enzymatic processing after secretion for synthesis into an
109 active form for their antimicrobial activity. For example, lactoferricin is derived by
110 pepsin digestion of lactoferrin post secretion (Wakabayashi et al. 2003) and pepsin
111 processing of lysozyme is responsible for generating lysozyme's antimicrobial
112 potency (Ibrahim et al. 2005). Post secretory processing of the mature cathelicidin
113 occurs once it has been secreted on to the skin surface to generate multiple AMPs that
114 display antimicrobial activity. Many AMPs lose their ability to undertake other
115 functions once processed from their parent form. The mechanisms that regulate post
116 secretory processing are uncertain, but it is reasonable to surmise that the processing
117 enzymes are regulated by the mucosal milieu and may be a mechanism that allows the
118 host to adapt to altered environmental circumstances. Other AMPs are secreted in
119 their processed form. Neutrophil defensins are stored as processed peptides in the
120 azurophilic granules of neutrophils. The secretion of peptides in a processed form
121 gives the body an immediate antimicrobial platform by which to attack pathogens,
122 while the ability to process peptides into different forms with various capabilities
123 provides the body with a broad spectrum of agents to protect host tissues.
124
125 As part of the innate immune system, AMPs do not show antigen specificity.
126 They do, however, discriminate between prokaryotic and mammalian cells. This
127 preferential selectivity is related to fundamental differences between the membranes

of the two types of cells, specifically membrane charge, microbe cell membranes have a net anionic charge while host cells are zwitterionic, and membrane lipid composition (Matsuzaki 1999). Traditionally the interaction between AMPs and microbes was thought to be as a result of electrostatic interaction caused by this difference in cell charges. More recent investigations with bomimetic structures indicate, however, that membrane lipid composition is more important a determinant than the overall net charge between the membranes in the ability of AMPs to preferentially select, and then interact with, microbe cells over host cells (Arnt et al. 2006). Microbe cell membranes contain phosphatidylglycerol (PG), cardiolipin and phosphatidylethanolamine (PE), which AMPs show high affinity toward. In contrast, mammalian cells are composed of phosphatidylcholine (PC) and cholesterol, which reduce the sensitivity of the membrane to the activity of AMPs. Furthermore, the lipids with negatively charged headgroups are in the inner leaflet of the membrane in mammalian cells, facing the cytoplasm. Targeting fundamentally common features of microbe cell membranes provides AMPs with their non-specific, broad capability, and contributes to the continuing effectiveness of AMPs against infectious agents. Changing the charge density on the membrane has been identified as one of the primary mechanisms by which bacteria evade AMPs (Devine and Hancock 2002). Targeting fundamentally different features between prokaryotic and eukaryotic cells also protects eukaryotic cells from antimicrobial activity.

The focus on AMPs has traditionally been on their antimicrobial properties. AMPs act against a broad spectrum of infectious pathogens *in vitro*, including Gram positive and Gram-negative bacteria, viruses and fungi. The antibacterial activity of AMPs has been measured against a range of bacteria, including *Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli* (Travis et al. 1999). While

154 there are conflicting reports, it appears that AMPs are effective anti-viral agents.
155 Lactoferrin inhibits the growth of respiratory syncytial virus, a common respiratory
156 virus, at a concentration ten-fold lower than that found in human milk (Grover et al.
157 1997). Lactoferrin also acts against human immunodeficiency virus (HIV) and
158 human cytomegalovirus (hCMV) *in vitro* (Harmsen et al. 1995). Both lactoferrin
159 (Arnold et al. 2002) and the cathelicidin LL-37 (Gordon et al. 2005) prevent
160 adenovirus, a respiratory virus, from replicating *in-vitro*. While AMPs act against a
161 broad spectrum of pathogens they display different selective activity for various
162 microorganisms. Lactoferrin's activity against adenovirus can be contrasted with its
163 action against another common respiratory virus, rhinovirus, which it did not inhibit
164 the growth of (Clarke and May 2000). AMPs have been shown to have fungicidal and
165 fungistatic effects, with processed forms of cathelicidin displaying activity against
166 *Candida albicans* at mucosal surfaces (Lopez-Garcia et al. 2005). Several AMPs are
167 induced *in vitro* by microbes (Duits et al. 2003). The evidence from *in-vitro* studies
168 suggests that AMPs display selective activity against a range of common infectious
169 pathogens. No published information appears available on the effect of common
170 stressors, such as exercise, on AMP functionality. Given the effect of exercise on
171 cellular activation, further investigation is warranted to determine if AMP
172 functionality is diminished, and susceptibility to infection altered, as a result of
173 intensive exercise training.
174
175 *2.1 Mechanisms of antimicrobial activity*
176 The mechanisms by which AMPs exert their antimicrobial activity is illustrated
177 in Figure 1. Whether AMPs are capable of attacking multiple targets simultaneously
178 or are target specific is a matter of debate. Direct attack can be lethal or have an
179 inhibitory effect on the growth and activity of microbes, with the concentration of the

