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 immunomodulatory 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.
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
binding capability, however, relates to the two homologous lobes at either end of the peptide. Kanyshkova and colleagues note that other enzymatic activities displayed by lactoferrin relate to different subfractions of the peptide (Kanyshkova et al. 2003). Similar observations have been made with other AMPs. Investigations into the human cathelicidin LL-37, an AMP secreted from leukocytes and epithelial cells, have identified several isoforms each of which has a different function (Murakami et al. 2002). Many AMPs require enzymatic processing after secretion for synthesis into an active form for their antimicrobial activity. For example, lactoferricin is derived by pepsin digestion of lactoferrin post secretion (Wakabayashi et al. 2003) and pepsin processing of lysozyme is responsible for generating lysozyme's antimicrobial potency (Ibrahim et al. 2005). Post secretory processing of the mature cathelicidin occurs once it has been secreted on to the skin surface to generate multiple AMPs that display antimicrobial activity. Many AMPs lose their ability to undertake other functions once processed from their parent form. The mechanisms that regulate post secretory processing are uncertain, but it is reasonable to surmise that the processing enzymes are regulated by the mucosal milieu and may be a mechanism that allows the host to adapt to altered environmental circumstances. Other AMPs are secreted in their processed form. Neutrophil defensins are stored as processed peptides in the azurophilic granules of neutrophils. The secretion of peptides in a processed form gives the body an immediate antimicrobial platform by which to attack pathogens, while the ability to process peptides into different forms with various capabilities provides the body with a broad spectrum of agents to protect host tissues.

As part of the innate immune system, AMPs do not show antigen specificity. They do, however, discriminate between prokaryotic and mammalian cells. This 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 biomimetic 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
there are conflicting reports, it appears that AMPs are effective anti-viral agents.

Lactoferrin inhibits the growth of respiratory syncytial virus, a common respiratory virus, at a concentration ten-fold lower than that found in human milk (Grover et al. 1997). Lactoferrin also acts against human immunodeficiency virus (HIV) and human cytomegalovirus (hCMV) \textit{in vitro} (Harmsen et al. 1995). Both lactoferrin (Arnold et al. 2002) and the cathelicidin LL-37 (Gordon et al. 2005) prevent adenovirus, a respiratory virus, from replicating \textit{in-vitro}. While AMPs act against a broad spectrum of pathogens they display different selective activity for various microorganisms. Lactoferrin's activity against adenovirus can be contrasted with its action against another common respiratory virus, rhinovirus, which it did not inhibit the growth of (Clarke and May 2000). AMPs have been shown to have fungicidal and fungistatic effects, with processed forms of cathelicidin displaying activity against \textit{Candida albicans} at mucosal surfaces (Lopez-Garcia et al. 2005). Several AMPs are induced \textit{in vitro} by microbes (Duits et al. 2003). The evidence from \textit{in-vitro} studies suggests that AMPs display selective activity against a range of common infectious pathogens. No published information appears available on the effect of common stressors, such as exercise, on AMP functionality. Given the effect of exercise on cellular activation, further investigation is warranted to determine if AMP functionality is diminished, and susceptibility to infection altered, as a result of intensive exercise training.

2.1 \textit{Mechanisms of antimicrobial activity}

The mechanisms by which AMPs exert their antimicrobial activity is illustrated in Figure 1. Whether AMPs are capable of attacking multiple targets simultaneously or are target specific is a matter of debate. Direct attack can be lethal or have an 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
model, where clusters of AMPs penetrate the membrane causing transient pores to form and the cell contents to leak out. While there is debate about the exact mechanism(s) of destabilization, there is agreement that disruption of the cell membrane would cause cell lysis if the permeability of the membrane overwhelms the ability of other mechanisms to maintain homeostasis within the cell. It has been suggested more recently that membrane permeabilisation is not the mechanism by which AMPs eliminate microbes but an activity that facilitates AMP access to internal targets, such as DNA/RNA (Kanyshkova et al. 2003) protein synthesis (Helmerhorst et al. 1999) and autolytic cell wall enzymes (Ginsburg 2004). The exact mechanism by which AMPs act is determined by a number of factors, including the strain of microbe, characteristics of the peptide and the way that AMPs interact with other immune factors, including other peptides, within the mucosal milieu (Matsuzaki 1999). Membrane permeabilisation is recognized, however, as a central feature to the antimicrobial properties of AMPs.

