IMBT

INSECT ANTIMICROBIAL PEPTIDES – SCIENCE and STRATEGIES

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1) Science.

Antimicrobial peptides (AMPs) were first discovered by Hans Boman and colleagues in 1981(Steiner H 1981) in the invertebrate Cecropia moth. Earlier work by John N Spitznagel had demonstrated the presence of antimicrobial proteins associated with the granules of polymorphonuclear leucocytes (PMN) that coated bacteria during phagocytosis[Zeya hi 1965]. Human macrophage outsource their alpha-defensins by imbibing fluid-phase defensins after their secretion from PMN or by ingesting apoptotic PMNs [Lehrer ri 2 2007]. The ingestion of apoptotic PMNs by macrophages demonstrates an example of cell-cell co-operation, but further it indicates how dendritic cells can acquire neutrophil sourced, pathogen-derived Ag in the activation and regulation of the adaptive immune response[Tan B H 2006]. It is proposed that human macrophage can ingest and be portal to insect AMPs and that insect AMP inactivated pathogen in neutrophils can be transferred to dendritic cells for further processing.

AMPs are present in diverse life forms ranging from bacteria to plants,insects and animals [Boman HG 1995]. In insects AMPs are induced on pathogen recognition. In plants and higher animals they may be induced or produced constitutively. In animals they represent the first line of defense against pathogen invasion. Their significance as first line defense against bacteria as compared to antibody response is underlined by the rate at which they are produced by an organism in response to a pathogenic challenge and their subsequent rate of kill. A dependance on an antibody response which takes many days to be up-regulated would see the pathogen war lost at a very early stage [Boman 2003]. Their activity has been assessed at two levels. Firstly, as pore formers in bacterial/pathogen membranes and secondly as inhibitors of intracellular macromolecular synthesis and cell division, and effecting changes in gene regulation. The activity and function of these two types of complementarily produced AMPs may well overlap [Otvos l 2005] and further research is required to define their role on early contact with pathogens/ bacteria/cancer cells. Their multifunctional and target specific interference of bacteria occurs at low concentrations eliminating toxic concerns and the potential for evolved bacterial counter strategies [Otvos l 2005] and resistance. Their specificities may vary according to the pH of the medium, and change accordingly. As bacterial AMPs they could be viewed as members of the quorum sensing inter and intra species communication molecules [Visick KL 2005], and in advanced, evolved and complex organisms, viewed as competitively superior developments of similar quorum sensing. Recent research indicates that targeting the quorum sensing pathway is a promising therapeutic approach against Staphylococcus infections [Iwatsuki K 2006][Harraghy N 2007]. The therapeutic potential of AMPs for a range of diseases is being increasingly appreciated through an understanding of the mechanisms nature has already evolved to overcome problems of protease susceptibility, such as through D amino-acid substitution and N-terminal glycosylation or through association with albumin (TP1). The ability of natural AMPs to penetrate bacterial plasma membranes whilst being or carrying multi-functional bio-active target specific domains, validates their potential as intracellular enzyme mediators[Otvos L 2005]. Insect AMPs also inhibit the ATPase action of bacterial heat shock protein DnaK and prevents chaperone assisted protein folding[Kragol 2001]. AMPs may be viewed as having dual activity in being antimicrobial as well as recruiting different monocytes [Wu Z 2003].

Recent insights into the evolution of species based on a shared genetic code, and the significance of widespread Horizontal Gene Transfer [HGT] in bacteria and viruses, provide an essential basis for understanding the evolution of complex life, such as human, but also, how its genetic integrity has evolved to preserve it against competing external DNA insertion. The significance of antimicrobial peptides being either constitutively expressed or induced has not been addressed. That insect, and some human defense peptides are induced, such as highly active hBD3, despite adequate food/energy reserves, etc. suggests fitness benefits that are not cost limiting [Harvell CD 1990, Schmid-Hempel P 2005]. These systems may provide as yet unknown advantages.

This understanding will lead to ideas of gene insulation [Junk DNA?] and protection, and maintenance of self, platforms which may well be undermined by unnatural gene transfer and manipulation,[genetic modification] whilst also providing further insights into the complexity of immune regulation and resistance to infection.[Goldenfold N 2007,Vetsigian K 2006].

The significance and potential of Antimicrobial Peptides as new anti-infectives is now fully recognised [Pereira H A 2006, Gordon Y J 2005], incorporating invertebrate AMPs [AMP4]; See review [Hancock rew 2006] for current commercial research projects. Significantly, the signalling and effector mechanisms of innate immune system have been phylogenetically conserved in insects and mammals [Hoffmann J A 1999]. Research using genomic and proteonomic analyses has established the complexity of innate and adaptive immune responses. These include a wide range of toll like receptors TLRs and pattern-recognition receptors PRRs which recognise pathogen molecular patterns including DNA and up-regulate inflammatory cytokines such as IL-12 p70. The TLRs can act synergistically when co-stimulated but lead to tolerance when sequentially stimulated. Advanced glycation end products (AGES) are nonenzymatically glycated or oxidated proteins,lipids and nucleic acids that are formed in an environment of oxidant stress. Binding of AGEs to their receptor RAGE initiates cellular signals that activate NF-Kb, resulting in the transcription of proinflammatory factors and has been implicated in chronic diseases such as diabetes, atherosclerosis and cancer [Lin Li 2006].

Rectification of mal-functions in immune functioning has led to individual drug provision for each abnormality. The targeting of enzyme pathways however, has not had the desired effect, due partly to the complexity and multi-functional role of participants within the pathway, and has led to various detrimental side effects. This has brought about a new paradigm in thinking based on whole systems [Kitano 2007]. These systems can be viewed either at the level of the individual or as integral parts of a simple or complex symbiotic working ecosystem. This has led to the formulation of concepts of robustness, that is, resilience to various perturbations, such as mutations, toxins, pathogenic insults and environmental changes, but also the concept of fragility of a biological system which is exposed to insults to which it has not been exposed or evolved with. The integration of these components provides the foundation for a sustainable approach to disease control [Kitano2007]. This latter viewpoint encapsulates IMBT concept and strategy which it will achieve through the provision of appropriate insect AMPs that act to substitute for the production of host intended effectors against pathogen and non-self, and also act as a feedback immunomodulator.