peptide the determining factor. In order to exert antimicrobial activity, AMPs must reach pathogen specific minimum concentrations. A key step in their antimicrobial activity is disruption of the microbe cell membranes. This occurs as a two-step process during the initial interaction between AMPs and bacteria. The first step involves electrostatic interaction that results in a depolarization of the microbe cell membrane. The loss of charge between the inside and outside of the cell membrane allows polar substances, which are usually tightly regulated under normal conditions, greater freedom to traverse into the cellular environment. The loss of charge also allows physical interaction between the peptide and the microbe. Following contact with pathogen membranes, AMPs form amphiphilic structures that have a polar hydrophilic and a non-polar hydrophobic section at opposite ends. This conformational change allows the peptide to insert into the membrane, further destabilizing its barrier function.

Destabilization of the microbe cell membranes has traditionally been thought to be the mechanism by which AMPs eliminate microbes. A number of models have been proposed to explain the process by which this may occur (Hancock 2001). The first is the carpet model, in which a layer of AMPs carpets the membranes, resulting in the membranes collapsing and eventually disintegrating as the concentration of AMPs reaches a critical threshold. The AMPs then gain direct access to the intracellular environment (Matsuzaki 1999). The second mechanism of destabilization is the barrel-stave model, which involves the formation of ion channels. These channels form after insertion of the hydrophobic section of the AMP into the membrane. The lipid membrane then separates, with the non-polar section of the AMP facing the membrane and a hydrophilic barrel forming that spans the membrane and exposes the cell to the external environment. The final model is the aggregate

206 model, where clusters of AMPs penetrate the membrane causing transient pores to
207 form and the cell contents to leak out. While there is debate about the exact
208 mechanism(s) of destablization, there is agreement that disruption of the cell
209 membrane would cause cell lysis if the permeability of the membrane overwhelms the
210 ability of other mechanisms to maintain homeostasis within the cell. It has been
211 suggested more recently that membrane permeabilisation is not the mechanism by
212 which AMPs eliminate microbes but an activity that facilitates AMP access to internal
213 targets, such as DNA/RNA(Kanyshkova et al. 2003) protein synthesis(Helmerhorst et
214 al. 1999)and autolytic cell wall enzymes (Ginsburg 2004). The exact mechanism by
215 which AMPs act is determined by a number of factors, including the strain of
216 microbe, characteristics of the peptide and the way that AMPs interact with other
217 immune factors, including other peptides, within the mucosal milieu (Matsuzaki
218 1999). Membrane permeabilisation is recognized, however, as a central feature to the
219 antimicrobial properties of AMPs.
220
221 The potency of AMPs against microbes is increased by their ability to act
222 synergistically, whereby AMPs interact to have a combined effect, and additively, in
223 which case the increased potency is the result of a number of AMPs working
224 independently on a pathogen simultaneously (Singh et al. 2000). This synergistic and
225 additive activity has a number of important effects. First, it reduces the minimum
226 concentration of AMPs required to eliminate microbes. A cocktail of AMPs working
227 in combination at mucosal surfaces lowers the concentration required to eliminate
228 microbes. Secondly, synergistic and additive interactions increase the spectrum of
229 infectious agents against which AMPs can act. Investigations have shown that
230 lactoferrin enhances the effectiveness of lysozyme to eliminate Gram-positive bacteria
231 (Leitch and Willcox 1999). The cell membrane of Gram-positive bacteria is protected

232 from lysozyme by lipotechoic acid. Lactoferrin neutralises the lipotechoic acid,
233 thereby rendering bacterial membranes of Gram positive bacteria more susceptible to
234 the activity of lysozyme (Leitch and Willcox 1999). Thirdly, synergistic and additive
235 interactions increase the speed at which AMPs eliminate infectious pathogens.
236 Combining lactoferrin, lysozyme and serum leukoprotease inhibitor resulted in a
237 faster rate of elimination of *E. coli* than any one protein used individually (Singh et al.
238 2000). Finally, synergistic and additive interactions provide an element of redundancy
239 to mucosal surfaces, lowering the likelihood that deficiencies will result in increased
240 clinical susceptibility to infection.
241
242 AMPs can also inhibit the growth and activity of microbes indirectly. One
243 mechanism by which this is done is by making the mucosal environment unsuitable
244 for colonization. In order to multiply and colonize epithelia, microbes require the
245 presence of nutrients on mucosal surfaces. One well characterized antimicrobial
246 agent, lactoferrin, binds free iron, a nutrient essential to the growth and multiplication
247 of microbes, at mucosal surfaces to restrict its use by bacteria (Legrand et al. 2004).
248 Making mucosal surfaces inhospitable reduces the ability of microbes to colonize
249 epithelia and slows their ability to multiply, thus giving the host tissues increased time
250 to marshal other defensive modalities. However, microbes have evolved and
251 developed mechanisms to overcome the ability of AMPs to reduce the availability of
252 nutrients. In the case of iron, some bacteria have evolved strategies to sequester it
253 from lactoferrin. This is one mechanism by which commensal microflora are able to
254 survive at mucosal surfaces.
255
256 Recent studies have reported inconsistencies in the direct antimicrobial
257 properties of AMPS between *in-vitro* and *in-vivo* conditions. *In-vitro* studies examine