The potency of AMPs against microbes is increased by their ability to act synergistically, whereby AMPs interact to have a combined effect, and additively, in which case the increased potency is the result of a number of AMPs working independently on a pathogen simultaneously (Singh et al. 2000). This synergistic and additive activity has a number of important effects. First, it reduces the minimum concentration of AMPs required to eliminate microbes. A cocktail of AMPs working in combination at mucosal surfaces lowers the concentration required to eliminate microbes. Secondly, synergistic and additive interactions increase the spectrum of infectious agents against which AMPs can act. Investigations have shown that lactoferrin enhances the effectiveness of lysozyme to eliminate Gram-positive bacteria (Leitch and Willcox 1999). The cell membrane of Gram-positive bacteria is protected
from lysozyme by lipotechoic acid. Lactoferrin neutralises the lipotechoic acid,
thereby rendering bacterial membranes of Gram positive bacteria more susceptible to
the activity of lysozyme (Leitch and Willcox 1999). Thirdly, synergistic and additive
interactions increase the speed at which AMPs eliminate infectious pathogens.
Combining lactoferrin, lysozyme and serum leukoprotease inhibitor resulted in a
faster rate of elimination of *E. coli* than any one protein used individually (Singh et al.
2000). Finally, synergistic and additive interactions provide an element of redundancy
to mucosal surfaces, lowering the likelihood that deficiencies will result in increased
clinical susceptibility to infection.

AMPS can also inhibit the growth and activity of microbes indirectly. One
mechanism by which this is done is by making the mucosal environment unsuitable
for colonization. In order to multiply and colonize epithelia, microbes require the
presence of nutrients on mucosal surfaces. One well characterized antimicrobial
agent, lactoferrin, binds free iron, a nutrient essential to the growth and multiplication
of microbes, at mucosal surfaces to restrict its use by bacteria (Legrand et al. 2004).
Making mucosal surfaces inhospitable reduces the ability of microbes to colonize
epithelia and slows their ability to multiply, thus giving the host tissues increased time
to marshal other defensive modalities. However, microbes have evolved and
developed mechanisms to overcome the ability of AMPs to reduce the availability of
nutrients. In the case of iron, some bacteria have evolved strategies to sequester it
from lactoferrin. This is one mechanism by which commensal microflora are able to
survive at mucosal surfaces.

Recent studies have reported inconsistencies in the direct antimicrobial
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 (Ardirty 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 (Elbsbach 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
substantial impact on the ability of a microbe to induce an immune response
(Brandenburg et al. 2001). With regard to LPS, the lipid A moiety is the pro-
inflammatory motif that induces an inflammatory response. Lipid A is characterized
by its shape and charge, having either a concave or conical shape and two or more
negative charges (Seydel et al. 2003). The depolarization and insertion of the AMP
into the cell membrane during the initial interaction between microbes and AMPs
cancels the negative charge and changes the shape of the lipid A section to render it
inactive (Brandenburg et al. 2001). AMPs can bind to other inflammatory inducing
factors, such as the unmethylated CpG dinucleotides in bacterial DNA (Britigan et al.
2001), that are also responsible for inducing an array of pro-inflammatory activities.
Blocking the ability of these immunoactivating motifs from inducing an inflammatory
response is thought to explain the anti-endotoxin activity (Bennett-Guerrero et al.
2001) of serum. It is reasonable to postulate that the presence of AMPs in mucosal
secretions would have the same effect.

Several AMPs also affect inflammatory activity by influencing the secretion of
cytokines from host cells. Lactoferrin inhibits the secretion of TNF-α, IL-1β, IL-6 and
IL-8 from monocytes whether added before or after an inflammatory inducing agent
(Haversen et al. 2002), and inhibits LPS from inducing the classical complement
pathway (Samuels et al. 2004). In contrast, the induction of AMPs during
inflammation facilitates local up-regulation of the immune response. The secretion of
AMPs increases significantly during inflammation. While this increase may mediate
antimicrobial activity it also serves to attract, recruit and activate other components of
the immune system central to an effective immune response. The increase in AMP
concentration establishes a chemical gradient, which attracts cells to the site of
infection. LL-37 is a chemoattractant for neutrophils, monocytes and T-cells to sites
of infection (De et al. 2000). AMPs also induce the secretion of pro-inflammatory
cytokines, such as IL-8 secretion from neutrophils and TNF-α from macrophages
(Shinoda et al. 1996), that in turn recruit cells to the source of the cytokine secretion.
These studies suggest AMPs are integral to the process of inflammation, although
their influence, either in promoting or resolving it, will depend on the interplay
between a variety of other factors, including cytokines and cellular activation.