The scientific platform on which IMBT technology is based has been independently researched in diverse areas of immune control in vertebrates, invertebrates and plants. IMBT is in a unique position to incorporate new research findings and understandings into the application of its concept of immunity generation. IMBT was ahead of its time when it was incorporated

2) **Benefits of IMBT production process and technology**

Most companies involved in the development of AMPs are following a path for size reduction, with improved stability, reduced toxicity, lowered costs, and as single drug candidates. The limitations of the singleï target-drug paradigm are now being highlighted. IMBT seeks to develop an array of full sized natural peptides, sourcing them from the immense variety of the worldôs insect fauna, and utilise the diverse functions that these peptides have, but which are only now being fully elucidated. Whereas current antibiotics are óhammers to crack a nutô, IMBT utilised insect AMPs are aimed at subverting the pathogens genetic functioning by stealth and then, through integration, facilitating the subsequent production and use of a repertoire of ónon-selfô regulator and control mechanisms, incorporating the innate and adaptive arms of the immune system.

As immune adjuvants to ensure a `clean sweep' after a pathogen infection.

Current use antibiotics may not eliminate completely an invasive pathogen, only reduce it to acceptable levels. Antimicrobial peptides working in tandem with the adaptive immune system [through chemotactic naive T cell recruitment] will ensure that the antibodies mop-up residual pathogens after the primary innate response has substantially reduced the pathogen load by targeting both pathogen exoskeleton, membrane or protein coat, as well as their intracellular enzymes, proteins RNA and DNA. It is the limited removal of a bacteria or virus subsequent to a course of current antibiotics that may cause ME through a subclinical stimulation of the immune system.
Recent research has established that current use antibiotics impairs and undermines the killing mechanisms of the hosts innate immunity, driving the evolution of resistant bacteria and causing pathogenic bacteria to persist in host tissues [Kristian S A 2007]. This impairment of hostsô immunity mechanism is due to a decrease in bacterial susceptibility to AMP killing. This in turn would lead to increased non co-ordination in adaptive immune responses, and enhance the potential for development of auto-immune diseases such as diabetes. The potential health ramifications for the transfer of such antibiotics through the food chain, [Antibiotics for farm animals] are inferred.
Inadvertent use of beta-lactam antibiotics to treat MRSA leads to enhanced toxin production. It is therefore imperative to target the protein-synthesis machinery of the pathogen through AMPs [Stevens D L 2007]. The utilisation of ultrashort antibacterial and antifungal peptides linked to lipophilic moieties that are produced by bacteria and fungi [Makovitzki A 2006], could, whilst being solely directed towards membrane permeation also lead to enhanced toxin production. Nature does not waste energy or resources, if a longer AMP is observed and found functioning, it is that size for a valid reason.

As a source of D amino acid substitution for enhanced protease resistance and Enhanced pathogen membrane binding

It has been established that D amino acid substitution in synthetic AMPs enhances their protease resistance, their ability to penetrate bacterial LPS membranes and their subsequent killing ability [Papo N 2005][LS1]. However, synthetic all D-enantiomer peptides substituted in HNPs inhibits T-cell activation [Gerber D 2005] compared to synthetic L-enantiomers. D amino-acid usage occurs in nature in the production of Bombinins from the frog Bombina variegate. These AMPs exhibit strong binding with Leishmania model membranes.

Insects have the capacity to provide multi-epimerisations with various amino acids greatly enhancing the therapeutic potential of their AMPs [Mangoni M L 2006] through targeting various aspects of the pathogen exoskeleton or membrane as well as intracellular enzyme pathways. The D-amino acids are sourced either from the proteolytic breakdown of gut bacteria cell walls [Pillai 2006] or through amino acid racemases [Yoshimura 2003]. The prescence of D-amino acids d-serine and d-aspartate in the human central nervous system in considerable quantities provide indications that insect D-amino acids may have additional therapeutic benefits[Fuchs 2005].

Non-targeter of beneficial, probiotic, gut bacteria

Many current use antibiotics are broad based and indiscriminate and target essential gut bacteria leading to loss of gut motility, IBS, restricted food digestion and associated reduced production of various bacterially produced, dietary fatty acids and other benevolent bi-products[Levy J 2000, Hoban D J 2003]. Pro-biotic bacteria are a source of butyrate, which induces the production of intestinal AMPs, to assist the host to battle infection such as shigellosis [Zasloff M 2006, Raqib R 2006]. Disrupting the natural gut fauna leads to excess growth of disease associated Clostridium difficile. The maintenance of a healthy gut bacterial fauna through the provision of probiotics has been used as therapy for inflammatory bowel disease, including antibiotic-associated diarrhea.[O`Sullivan G C 2005]. Residual antibiotics in the food chain may exacerbate these conditions.

As a Continuum/replacement of immune enhancers in maternal milk

The presence of maternally produced antibodies in mammalian motherôs milk has long been established. The time window for presentation and utilisation is limited to the young neonate after which the developed neonateôs digestive system breaks down all ingested maternal and food sourced antibodies.

The presence of maternal AMPs in the form of beta-defensin-1 has been established [Jia H P 2001]. These act to augment the neonateôs innate immune status, whilst facilitating the development of the adaptive immune response, and providing the wherewithal for a natural attenuated live vaccination. The supplementation of insect derived AMPs formulations augments the maternal colostrum supply, an evolutionary development of insect fatty-tissue? The synergetic effect of the multiple immune components of maternal milk is only now being fully appreciated [Isaacs C E 2005, Newburg D S 2005]. It is within this context that the significance and benefits to an ingestor, of an array of induced antimicrobial components from insects, should be viewed.

Patient receptivity

Many therapeutics can be disadvantagious to immuno- compromised persons, such as the use of attenuated viral vaccines. For individuals who are healthy and do not have an infection, there should be no harm in consumption. For normally healthy persons with an infection, this will be addressed without incurring unwanted side effects, such as sepsis, and for those whose immune system is lacking, a solution is provided. IMBT products can be provided to individuals at all stages of their lifespan, in health and in sickness, in readiness for when they are needed as a support to the hosts immune surveillance against and development of auto immune diseases and pathogen attack

Production of wide diversity of AMPs for broad therapeutic use.

Current research on antibacterials has been centred on producing antibiotics that are active against gram positive and gram negative bacteria. There are no novel mode of action(MOA) gram-negative antibacterials in Phase 1, nor are there any good preclinical candidates. Insect AMPs can be sourced from an immense diversity of species each with a variety of specific peptides with various subsets.