AMPs as a stand alone factor, which is not easily translated to their action *in-vivo*, where agonistic and antagonistic factors in the local environment can exert considerable influence on AMP activity. For example, AMPs lose their antimicrobial activity in the presence of physiological levels of NaCl or serum (Travis et al. 1999). Furthermore, there are discrepancies between the concentrations of AMPs used in *in-vitro* studies compared with the concentrations in the physiological milieu present *in-vivo* (McPhee and Hancock 2005). The concentrations at which AMPs display their antimicrobial activity *in-vitro* is in the μg-mg/ml range, far higher than that found in many locations in the body, especially in non-inflammatory conditions, where the concentration of AMPs is below the minimum inhibitory concentration used *in vitro*. This suggests that the direct antimicrobial properties demonstrated by AMPs may not be their sole, or even primary, role in host defense *in-vivo*. Instead, it may be an activity that occurs only under inflammatory conditions, where the substantially higher concentrations of AMPs are reached which would overcome the inhibitory effect of physiological salt concentrations.

2.2 Immunodulatory activity of the AMP family

There is evidence that AMPs have strong immunomodulatory influence (Table 1). These activities have fueled debate about the mechanism by which AMPs exert their protective effect at mucosal surfaces because they have been conducted under relevant *in-vivo* conditions (Bowdish et al. 2005). AMPs exert their anti-inflammatory activity by preventing interaction between microbes and host cells and stimulating the secretion of cytokines. AMPs prevent interaction between microbes and host cells by interfering with cell receptors that recognize microbes and by neutralizing microbe specific immuno-activating structures. The ability of AMPs to block binding between host cells and microbes has been demonstrated against a variety of pathogens. Human

β-defensin (hBD-2) prevents interaction between host cells and the HIV virus by down-regulating receptors on host cells involved in viral transmission (Quinones-Mateu et al. 2003) while lactoferrin binds to host cell receptors and blocks their interaction with viral pathogens, such as adenovirus (Arnold et al. 2002). AMPs bind with CD14 and lipopolysaccharide (LPS) binding protein (LBP) to impair the binding of inflammatory components on bacterial membranes to host cells (Kirkland et al. 1993). CD14 is a receptor found on monocyte, macrophage and neutrophil membranes (mCD14) and in serum (sCD14) and is the primary mechanism of immune activation to small concentrations of lipopolysaccharide (LPS) (Le Roy et al. 2001). mCD14 facilitates binding of immune cells and LPS, while sCD14 mediates binding between LPS and cells involved in immune activation that do not have a membrane bound CD14 receptor, such as epithelial cells (Arditi et al. 1993) . Each of the major structural classes of AMPs block binding between LPS and LBP (Scott et al. 2000). This prevents the transfer of LPS to CD14 that would otherwise initiate an LPS induced inflammatory response. The ability of LPS to induce an inflammatory response is further impaired by the ability of AMPs, such as lactoferrin, to also bind with high affinity to CD14 to prevent a LBP-LPS complex from binding to it (Baveye et al. 2000). Preventing interaction between a potential host cell and microbes in this way prevents the initiation of an immune response.

The anti-inflammatory influence of AMPs is also mediated by the inactivation of microbe-specific pro-inflammatory motifs (Elsbach 2003). The innate immune system is alerted to the presence of an infectious pathogen by structural features that are microbe-specific. These motifs are recognized by a variety of receptors, such as the toll like receptor, which then induce the release of inflammatory mediators. Altering the characteristics of these microbe specific pro-inflammatory features may have a

310 substantial impact on the ability of a microbe to induce an immune response
311 (Brandenburg et al. 2001). With regard to LPS, the lipid A moiety is the pro-
312 inflammatory motif that induces an inflammatory response. Lipid A is characterized
313 by its shape and charge, having either a concave or conical shape and two or more
314 negative charges (Seydel et al. 2003). The depolarization and insertion of the AMP
315 into the cell membrane during the initial interaction between microbes and AMPs
316 cancels the negative charge and changes the shape of the lipid A section to render it
317 inactive (Brandenburg et al. 2001). AMPs can bind to other inflammatory inducing
318 factors, such as the unmethylated CpG dinucleotides in bacterial DNA (Britigan et al.
319 2001), that are also responsible for inducing an array of pro-inflammatory activities.
320 Blocking the ability of these immunoactivating motifs from inducing an inflammatory
321 response is thought to explain the anti-endotoxin activity (Bennett-Guerrero et al.
322 2001) of serum. It is reasonable to postulate that the presence of AMPs in mucosal
323 secretions would have the same effect.
324
325 Several AMPs also affect inflammatory activity by influencing the secretion of
326 cytokines from host cells. Lactoferrin inhibits the secretion of TNF-α, IL-1β, IL-6 and
327 IL-8 from monocytes whether added before or after an inflammatory inducing agent
328 (Haversen et al. 2002), and inhibits LPS from inducing the classical complement
329 pathway (Samuelsen et al. 2004). In contrast, the induction of AMPs during
330 inflammation facilitates local up-regulation of the immune response. The secretion of
331 AMPs increases significantly during inflammation. While this increase may mediate
332 antimicrobial activity it also serves to attract, recruit and activate other components of
333 the immune system central to an effective immune response. The increase in AMP
334 concentration establishes a chemical gradient, which attracts cells to the site of
335 infection. LL-37 is a chemoattractant for neutrophils, monocytes and T-cells to sites

