Instituto Juan March
de Estudios e Investigaciones

91 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Eukaryotic Antibiotic Peptides

Organized by

J. A. Hoffmann, F. García-Olmedo and L. Rivas

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Introduction

J. A. Hoffmann, F. García-Olmedo and L. Rivas

Until few years ago, antibiotic peptides in eukaryotes were considered as an evolutionary relic, especially when compared with the powerful and complex immune response present in higher mammals. In 1972 the first experimental evidence of plant peptides (thionins) active against plant pathogens was published, but it was not until the end of the 70's that this field started an exponential growth on a broad variety of different sceneries. Nowadays, several antibiotic peptides and their analogues are in advanced clinical trials, and some human pathological disorders have been related to their absence or lack of function.

Eukaryotic antibiotic peptides contribute both to the control of local bacterial flora and as a first-line barrier against pathogen invasion, as demonstrated in insect mutants deficient in some antibiotic peptides or in transgenic plants overexpressing particular antibiotic peptides. In higher vertebrates, their importance in health is less known; as aforementioned, the strength of antigen-specific immunity, as well as the simultaneous presence of other components of innate immunity, hamper the definition of the roles of specific peptides, and although some transgenic mice expressing defensins have been recently developed, overexpression and knock-out experiments with mice are lagging behind those with plants. The protective role of antibiotic peptides in human health is emphasized by the recently found correlation between recurrent bacterial lung infections in cystic fibrosis and lack of activity of local antibacterial peptides because of the high salinity in the alveolar fluid, due to the genetic default in chloride transport.

Besides constitutive expression of some antibiotic peptides in mammals, infectious and inflammatory processes can modulate their expression, mediated by LPS, IL-1, IL-2 , or TNF-α. Furthermore, an evolutionary convergence in some of their signal transduction pathways, involving members of the Rel/NF-κB family occurs among plants, insects, amphibia and mammals.

To date, more than 400 sequences of both natural and derived antibiotic peptides have been described; despite large differences in size and amino acid sequences, most of the antibiotic peptides share a strong cationic character and adopt an amphipatic structure in their interaction with model or biological membranes. Their mechanism of action is

often based on permeabilization of the plasma membrane of the pathogen with dissipation of the ionic gradients across the membrane with subsequent bioenergetic collapse of the organism; the permeabilization is brought about by disruption of phospholipid bilayer rather than by interaction with chiral receptors or channels; hence discrimination between self and non-self is mostly based on membrane lipid composition, as prokaryotes are devoid of sterols and possess a higher percentage of anionic phospholipids when compared with eukaryotes. This mechanism is double-edged; the design of peptides with higher membrane affinity risks a substantial loss of specificity and production of self-damage; by contrast and in support of a pharmaceutical application, resistance against membrane-active antibiotic peptides prompted by continuous culture at sub-lethal concentrations has not been reported yet, even when some pathogens possess a natural resistance to some of these peptides. To reach the inner membrane of Gram negative bacteria, antibiotic peptides need first to disrupt the outer membrane by interaction with lipopolysaccharide. In most cases, this leads to inhibition of the events triggered by LPS, such as endotoxic shock, hence the peptides can also work as antiendotoxemic agents. In many cases, chemical synthesis of these peptides or their analogues is easily afforded, giving access to studies of structure-activity relationships, aiming at the design of better and more active analogues.

In summary, the workshop has succeeded in updating our knowledge on this fascinating field, where many different biological disciplines, as well as organisms from very diverging origins, converge to provide answers and to create great expectations for future pure and applied research.

J. A. Hoffmann, F. García-Olmedo and L. Rivas

Session 1

Chair: Francisco García-Olmedo

Peptide Antibiotics - New Rendez-vous with Old Animalcules

Anita Boman and Hans G. Boman

Microbiology and Tumor Biology Center. Karolinska Institutet, Box 280 SE-171 77 Stockholm

In this talk I will show results from two projects, one initiated for intellectual reasons, one started by serendipity then pursued as normal. The year 1996 became a turning point in my research. Together with Viktor Mutt and his coworkers, our group at Stockholm University during 1987-95 isolated several new peptide antibiotics from pig intestine. This fruitful collaboration gave the porcine cecropin P1, then PR-39 and towards the end and in parallel the cloning of FALL-39 and NK-lysin, both the peptide and the clone. While apoptosis ended this line of work, I survived myself by a move to a program for biomedical ecology initiated at
MTC.

Happily, the beginning of 1996 was spent in Rome and a productive collaboration was established with Donatella Barra and Maurizio Simmaco with focus on the in vivo function of peptide antibiotics. Soon we realized the potential of frogs as model system for the study of natural infections and Maria Luisa Mangoni became an import link between Rome and Stockholm.

The very first step was the isolation of bacteria from the skin and mouth of two frogs living together, Rana temporaria and Bombina orientalis. By now we have isolated and identified 6-7 rather closely related gram negative bacteria from six different species of frogs. From five different isolates we have made mutants resistant to streptomycin, nalidixic acid or rifampicin (to be used as marked strains). So far the predominant species found in every frog has been Aeromonas hydrophila, a bacterium which is very common in soils and waters.

As the result of this long-term planning, we first started to infect frogs with an *Aeromonas* mutant. Instead of injecting the bacteria, we either bathed the frogs in bacteria or pipetted bacteria into their mouths. Due to the marker, we were able to study how the natural balance is readjusted. The rate of elimination is always fast - within one doubling time for the bacteria. All evidence indicate that the adjustment is due to the antibacterial peptides secreted from the skin gland and bacteria-bathing does induce the peptide synthesis and cortisone blocks this induction. We obtained no convincing evidence for the role of immunoglobulins - using a rabbit antisera as positive control.

The second project steams from Staffan Normark and Katrin Pütsep and their research on *Helicobacter pylori*. My involvement began with adjusting an erroneous sequence of a peptide that turned out to be active when synthesized. To cut a long story short, we have now found that *H. pylori* can produce several cecropin-like peptides. They all originate from the N-terminal part of ribosomal protein L1. Synthetic peptides based on this sequence are antibacterial, but they do not act on *H. pylori* itself.
Antisera made against a synthetic peptide can identify fragments in HPLC fractions from a lysate were they coelute with the antibacterial activity.
These results may give a hint on the evolution of the cecropins. The antibiotic activity may also compensate *H. pylori* for its slow growth rate and provide a mean to out-compete faster growing bacteria in the complex human gastro-intestinal ecosystem. In fact, *H. pylori* may be part of the human natural flora and in a useful manner contribute to a healthy balance in our ecosystem.

GENETIC AND ABIOTIC MODULATION OF THE SALICYLATE-MEDIATED DEFENSE RESPONSE IN PLANTS

Antonio Molina, Michelle Hunt, Sandra Volrath, Dave Guyer, Klaus Maleck, Eric Ward and John Ryals

Novartis Agricultural Biotechnology Research

3054 Cornwallis Road, Research Triangle Park, North Carolina 27709, USA

Salicylic acid (SA) plays a key role in the activation of plant defense responses such as the gene-for-gene resistance and the so-called systemic acquired resistance (SAR), a broad-spectrum systemic defense response that is activated following pathogen infection (Ryals et al. 1996). SA levels increase after pathogen infection, which in turn leads to the induction of the expression of a number of pathogenesis-related (PR) genes. Depletion of endogenous SA levels by overexpression of the bacterial enzyme salicylate hydroxylase, results in a breakdown of SAR and gene-for-gene resistance (Delaney *et al.*, 1994; Gaffney *et al.*, 1993). In addition to pathogens, SA-mediated defense response can also be activated by genetic or chemical disruption of a primary metabolic pathway, or by exogenous treatment with SA and chemical inducers such as 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole (BTH). These chemical inducers do not increase SA concentration in the plant, and can activate SAR in both wild-type and NahG plants. In *Arabidopsis*, mutations in the *NIM1/NPR1* gene have been shown to impair the capacity of plants to respond to both pathogens and chemical inducers and to undergo an SAR response (Cao *et al.*, 1994; Delaney *et al.*, 1995). The *NIM1/NPR1* gene have been cloned (Cao *et al.*, 1997; Ryals *et al.*, 1997), and the predicted NIM1/NPR1 protein have been shown to share significant homology with the mammalian IκBα subclass of transcription factor inhibitors, suggesting that SA-dependent signal transduction pathway may share mechanistic parallels to the mammalian NF-κB signal transduction pathway (Ryals *et al.*, 1997). SA-dependent plant defense responses contribute to the effectiveness of fungicides*in planta* . In NahG and *nim1* plants, fungicides fail to control fungal growth (Molina *et al.* 1998). Combinations of fungicides with the SAR activator BTH results in a synergistic effect on pathogen resistanec in wild-type plants (Molina *et al.* 1998). These observations are strinkingly reminiscent of the reduced efficacy of antifungal compounds in immunocompromised animals.

Cao *et al.* (1997). Cell, 88: 57-63.

Cao *et al.* (1994). Plant Cell, 6: 1845-1857.

Delaney *et al.* (1995). Proced. Natl. Acad. Sci. USA, 92: 6602-6606.

Delaney *et al.* (1994). Science, 266: 1247-1250.

Gaffney *et al.* (1993). Science, 261: 754-756.