Provides a sustainable resource in healthcare.

The incorporation of IMBT technology utilises the results of evolutionary digression in molecular bacterial communication to provide a solid template for reinforcing the hostôs own immune system and in doing so ensure a sustainable solution for future health provision. IMBT technology acknowledges the complexity of evolved immune systems and their regulation and does not concur with the simplistic approach of solitary use antibiotics or on restricted targeting. It promotes viable integrated life systems to maintain human and animal DNA functional integrity

Routes to market.

As a feedstuff for farm animals
As a nutraceutical for humans
As an extracted topical agent for dermal as well as transdermal application
As a nasal agent
As an oral medicament

Marketing

The market for lifestyle choices, naturally produced food, nutritional supplements and effective medicinal preparations is growing and offers significant opportunities for investment in sustainable technologies. The potential is enormous. The upcoming market in healthcare will be in prevention as compared to current regimes of treatment. The preventative strategy applied across a broad spectrum of consumers will, provide a safeguard, having no adverse effects on those for whom it might at that time not be required, though at a later time it might. There is also a growth in demand for technologies that can safely **regenerate organic biomass**. IMBT can satisfy the demand for sustainable

solutions to these and other opportunities using environmentally friendly technologies.

The cost of production of synthetic peptides is of the order of $100-$800 per gram[an average daily dose]. Attempts to produce peptides by various recombinant DNA methods using bacteria, fungi, plant and animal systems have proven commercially unfeasible [Hancock 2006]. IMBT costs should be at least 100 times lower. The daily production rate of endogenous HNPs 1-3 is in the micromolar order of 10-15mg/kg body weight /day [LEHRER 2 2007], compared to peptide hormones which are effective at nanomolar concentrations. Supplication with exogenous insect AMPs would be expected to be required at a similar order of magnitude to host HNP production.

ADVANTAGES OVER COMPETING TECHNOLOGIES

i) Genetically Modified Plants.

The generation of new modified, insect immune based plant genes and their insertion into the plant genome ignores the importance of hundreds of millions of years of evolution which has created a diversification of species based on their DNA. Safeguards have been built in to this DNA structure to ensure species separation and to prevent horizontal gene transfer between species. The GM construct facilitates gene transfer between other plant species and also potentially to bacteria and animal species whist undermining the evolved complexity of the insects diverse immune response. The limited transfer of genes may well promote/induce bacterial resistance through an inadequate antimicrobial response. To prevent evolution of phytopathogens to insect antimicrobial peptides expressed in transgenic crops, multiple expression of different AMPs and their homologues would be required as well as appropriate proteinase inhibitors [Vilcinskas A 2005]. This will be difficult to achieve due to the increasing complexity of cellular regulations required.

New bacterial antigen based GM plant vaccines [Streatfield S J 2006] that are being proposed have the potential to elicit a biased immune response in immuno-compromised individuals. This would be exhibited as a positive adaptive response but lacking an innate response [due either to genetic predisposition or through pathogen suppression of immune regulatory mechanisms] in particular antimicrobial peptides, leading to non co-ordinated immune functioning and induction of antibodies against other plant antigens present with the vaccine. This allergic induction to food would be further exacerbated by horizontal gene transfer to other plant/species [Perron G G 2006, Chan V S 2006, Rice D W 2006, Celec P 2005 gm]

ii**) Genetically Modified Bacteria**

Microbial resistance to GM bacteria produced antimicrobial agents such as Avoparcin has been demonstrated. The modified bacterial DNA was found in animal feed from where the resistant genes are transferred to the animals commensal bacteria and subsequently to humans [Lu K 2004].

The expression of AMPs in E-coli bacteria is limited by problems of, limited size, toxicity to host cells, susceptibility to proteolytic degradation, inability to provide appropriate modified amino acids or their post translational modifications, or to ensure the correct peptide folding. Some of the separation techniques involve the use of highly toxic compounds ie. CNBr and hydroxylamine [Quande W 2005].

iii**) Therapeutic Monoclonal Antibodies**

Mammalian neonates ingest immunoglobulins from maternal colostrum and milk, the process is aided by protease inhibitors. Gut transmission is arrested after 18 days, after which, during the weaning period, the neonate may be exposed to non maternal antibodies in its food, which may or may not have beneficial attributes. Nature plays safe and destroys them in the gut with proteases, or produces an immune response if introduced into the blood. The mammalian digestive and immune system has evolved for self immune regulation and to maintain its integrity. Appropriately, it has not evolved a process for utilisation of diverse dietary IGs [Telemo E 1987].

Parenteral infliximab use in some patients leads to the production of antibodies against the therapy and is associated with fever, chills, nausea, myalgias, arthralgies and dysphagia. Infliximab use can also result in either attenuated or loss of response [Baidoo L 2005]. To achieve immune tolerance to the drug, immunomodulators are administered, further undermining hostôs capacity to deal with infections [Han P D 2004]. The cytokine storm that resulted from the trial use of the anti-CD28 monoclonal antibody TGN1412 [Suntharalingham G et al 2006] demonstrates the complexity of a self regulating, multi-faceted aspects of a biological systems immune network that reacts violently against this type of disruptive therapy.

The significance of immunogenic responses to monoclonal antibodies is addressed by the American FDA under Development and regulation of monoclonal products: challenges and opportunities [Weinberg W C 2005]. The immunogenic response is in part a result of inappropriate or non posttranslational glycosylation of peptides and proteins which differs in diverse cell systems [Brooks S 2006].

The serum levels of IgG are maintained by a cellular homeostasis mechanism involving the óneonatalô Fc receptor (FcRn).[Ward E S 2003]. Attempts have been made to modulate the interaction and effectiveness of this receptor to increase monoclonal antibody recycling and reduce clearance. It is however, proving very difficult to define a common set of parameters for the optimisation of the pharmokinetic properties of a range of monoclonal antibodies to achieve this goal [Data-Mannan A 2007]. Increasing the binding of monoclonal antibodies to the FcRn receptor increases the rate of clearance in primates and not decrease as anticipated. Additional factors other than FcRn-mediated antibody recycling are involved, and will have to be considered when advancing monoclonal antibody recycling as an enhanced therapy.