336 of infection (De et al. 2000). AMPs also induce the secretion of pro-inflammatory
337 cytokines, such as IL-8 secretion from neutrophils and TNF-α from macrophages
338 (Shinoda et al. 1996), that in turn recruit cells to the source of the cytokine secretion.
339 These studies suggest AMPs are integral to the process of inflammation, although
340 their influence, either in promoting or resolving it, will depend on the interplay
341 between a variety of other factors, including cytokines and cellular activation.
342
343 The secretion of AMPs during inflammation acts as a link between the innate and
344 adaptive components of the immune system (Yang et al. 2001). Activation of the
345 adaptive immune system is mediated by the uptake of antigen at sites of infection and
346 presentation of antigen to T and B cells in lymph nodes. Dendritic cells have been
347 identified as key cells in the respiratory tract that take up and present antigen to
348 activate the adaptive immune system. There is a broad spectrum of stimuli that induce
349 the trafficking of dendritic cells to sites of infection. Both α and β defensins recruit
350 immature dendritic cells to sites of infection through chemoattraction and induce their
351 maturation by binding with CCR6 receptors on the dendritic cell surface (Yang et al.
352 1999). The activation of dendritic cells via the CCR 6 receptor also induces the
353 secretion of IL-8, which serves to further promote an inflammatory response. AMPs
354 clearly have an important role in activating the adaptive immune response and
355 recruiting adaptive immune effector cells toward sites of infection.
356
357 The immunomodulatory action of AMPs has been shown to be independent of their
358 antimicrobial activity, suggesting that this may be the mechanism by which AMPs
359 exert their protective effect. The ability of AMPs to neutralize immunoactivating
360 structures on microbes and block microbes from binding to host cells prevents

361 infectious agents from inducing an inflammatory response. This activity may play a
362 part in preventing unintended or constant inflammation at mucosal surfaces. The
363 mucosal surfaces of the body are constantly exposed to a high antigenic load and a
364 balance must be maintained between active and passive immunity, so these sites are
365 not in a permanent state of inflammation. This activity may be particularly relevant to
366 AMPs expressed constitutively at mucosal surfaces. Given their immediate induction
367 during an innate immune response, the ability of AMPs to modulate inflammation
368 may contribute to an appropriate immune response at sites of infection. The resolution
369 of inflammation is a crucial aspect of an immune response and through their anti-
370 inflammatory influence, AMPs may act as a counter-regulatory mechanism that
371 dampens the immune response (Bowdish et al. 2005).
372
373 While much of the evidence for AMPs having a protective role in host defense is
374 inferred from *in-vitro* studies, *in-vivo* studies have confirmed that AMPs play a
375 prophylactic role at mucosal surfaces. In an *in-vivo* animal study, selective inhibition
376 of cathelicidin in mutant mice, via deletion of the relevant gene, resulted in severe
377 necrotic infection following inoculation with group A Streptococcus, which did not
378 occur in wild-type mice (Nizet et al. 2001). Dysfunctional AMP secretion is also
379 associated with greater susceptibility to infection. Overproduction of AMPs *in-vivo*,
380 as occurs with psoriasis, reduces the risk of secondary infection, which contrasts with
381 the decreased expression of AMPs in patients suffering atopic dermatitis, who
382 experience increased susceptibility to secondary infection (Ong et al. 2002).
383 Reversing conditions that inhibit the antimicrobial activity of AMPs reduces
384 susceptibility to common infectious pathogens in disease states. A link has been
385 proposed between AMPs and an increased susceptibility to infection in cystic fibrosis
386 (CF). CF patients have high salt concentration in respiratory secretions that are

thought to inactivate the antimicrobial activity of AMPs, thus leading to a heightened susceptibility to infection. Reducing the salt concentration resulted in CF secretions being able to kill common pathogens that it was unable to previously (Travis et al. 1999). Collectively these studies indicate that AMPs play an important role in susceptibility to infection *in-vivo.*