Molina *et al.* (1998). Plant Cell, 10: 1903-1914.

Ryals *et al.* (1996). Plant Cell, 8: 1809-1819.

Ryals *et al.* (1997) Plant Cell, 9: 425-439.

Functional analysis of the PDF1.2 plant defensin gene of *Arabidopsis thaliana*.

Bart P.H.J. THOMMA, Iris A.M.A. PENNINCKX, Koenraad TIERENS, Kristel EGGERMONT, Willem F. BROEKAERT and Bruno P.A. CAMMUE

F.A.Janssens Laboratory of Genetics, Catholic University of Leuven, K. Mercierlaan 92, B-3001 Leuven, Belgium

The *Arabidopsis thaliana* gene *PDF1.2* encodes a 51 amino acid peptide that belongs to the family of antifungal plant peptides termed "plant defensins". The *PDF1.2* gene is activated systemically upon challenge of plants with fungal pathogens. Pathogen-induced activation of this gene depends on amplification of pathogen-detection events by the plant hormones ethylene and jasmonate. We have established that the ethylene and jasmonate signalling pathways need to be triggered concomitantly for efficient activation of *PDF1.2*. Arabidopsis mutants impaired in either the ethylene or jasmonate signalling pathways are unable to accumulate *PDF1.2* and such mutants are more susceptible to infection by the grey mold fungus *Botrytis cinerea*. The effect of the mutations in ethylene or jasmonate signal transduction components are, however, pleiotropic and therefore the susceptibility of such mutants may be due to the inability to produce many effector molecules, including *PDF1.2*.

To study more precisely the contribution of *PDF1.2* to disease resistance we have generated transgenic Arabidopis plants in which expression of *PDF1.2* is downregulated by antisense inhibition. The disease susceptibility of these lines is currently under investigation. In addition, we are generating Arabidopsis mutants by random chemical mutagenesis in which expression of *PDF1.2* is either downregulated or constitutively upregulated.

MECHANISMS OF BACTERIAL RESISTANCE TO HOST DEFENSE PROTEINS

LÓPEZ-SOLANILLA, E., GARCÍA-OLMEDO, F. AND RODRÍGUEZ-PALENZUELA, P.

Dep. Biotechnology. E.T.S.Ingenieros Agrónomos, UPM. Av.Complutense 28040 Madrid, Spain.

An effective defense mechanism against invading pathogens, shared by plants and animals, is mediated by small cationic proteins, as cecropins, magainins and defensins from animals, and thionins and snakins from plants. These proteins exert a toxic action against bacteria, possibly through the alteration of membrane permeability. The role of antimicrobial peptides in pathogenesis has been highlighted by the observation of increased susceptibility to infection in *Drosophila* mutants affected in their synthesis, but no such evidence is available with respect to plant peptides because appropriate plant mutants with decreased peptide levels have not yet been obtained. An alternative line of evidence about the role of antimicrobial peptides is based in the production of peptide-sensitive mutants of the pathogen. The hypothesis that the peptides are involved in defence would be supported by a decrease of virulence in this type of mutants. Indeed, in the animal pathogen *Salmonella typhimurium*, both rough lipopolysaccharide (LPS) mutants and *sapA-F* mutants, which show increased sensitivity to antimicrobial peptides (*sap* stands for sensitive to antimicrobial peptides), have reduced virulence, suggesting that resistance to host peptides has a direct role in *Salmonella* pathogenesis [1]. Similarly, we have previously found that thionin- and LTP-sensitive mutants of *Ralstonia (Pseudomonas) solanacearum* were both altered in their LPS structure and are avirulent in tobacco [2].

Erwinia chrysanthemi, is an economically important phytopathogenic bacterium that causes soft-rot diseases in a wide range of crops. Little is known about the mechanisms that enable *E. chrysanthemi* to resist the action of antimicrobial agents from the plant host. Although *S.typhimurium* and *E.chrysanthemi* have very different pathogenic behaviour, the fact that they are phylogenetically related and that antimicrobial peptides occur in their respective animal and plant hosts lead us to investigate the possible role of the Sap system in plant-pathogen interactions.

The *sapA-F* (sensitive to antimicrobial peptides) operon from the pathogenic bacterium *Erwinia chrysanthemi* has been characterized. It has five ORFs that are closely related (71% overall amino acid identity) and are in the same order as those of the *sapA-F* operon from *Salmonella typhimurium*. An *E. chrysanthemi sap* mutant strain, BT105, was obtained by marker-exchange. Mutant BT105 was more sensitive than the wild type to wheat α-thionin and to snakin-1, the most abundant antimicrobial peptide from potato tubers. Mutant BT105 was less virulent than the wild type in potato tubers: lesion area was 37% of control and growth rate was two orders of magnitude lower. The magnitude of the effect of *sapA-F* inactivation on virulence in potato tubers

and chicory leaves was greater than the effects of mutations affecting the *pel* operon and the Hrp system in the same pathogen.

These results indicate that the interaction of antimicrobial peptides from the host with the *sapA-F* operon from the pathogen plays a similar role in animal and in plant bacterial pathogenesis. They also show that this operon is important for the pathogenicity of *E. chrysanthemi*.

References

[1] Parra-Lopez C, Baer MT, Groisman EA, (1993). The EMBO Journal 12, 4053-4062.

[2] Titarenko E, López-Solanilla E, García-Olmedo F, Rodríguez-Palenzuela P, (1997). Journal of Bacteriology 179, 6699-6704.

Session 2

Chair: Jules A. Hoffmann

The Antimicrobial Host Defense Of Drosophila

Jules A. Hoffmann.
Institute of Molecular and Cellular Biology (IBMC) CNRS and University Louis Pasteur, Strasbourg, France

The presentation will review recent developments in our understanding of the remarkably efficient antimicrobial defenses of insects. Research in this field is spearheaded by studies on *Drosophila* which provides exceptional opportunities thanks to the powerful genetics. In short, septic injury results in the nearly immediate activation of proteolytic cascades leading to coagulation, melanization and opsonization, in cellular reactions such as phagocytosis and capsule formation, and in the induction of a battery of potent, mostly small-sized, antimicrobial peptides with large activity spectra. The latter aspect of the *Drosophila* host defense first described by H.G. Boman and J. Postlethwait in 1976 has been the focus of intense investigation over the last few years. Nine distinct antimicrobial peptides (plus their isoforms) have been fully or partially characterized. Their induction relies on as yet poorly understood recognition mechanisms by so-called pattern recognition receptors which probably can activate proteolytic cascades. In one case it is documented that this results in the cleavage of a cytokine-like polypeptide, Spaetzle, enabling it to bind to the transmembrane receptor Toll, a prototype of a large family of multifunctional regulators which mediate their effects via inducible transactivators of the Rel/NF-κB family. Genetic and biochemical evidence points to remarkable similarities between the Spaetzle-induced transcription of antimicrobial peptides in *Drosophila* and the cytokine-induced transcription of many immune responsive genes in mammals. Several recently isolated mutants now provide some insights into the biological relevance of the recognition, signaling and effector mechanisms of the antimicrobial host defense of *Drosophila* and this will be discussed in the presentation.

The Antimicrobial Peptide From Insects. Recent Progress

Charles Hetru

Institute of Molecular and Cellular Biology (IBMC) CNRS and University Louis Pasteur, Strasbourg, France

Antimicrobial peptides are pivotal elements of the innate immune defense against bacterial and fungal infections. Within the impressive list of antimicrobial peptides available at present, more than half have been characterized in arthropods. Cysteine-rich antimicrobial peptides represent the most diverse and widely distributed family among arthropods and to a larger extent, among invertebrates. Proeminent groups of cysteine-rich peptides are peptides with the Csαβ motif and peptides forming an hairpin-like β-sheet structure. Although these substances exhibit a large structural diversity and a wide spectrum of activity, most of them have in common the ability to permeabilize microbial cytoplasmic membranes.

Innate immunity and the interaction of the malaria parasite with the mosquito vector

Fotis C. Kafatos

The *Plasmodium* parasites that cause human malaria are transmitted exclusively by a few mosquito species of the genus *Anopheles*. Even closely related species of anopheline mosquitoes differ markedly in their capacity to act as vector. For example, in contrast to the high vectorial capacity of *A. gambiae*, the sympatric sibling species *A. arabiensis* is normally a much less important vector. Moreover, field studies have established that intraspecific variants of *A. gambiae* may differ in their importance as vectors[1].

Mosquito susceptibility or refractoriness to the parasite can be determined at multiple points during infection[2]. Documented examples include ookinete lysis, melanotic encapsulation of oocysts, and inability of sporozoites to invade salivary glands. Genetic factors in both mosquito and parasite determine compatibility of a given *Anopheles-Plasmodium* interaction[3]. Understanding the mechanisms of refractoriness of different mosquito species to a given parasite could lead to the identification of weak links in the parasite's armor, thereby facilitating the design of novel methods for control of *Plasmodium* transmission through genetic engineering of the mosquito vectors.

Our laboratory, in collaboration with that of F.H. Collins, has taken a genetic and genomic approach to elucidate the molecular basis of a dramatic example of refractoriness, namely encapsulation of Malaria parasites (of many species) in a melanized capsule at the early oocyst stage, on the basal surface of the midgut[3]. To this effect, a genetic map based on microsatellite markers was constructed[4] and used to analyze crosses involving refractory and susceptible mosquitoes[5]. Thus, three quantitative trait loci (QTLs) located at dispersed chromosomal loci were identified, which together account for ca. 75% of the melanotic encapsulation phenotype. Genomic approaches are being used currently to positionally clone these loci.