The HAMLET alpha-lactalbumin oleic acid complex found in mammalian milk is kinetically unstable, and does not lend itself to manipulation or synthesis; it represents an appropriate in vivo evolved system designed to protect lymphoid cells in the gut associated lymphoid tissue from viral transformation or premalignancy, ensuring production of the lymphocyte repertoire of the adaptive immune system in the neonate gut [Fast J 2004]. This prepares the neonate for weaning when safe maternal antibodies are not available and those that may be encountered in the diet may well not be safe or cause disruption to cellular momeostasis and must be digested through appropriate proteolytic pathways that develop during the maturation of the gut. This demonstrates the evolved working of a developed human gut and its resilience to challenge or insult of non-native complex proteins. Maternal milk based innate antimicrobial peptides will also not be available after weaning though dietary insect AMPs provide an exogenous utilisable alternative.

iv**) Synthetic AMPs**

The development of synthetic antimicrobial peptides has been hampered by their loss of function that occurs in sera, they may also be ion sensitive i.e. to Ca, Mg, Na or sera components. Synthetic AMPs production is limited to small non-complex peptides and cannot provide the long chain peptides with appropriate secondary modifications and tertiary configurations, necessary for their immuno-modulatory function.

It is important for AMPs to be immuno-modulatory at low concentrations and to synergise with the hostôs immuno-regulatory mechanisms both at the innate level and adaptive level [Phadke S M 2005]. Current AMP product portfolios do not achieve this.

v)**Fungal Peptides**

Various fungal peptides that have insecticidal as well as anti-viral properties are being investigated for their toxicity to cancer cells. Destruxins from the entomopathogen Metarhizium anisopliae have been shown to down- regulate the production of antimicrobial peptides when injected into Drosophila, suppressing the humoral immune response and subsequently exposing the insect to bacterial infection and death to previously innocuous bacteria. The adverse health implications for human use are inferred [FP1]. This research demonstrates an example of a peptide from a distant phyla having multi-functions, the overall effect being the suppression of the immune system and the exposure of a recipient to pathogenic infection. It is imperative that an AMP that is used therapeutically and down regulates the production of self AMPs through feedback mechanisms should be capable of replicating with similar specificity the activity profile of the AMP it is substituting for.

vi) **Chemo-therapeutic drug use in cancer.**

The majority of drugs used in anticancer chemotherapies have the function of inhibiting nucleic acid synthesis. However, at the concentrations required to control tumour growth, this frequently also affects healthy cells causing undesirable side effects [Dennison S R 2006]. Furthermore, chemotherapy for cancer has been thwarted by the development, in cancer cells of resistant immunity to the drugs being used. This resistance is accompanied by a simultaneous resistance to related or co-present bioactives molecules, and is known as multidrug resistance (MDR). One of the most important mechanisms for this resistance is that due to transporter proteins such as MDR1/P-glycoprotein. Current efforts are being investigated to suppress the expression, or inhibit the function of this protein [Takara 2006]. MDR1/P-glycoprotein and other transporter proteins are essential requirements for toxin removal from cells, for maintaining appropriate blood / tissue barriers such as blood/brain and blood /cerebral spinal fluid and in tissue defense [Chang 2007], targeting these proteins may have serious adverse effects. The self clearance of tumours is promoted by p53, a tumour suppressor that restricts proliferation in response to DNA damage or deregulation of mitogenic oncogenes. P53 acts primarily in the up-regulation of inflammatory cytokines, triggering an innate immune response that targets the tumour cells in vivo [Xue 2007]. Efforts to reactivate p53 activity by means of pharmacological drugs are being explored.[Ventura A 2007]. P53 is up-regulated by human neutrophil peptide (HNP) in epithelial A549 carcinoma line cells [Vaschetto R 2007]. HNP increases the expression of CD28 and CD152 in CD4+ lymphocytes, which are the receptors for the costimulatory ligands CD80 and

CD86 on lung epithelial cells. CD80 and CD86 expression is up-regulated in the carcinoma cell line, leading to the targeting of the cancer cell by lymphocytes. Thus a linkage is established between the innate and adaptive arms of the immune system in tumour suppression. MUC1 is a heterodimeric protein that is aberrantly overexpressed in human breast cancers and induces transformation. It interacts with p53 and regulates p53 transcription [Wei X 2007]. It is responsible for the downregulation of innate immunity and thus hindering the co-ordinated production of an effective MUC1-specific Th response [Turner M S 2007] through an expansion of regulatory T-cells. The addition of exogenous AMPs should address p53 restricted transcription allowing full innate inflammatory response and down regulation of regulatory T-cells.

Low dose use of interferon in melanoma results in the auto-immune induction of antinuclear antibodies.

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3**) Therapeutic Potential**

a) **As oral antimicrobial agents**

Modern day Antibiotics stem from the development of tyrothricin in 1939; in the interceding years, penicillin, tetracyclines, macrolides, aminoglycosides, cephalosporins and other new antibiotics have been discovered and commercialised. However, their use has led to the creation of multi resistance in bacteria, and the protective arsenal of antibiotic therapy is rapidly waning. The research effort into new antibiotic therapies by large pharmaceutical companies has been sidelined in favour of more profitable lines in chronic disease control. For a sustainable solution to providing enhanced resistance against pathogens a new or revised paradigm in thinking is required, one that incorporates evolved systems and systems biology - ***Immunity Generation.***

i) For use against bacteria and in providing protection against toxic shock.

The phylogenetically conserved innate immune effectors that are insect antimicrobial peptides, which can target human and animal pathogens, provide an ideal, diverse and sustainable source of supplementary health defense peptides.