Beyond their protective capability there is an accumulating body of evidence indicating that AMPs are involved in wound healing. This activity is mediated through a variety of mechanisms, including promotion of angiogenesis/arteriogenesis, the proliferation and migration of epithelial cells and, indirectly, by attracting immune cells that secret factors promoting wound closure to sites of infection (Zanetti 2004). Vascularisation is an essential component to restoring tissue integrity after injury by allowing, among other things, the trafficking and migration of cells and molecules to sites of injury from the bloodsteam. *In-vivo* and *ex-vivo* studies have shown that the cathelicidin LL-37 induces vascularisation by binding to a formyl peptide receptor like 1 (FPRL1) on epithelial cells (Koczulla et al. 2003). In addition human epithelial cell lines treated with synthetic biologically active LL-37 peptide showed a significant increase in cell proliferation, while reduced expression of this AMP delays healing. Similar findings have been made with regard to defensins. Human neutrophil defensins induce airway epithelial cell proliferation and cell migration. The recruitment of immune cells to sites of infection may form an indirect mechanism by which AMPs enhance wound healing. The recruitment of immune cells improves the body's ability to mount an immune response and upregulate inflammation by the release of chemokines. Chemokines also affect wound healing, by acting as growth factors and angiogenic agents (Sorensen et al. 2003). The finding that AMPs play a

role in wound closure supports an expanded functional role beyond that of host defense.

2.3 AMPs and the Upper Respiratory Tract

The upper respiratory tract is a key entry point for viral pathogens. Upper respiratory tract infections (URTI) are associated with a high morbidity burden and may have sequelae that lead to death, especially in infants and in those aged over 70 years (Hashem and Hall 2003), and form the most common presentation to general medical practice. Essentially there are five main causes of respiratory illness: viral infection, bacterial infection, allergic responses, exercise-induced asthma and non-infectious inflammation (Pyne and Gleeson 1998). Most infectious causes of illness are viral, with the most common being caused by rhinovirus, coronavirus, respiratory syncitial virus, parainfluenza virus or adenovirus. Many of these viruses are enveloped viruses that are susceptible to the antimicrobial activity of AMPs. URTI of an infective nature is accompanied by a variety of symptoms, including sore throat, cough, runny nose, congested sinuses, headache, myalgia and fibralgia (Barrett et al. 2002).

URTIs are associated with substantial burden and are a primary reason for absence from work and school, and, when associated with health care costs, driven largely by physician visits and over the counter products to remedy associated symptoms, URTI is a significant economic burden (Hashem and Hall 2003). An economic analysis in the United States estimated the direct cost of respiratory tract infections was $9 billion, not including lost work days (Dixon 1985). The human cost of URTI is less clear but no less significant. In many cases, these illnesses necessitate reduced social interaction and rest, reduced feelings of health and wellbeing and reduced quality of life (Hashem and Hall 2003). For various population groups, such as athletes, URTIs

438 may have increased significance. While evidence is thus far inconclusive, there is data
439 suggesting that athletes remaining free of URTI in the lead up to and during
440 competition perform marginally better than athletes reporting illness (Pyne et al.
441 2001).
442
443 The innate mucosal immune system is a key element in the maintenance of an
444 infection free state in the upper respiratory tract. One mechanism by which the role of
445 AMPs could be assessed in the upper respiratory tract in human subjects is through
446 experimental manipulation of physical stress. A laboratory or field-based model of
447 physical stress that elicits substantial variations in the concentration and/or function of
448 AMPs is required. This approach could be useful in studying the relationships
449 between AMPs and clinical consequences in terms of the incidence, severity or
450 duration of infection. This method of investigation is warranted because substantial
451 perturbations in immunity would be expected to alter susceptibility to illness, and
452 there is evidence that the aetiology of some infectious episodes after exercise are from
453 pathogens susceptible to AMPs (Spence et al. 2004). While there are established links
454 between immunodeficiency and infection, the literature is less clear about the way in
455 which normal perturbations in immunity affect risk of infection. There is a large
456 variation in susceptibility to URTI among healthy individuals, with the incidence of
457 URTI lower in some individuals than others (Gwaltney 2002). Further work is
458 required to better characterize the way in which clinically normal variations in
459 immunity in healthy people, including changes in the concentration of AMPs, affect
460 susceptibility to common illnesses and infections.
461