In parallel, with genomics, molecular analysis of mosquito immune response is underway, informed by the study of *Drosophila* immunity[6]. In these studies, we have collaborated extensively with the laboratory of J.H. Hoffmann in Strasbourg. Among the pertinent questions is whether the parasite is perceived by the immune system in susceptible as well as refractory mosquitoes. A combination of biochemical and molecular approaches have identified *A. gambiae* genes that may play important roles in the innate immune response to *Plasmodium* infection. These include a homologue of insect *defensin*[7], encoding a peptide immune factor identified originally on the basis of activity against bacteria. A second gene, designated *AgGNBP* (for *Anopheles gambiae* Gram-Negative Binding Protein) encodes a protein potentially involved in binding to microorganisms[8]. A number of other genes that are induced by bacterial challenge have been isolated and characterized[8,9]. Most recently, an *Anopheles* cecropin gene has been identified (J. Vizioli, P. Bulet, A. Richman et al.).

We have used these genes as 'molecular markers' to obtain clear evidence that the presence of *Plasmodium* parasites, ingested during a bloodmeal, triggers an immune response[8,10]. The response is observed both in refractory (encapsulating) and susceptible strains of *A. gambiae*. The parasite must be infected to trigger this response: ingestion of a strain of *Plasmodium* that does not produce gametocytes (and

is therefore incapable of infecting the mosquito) falls to provoke an immune response.

Induction of *defensin* gene expression reflects the kinetics of *Plasmodium* invasion. Thus, defensin RNA levels are elevated 18 to 30 hours post-feeding, when motile ookinetes are traversing the midgut epithelium. Analysis of immune marker gene expression in dissected tissues further reveals that the inducible immune response occurs both 'locally' in the midgut and 'systematically', presumably in cells of the fat body and in hemocytes (those tissues traditionally considered to be the principal sites of insect immune function[10]. More recently, a multi-site, multiphasis immune response to the parasite has been demonstrated, involving in succession the midgut, miscellaneous abdominal tissues, and the salivary where the sporozoites ultimately lodge[9]. Evidently, the parasite encounters immune reaction throughout its development in the vector. Whether these reactions significantly limit the level of *Plasmodium* infection, and how they may be enhanced to prevent infection altogether, remains to be determined.

A more extensive recent review, from which this abstract was partly extracted, can been found in reference 11.

1. Coluzzi, M., Malaria vector analysis and control. *Parasitol. Today* **8**, 113-118 (1992).
2. Billingsley, P.F. & Sinden R.E., Determinants of malaria-mosquito specificity. *Parasitol. Tody* **13**, 297-301 (1997).
3. Collins, F.H. *et al.*, Genetic selection of a *Plasmodium*-refractory strain of the malaria vector *Anopheles gambiae*. *Science* **234**, 607-610 (1986).
4. Zheng, L. *et al.*, An integrated genetic map of the African human malaria vector mosquito, *Anopheles gambiae*. *Genetics* **143**, 941-952 (1996).
5. Zheng, L. *et al.*, Quantitative trait loci for refractoriness of *Anopheles gambiae* to *Plasmodium cynomolgi B*. *Science* **276**, 425-428 (1997).
6. Hoffmann, J.A., Reichhart, J-M & Hetru, C., Innate immunity in higher insects. *Curr. Opin. Immunol*, **8** 8-13 (1996).
7. Richman, A.M. *et al.*, Inducible immune factors of the vector *mosquito Anopheles gambiae*: biochemical purification of a defensin antibacterial peptide and molecular cloning of prrprodefensin cDNA. *Insect Mol. Biol.* S, 203-210 (1996).
8. Dimopoulos, G. *et al.*, Molecular immune response of the mosquito *Anopheles gambiae* to bacteria and malaria parasites. *Proc. Natl. Acad. Sci. USA* **94**, 11508-11513 (1997).
9. Dimopoulos, G. *et al.*, Malaria infection of the mosquito *Anopheles gambiae* activates immune responsive genes during critical transition stages of the parasite life cycle. *EMBO J* **17**, 6115-6123 (1998).
10. Richman, A.M. *et al.*, *Plasmodium* activates the innate immune response of *Anopheles gambiae* mosquitoes. *EMBO J* **16**, 6114-6119 (1997).
11. Richman, A.M. & Kafatos F.C., Malaria: In the belly of the beast. *Nature Medicine* **4**, 552-553 (1998).

Insect-Derived Peptides as Leads for Future Drugs

Shunji Natori
Graduate School of Pharmaceutical Sciences, University of Tokyo, Bunkyo-ku, Tokyo 113-003, Japan

We have been studying the self-defense system of the flesh fly, *Sarcophaga peregrina*. So far, we have isolated and characterized several antibacterial proteins, one antifungal protein (AFP) and two lectins that are assumed to play roles in this defense system. Recently, we isolated a novel inducible antibacterial substance named 5-S-GAD. In this workshop, I would like to discuss the possibility of utilizing these defense molecules for the development of novel drugs, especially focusing on sapecin B and 5-S-GAD.

Sapecin B : Sapecin B is a sapecin family antibacterial protein that consists of 34 amino acid residues. On 1H-NMR analysis, sapecin B was found to contain four domains: the 6 residues at the amino terminal form a loop, and the adjacent 11 residues an α-helix, and finally two β-sheets are located at its carboxyl terminal. We found that the a-helix domain (residues 7-17) is the core of the antibacterial activity of sapecin B. We further modified this peptide and synthesized a series of peptides consisting of only Lys and Leu. We found that one of these peptides, KLKLLLLLKLK-NH2, is potentially useful for treatment of infectious diseases caused by bacteria, even those such as MRSA which are resistant to various antibiotics. 5-S-GAD : 5-S-GAD was originally found as an inducible antibacterialsubstance in Sarcophaga, and was shown to be a key molecule in the defense system of this insect. The compound is assumed to act as an antibacterial substance as well as an activator of Rel family transcription factor by producing H2O2. 5-S-GAD was found to be a potent inhibitor of protein tyrosine kinase. Therefore, we examined its effect on the growth of various human cancer cells in vitro. Of 38 tumor cell lines examined, the growth of three was significantly suppressed by 5-S-GAD. These were 2 breast cancer cell lines and 1 melanoma cell line. The reason why 5-S-GAD selectively suppresses the growth of these cancer cells is still not known. However,using 5-S-GAD as a starting material, it might be possible to develop antitumor drugs with selective toxicity against certain tumor cells. We also found that 5-S-GAD significantly inhibited the differentiation of osteoclasts in vitro, suggesting that it has the potential to suppress osteoporosis.

References

1. Y. Nakajima, J. Alvarez-Bravo, J-H. Cho, K. Homma, S. Kanegasaki and S. Natori Chemotherapeutic Activity of Synthetic Antimicrobial Peptides: Correlation between Chemotherapeutic Activity and Neutrophil-Activating Activity. FEBS Letts. (1997) 415, 64-66.

2. M. Hijikata, A. Kobayashi, J-Y., Leem, H. Fukasawa, Y. Uehara and S. Natori Inhibition of Protein Tyrosine Kinase by 5-S-GAD, a Novel Antibacterial Substance from an Insect. Biochem. Biophys. Res. Commun. (1997) 237, 423- 426.

"THE ANTIFUNGAL PROTEIN (AFP) FROM *Aspergillus giganteus* BINDS TO NUCLEIC ACIDS".

Lacadena, V., Martínez del Pozo, A., Oñaderra, M. and Gavilanes, J.G.

Departamento de Bioquímica y Biología Molecular I, Facultad de Química, Universidad Complutense, 28040 Madrid.

The mold *Aspergillus giganteus* MDH 18894 was isolated from the soil of a farm in Michigan during an antitumor screening program (1). This imperfect ascomycete produces two extracellular polypeptides (1,2). One of them is α-sarcin, a cytotoxic ribonuclease which has been thoroughly characterized (3-5). The other one is a very basic small-sized protein (51 amino acids), with a high conten of disulfide bridges and antifungal activity (2, 5-8). This polypeptide has been deeply characterizated from the structural point of view (6-9). However, its mode of action remains unknown. The solution structure of AFP was determined by using experimentally derived interproton distance constrains from NMR spectroscopy (4). The folding of AFP consists of five antiparalell ß-strands definig a small and compact ß-barrel stabilized by four internal disulfide bridges (9). When this structure was solved, the AFP-fold was only found as a domain of the *Staphylococcal aureus* nuclease, which does not present disulfide bridges (10). However, this structure has been recently detected, by means of three-dimensional structures comparison, in several other unrelated proteins, most of them with nucleic acid related functions. Actually, we now know that AFP is a member of the proteins containing an OB-fold, for oligonucleotide binding motif (11). This discovery suggest that AFP function must be in close connection to interacting with nucleic acids. Consequently, we have explored this possibility and, in fact, we have demonstrated how AFP binds to plasmid DNAs, by means of agarose gel retardation assays. This binding does not show any sequence specificty but rather seems to be related to a electrostatic interaction between AFP posite charges and the negative phosphates of DNA. Important changes in the circular dichroism and fluorescence spectra of AFP are observed when this interaction takes place. In addition, we have also investigated the interaction established between AFP and the 23S rRNA, the natural target of α-sarcin, the protein coproduced with AFP by *A.giganteus*.