Lipopolysaccharide (LPS) or endotoxin is a major component of the outer membranes of gram negative bacteria. It activates monocytes and macrophages to produce TNF-alpha, interleukin (IL)-1 and IL-6, mediators of inflammation. Excessive release of LPS through antibiotic use such as beta-lactam leads to the pathophysiology of septic shock. In addition, beta-lactam usage induces and enhances alpha-toxin, toxin1 and the leukotoxin Panton-Valentine leukocidin production in staphylococcus infections contributing to worse outcomes [Stevens D L 2007]. Human Cathelicidin Antibacterial Peptides [CAP] inhibit TNF-alpha expression by binding to LPS , suppressing its binding to LPS binding protein and subsequent transport to CD14+ cells [Nagaoka I 2001]. AMPs derived from the beetle Allomyrina dichotoma defensin suppress TNF-alpha production and provide protection against LPS- induced inflammation [Motobu M 2003, Koyama Y 2006]. Insect AMPs target the quorum sensing pathway of staphylococcus and are prime candidates for therapeutic targeting and control of pathogenic bacteria.

ii)**Virus**

The human antimicrobial peptides HNP1-3 inactivate herpes simplex, cytomegalovirus, influenza A and vesicular stomatitis virus. They are active against adenovirus as well as enveloped viruses [Lehrer 2007]. Antimicrobial peptides are induced in Drosophila in response to viral challenge, both the Toll and IMD pathways being activated, expression of any individual AMPs does not confer viral resistance [Zambon R A 2005], as expected. Each AMP contributes to the overall immune response along with accessory effectors. The specific activity of different AMPs in anti-viral activity has been demonstrated with human alpha-defensins (HNP1-4,HD5 andHD6) and beta-defensins (hBD), hBD1 is produced constitutively hBD2 and hBD3 are induced. Their activity against bacterial toxins [see below] is replicated with activity against virally encoded peptides and proteins. Alpha-defensins bind the viral protein gB2 of Herpes simplex /or itôs receptor,heparin sulphate, and prevent cell binding. The hBD3 binds both gB2 and its receptor giving it strong protective activity. They can also inhibit viral penetration, should binding occur. They also inhibit viral DNA gene expression and replication [Hazrati E 2006]. The protection afforded by defensins to cells that do not produce them is assisted by some means of receptor-mediated uptake or endocytosis. Provision of exogenous AMPs in addressing the viral B2 function of suppression of host RNAi [Marques J T et al 2007] may then also assist in preventing increased susceptibility to bacterial infections as a consequence of viral infections [Navarini A A et al 2006]. This strategy would overcome the viral mechanism of inhibitor kappaB-like protein production that inhibits NF-kappaB activation and suppression of the hosts innate immune system including AMP synthesis [Thoetkiattikul H et al 2005].

iii) **Bacterial and Viral Toxins**

HNP 1 neutralizes Bacillus anthrax lethal toxin[LT], Diphtheria toxin and Pseudomonas exotoxin.[Kim C 2006]. Though viewed as toxins per se, these bacterial products should be viewed as immune suppressors, whose function it is to subvert both innate and adaptive arms of the immune system [Rossi Paccani S 2007]. LT inhibits the migration of AMP containing PMNs as well as other immune cells, through inhibition of actin based motility as a result of mitogen-activated protein kinase kinases (MAPKK) targeting. Addressing the production of the toxins through targeting the encoding bacterial DNA or their neutralization by (insect) antimicrobial peptides will facilitate adaptive and innate responses and subsequent elimination of the pathogen. Targeting bacterial/viral toxins that are RNAi suppressors, by antimicrobial peptides, will enhance the effectiveness of RNAi [Wang X H 2006] machinery in viral resistance.

iv) **HIV1- AIDS**

Current use antiretroviral therapies are represented by a range of nucleoside reverse transcriptase inhibitors such as zidovudine, lamivudine, tenovir and emtricitabane. Dual treatment regimes reduce RNA plasma levels to acceptable levels in 73 to 84% of patients. There are concerns over resistance development, cumulative renal dysfunction, diabetes, lower CD4+ cell count and mitochondrial damage.
Triple use nRTIôs leads to higher virologic failure rates due to increased resistance. The significance of rapid, profound suppression of viral replication must be emphasised for viral clearance and resistance avoidance [Hammer S M 2006].
The HIV-1 nef gene encodes a 27-kDa protein that plays a crucial role in AIDS pathogenesis. It has been established that it regulates signalling molecules in the JNK

and NF-kB signalling pathways in Drosophila. Melanization and the transcriptional expression of the antimicrobial peptide diptericin are inhibited after bacterial infection by nef expression [Lee S B 2005].
Synthetic alteration to human AMPs to improve activity against a fungal pathogen has the unwanted and unpredictable effect of enhancing HIV1 replication [aids3].
The insect antimicrobial peptides melittin and cecropin have been shown to inhibit replication of human immunodeficiency virus1 by suppressing viral gene expression [Wachinger M 1998] Human defensins also have anti-HIV activity.

I propose that a strategy that seeks to counter the human AIDS virus undermining host innate immune response, by providing natural supplementary exogenous AMPs, which at the same time have the potential to synergise with the adaptive immune system, **is** the most effective and most natural way of ensuring viral suppression whilst preventing viral resistance. Exogenous insect AMPs are not expected to have any deleterious effects on patients. Viral clearance using insect AMPs is expected to be significantly higher than the 84% achieved with nRTIs.

vi**) Avian Flu**
Current strategies to counter the threat posed by avian flu virus include the production of vaccines. There are, however significant problems associated with this strategy. The virus is capable of existing within various envelope proteins, haemagglutinin(HA), neuraminidase(NA), and an ion channel protein(matrix protein 2M2) that may vary in their amino acid composition through genetic and antigenic variation. This necessitates changes in antibody induction for each variation. It is difficult to devise vaccines that target all variable antigen combinations. There are problems in eliciting immunogenic responses from inactivated H5 virus, though an enhanced response is achieved using adjuvants. Split-virion vaccines are not optimally immunogenic and require multiple doses [Subbarao K 2007]. Live attenuated virus vaccines are potentially dangerous to immuno compromised persons. Research demonstrating safe use of attenuated influenza vaccine in immuno-compromised ferrets [Huber V C 2006] is flawed since the two immuno supressors used, namely dexamethasone, enhances human beta-defensin-2 transcription and antimicrobial capacity [Witthoft T 2005] in humans and would be expected to achieve the same in ferrets. This is the opposite effect to what is required to demonstrate innate immuno-compromisation. Also, cytarabine has been shown to synergise with the insect antimicrobial peptide cecropin [Hui L 2002] which does not indicate a suppressive role for cytarabine in innate immune function. The antiviral drugs Tamiflu and zanamivir are neuraminidase inhibitors that are being stockpiled in the event of pandemic flu-outbreak The potential for resistance through single amino-acid substitution has been established [de Jong MD 2005]. There are potential risks associated with widespread use of Tamiflu due to its apparent resistance to biodegradation. It is predicted that the accumulation of oseltamivir carboxylate[OC], the active antiviral metabolite of Tamiflu, in waterways linked to sewage treatment works, poses a potentially significant ecotoxicologic risk. The concentration of OC in wildfowl could be sufficient to drive the generation of Tamiflu-resistant mutants [Singer AC 2007]. There is a growing appreciation that the most efficient way to target viral infection is to recruit cell-mediated immunity with its antiviral activity[Subbaro L 2007] including antimicrobial peptides that can both target variations in protein antigens for correct antibody response whilst also targeting the viral replication machinery. Providing dietary AMPs in chickens could also alleviate

the problem at source and is a preferred strategy against specific vaccines in livestock that may be drivers of evolutionary resistance and pathogenicity of this and other virus.