462 *2.4 Exercise as a model by which to study AMPs*

463 The effect of stress on the immune system has been well documented. By
464 affecting the secretion of various neuropeptides, or stress hormones, stress has a direct
465 effect on the immune system by causing changes to the trafficking and activity of
466 effector cells (neutrophils, lymphocytes, macrophages), the secretion of cytokines,
467 and the induction of endogenous factors that regulate immune activity (heat shock
468 proteins). Many of the acute changes that occur in response to stress enhance
469 immunity. However, the effects of stress hormones on immune function may
470 suppress immunity if elevated too acutely, for long periods of time, or too frequently.
471 Psychological stress can impact negatively on adaptive immune parameters, resulting
472 in the reactivation of three latent herpesviruses, Epstein-Barr virus (EBV), herpes
473 simplex virus type-1 (HSV-1), and human herpesvirus 6 (HHV-6) (Glaser et al. 1999).
474 Similar findings are reported on the effect of stress on factors of innate immunity.
475
476 Exercise, particularly prolonged intense exercise, is known to cause a transient
477 perturbation in cellular and humoral aspects of immunity, which is consistent with our
478 understanding about the effects of stress on the body. The extent of the disturbance to
479 immunity is determined by the intensity, duration and frequency of the exercise
480 workload (Gleeson et al. 2003). The acute immune response to prolonged intense
481 exercise in blood is characterized by a biphasic increase in leucocyte numbers,
482 particularly neutrophils, macrophages and NK cells, during and immediately after
483 exercise (Pyne 1994). Lymphocyte numbers then decrease in the period after exercise
484 to concentrations below resting values (Nieman et al. 1995). The secondary increase
485 in leucocyte cell numbers over the following hours is largely attributable to the
486 mobilization of neutrophils. Prolonged intense exercise is associated with substantial
487 changes in cell functional activity. Neutrophil respiratory burst activity and

488 degranulation increase during and immediately after exercise, before decreasing to
489 levels below the pre-exercise period. High intensity exercise is also associated with a
490 reduction in the expression of neutrophil cell surface receptors immediately and for an
491 hour post exercise (Peake et al. 2004). These studies indicate that discrete aspects of
492 neutrophil function can be negatively affected by prolonged intense exercise.
493 Variations have also been found in NK cell activity (NKCA). However, these
494 perturbations in activity appear dependent on the training history of the subjects, with
495 healthy and well conditioned subjects experiencing a fall below pre-exercise values in
496 NKCA, while prolonged intense exercise had no effect on NKCA in highly trained
497 athletes. The changes associated with prolonged intense exercise, while only transient,
498 reflect a period of immunosuppression.
499
500 Prolonged intense exercise has a negative effect on mucosal immunology. The
501 effect of exercise on mucosal immunology has been assessed by quantifying changes
502 in salivary immunoglobulin A (SIgA) between the pre- and post-exercise period
503 (Tomasi et al. 1982). SIgA is secreted by B-cells and constitutes a humoral
504 component of the adaptive immune system that provides antigen specific immunity at
505 mucosal surfaces. There is an acute and chronic decrease in SIgA following a session
506 of prolonged intense exercise or over a heavy training period (Gleeson et al. 1999).
507 Considerable change has also been observed in innate mucosal defences following
508 prolonged intense exercise, including impaired cilia beat frequency and mucocilliary
509 transit time (Muns et al. 1995), an influx of PMNs to the respiratory tract for several
510 days and reduced phagocytic activity for up to 24 hours in the URT (Muns 1994).
511 Collectively this data suggests that prolonged intense exercise has a suppressive effect
512 on mucosal immunity.
513

514 There are a diverse range of AMPs and proteins in saliva, including lactoferrin,
515 lysozyme, secretory leukocyte protease inhibitor (SLPI), defensins, LL-37 and
516 histatins. Similar to other mucosal surfaces, many of these AMPs form a constitutive
517 barrier to foreign objects entering the oral, nosocomial and upper respiratory tract
518 (Singh et al. 2000). These factors are secreted from surface epithelial cells and
519 salivary glands (Dubin et al. 2004). Following infection, the concentration of these
520 AMPs, and other non-constitutive peptides, increases as they are induced from
521 epithelial and immune cells. To date there are no studies that have systematically
522 examined the acute (minutes to hours) and chronic (days to weeks) changes in AMP
523 concentration in saliva after exercise or training.
524
525 There are a diverse number of mechanisms by which exercise could alter the
526 concentration of AMPs in the respiratory tract (Table 2). Prolonged intense exercise is
527 associated with hyperventilation, that, during exercise, would dry the respiratory tract,
528 potentially reducing the protective shield provided by AMPs. However, in the post
529 exercise period, hyperventilation may increase the secretion of AMPs by inducing an
530 inflammatory response. Airway epithelial cells lining the respiratory tract may
531 experience mechanical trauma as large amounts of air are forcefully inspired. The
532 greater volume of ventilation will increase exposure to environmental irritants and
533 microbes. Epithelial cells increase their expression of AMPs following physical
534 damage (Dorschner et al. 2001) and contact with microbes (Duits et al. 2003). The
535 recruitment and activation of neutrophils during exercise could increase the
536 concentration of AMPs at mucosal surfaces during and immediately after exercise, as
537 neutrophils secrete soluble proteins, including AMPs, when activated. Inhaled
538 particles may in-directly stimulate the expression of AMPs by inducing pro-
539 inflammatory cytokines. The expression of AMPs is increased in the presence of

proinflammatory chemokines, especially interferon-gamma, interleukin (IL) 1-β and IL-8. This suggests that in the immediate period after exercise (minutes to hours), local mechanisms may increase the concentration of AMPs in the respiratory tract.