BIBLIOGRAPHY

(1)Olson and Goerner (1965) *Appl. Microbiol.* **13**, 314-321.
(2)Olson *et al.* (1965) *Appl. Microbiol.* **13**, 322-326.
(3)Wool *et al.* (1992) *TIBS* **17**, 266-269.
(4)Gasset *et al.* (1994) *Cur. Top. Pept. Protein Res.* **1**, 99-104.
(5)Pérez-Cañadillas *et al.* (1998) *Biochemistry (en prensa).*
(6)Nakaya *et al.* (1990) *Eur. J. Biochem.* **193**, 31-38.
(7)Wnendt *et al.* (1994) *Curr. Genet.* **25**, 519-523.
(8)Lacadena *et al.* (1995) *Arch. Biochem. Biophys.* **324**, 273-281.
(9)Campos-Olivas *et al.* (1995) *Biochemistry* **34**, 3009-3021.
(10)Arnone *et al.* (1971) *J. Biol. Chem.* **246**, 2302-2316.
(11)Murzin (1993) *EMBO J.* **12**, 861-867.

Session 3

Chair: Michael E. Selsted

TRANSCRIPTIONAL REGULATION OF THE EXPRESSION OF ANTIMICROBIAL PEPTIDE GENES IN AMPHIBIA

Maurizio Simmaco*, Hans G. Boman# and Donatella Barra

Dipartimento di Scienze Biochimiche, Università La Sapienza, 00185 Roma, Italy; *Dipartimento di Scienze Biomediche, Università G. D'Annunzio, 66100 Chieti, Italy, #Microbiology and Tumor Biology Center, Karolinska Institutet, SE-171 77 Stockholm, Sweeden.

Animal antibacterial peptides are widely distributed in living organisms where they represent effector molecules of innate immunity (1). They are all synthesized by ribosomes as preproproteins and proteolytically processed to the active form. Despite the high structural variability, the proposed mechanism of action for most of them is mainly based on the selective permeabilization of bacterial cell membranes.

It has been demonstrated that in insects the production of antimicrobial peptides is induced as a response to a bacterial infection and this seems to be the main mechanism by which infections are counteracted (2). Also in vertebrates an immediate host response is needed, which should be active before the adaptive immune system is ready. In mammals, phagocytic cells, like macrophages, PMN and NK cells, as well as the antimicrobial peptides, such as defensins, PR-39, NK-lysin, etc., are indeed linked to innate immunity.

It is difficult to demonstrate experimentally the role of antimicrobial peptides in controlling the natural flora. Several approaches have been attempted to obtain such evidence, mainly on cell coltures.

Antibacterial peptides are abundant in skin secretion and in the digestive system of frogs (3), which, in addition, are known to have immunoglobulins, complement and major histocompatibility antigens (4). Thus, frogs can be considered suitable model systems for the study of factors controlling both natural infections and the natural flora.

We have studied the role of antimicrobial peptides in innate immunity in two amphibian species, *Rana esculenta* and *Bombina orientalis*. Some years ago, the antimicrobial peptides present in their secretions were isolated and well characterized, also by molecular cloning (5-7).

This knowledge is now used as the platform for our present studies of the gene control and *in vivo* functions of the peptides. In this talk, I will concentrate on our results on Bombina and leave to Hans G. Boman to review our recent joint work on Rana.

We have demonstrated that glucocorticoids (GC), earlier known to suppress immune functions in mammalian cell cultures (8, 9), can give a total block of peptide synthesis both in Rana and in Bombina (10, 11). The

blocking of peptide synthesis was demonstrated by the absence of both the peptides (with HPLC) and the mRNA (Northern blot analyis). That these effects were due to an induced synthesis of IκBα was demonstrated using an antibody made against a synthetic peptide with a sequence derived from human IκBα. Thus the NF-κB/IκBα machinery for gene control is extremely well conserved and support our use of frogs as model system.

As the next step we have sequenced the gene for bombinin, one of the major antibacterial peptides in *Bombina orientalis* (11). The upstream region contains potential binding sites not only for NF-κB but also for NF-IL6. These two regulatory elements have so far been found in all genes for inducible peptides with antibacterial activities. That the Bombina promoters are functional is documented in a Poster at this meeting (12).

Taken together, all our results are consistent with the hypothesis that innate immunity is mediated by peptides as effector molecules. Even if the sequences of the effectors show a large variability, the general concept and the transcriptional control are surprisingly well conserved in different animals.

References

1. Boman, H.G. (1995) *Annu. Rev. Immunol.* 13, 61-92.
2. Hoffmann, J.A. and Reichhart, J.M. (1997) *Trends Cell. Biol.* 7, 309-316.
3. Barra, D. and Simmaco, M. (1995) *Trends Biotechnol.* 13, 205-209.
4. Du Pasquier, L. (1993) in *Evolution of the immune system*, Paul, W., ed., Raven Press, New York, pp. 199-233.
5. Simmaco, M., Mignogna, G., Barra, D. and Bossa, F. (1994) *J. Biol. Chem.* 269, 11956-11961.
6. Gibson, B.W., Tang, D., Mandrell, R., Kelly, M. and Spindel, E.R. (1991) *J. Biol. Chem.* 266, 23103-23111.
7. Mignogna, G., Simmaco, M. and Barra, D. (1998) in *D-Amino Acids in Sequences of Secreted Peptides of Multicellular Organisms*, P. Jollès, ed., Birkhäuser Verlag, Basel, pp. 29-36.
8. Scheinman, R.I., Cogswell, P.C., Lofquist, A.K. and Baldwin, A.S. (1995) Science 270, 283-286.
9. Auphan, N., DiDonato, J.A., Rosette, C., Helmberg, A. and Karin, M. (1995) Science 270, 286-290.
10. Simmaco, M., Boman, A., Mangoni, M.L., Mignogna, G., Miele, R., Barra, D. and Boman, H.G. (1997) *FEBS Lett.* 416, 273-275.
11. Miele, R., Ponti, D., Boman, H.G., Barra, D. and Simmaco M. (1998) *FEBS Lett.* 431, 23-28.
12. Miele, R., Bjorklund, G., Barra, D., Simmaco, M. and Engström, Y. (1999) Abstract of the Workshop on Eukaryotic Antibiotic Peptides, Madrid, February 8-10.

HUMAN DEFENSINS

Tomas Ganz, PhD, MD, University of California, Los Angeles

As new members of the human defensin family are identified, the biological variety of these peptides is becoming apparent. Previous studies characterized α-defensins HNP1-3 (Ganz *et al.*, 1985) and HNP-4 which are major constituents of the microbicidal granules of human blood granulocytes, and HD-5 and HD-6 which are abundantly expressed in Paneth cells, intestinal epithelial cells specialized in host defense functions. More recent studies identified two β-defensins: a constitutively expressed human epithelial defensin HBD-1 which is particularly abundant in the kidney and the urogenital tract (Valore *et al.*, 1998), and an infection/cytokine-inducible epithelial defensin HBD-2 which is very abundant in the skin. All known human defensin genes are clustered on chromosome 8p23 within about 500 kb (Liu *et al.*, 1997; Liu *et al.*, 1998). From the telomere to the centromere, the order of defensin genes (peptides in parentheses) is: HDEFA5 (HD-5), multiple copies of HDEFA1 (HNP1-3), HDEFA4 (HNP-4), HDEFA6 (HD-6), HDEFB1 (HBD-1) all separated by 20 kb or less from each other. Located another 300-400 kb towards the centromere is HDEFB2 (HBD-2). In this HUGO nomenclature H designates a human gene, DEF stands for defensin, and A or B designate an α- or β-defensin. The separation of the Paneth cell genes HDEFA5 and HDEFA6 by the myeloid defensin genes supports the conjecture (Bevins *et al.*, 1996) that granulocyte (myeloid) α-defensins HNP1-3 and HNP-4, encoded by HDEFA1 and HDEFA4 evolved from an ancestral Paneth cell defensin by reduplication and divergence. Since β-defensins but not α-defensins have been identified in birds (gallinacins, avian β-defensins) and crotalid snakes, they may constitute the oldest branch of the defensin family, from which the Paneth cell α-defensins evolved. Some functional similarity of the genes encoding epithelial β-defensins and Paneth cell α-defensins is suggested by the coexpression of HD-5 and HBD-1 in reproductive epithelia (Quayle *et al.*, 1998; Valore *et al.*, 1998).

Although homology cloning has been useful in the discovery of new defensins, it does not provide information about the posttranslational processing that takes place *in vivo*. Some defensins are readily purified in large amounts from natural sources but this has been difficult for human Paneth cell defensins. We recently purified forms of HD-5 and HD-6 from the urine of patients whose bladders were replaced by reservoirs constructed from human ileum (Porter *et al.*, 1998) and showed that several of the peptide forms are generated by cleavage with trypsin or another trypsin-like protease. Further studies will be required to characterize the processing pathways that generate active defensins.