The secretion of AMPs during inflammation acts as a link between the innate and
adaptive components of the immune system (Yang et al. 2001). Activation of the
adaptive immune system is mediated by the uptake of antigen at sites of infection and
presentation of antigen to T and B cells in lymph nodes. Dendritic cells have been
identified as key cells in the respiratory tract that take up and present antigen to
activate the adaptive immune system. There is a broad spectrum of stimuli that induce
the trafficking of dendritic cells to sites of infection. Both α and β defensins recruit
immature dendritic cells to sites of infection through chemotraction and induce their
maturation by binding with CCR6 receptors on the dendritic cell surface (Yang et al.
1999). The activation of dendritic cells via the CCR 6 receptor also induces the
secretion of IL-8, which serves to further promote an inflammatory response. AMPs
clearly have an important role in activating the adaptive immune response and
recruiting adaptive immune effector cells toward sites of infection.

The immunomodulatory action of AMPs has been shown to be independent of their
antimicrobial activity, suggesting that this may be the mechanism by which AMPs
exert their protective effect. The ability of AMPs to neutralize immunoactivating
structures on microbes and block microbes from binding to host cells prevents
infectious agents from inducing an inflammatory response. This activity may play a
part in preventing unintended or constant inflammation at mucosal surfaces. The
mucosal surfaces of the body are constantly exposed to a high antigenic load and a
balance must be maintained between active and passive immunity, so these sites are
not in a permanent state of inflammation. This activity may be particularly relevant to
AMPS expressed constitutively at mucosal surfaces. Given their immediate induction
during an innate immune response, the ability of AMPS to modulate inflammation
may contribute to an appropriate immune response at sites of infection. The resolution
of inflammation is a crucial aspect of an immune response and through their anti-
inflammatory influence, AMPS may act as a counter-regulatory mechanism that
dampens the immune response (Bowdish et al. 2005).

While much of the evidence for AMPS having a protective role in host defense is
inferred from in-vitro studies, in-vivo studies have confirmed that AMPS play a
prophylactic role at mucosal surfaces. In an in-vivo animal study, selective inhibition
of cathelicidin in mutant mice, via deletion of the relevant gene, resulted in severe
necrotic infection following inoculation with group A Streptococcus, which did not
occur in wild-type mice (Nizet et al. 2001). Dysfunctional AMP secretion is also
associated with greater susceptibility to infection. Overproduction of AMPS in-vivo,
as occurs with psoriasis, reduces the risk of secondary infection, which contrasts with
the decreased expression of AMPS in patients suffering atopic dermatitis, who
experience increased susceptibility to secondary infection (Ong et al. 2002).
Reversing conditions that inhibit the antimicrobial activity of AMPS reduces
susceptibility to common infectious pathogens in disease states. A link has been
proposed between AMPS and an increased susceptibility to infection in cystic fibrosis
(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 \textit{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 bloodstream. \textit{In-vivo} and \textit{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
may have increased significance. While evidence is thus far inconclusive, there is data suggesting that athletes remaining free of URTI in the lead up to and during competition perform marginally better than athletes reporting illness (Pyne et al. 2001).

The innate mucosal immune system is a key element in the maintenance of an infection free state in the upper respiratory tract. One mechanism by which the role of AMPs could be assessed in the upper respiratory tract in human subjects is through experimental manipulation of physical stress. A laboratory or field-based model of physical stress that elicits substantial variations in the concentration and/or function of AMPs is required. This approach could be useful in studying the relationships between AMPs and clinical consequences in terms of the incidence, severity or duration of infection. This method of investigation is warranted because substantial perturbations in immunity would be expected to alter susceptibility to illness, and there is evidence that the aetiology of some infectious episodes after exercise are from pathogens susceptible to AMPs (Spence et al. 2004). While there are established links between immunodeficiency and infection, the literature is less clear about the way in which normal perturbations in immunity affect risk of infection. There is a large variation in susceptibility to URTI among healthy individuals, with the incidence of URTI lower in some individuals than others (Gwaltney 2002). Further work is required to better characterize the way in which clinically normal variations in immunity in healthy people, including changes in the concentration of AMPs, affect susceptibility to common illnesses and infections.
2.4 Exercise as a model by which to study AMPs

The effect of stress on the immune system has been well documented. By affecting the secretion of various neuropeptides, or stress hormones, stress has a direct effect on the immune system by causing changes to the trafficking and activity of effector cells (neutrophils, lymphocytes, macrophages), the secretion of cytokines, and the induction of endogenous factors that regulate immune activity (heat shock proteins). Many of the acute changes that occur in response to stress enhance immunity. However, the effects of stress hormones on immune function may suppress immunity if elevated too acutely, for long periods of time, or too frequently.