In addition to exerting local influence on the expression of AMPs, intense exercise may exert indirect effects by increasing the secretion of neuropeptides. Exercise stimulates the hypothalamic-pituitary-adrenal axis to secrete stress hormones, thus increasing the body's ability to meet the physical and metabolic demands of exercise. Prolonged intense exercise is associated with substantially increased secretion of human growth hormone, β-endorphin, catecholamines and glucocorticoids. These factors have a strong influence on the immune system by activating specific receptors on host cells. Exercise-induced changes in plasma concentrations of stress hormones have been associated with changes in circulating leucocyte distribution and activity. The effect of stress hormones on AMPs, however, is somewhat less certain. Catecholamines could induce the expression of AMPs by activating the transcription factor NF-kappa B, which is a pathway involved in upregulating the secretion of peptides such as defensins. Neuropeptides can induce expression of AMPs from glands in animal models , however, they have little impact on AMP secretion from epithelial cells (Dubin et al. 2004). While further studies confirming the effects of neuropeptides on AMP secretion are required, it is plausible to suggest that their secretion during exercise may alter the concentration of AMPs in the post exercise period.

The clinical significance of these changes would suggest that, except during the period when exercise is undertaken and the antimicrobial shield may be diminished by

565 drying, there would be a reduced susceptibility to infection in the period (1-3 hours)
566 post exercise. However, exercise-induced secretion of AMPs may result in a
567 refractory period, where the ability of host cells to secrete AMPs in the immediate
568 post exercise period is reduced. Neutrophils have a transient reduction in the ability to
569 undertake further activity once activated. Given neutrophils are a substantial source of
570 AMPs, this may have implications for the ability of the upper respiratory tract to
571 respond to pathogenic challenge should it occur in the post exercise period. This may
572 be similar for epithelial cells. The increased secretion of lysozyme from glands is
573 because of a prior accumulation of the protein over time (Dubin et al. 2004). This
574 suggests the existence of a refractory period where innate defences are suppressed
575 until recovery and restoration is achieved. The significance of a post-exercise
576 refractory period in AMP protection may be negligible after one bout of exercise but
577 become more significant over a training period of several weeks to months. Elite
578 athletes participating in sports such as rowing, swimming, cycling and running
579 undertake a multitude of high intensity training sessions on a weekly basis. Indeed,
580 chronic stress is associated with a reduced secretion of salivary lysozyme (Koh et al.
581 2002). Hence, it could be postulated that prolonged intense exercise may have a
582 negative effect on the concentration of AMPs in the upper respiratory tract.
583
584 In addition to the suppressive effects of exercise on mucosal immunity there are
585 other reasons justifying its use as an experimental model. Exercise can easily be
586 controlled and reproduced in animal and human settings. This means that the exercise
587 load (physical stress) applied can be prescribed relative to an individual's capability.
588 While prolonged intense exercise causes transient perturbations in immunity, there are
589 individual differences in the relative load or intensity of exercise required to achieve
590 such an effect. The magnitude of this between-subject variation is influenced by

591 physical capacity, training history and fitness. These factors need to be considered
592 when determining the load (intensity and duration) to be applied in experimental
593 settings. Failure to apply the relevant exercise load might confound study results.
594
595 The use of exercise as a model to study the role of AMPs forms part of the
596 discipline of exercise immunology, which has extensively studied the effect of
597 prolonged intense exercise on the immune system in athletic populations to determine
598 why this sub-group of athletes appears to be at greater risk of illness. Studies
599 examining the incidence of URTI in elite athletes engaging in prolonged intense
600 exercise have had variable outcomes, with some studies reporting a heightened
601 incidence (Spence et al. 2004) and others reporting no change (Pyne et al. 2001) in
602 comparison with sedentary control groups. There is general consensus that athletes
603 may experience higher rates of illness during critical training periods and competition.
604 This relationship has been characterized as a theoretical J-shaped curve. The J curve
605 relates the incidence of illness to exercise load. According to this model, individuals
606 engaging in moderate exercise have a reduced risk of illness compared to sedentary
607 individuals or athletes undertaking a high exercise load. Whether this model also
608 applies to AMPs is unclear and worthy of investigation to shed insight on underlying
609 mechanisms and clinical outcomes for the altered susceptibility to illness. A pilot
610 investigation conducted at the Australian Institute of Sport has demonstrated that the
611 concentration of salivary lactoferrin, one of the most abundant AMPs, decreased
612 during a season of training in highly trained elite rowers (unpublished data).
613
614
615

3. Future directions

Further in-vivo studies of AMPs are required to elucidate their role with regard to susceptibility to infection at mucosal surfaces. Given that changes in the concentration of AMPs have substantial implication for their interaction with microbes, there is a need to employ investigative models that physiologically suppress their presence at mucosal surfaces. Exercise may be an appropriate mechanism to further our understanding of the role of AMPs in the control of upper respiratory tract immune status. Further studies are required to examine acute and long-term changes in AMPs in recreational subjects and highly trained athletes undertaking intense, prolonged training. These studies need to address the relationship between intense prolonged exercise on AMP concentrations and determine whether acute or long-term alterations in the concentration and/or function of AMPs is associated with increased incidence of infection. A methodological approach that takes into account confounding variables of exercise such as frequency, intensity and duration is required.