The evidence for a microbicidal function of human neutrophil defensins is indirect but strong. They are present in neutrophil granules at sufficiently high amounts to generate ~mg/ml concentrations in phagocytic vacuoles. At ~μg/ml concentrations, defensins are inhibited by several constituents of plasma, including salts, but this inhibition can be overcome by increasing peptide concentrations. The concentrations of defensins generated by epithelia are less well known. We defined the geometry of the porcine β-defensin-1 (PBD-1) layer on the filiform papillae of the pig tongue and determined the amount of defensin present by scraping

and semiquantitative Western blotting. The concentration of PBD-1 is at least 100 μg/ml, sufficient for both direct and synergistic activity in the low salt milieu of saliva.

Despite a similar structural framework, defensins exhibit a variety of antimicrobial spectra. Of the newest defensins, the recombinant baculovirus-derived β-defensin HBD-2 was more potent (Singh *et al.*, 1998) than equimolar amounts of HBD-1 and was active against many gram-positive and gram-negative bacteria including strains of *E. coli, Ps. aeruginosa,* gp B streptococcus and *Staph. epidermidis* but was remarkably and consistently ineffective against *Staph. aureus*, as previously described for skin-derived HBD-2 by Harder et al. The structural basis of these differences in activity is of much interest.

Reference List

Bevins, C.L., Jones, D.E., Dutra, A., Schaffzin, J. & Muenke, M. (1996). Human enteric defensin genes: chromosomal map position and a model for possible evolutionary relationships. *Genomics* **31**, 95-106.

Ganz, T., Selsted, M.E., Szklarek, D., Harwig, S.S., Daher, K., Bainton, D.F. & Lehrer, R.I. (1985). Defensins. Natural peptide antibiotics of human neutrophils. *J.Clin.Invest.* **76**, 1427-1435.

Liu, L., Wang, L., Jia, H.P., Zhao, C., Heng, H.H.Q., Schutte, B.C., McCray, P.B.J. & Ganz, T. (1998). Structure and mapping of the human β-defensin HBD-2 gene and its expression at sites of inflammation. *Gene* **222**, 237-244.

Liu, L., Zhao, C., Heng, H.H.Q. & Ganz, T. (1997). The human β-defensin-1 and α-defensins are encoded by adjacent genes: two peptide families with differing disulfide topology share a common ancestry. *Genomics* **43**, 316-320.

Porter, E.M., Poles, M.A., Lee, J.S., Naitoh, J., Bevins, C.L. & Ganz, T. (1998). Isolation of human intestinal defensins from ileal neobladder urine [In Process Citation]. *FEBS Lett* **434**, 272-276.

Quayle, A.J., Porter, E.M., Nussbaum, A.A., Wang, Y.M., Brabec, C., Yip, K.P. & Mok, S.C. (1998). Gene expression, immunolocalization, and secretion of human defensin-5 in human female reproductive tract. *Am J Pathol* **152**, 1247-1258.

Singh, P.K., Jia, H.P., Wiles, K., Hesselberth, J., Liu, L., Conway, B.D., Greenberg, E.P., Valore, E.V., Welsh, M.J., Ganz, T., Tack, B.F. & McCray, P.B.J. (1998). Production of β-defensins by human airway epithelia. *Proc Natl Acad Sci U S A* **95**, 14961-14966.

Valore, E.V., Park, C.H., Quayle, A.J., Wiles, K.R., McCray, P.B. & Ganz, T. (1998). Human beta-defensin-1: An antimicrobial peptide of urogenital tissues. *J Clin Invest* **101**, 1633-1642.

CATHELICIDIN-DERIVED PEPTIDES AND ANALOGUES: ANTIMICROBIAL AND CYTOTOXIC ACTIVITIES.

Gennaro, R.[1], Benincasa, M.[1], Dolzani, L.[2], Skerlavaj, B.[3], Risso, A.[3], Scocchi, M.[4] and Zanetti, M.[3,4]

[1]Dip. di Biochimica, Biofisica e Chimica delle macromolecole e [2]Dip. di Scienze Biomediche, Univ. di Trieste; [3]Dip. di Scienze e Tecnologie Biomediche, Univ. di Udine; [4]Laboratorio Nazionale CIB, AREA Science Park, Trieste, Italy.

The emergence and widespread diffusion of bacterial strains resistant to conventional antibiotics is of great concern in clinical settings and is an especially severe problem for immunodepressed patients. To cope with this problem new classes of antimicrobial agents are needed and pharmaceutical companies are in search of new lead compounds. A promising source of such compounds is the variety of gene encoded antimicrobial peptides that are widespread in the living organisms as part of the natural host defense. These peptides exert a potent antimicrobial activity *in vitro* with broad activity spectra, and rapidly kill target cells, including antibiotic-resistant pathogens.

In recent years we have identified a number of myeloid antimicrobial peptides from several mammalian species and derived from a family of precursors named cathelicidins (1). Members of these family are characterized by a conserved N-terminal preproregion, followed by structurally varied C-terminal sequences corresponding to active peptides after proteolytic cleavage. Cathelicidin-derived peptides vary significantly by sequence, structure and length and include α-helical, Cys-rich, Pro- and Arg-rich, and Trp-rich peptides. Only one congener is present in man while other species, in particular artiodactyl species, show several members.

We have chemically synthesized and characterized the activity of a number of these peptides and of their fragments and analogues. The *in vitro* effectiveness of cathelicidin-derived peptides representative of α-helical (SMAP-29, BMAP-27, BMAP-28 and N-terminal fragments of the latter two), Trp-rich (indolicidin), Cys-rich (Protegrin PG-1) and PR-rich (N-terminal fragments of Bac7) structural classes, has been tested against multiresistant clinical isolates with the aim to select the most promising for further *in vivo* studies. The antimicrobial activity was evaluated by the microdilution susceptibility test, to determine the minimum inhibitory concentration (MIC) values. Over sixty clinical isolates, frequently resistant to several classes of antibiotics have been used. The strains tested included methicillin-resistant *Staphylococcus aureus*, vancomicin-resistant *Enterococcus faecalis*, and, among Gram-negative species, several strains of *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Burkholderia cepacia* and *Serratia marcescens*.

The results indicate that the peptides have different potencies against the strains used. SMAP-29 and PG-1 are the most potent and are active against most of the strains tested, with MIC values in the range 0.5-2 μM. SMAP-29 is the only one active against the clinical isolates of *S. marcescens* tested (MICs of 0.5-8 μM). BMAP-27 is more active against Gram negative and BMAP-28 against Gram positive strains. A reversed behaviour is shown by their N-terminal 1-18 fragments that also display a lower cytotoxic activity against eukariotic cells with respect to the parent peptides. The spectrum of activity of Bac7 fragments is restricted to Gram negative species, with Bac7(1-35) particularly active against *A. baumannii* strains. SMAP-29 is the most potent among the peptides tested and has been further characterized for its tendency to select resistant mutants. The results indicate that MIC values of SMAP-29 against the *E. coli* ML-35 strain are unchanged after 10 and 20 passages in the presence of sublethal concentrations

of the peptide. When tested under the same conditions, the *E. coli* strain shows a relevant increase of the MIC values for nalidixic acid, used as a positive control.

These results prompted us to test the efficacy of some of these peptides against systemic infections. After determination in mouse of the LD_{50}, the peptides SMAP-29, BMAP-28(1-18) and BMAP-27(1-18) have been tested in a peritoneal infection model induced in mice by i.p. injection of *P. aeruginosa*. Preliminary results indicate that SMAP-29 and BMAP-27(1-18), injected i.p. respectively at 1 and 4 mg/Kg. are effective in protecting mice from *P. aeruginosa* infection causing a 90% mortality of control mice in 24 h.

REFERENCES

1. Zanetti, M., Gennaro, R. and Romeo, D. (1995) FEBS Lett. 374, 1-5.

Insights into Mammalian Antimicrobial Peptide Structure-Activity Relationships

Michael E. Selsted, Department of Pathology, College of Medicine, University of California, Irvine, CA 92612

Studies in this laboratory have focused on the antimicrobial mechanisms of α- and β defensins and of indolicidin, a member of the cathelicidin peptide family. α and β-defensins are tridisulfide peptides of 29-42 amino acids that are expressed in circulating granulocytes, granulated epithelial cells, and in other epithelial (1). Though encoded by distinct gene families, and despite their differing disulfide patterns, α and β defensins have nearly identical folded conformations (2).

The α-defensins characterized in phagocytes and Paneth cells behave in solution as monomers. *In vitro* antimicrobial assays demonstrate a general correlation between microbicidal potency and peptide cationicity, though many exceptions to this relationship are known. Moreover, net charge alone is inadequate to explain the microbicidal effect, as preparations of linearized defensins possessing native charge are virtually inactive. Experiments employing model membranes demonstrate that monomeric defensins can permeabilize large unilamellar vesicles (LUVs) in a dose-dependent that is consistent with graded disruption of the bilayer (3).

Studies on naturally-occurring defensin structural variants have provided insight into molecular features important for activity against various microbes. Notably, the mouse enteric defensin family includes at least 20 distinct isoforms as determined by protein and/or cDNA sequence analysis (4). Within this mouse peptide family, 17 defensin peptides differ from each other by 7 or fewer amino acid substitutions. However, the biological properties of variants differing even by one residue are remarkably different. In addition, the activities of mouse enteric defensins are modulated *in vivo* by proteolytic processing events in the bowel lumen.