vi**) Pseudomonas aeroginosa (PA) in Cystic Fibrosis**

Cystic Fibrosis is a lung disease that becomes subject to a defect in mucociliary clearance and a cycle of infection and inflammation that has been interpreted as due to, amongst others, the non-functioning of the cystic fibrosis transmembrane conductance regulator [Machen T E 2006], and the formation of antimicrobial hypothiocyanate [Moskwa P 2007]. The underlying cause of CF is an inability to address a pathogen challenge, that is Pseudomonas aeroginosa in 90% of all cases. This may be due to either an underlying compromised innate immune system or its being subject to a PA/pathogen invoked suppressive mechanism of AMP expression [Apidianakis Y 2005]. Current therapies are targeted at reducing inflammation, reducing obstruction or reducing infection. Antimicrobial peptide therapy has been advanced for CF control [Zhang L 2005, Mason A J 2006]. Overexpression of beta-defensin-2 in rats provided protection against Pseudomonas aeroginosa [Shu Q 2006].

vii)**Parasites**

The observation that the antimicrobial peptide stomoxyn in the gut of the blood sucking insect Stomoxys calcitrans was associated with the insect being a non-cyclical vector of trypanosomes [Boulanger N 2002] and the significance of the killing of trypanosomes by antimicrobial peptides as reviewed [Mcgwire B S 2003] indicates a strategy for parasitic control. It might not be necessary to eliminate all malarial carrying mosquitos, i.e. through GM or sterilisation technologies, but simply to provide a defense against them. The innate response production of AMPs in potential but non-vectors indicates either an effective pathway for their production or that the AMPs produced are effective against the parasite. These insect AMPs can be harnessed to provide exogenous first line defense in humans, but where then, additionally, they recruit adaptive immune machinery for the provision of antibodies. Alternatively, AMPs in correct linkage with the parasites can provide vaccine strategies. In nature, such a mechanism may be the ingestion of non-vectorial ectoparasites of primate endoparasites such as malaria as demonstrated in primate grooming. Specifific ectoparasites are non-vectors through their innate immune responses covering AMP and lipid production. The ectoparasites would produce an effective antigen complex that is receptive to the consumer for appropriate adaptive processing and antibody production. Presentation of the complex is then a function of its presence in the community.

b) **As Topical Antimicrobial Agents**

i) against acne vulgaris

Cationic antimicrobial peptides are active against Propionibacterium acnes and are also anti-inflammatory through their binding of proinflammatory bacterial factors such as lipoteichoic acid and inhibiting secretion of proinflammatory cytokines such as TNF-alpha and IL1. [Marta Guarna M 2006].

c) **As Topical Antimicrobial Agents and in multi-functional cell recruitment in wound repair**

Besides their microbicidal functions, antimicrobial peptides are known to recruit macrophages to sites of cutaneous injury. hBD-2,-3 and-4 but not hBD-1 stimulate human keratinocytes to increase gene expression and protein production of IL-6, IL-10, IP-10, monocyte chemoattractant protein-1, macrophage inflammatory protein-3alpha, and rantes. Hbd increase keratinocyte migration and proliferation. In addition these peptides induce phosphorylation of EGFR, signal transducer and activator of transcription (STAT)1 and STAT3 which are intracellular molecules involved in keratinocyte migration and proliferation [Niyosaba F 2007, Shauber J 2007]. Wound repair is achieved successfully in most individuals, but in those that suffer from diabetes, wounds may become chronic. I propose that this is due to the same underlying limitations of expression of innate immune surveillance that are for example the gene polymorphisms DQ8 and DQ2 [Sanjeevi C B 2006]. The topical provision of insect AMPs can address these problems. I believe that this is demonstrated by the óinduced production of AMPsô by MRSA in in situ maggot therapy in diabetic patients. These óAMPsô are active against Pseudomonas aeroginosa, Klebsiella pneumoniae and MRSA [Huberman L 2007]. In these patients their compromised immune system is evident in their auto-immunity and poor immune response to cutaneous wound pathogenic bacteria.

Additionally, skin that has been damaged by burns that have caused disruption to host defense peptide production, can be assisted in its recovery by the provision of topical insect AMPs through their prevention of infection and assisted repair cell recruitment.

d**) As vaccine adjuvant.**

Preferred vaccine candidates should provide an innocuous molecular template for the production of an appropriate adaptive immune response and subsequent therapeutic control of the pathogen. Strategies that involve the delivery of attenuated virus as a vaccine to immune compromised individuals, run the risks that even the attenuated form is not held in check, and becomes profligate. Killing of bacteria or virus by heat treatment or chemical methods to prevent replication often disrupts the antigen template.

The provision of an antimicrobial peptide killed pathogen achieves 3 goals. Firstly, the pathogen is dead and cannot reproduce and is thus safe. Secondly, the membrane template is not disrupted and appropriate antigens can be viewed and correctly responded to by the adaptive system. Thirdly, the chemo-attraction between AMPs and immature dendritic cells and memory T-cells promotes and enhances antibody production [Yang D 1999].

The transfer of mucal encased, host peptide inactivated lung pathogens, and tonsil released antigen complexes to the gut for secondary processing provides an evolutionary indication of the preferred route for administration of vaccine candidates, i.e orally. A direct pathway for immediate excretion of these products has not evolved.