Psychological stress can impact negatively on adaptive immune parameters, resulting in the reactivation of three latent herpesviruses, Epstein-Barr virus (EBV), herpes simplex virus type-1 (HSV-1), and human herpesvirus 6 (HHV-6) (Glaser et al. 1999). Similar findings are reported on the effect of stress on factors of innate immunity.

Exercise, particularly prolonged intense exercise, is known to cause a transient perturbation in cellular and humoral aspects of immunity, which is consistent with our understanding about the effects of stress on the body. The extent of the disturbance to immunity is determined by the intensity, duration and frequency of the exercise workload (Gleeson et al. 2003). The acute immune response to prolonged intense exercise in blood is characterized by a biphasic increase in leucocyte numbers, particularly neutrophils, macrophages and NK cells, during and immediately after exercise (Pyne 1994). Lymphocyte numbers then decrease in the period after exercise to concentrations below resting values (Nieman et al. 1995). The secondary increase in leucocyte cell numbers over the following hours is largely attributable to the mobilization of neutrophils. Prolonged intense exercise is associated with substantial changes in cell functional activity. Neutrophil respiratory burst activity and
degranulation increase during and immediately after exercise, before decreasing to
levels below the pre-exercise period. High intensity exercise is also associated with a
reduction in the expression of neutrophil cell surface receptors immediately and for an
hour post exercise (Peake et al. 2004). These studies indicate that discrete aspects of
neutrophil function can be negatively affected by prolonged intense exercise.
Variations have also been found in NK cell activity (NKCA). However, these
perturbations in activity appear dependent on the training history of the subjects, with
healthy and well conditioned subjects experiencing a fall below pre-exercise values in
NKCA, while prolonged intense exercise had no effect on NKCA in highly trained
athletes. The changes associated with prolonged intense exercise, while only transient,
reflect a period of immunosuppression.

Prolonged intense exercise has a negative effect on mucosal immunology. The
effect of exercise on mucosal immunology has been assessed by quantifying changes
in salivary immunoglobulin A (SIgA) between the pre- and post-exercise period
(Tomasi et al. 1982). SIgA is secreted by B-cells and constitutes a humoral
component of the adaptive immune system that provides antigen specific immunity at
mucosal surfaces. There is an acute and chronic decrease in SIgA following a session
of prolonged intense exercise or over a heavy training period (Gleeson et al. 1999).
Considerable change has also been observed in innate mucosal defences following
prolonged intense exercise, including impaired cilia beat frequency and mucociliary
transit time (Muns et al. 1995), an influx of PMNs to the respiratory tract for several
days and reduced phagocytic activity for up to 24 hours in the URT (Muns 1994).
Collectively this data suggests that prolonged intense exercise has a suppressive effect
on mucosal immunity.
There are a diverse range of AMPs and proteins in saliva, including lactoferrin, lysozyme, secretory leukocyte protease inhibitor (SLPI), defensins, LL-37 and histatins. Similar to other mucosal surfaces, many of these AMPs form a constitutive barrier to foreign objects entering the oral, nosocomial and upper respiratory tract (Singh et al. 2000). These factors are secreted from surface epithelial cells and salivary glands (Dubin et al. 2004). Following infection, the concentration of these AMPs, and other non-constitutive peptides, increases as they are induced from epithelial and immune cells. To date there are no studies that have systematically examined the acute (minutes to hours) and chronic (days to weeks) changes in AMP concentration in saliva after exercise or training.

There are a diverse number of mechanisms by which exercise could alter the concentration of AMPs in the respiratory tract (Table 2). Prolonged intense exercise is associated with hyperventilation, that, during exercise, would dry the respiratory tract, potentially reducing the protective shield provided by AMPs. However, in the post exercise period, hyperventilation may increase the secretion of AMPs by inducing an inflammatory response. Airway epithelial cells lining the respiratory tract may experience mechanical trauma as large amounts of air are forcefully inspired. The greater volume of ventilation will increase exposure to environmental irritants and microbes. Epithelial cells increase their expression of AMPs following physical damage (Dorschner et al. 2001) and contact with microbes (Duits et al. 2003). The recruitment and activation of neutrophils during exercise could increase the concentration of AMPs at mucosal surfaces during and immediately after exercise, as neutrophils secrete soluble proteins, including AMPs, when activated. Inhaled particles may in-directly stimulate the expression of AMPs by inducing pro-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
drying, there would be a reduced susceptibility to infection in the period (1-3 hours)
post exercise. However, exercise-induced secretion of AMPs may result in a
refractory period, where the ability of host cells to secrete AMPs in the immediate
post exercise period is reduced. Neutrophils have a transient reduction in the ability to
undertake further activity once activated. Given neutrophils are a substantial source of
AMPS, this may have implications for the ability of the upper respiratory tract to
respond to pathogenic challenge should it occur in the post exercise period. This may
be similar for epithelial cells. The increased secretion of lysozyme from glands is
because of a prior accumulation of the protein over time (Dubin et al. 2004). This
suggests the existence of a refractory period where innate defences are suppressed
until recovery and restoration is achieved. The significance of a post-exercise
refractory period in AMP protection may be negligible after one bout of exercise but
become more significant over a training period of several weeks to months. Elite
athletes participating in sports such as rowing, swimming, cycling and running
undertake a multitude of high intensity training sessions on a weekly basis. Indeed,
chronic stress is associated with a reduced secretion of salivary lysozyme (Koh et al.
2002). Hence, it could be postulated that prolonged intense exercise may have a
negative effect on the concentration of AMPs in the upper respiratory tract.