Three human myeloid α-defensins (HNP 1-3) are the only defensins known to exist as stable non-covalent dimers (5). Their dimeric conformations confer an amphiphilic topology that promotes further aggregation in solution. Studies of the interactions of defensins with anionic (POPG) LUVs demonstrated the formation of stable pores that accommodated the transit of macromolecular probes across the bilayer, and provides the basis for modeling a stable oligomeric pore (6). Additional structural elements involved in HNP 1-3 microbicidal function were revealed by comparing the antimicrobial properties of natural variants differing by only a single amino acid.

In contrast to the disulfide rich defensins, indolicidin is a tridecapeptide amide that lacks cysteine, but it is unusually rich in tryptophan (five residues; 7). Indolicidin binds strongly to LUVs formed from neutral or anionic phospholipids, and permeabilizes the bilayer in a dose dependent manner that differs from that

observed with the dimeric defensins (8). While the atomic structure of indolicidin has not yet been reported, the peptide chain length alone indicates that the peptide monomer cannot span a biological bilayer. However, recent studies indicate that indolicidin may assemble to form multimers. The concentrations at which this occurs approximate those required for microbicidal activity.

1. Selsted M.E. and Ouellette A.J. (1995) *Trends in Cell Biology* 5, 114-119.
2. Zimmermann, G.R., Legault, P., Selsted, M.E., and Pardi, A. (1995) *Biochemistry* **34**, 13663-13671
3. Hristova, K., Selsted, M.E., and White, S.H. (1996) *Biochemistry* **35**, 11888-11894
4. Ouellette A.J., Hsieh M.M., Nosek M.T., Cano-Gauci D.F., Huttner K.M., Buick R.N., and Selsted M.E. (1994) *Infect.Immun.* **62**, 5040-5047.
5. Hill, C.P., Yee, J., Selsted, M.E., and Eisenberg, D. (1991) *Science* **251**, 1481-1485
6. Wimley, W.C., Selsted, M.E., and White, S.H. (1994) *Protein Science* **3**, 1362-1373
7. Selsted, M.E., Novotny, M.J., Morris, W.L., Tang, Y.-Q., Smith, W., and Cullor, J.S. (1992) *J.Biol.Chem.* **267**, 4292-4295
8. Ladokhin, A.S., Selsted, M.E., and White, S.H. (1997) *Biophys.J.* **72**, 794-805

Biological Properties of Structurally Related α-Helical Cationic Antimicrobial Peptides

Monisha G. Scott and R.E.W. Hancock

One prominent class of cationic antimicrobial peptides comprises the α-helical class, which is unstructured in free solution but folds into an amphipathic α-helix upon insertion into the membranes of bacteria. In this study a series of α-helical cationic peptides, were created based on the antibacterial and anti-endotoxic peptides CEME, a cecropin-melittin hybrid and its derivative CEMA, as well as two novel variants CP26 and CP29 which were designed to be more amphipathic. From these four peptides, a series of 20 variants with small amino acid changes were designed. The antimicrobial activity of the peptides against Gram-negative bacteria was studied by MIC determination. Alterations in the charge, hydrophobicity, or length of the variant peptides did not improve the antimicrobial activity, and there was no statistically significant correlation between any of these factors and MIC for *Pseudomonas aeruginosa* or *Escherichia coli*. None of the peptides were effective against *Burkholderia cepacia*. Individual peptides demonstrated synergy with conventional antibiotics against antibiotic resistant strains of *P. aeruginosa*. The peptides were also tested for their anti-endotoxic potential by assessing their ability to bind LPS and block the production of the cytokines, IL-6 and TNF, by LPS-stimulated murine macrophages. The peptides varied considerably in their ability to bind *E.coli* smooth strain O111:B4 LPS and this correlated significantly with their antimicrobial activity and ability to block LPS-stimulated TNF and IL-6 production. The effect of individual peptides on LPS stimulated IL-6 production was very similar to that seen with for TNF production. The effect of the peptides on CpG DNA stimulation of the macrophages was also studied since bacteria not only release LPS during infection but also their DNA. Although the peptides blocked production of cytokines in response to CpG DNA stimulation of macrophages, the effect was not as significant as seen with LPS. In general, the peptides studied here demonstrated a broad range of activities including antimicrobial, anti-endotoxin and enhancer activities.

Session 4

Chair: David Andreu

Regulatory Aspects Of Epithelial Antimicrobial Peptide Expression In Mammals

Charles L. Bevins MD PhD
Department of Immunology
The Cleveland Clinic Foundation
Cleveland, OH

In the animal kingdom, highly specialized epithelial surfaces of several organ systems are charged with vital physiologic functions including gas exchange, digestion, water conservation, and reproduction. Despite continual encounters with a wide range of microbes, the low incidence of infectious and inflammatory complications at these epithelial mucosal surfaces suggests that local host immunity includes highly effective, broad-spectrum, non-inflammatory antimicrobial defenses.

Recent studies by our group and others have focussed on one component of mammalian mucosal defense, antimicrobial peptides. Antimicrobial peptides are distributed in several organ systems and the predominant class of molecules found at a single site appears to vary between mammalian species. Within a species, dramatic tissue specific expression is often observed. Some peptides are brought to mucosal sites by circulating phagocytic white cells, while others are locally produced by resident epithelial cells. Our current interest is on the regulation of the defensin peptides expressed by mucosal epithelial cells.

We are addressing aspects of tissue specific expression of defensins via the study of defensin peptides that are expressed in Paneth cells, epithelial cells that are located at the base of small intestinal crypts. Two human defensins, HD5 and HD6, appear to evolutionarily distantly related but are both coordinately expressed in Paneth cells at high levels. Despite significant variation through much of the gene sequence, these genes show remarkable sequence similarity in their 5' flanking regions, suggesting that important regulatory elements for gene expression are located in this area. To test this hypothesis, we generated transgenic mouse lines using 3 kb of human genomic DNA, which spans the HD5 gene and includes 1.5 kb of 5' flanking sequence. Northern blot analysis of multiple tissues of the transgenic mice demonstrated HD5 mRNA expression limited to the small intestine, with greater expression noted distally. *In situ* hybridization localized the HD5 mRNA to the transgenic mouse Paneth cell. Immunohistochemistry, using polyclonal HD5 antiserum (provided by E.M. Porter and T. Ganz), detected HD5 protein in Paneth cells. No antibody staining was detected in the wild type mouse Paneth cells. The data support the hypothesis that cis-acting elements necessary for tissue specific expression of HD5 in the transgenic mouse are located in 3 kb of DNA encompassing the HD5 gene.

The first mammalian defensin gene to demonstrate significant inducibility upon infectious challenge is Tracheal Antimicrobial Peptide (TAP). TAP is a defensin peptide expressed in ciliated respiratory epithelial cells. TAP gene expression is dramatically upregulated in primary cultures of bovine tracheal epithelial cells by the addition of bacterial lipopolysaccharide (LPS). The induction is via a CD14-dependent pathway. Others have shown that this gene also is dramatically induced upon bacterial infection *in vivo*. Examination of the 5' flanking region of the TAP gene indicates several potential *cis*-acting regulatory sequences which may be key to this induction, including the consensus sequences for NF-κB and NF-IL6. Our *in vitro* studies indicate that induction of TAP gene expression by LPS occurs by increased transcription and is mediated by the binding of the activated p65/p50 heterodimeric form of NF-κB to the consensus sequence in the promoter region. Our results suggest a mechanism for regulation of inducible gene expression, which shares common elements with other host defense genes.

CYSTIC FIBROSIS LUNG DISEASE: A DEFECT IN MUCOSAL DEFENSE

Jeffrey J. Smith, Department of Pediatrics, University of Iowa College of Medicine, 200 Hawkins Drive, Iowa City, IA 52242 (USA). Tel.: 1 319 356 7265. Fax: 1 39 356 7171. E-mail: smithjj@blue.weeg.uiowa.edu

The airway mucosal surface is continuously exposed to bacteria in the air we breathe. To prevent bacterial infections, the airways use a variety of defense mechanisms. For example, peptides with broad-spectrum antibacterial activity are secreted into airway surface liquid (ASL) to prevent bacteria from proliferating on the airway surface. These peptides include: lysozyme, lactoferrin, secretory leukocyte protease inhibitor (SLPI), phospholipase A_2, the ß-defensins hBD-1 and hBD-2, the cathelicidin LL-37, the neutrophil-derived α-defensins, and small, anionic antimicrobial peptides. Because of technical difficulties in obtaining undisturbed ASL, the actual *in vivo* concentrations of these antimicrobial peptides are unknown, but lysozyme, lactoferrin, and SLPI appear to be the most abundant (1).

Several factors may influence the antibacterial activity of ASL besides the concentration of the individual peptides. First, these peptides have synergistic antibacterial activity (2). Thus, the concentrations that provide physiologic benefit *in vivo* may be considerably less than the concentrations needed to function as a sole antimicrobial peptide. Second, antibacterial activity may also be influenced by physiologic concentrations of salt in ASL. That is, the *in vitro* activity of these peptides decreases as the concentration of NaCl increases (up to 150 mM NaCl) (1,3). In addition, the synergy of these peptides also decreases as NaCl concentration increases. These observations suggest that the NaCl concentration of ASL may play a key role in airway mucosal defense by influencing the antibacterial activity of ASL.