Collection of saliva to study mucosal immunity is well established. Saliva collection is non-invasive and straight-forward and can be more easily standardized in relation to other secretions of the mucosal immune system (Gleeson 2000). Ease of collection and validity as a marker of the mucosal immune system, particularly with respect to IgA, makes saliva collection the preferred method of antimicrobial assessment in athletes, especially in comparison to the collection of other mucosal secretions. Nasal secretions have been used to study AMPs in respiratory secretions. However, most collection techniques for nasal secretions are complex and invasive, such as nasal lavage and suction, because spontaneously secreted fluid is not released

642 in a large enough volume at a constant rate in healthy individuals. The detection of
643 AMPs in nasal lavage is also markedly diminished by dilution of airway secretions
644 (Cole et al. 1999). The collection of tear fluid may also be an effective method for
645 studying changes in mucosal immunity. However, changes in the concentration of tear
646 fluid may not accurately reflect changes occurring in respiratory tract AMPs. Given
647 their role in protection of the upper respiratory tract and the ease with which their
648 status can be collected, salivary AMPs offer promise as useful parameters in
649 monitoring the status of the mucosal immune system.
650
651 **4. Conclusion**
652
653 AMPs play a diverse role in the innate mucosal immune system. As a
654 constituent product at mucosal surfaces, AMPs participate in the barrier function that
655 prevents microbes from causing infection. This activity is mediated by acting directly
656 on microbes, which can be lethal or inhibit their growth and activity, or by preventing
657 them from initiating an inflammatory response. There should also be recognition that
658 AMPs act more broadly to participate in an immune response by recruiting cells,
659 inducing cytokines and aiding in tissue repair. While there is a growing body of
660 evidence that AMPs play a role in mucosal immunity, further research is required to
661 quantify their role with regard to susceptibility to infection. One mechanism by which
662 this can be explored is through prolonged intense exercise, which causes a transient
663 suppression of immunity. Individuals undertaking heavy prolonged exercise appear to
664 suffer an increased incidence of URTI. As yet, however, no link has been found
665 between exercise induced immunosuppression and increased incidence of illness.
666 Further prospective, well-designed and controlled studies are required to clarify the
667 relationships between exercise-induced perturbations in AMPs and incidence of

668 illness. This line of investigation should enhance our understanding of the role of

669 AMPs in mucosal immunity.

Table 1

Immunomodulatory functions of AMPs

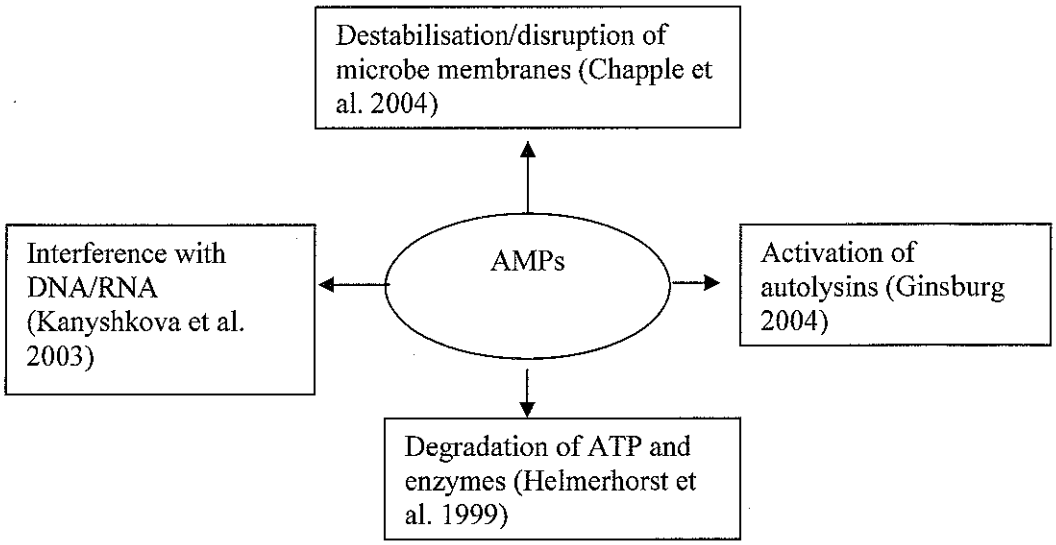
• neutralizing immuno-activating structures
• acting directly on cellular cytokine expression
• regulating receptor expression
• binding host cell proteins
• chemoattractant for immune cells

Table 2

Mechanisms by which acute exercise may effect the concentration of AMPs

- increased secretion of neuropeptides
- induction of AMP secretion by pro-inflammatory cytokines
- damaged epithelial cells releasing AMPs
- neutrophil secretion of AMPs

Fig 1: Mechanisms by which AMPs exert their antimicrobial activity.



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