The evolutionary emergence of the adaptive immune system is generally linked to that of the jawed vertebrates, during a period when variable (V) type Ig superfamily genes were invaded by transposable elements that contain RAG1 and RAG2 genes [Agrawal et al 1998]. It has been argued that the acquisition of a jaw imposed selective pressure for the emergence of the adaptive immune system since the ability to swallow foods increased the incidence of injury to the digestive organ wall from

sharp bones [Matsunaga 1998]. It has also been proposed that the adaptive immune system evolved due to greater exposure to pathogens [Kitano 2005]. I argue that the latter is of greater significance but through mutual co- evolution with food sourced, ingested AMP inactivated pathogens.
The difficulty in providing adequate vaccines for persistant infections [Zinkernagel R 2006 (b)] is a reflection on the pathogens ability to prevent an effective combined innate and adaptive response through downregulation of host response or partial invisibility to surveillance. Utilise the immune machinery of non-vectors and supplementary sources of insect AMPs! The use of AMP inactivated pathogens provides an alternative to repetitive use of attenuated vaccines in early childhood, whilst the protective effects of maternal antibodies wane.

e) **As curative agents in auto-immune diseases**
Most research on addressing the causes of auto-immune diseases centres on the control mechanisms related to internal immune regulation in both the adaptive and innate immune systems [aa1, aa2, aa3,]. Most have approached the problem by down-regulating the immune system. In nature, such a strategy is exemplified by the down-regulation of beeôs immune system by ectoparasitic varroa mites [Yang X 2005]. This leads to bacterial and viral infection and death in the bee. This demonstrates the potential pitfalls of immune suppression when applied to humans. The understanding that auto-immune diseases are the óconsequences of infection-triggered immunopathologies causing and maintaining inflammatory processes at low levels for a long period of timeô, is now being promoted [Zinkernagel R 2006 (A)] and that they can be addressed by appropriate anti-bacterials, antivirals and anti-parasitic treatments. These treatments should at the same time not interfere with the natural promotion of self vaccination with antibody production.
A developing understanding of gene regulation, innate and adaptive immunity provides new insights for a therapeutic approach based on immune effector i.e. AMP supplementation, that can address chronic inflammation by facilitating a comprehensive innate/adaptive effective response. Research on the evolution of the immune system in response to its environment will assist this development. A successfully evolved species incorporates the appropriate DNA machinery for self preservation and reproduction, whilst embracing opportunities provided by its environment to counteract subversive pathogen strategies. I believe that humans have evolved an immune system that is self regulating, that does not import by ingestion óregulatory mechanismsô or molecules i.e. Interleukins or Antibodies, as then host immune control could be lost or usurped. However, higher animals ingest and can accept and incorporate effector antibiotic-type molecules such as AMPs to supplement their regulatory network. I believe that humans have not suddenly evolved a disease scenario of auto-immunity but have had this imposed on them through a diet lacking AMPs [Vitamins?]

i) **Diabetes**
Diabetes is an auto-immune disease in which the Isle of Langerhans cells of the pancreas are mistakenly targeted by the adaptive immune system, as a subsequence, insulin production is curtailed, and glucose metabolism is disrupted. Insulin itself may also be a mistaken target. Current therapy has been the exogenous supply of injected synthetic insulin, though this is associated with the major constraint of multiple daily parenteral administration. It had previously been thought that insulin and glucose dis-regulation in diabetes was the cause of

associated degenerative complications including atherosclerosis and chronic wound ulcers. It is now being increasingly realised that a common underlying malfunction in innate immune regulation is the culprit. Therapies that are under investigation include Beta-cell transplant, though this suffers from an inflammatory response [Montolio M 2007] from the host and the subsequent requirement for immune suppressors, Beta-cell mass is not maintained and there is difficulty in obtaining adequate supplies of beta-cells for transplant [Pileggi A 2007]. Anti-CD3 antibodies have shown a brief period of respite for non-producing Beta cells but this has been short lived, and has not led to Islet cell regeneration [Phillips J M 2007]. Stem cell production is limited by the inability to replicate by cultured, expanded or otherwise manufactured cells with the same precision and compexity that native Beta cells exhibit in producing insulin in response to glucose levels, a mechanism that is not yet fully understood. Porcine xenografts need so much immuno-suppression that the benefits are outweighed by the risks [Ren J 2007]. The observation that amyloid polypeptide oligomers are present in type2 diabetes cells and that this leads to membrane disruption and disruption of islet architecture and a decrease in coordinate function [Ritzel R A 2007] demonstrates similar pathology to Alzheimers Disease and its associated pathogen induced basis [see Alzheimer section below].

ii) Rheumatoid arthritis. RA

Recent additions in the arsenal of therapeutic agents for RA are a range of monoclonal antibodies to TNF-alpha. However, there have been increasing reports on the reactivation of dormant diseases such as TB and cancer. TNF antagonists decrease the frequency of the subpopulation of CD4+ T lymphocytes releasing IFgamma upon challenge with mycobacterial antigens. They also inhibit the activation of CD4+T lymphocytes by mycobacterial antigens [Hamdi H 2006][Bongartz T 2006]. Derivatives of beetle defensin inhibit tumour necrosis factor-alpha production [Koyama Y 2006].

iii) **Atherosclerosis and vascular diseases.**

Recent research findings have indicated the role that infectious agents play in the development and progression of atherosclerosis [Sawayama Y 2006] [Gredmark S 2007]. The limited benefits of cholesterol lowering drugs are frequently paralleled with an ongoing deterioration of cell metabolism. Cholesterol is generated as part of the inflammatory response to infectious agents. The anticrobial peptide CAP37 is induced in atherosclerotic lesions through the alarm signals of either lipopolysaccharide LPS or tumour necrosing factor-alpha. CAP37 is an important regulator of endothelial cells, smooth muscle cells and monocyte functions, these are central to the development of atherosclerosis. CAP37 expressed higher levels of the intercellular cell adhesion molecule-1(ICAM-1) and induced monocyte migration and adhesion to endothelium [Pereira H A2006]. It cannot alone address a pathogen challenge, and requires supplementary AMPs. AMPs use against the infection and inflammatory agent will address the underlying cause of atherosclerosis by eliminating the LPS and TNF-alpha alarm signals.