Cystic fibrosis (CF) is a genetic disease characterized by chronic bacterial lung infections, neutrophil infiltration, and recurrent obstruction of the airways which slowly progresses to respiratory failure in most persons with CF. Because CF is a disorder of NaCl transport by epithelia, it has been hypothesized that CF lung disease may be due to a defect in airway mucosal defense that results from increased NaCl concentration in ASL (4). CF is caused by mutations in the gene that codes for a chloride channel called the cystic fibrosis transmembrane conductance regulator (CFTR). In the airways, a lack of CFTR chloride channels reduces the ability of CF epithelia to absorb NaCl (5,6). Reduced NaCl absorption may lead to increased NaCl concentrations in CF ASL (6) and an inhibition of the ASL antibacterial activity (1,4,7). Perhaps to compensate for this reduced antibacterial activity, pulmonary macrophages stimulate neutrophil infiltration into the airways (8,9) and enhanced mucus secretion (goblet cell metaplasia and submucosal gland hypertrophy). Persistent inflammation in CF airways releases excessive DNA from senescent neutrophils into the airway mucus which alters its visco-elastic properties and impairs its clearance. This impaired mucociliary clearance leads to peripheral airway obstruction which further accentuates the infection and inflammation. This scenario accounts for the clinical pattern that characterizes CF lung disease: recurrent exacerbations of airway infection, inflammation and obstruction that damage the airways (bronchiectasis) and cause a progressive decline in lung function.

References:

(1) Travis SM, BD Conway, J Zabner, JJ Smith, NN Anderson, P Singh, EP Greenberg, and MJ Welsh. Activity of abundant antimicrobials of human airway. *Am. J. Respir. Cell Mol. Biol.* (In

press, 1999).

(2) Singh P, and MJ Welsh. Components of airway surface fluid have synergistic antimicrobial activity. *Pediatr. Pulmonol.* Supplement 14: 323, 1997.

(3) Bals R, X Wang, M Zasloff, and JM Wilson. The peptide antibiotic LL-37 /hCAP-18 is expressed in epithelia of the human lung where it has broad antimicrobial activity at the airway surface. *Proc. Natl. Acad. Sci. USA* 95: 9541-9546, 1998.

(4) Smith JJ, SM Travis, EP Greenberg, and MJ Welsh. Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. *Cell* 85: 229-236, 1996.

(5) Uyekubo SN, H Fischer, A Maminishkis, B Illek, SS Miller, and JH Widdicombe. cAMP-dependent absorption of chloride across airway epithelium. *Am. J. Physiol.* 275: L1219-L1227, 1998.

(6) Zabner J, JJ Smith, PH Karp, JH Widdicombe, and MJ Welsh. Loss of CFTR chloride channels decreases salt absorption by cystic fibrosis airway epithelia. *Mol. Cell* 2: 1-7, 1998.

(7) Goldman MJ, GM Anderson, ED Stolzenberg, UP Kari, M Zasloff, and JM Wilson. Human beta-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis. *Cell* 88: 553-560, 1997.

(8) Khan TZ, JS Wagener, T Bost, J Martinez, FJ Accurso, and DWH Riches. Early pulmonary inflammation in infants with cystic fibrosis. *Am. J. Respir. Crit. Care Med.* 151: 1075-1082, 1995.

(9) Konstan MW, KA Hilliard, TM Norvell, and M Berger. Bronchoalveolar lavage findings in cystic fibrosis patients with stable, clinically mild lung disease suggest ongoing infection and inflammation. *Am. J. Respir. Crit. Care Med.* 150: 448-454, 1994.

Jeffrey J. Smith, M.D.
Associate Professor
Department of Pediatrics
University of Iowa College of Medicine
Iowa City, IA 52242

Phone 319 356-7265
FAX 319 356-7171
email address: smithjj@blue.weeg.uiowa.edu

ANTIFUNGAL AND ANTITUMORAL PROPERTIES OF SYNTHETIC CECROPIN-LIKE PEPTIDES

D. Andreu[1], L. Cavallarin[2], A. Morilla[3], S. Nos-Barberà[3] & B. San Segundo[2]
[1] Department of Organic Chemistry, University of Barcelona, Spain
[2] Center for Research and Development, CSIC, Barcelona, Spain
[3] Institute of Applied Ophthalmobiology, University of Valladolid, Spain

The cecropins have generated more interest (measured in terms of reported structure-activity studies) among peptide chemists than any other family of antimicrobial peptides. Using chemical sense aided by conformational considerations, numerous analogs, enantiomers, topoisomers, as well as chimeric constructions with other active structures have been prepared and evaluated. The sequence hybridization concept, first tested on cecropin-melittin peptides, has proven particularly useful for developing promising lead compounds. This presentation will recapitulate some earlier findings in this field and then focus on recent work concerning application of this class of peptides as antifungal and antitumoral agents.

Potent inhibition of several fungal plant pathogens can be achieved by short (11- and 12-residue) structures that reproduce the N-terminal part of cecropin A. Minimal inhibitory concentrations in the 10^{-5} M range have been obtained against pathogens such as *Phytophora*, *Fusarium* or *Trichoderma*. Tandem repeat constructions based on the active sequences retain potency and provide guidelines for engineering fungal resistance in crop plants.

Another group of synthetic peptides with some resemblance to cecropin A has been tested for antitumor activity against uveal melanoma, the most frequent malignant intraocular tumor and one of very few eye diseases with a high fatality rate. About ten peptides with adequate in vitro profiles (high cytolytic activity for tumoral cells, intermediate activity for melanocytes, minimal activity for corneal epithelial cells) have been identified as potential candidates for preclinical studies.

Leishmanicidal Activity Of Eukaryotic Antimicrobial Peptides And Their Analogues

Luis Rivas
Centro de Investigaciones Biológicas (CSIC) Velázquez 144. 28006-Madrid (Spain).

Leishmania is a human and animal parasitic Protozoa responsible for leishmaniasis, an infection prevalent mainly in tropical and subtropical regions, with a broad range of clinical manifestations depending both on the *Leishmania* specie and on immunological status of the host. Interaction between *Leishmania* and antibiotic peptides raises interest from a clinical perspective, as alternative drugs againts the growing cases of multirresistant strains , and from a biochemical point of view, due to the oddities of the plasma membrane of this parasite, such as abundant GPI compounds, including a metalloproteinase and a highly anionic oligosaccharide, the lipophosphoglycan (LPG), and low rate of endocytosis.

During the last years we have been studying several eukaryotic antibiotic peptides on this parasite; our work has been mainly focused on cecropin A-melittin hybrids; the leishmanicidal effect of a selected set of these analogues did not show a direct correlation with their corresponding antibactericidal or fungicidal activities. CA(1-8)M(1-18) showed the highest leishmanicidal activity, with a MIC in the micromolar range . The mechanism of action of CA(1-8)M(1-18) on the promastigote, the insect extracellular form of the parasite, was studied first; the main target was permeabilization of the plasma membrane, with a fast kinetics, dissipation of membrane potential and drop in intracellular levels of ATP; the first step is an electrostatic interaction beween the polycationic peptide with the anionic phospholipids of the membrane, as it is quenched by both foreign polyanions (heparin) and the own LPG present in the promastigote, representing a defense mechanism for the parasite.

By contrast, purified amastigotes, the pathological form of this parasite for vertebrates, which dwells in the parasitophorus vacuole of the macrophage, were much more resistant resistant than the promastigote *in vitro* against all these peptides, despite its lack of LPG and poor proteolytic activity associated with its plasma membrane, consequently, this resistance was possibly due to the phospholipid composition of its plasma membrane. Nevertheless, CA(1-8)M(1-18) was active on amastigotes, either when applied as ointment on *Leishmania* ulcers of infected mice, or *in vitro* on intracellular amastigotes inside infected macrophages, due to an indirect effect on the macrophage, by induction of the iNOS and production of NO, which implied induction of NF-κB elements. NO production synergized with Th1-cytokines, natural inducers of iNOS expression, but it did not overcome anergy induced by Th2 cytokines.

New methods to assay leishmanicidal activity of other antibiotic peptides, as well as comparison of cecropin A-melittin peptides with other antibiotic peptides active on *Leishmania* will be discussed.

1.-Velasco, M., Díaz-Guerra, M.J.M., Díaz-Achirica, P., Andreu, D., Rivas, L. and Boscá, L. (1997) ✌Induction of nitric oxide synthase and apotosis in RAW cells by cecropinA-melittin hybrid peptide CA(1-8)M(1-18).J.Immunol. 258:4437-4443

2.- Diaz-Achirica, P., Guinea, A., Ubach, J., Andreu, D. and Rivas, L. (1998) ✌ The plasma membrane from Leishmania donovani promastigotes is the main target fror CA(1-8)M(1-18), a synthetic cecropin A-melittin hybrid . Biochem. J.330,453-460.