In addition to the suppressive effects of exercise on mucosal immunity there are
other reasons justifying its use as an experimental model. Exercise can easily be
controlled and reproduced in animal and human settings. This means that the exercise
load (physical stress) applied can be prescribed relative to an individual's capability.
While prolonged intense exercise causes transient perturbations in immunity, there are
individual differences in the relative load or intensity of exercise required to achieve
such an effect. The magnitude of this between-subject variation is influenced by
physical capacity, training history and fitness. These factors need to be considered when determining the load (intensity and duration) to be applied in experimental settings. Failure to apply the relevant exercise load might confound study results.

The use of exercise as a model to study the role of AMPs forms part of the discipline of exercise immunology, which has extensively studied the effect of prolonged intense exercise on the immune system in athletic populations to determine why this sub-group of athletes appears to be at greater risk of illness. Studies examining the incidence of URTI in elite athletes engaging in prolonged intense exercise have had variable outcomes, with some studies reporting a heightened incidence (Spence et al. 2004) and others reporting no change (Pyne et al. 2001) in comparison with sedentary control groups. There is general consensus that athletes may experience higher rates of illness during critical training periods and competition. This relationship has been characterized as a theoretical J-shaped curve. The J curve relates the incidence of illness to exercise load. According to this model, individuals engaging in moderate exercise have a reduced risk of illness compared to sedentary individuals or athletes undertaking a high exercise load. Whether this model also applies to AMPs is unclear and worthy of investigation to shed insight on underlying mechanisms and clinical outcomes for the altered susceptibility to illness. A pilot investigation conducted at the Australian Institute of Sport has demonstrated that the concentration of salivary lactoferrin, one of the most abundant AMPs, decreased during a season of training in highly trained elite rowers (unpublished data).
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
in a large enough volume at a constant rate in healthy individuals. The detection of
AMPs in nasal lavage is also markedly diminished by dilution of airway secretions
(Cole et al. 1999). The collection of tear fluid may also be an effective method for
studying changes in mucosal immunity. However, changes in the concentration of tear
fluid may not accurately reflect changes occurring in respiratory tract AMPs. Given
their role in protection of the upper respiratory tract and the ease with which their
status can be collected, salivary AMPs offer promise as useful parameters in
monitoring the status of the mucosal immune system.

4. **Conclusion**

AMPs play a diverse role in the innate mucosal immune system. As a
constituent product at mucosal surfaces, AMPs participate in the barrier function that
prevents microbes from causing infection. This activity is mediated by acting directly
on microbes, which can be lethal or inhibit their growth and activity, or by preventing
them from initiating an inflammatory response. There should also be recognition that
AMPs act more broadly to participate in an immune response by recruiting cells,
inducing cytokines and aiding in tissue repair. While there is a growing body of
evidence that AMPs play a role in mucosal immunity, further research is required to
quantify their role with regard to susceptibility to infection. One mechanism by which
this can be explored is through prolonged intense exercise, which causes a transient
suppression of immunity. Individuals undertaking heavy prolonged exercise appear to
suffer an increased incidence of URTI. As yet, however, no link has been found
between exercise induced immunosuppression and increased incidence of illness.

Further prospective, well-designed and controlled studies are required to clarify the
relationships between exercise-induced perturbations in AMPs and incidence of
illness. This line of investigation should enhance our understanding of the role of 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
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.

- Destabilisation/disruption of microbe membranes (Chapple et al. 2004)
- Interference with DNA/RNA (Kanyshkova et al. 2003)
- Degradation of ATP and enzymes (Helmerhorst et al. 1999)
- Activation of autolysins (Ginsburg 2004)
References