iv) **Alzheimers, Parkinsons and Neurodegenerative diseases**

Gene expression of the inflammatory major histocompatibility complex (MHC), paralleled by a decrease in T cell number, indicate immune dysfunction [Parachikova A 2006]. The association of beta-amyloid formed senile plaques with the inflammatory cytokine interleukin-1beta and the activated nuclear factor kappaB (NF-kappaB) [Samuelsson M 2005, Zhao Y 2006] indicate a response to pathogenic stimuli. This response may be initiated by the activation of toll-like receptor2 (TLR2) on the microglia. TLR2 when stimulated by peptidoglycan from staphylococcus aureus induces considerable formyl peptide receptor-like 1(FPRL1)production, which in turn enhances uptake of beta-amyloid by the microglia cells[Chen K2006]. The antimicrobial peptide CAP37 is induced in endothelium of Alzheimerôs brain microvessels in response to LPS or TNF-alpha and its recruitment of microglia and up-regulation of the chemokine regulated upon activation normal T-cell expessed and secreted (RANTES) contribute to the inflammatory response that is Alzheimers [Periera H A 2006]. The aim should be to address the underlying cause of plaque formation, namely pathogens, by the provision of appropriate defense AMPs at an early preventative stage. Vaccination against the immunogen beta-amyloid has resulted in meningoencephalitis and may lead to associated microhemorrhages [Asuni A A 2006].

v) **Multiple sclerosis**

The onset of multiple sclerosis is associated with infectious mononucleosis caused by the Epstein-barr virus [Nielson T R 2007]. Attempts to regulate cytokine levels and inflammation with interferon beta [IFN-beta] have had limited success. Combining this therapy with alpha4 antagonists such as Natalizumab has improved the relative risk of sustained disability progression but has had the drawbacks of pharyngitis, sinus congestion, peripheral edema and multi-focal leukocephalopathy [Ruddick R A 2006]. The preferred candidate(s) for treatment of MS needs to combine both anti-inflammatory properties in addition to neuroprotective properties [Sorensen ps 2007]. The modulation of immune genes by the antimicrobial peptide lactoferrin,(whose activity is restricted to gut lining epithelia and undergoes proteolytic digestion on absorption into the blood), demonstrates through its up-regulation of IFN-beta [anti-inflammatory activity through IL-10 production P, and IL-12p40 [Wakabayashi H 2006] and its inherent antimicrobial capacity, that an (insect) AMP that is transferred across the gut into the blood and thus available to all tissues throughout the body would fulfil the requirements for effective control of MS.

x) **Allergy,Asthma and Atopic dermatitis**

These are auto-immune diseases of the epithelia. In Atopic dermatitis it is associated with a deficiency of dermcidin-derived antimicrobial peptides in sweat [Rieg S 2005] or deficiency of HBD3 [Howell M D 2006]. Little research has been published on the levels of AMPs in the lung epithelia of asthma suffers, but early childhood susceptibility to viral infection indicates a compromised innate immune system and underperformance in AMP production. The demonstration of the presence of Staphylococcus aureus in lung epithelia, with its ability to release superantigens into the tissue provides evidence for an immune defect in innate immunity, and a rationale for the damaging effects of an inflammatory response [Bachert C 2007], that is not fully addressed. The invasiveness of certain S aureus strains in the nasal environment may be attributable to their ability to suppress two principal antimicrobial peptides, human beta-defensin-2 and human beta-defensin-3 [Quinn G A 2007].

xi) **Psychiatric disorders**

Growing evidence suggests that psychiatric disorders such as schizophrenia and autism are caused by autoantibodies [Margutti P 2006].

f) **Anticancer Agents**

Recent research has established the significance of the down-regulation of innate immune surveillance [TLR9 expression] by the Cervical Cancer ï Associated Papillomavirus Type 16, in the non- clearance of the virus and its associated cell mal-functioning and tumour development [Hasan U A et al 2007]. [Braakhuis B J 2004]. Antimicrobial peptides are induced on viral infection in Drosophila,[Zambon R A et al 2005], their role in viral suppression is still being researched. AMPs may be associated with the control of virally expressed suppressor proteins. These proteins bind to long double-stranded RNA(dsRNA) and inhibit Dicer-2 mediated processing of dsRNA into short interfering RNA(siRNA).[van Rij R P 2006].

Insect AMPs such as synthetic cecropins have been shown to induce transient ion channel pores in cancer cells [Ye J S 2004] and lyse leukaemia cells [Hui L 2002] and have been proposed as novel cytotoxic agents for cancer treatment [Mader I S 2006, Dennison S R 2006]. Many drugs have been developed for the control of cancer cells that are produced as a result of aberrant cell functioning and that have not lead to appropriate self apoptosis. However, the development of Multi Drug Resistance (MDR) through membrane bound efflux pumps, such as P-glycoprotein, is a major problem in cancer therapy. These efflux pumps provide a transport defense mechanism against diverse toxicants including the perceived toxins that are pharmaceutical drugs. AMPs do not appear to be subject to the same removal mechanism thus facilitating their anti-cancer activity. Insect AMPs can be used in a preventative pre-tumour status to eradicate the cancer at the malignant cell level. This approach eliminates the need to address tumour blood supply control, as in angiogenesis inhibitors, where resistance to the drugs is occurring, or with anti-angiogenic gene therapy, where efficacy is in early treatment or use of radiotherapy. It is becoming clear that the next step to anti-angiogenesis therapy is to control its initiation [Harper J 2006] but better still nip it in the bud of single cell aberration.

g) **As replacement Defensin therapy in Crohn's disease.**

A deficiency in the production of HD-5 and HD-6 linked to a mutation in the NOD2 cytoplasmic peptidoglycan sensor is associated with ileal Crohnôs disease. Colon Crohns disease is characterised by an impaired induction of beta defensins in enterocytes [Sambhara S 2007]. Future therapeutic strategies aimed at restoring the host-microbe balance at the intestinal mucosa may prove superior to those that broadly suppress inflammation and adaptive immunity [Wehkamp J 2006] which can be achieved through exogenous supply of defensins [Sambhara J 2007].

g**)Immunomodulators**

The approach to assisting host immune system regulation, in the face of virulent attack has been either, to supplement with inflammatory mediators in instances of an underpowered immune system or, with anti-inflammatory mediators in an overactive immune system. I maintain that, expressions of an overactive immune system represents a non-addressed, un-satisfied response to an immune challenge, from any of the diverse parts of the hostôs immune surveillance systems. Down-regulation is achieved by supplementing with insect AMPs, which parallels the anti-inflammatory

effects of macrolide antibiotics [Amsden G W2005]. AMPs that are not antigen linked may link with a signalling anti-inflammatory ligand cascade in a feedback control loop. IMBTôs approach is unique in that inflammatory and anti-inflammatory mediation is achieved with the same therapeutic AMP regime: Its direction being determined by the immune status of the recipient subject to the pathogen challenge. Its anti-inflammatory action does not lead to an immuno-compromised status as with TNF-alpha antibodies.