Expression Of Human Beta-Defensin 1 And 2 In Normal Human Skin

Amy Falconer, Rozina Ali, Anthony G Quinn

Centre for Cutaneous Research, St Bartholomew's and the Royal London Hospital School of Medicine & Dentistry, 2 Newark St , Whitechapel, London

Skin infections make a substantial contribution to the total burden of infectious disease worldwide. In addition to their role as primary pathogens many organisms aggravate inflammatory skin conditions such as atopic eczema or contribute to the delayed healing of chronic ulcers and infected surgical wounds. Natural antibiotics are conserved peptides with broad antimicrobial activity which contribute to the innate resistance of insects, plants and mammals to invading pathogens. The identification of beta defensins and other antimicrobial peptides in epithelial tissues suggests that these peptides may play an important role in the innate resistance of the skin to infection. Clinical studies suggest that there are marked differences between individuals in their susceptibility to skin infections. As part of our research investigating the role of antimicrobial peptide antibiotics in skin disease we have examined the expression of human beta defensin 1 and 2 (hBD-1 and hBD-2) in normal skin samples to look for evidence of inter-individual and site specific differences in expression.

Total RNA was isolated from normal skin samples from a variety of sites. For some samples, tissue was fixed in paraformaldehyde and processed for *in situ* hybridisation. hBD-1 and hBD-2 expression was examined using RT-PCR. For *in situ* hybridisation amplified product was directionally cloned into pBluescript and/or pGEM and sequenced.

hBD-1 was detected by RT-PCR in 21 of 23 normal skin samples. hBD-2 was detected in 13 of 23 samples. *In -situ* hybridisation localised hBD-1 and 2 transcripts to the suprabasal keratinocytes in the skin samples and there was a good correlation with the result from the *in situ* analysis. Site specific differences were seen for hBD-2 but not hBD-1 with strong hBD-2 expression in 7 of 7 foreskin samples but only 6 of 16 samples from other sites. Cultured keratinocytes from foreskin and other skin sites expressed both hBD1 and hBD-2.

Our results suggest that both HBD-1 and HBD-2 are constitutively expressed in normal skin and that these genes may contribute to the innate resistance of skin to infections. The inter-individual and site specific differences in beta-defensin expression identified in this study will need to be taken into account when assessing the significance of alterations in defensin expression in inflammatory skin conditions. Further studies are required to investigate the relationship between beta-defensin expression and susceptibility to skin infections.

Session 5

Chair: Luis Rivas

MULTIPLE AND COMBINATORIAL PEPTIDE SYNTHESIS APPLIED TO THE DEVELOPMENT OF ANTIBACTERIAL PEPTIDES

Sylvie Blondelle[1], Richard A. Houghten[1], Concepción Abad[2] and Enrique Pérez Payá[1,2]

[1] Torrey Pines Institute for Molecular Studies, San Diego, CA 92121 USA

[2] Departament de Bioquímica i Biologia Molecular, Universitat de Valencia, E-46100 Burjassot, Valencia, Spain

The need to discover novel antibacterial compounds with new and improved activities provides interesting challenges for the design of synthetic pathways with a high chance of success, together with a good likelihood of discovering exciting new lead compounds that would allow the development of new antibacterial compounds of great scientific and commercial value. One of the most effective ways of finding new and useful antibacterials is by screening of combinatorial libraries. The chemical diversity generated by combinatorial methods offers microbiologists a potential set of unseen leads. When a lead is obtained, strategies of peptide analoging can be developed to further understand the structure-activity relationship. Furthermore, the recent successes in generating peptidomimetic and organic compound libraries make the combinatorial technology a valuable tool in the search for novel antimicrobial agents. These developments take on increased significance because of the alarming emergence of strains resistant to existing therapeutic regimens.

References:

Blondelle, S.E., Pérez Payá, E. and Houghten, R.A. (1996) *Antimicrob. Agents Chemother.*, 40 (5) 1067.

Blondelle, S.E., Takahashi, E., Houghten, R.A. and Pérez-Payá, E. (1996) *Biochem. J.*, 313, 141.

Salom, D., Pérez-Payá, E., Pascal, J. and Abad, C. (1998) *Biochemistry* 37, 14279.

HOW DO ANTIMICROBIAL PEPTIDES LYSE BACTERIAL MEMBRANE? FROM NATIVE MOLECULES TO DE-NOVO DESIGNED

Yechiel Shai. Department of Biological Chemistry, The Weizmann Institute of Science, Rehovot, 76100, Israel.

The increasing resistance of bacteria to conventional antibiotics resulted in a strong effort to develop antimicrobial compounds with new mechanisms of action. Antimicrobial peptides seem to be a promising solution to this problem. The most studied group includes the linear, mostly amphipatic α- helical peptides. Although the exact mechanism by which they kill bacteria is not clearly understood, it has been shown that peptide-lipid interaction leading to membrane permeation plays a role in their activity. Membrane permeation by amphipatic α-helical peptides has been proposed to proceed via several mechanisms described by the following models: (i) transmembrane pore formation via a "barrel-stave" model; (ii) the "self-promoting uptake" process; (iii) membrane destruction / solubilization via a "carpet-like" (detergent-like) model; and (iv) the torodial model. Studies on the antimicrobial peptides cecropins, dermaseptins and derivatives of pardaxin using fluorescence, Attenuated Total Reflectance Fourier-Transform Infrared (ATR-FTIR), and CD spectroscopy revealed a non "barrel-stave", but rather a "carpet" mechanism as their mode of action. The later mechanism agrees also with the torodial model and the "self-promoting uptake" process. In order to confirm the carpet mechanism, D-amino acids were incorporated into native and de-novo designed cytolytic amphipatic α-helical peptides, thus preventing the resulting diasteriomers to permeate the bacterial wall by forming transmembrane pores but rather by a detergent like manner. These novel group of diasteriomers lost their cytotoxic effects on mammalian cells but retained a high antibacterial activity which was expressed in a complete lysis of the bacteria. These findings open the way for a new strategy in developing a class of highly potent antibacterial polypeptides for the treatment of infectious diseases.

Interaction Of Wheat α-Thionin With Large Unilamellar Vesicles

José M. Caaveiro[1], Antonio Molina[2], Pablo Rodríguez-Palenzuela[2], Félix M. Goñi[1] and Juan Manuel González-Mañas[1]

[1]Grupo Biomembranas (Unidad asociada al CSIC), Departamento de Bioquímica y Biología Molecular, Universidad del país Vasco, Aptdo 644, 48080 Bilbao, Spain

[2]Laboratorio de Bioquímica y Biología Molecular, Departamento de Biotecnología UPM, ETS Ingenieros Agrónomos, 28040 Madrid, Spain.

The interaction of the wheat antibacterial peptide α-thionin with large unilamellar vesicles has been investigated by means of fluorescence spectroscopy. Binding of the peptide to the vesicles is followed by the release of vesicle contents, vesicle aggregation, and lipid mixing. Vesicle fusion, i.e., mixing of aqueous contents, was not observed. Peptide binding is governed by electrostatic interaction and shows no cooperativity. The amphipatic nature of wheat α-thionin seems to desestabilize the membrane bilayer and trigger the aggregation of the vesicles and lipid mixing. The presence of distearoylphosphatidylethanolamine-poly(ethylene glycol 2000) (PEG-PE) within the membrane provides a steric barrier that inhibits vesicle aggregation and lipid mixing but does not prevent leakage. Vesicle leakage through discrete membrane channels is unlikely, because the release of encapsulated large fluorescent dextrans is very similar to that of 8-aminonaphthalene-1,3,6 trisulfonic acid (ANTS). A minimum number of 700 peptide molecules must bind to each vesicle to produce leakage, which suggest a mechanism in which the overall destabilization of the membrane is due to the formation of transient pores rather than discrete channels

Supramolecular complex formation of antimicrobial peptides in lipid bilayers

Katsumi MATSUZAKI

Graduate School of Pharmaceutical Sciences, Kyoto University
Sakyo-ku, Kyoto 606-8501, Japan

A class of antimicrobial peptides, such as magainin 2 and PGLa, is considered to kill bacteria by permeabilizing cell membranes [1, 2]. We have proposed that the peptides recognize acidic phospholipids to form dynamic, peptide–lipid supramolecular complex pores, which induce mutually coupled transbilayer transport of ions, lipids, and the peptides per se [3–8]. This physicochemical process should be regulated by the physicochemical properties of both peptides and lipids. Comparison of data obtained for various amphiphathic peptides concluded that 1) an increase in peptide positive charge decreased pore formation rate and pore stability because of interpeptide electrostatic repulsions and 2) an decrease in polar angle facilitated pore formation [9, 10]. The presence of lipids imposing negative curvature strain on the membrane, such as phosphatidylserine and phosphatidic acid, inhibits pore formation [11].

Magainin 2 and PGLa also form peptide–peptide supramolecular complexes [10]. Resonance energy transfer experiments elucidated that magainin 2 forms a dimer with an association free energy (ΔG) of –9 kJ/mol. Magainin 2 and PGLa are complexed into a 1:1 heterodimer of high activity (ΔG = –15 kJ/mol), which is responsible for synergism between these peptides.

References

1. Matsuzaki, K., *Biochim. Biophys. Acta (Reviews on Biomembranes)*, **1376**, 391–400 (1998).
2. Matsuzaki, K., in *Biomembrane Structures* (Haris, P. I., Chapman, D., Eds.), pp205–227, IOS Press, Amsterdam (1998).
3. Matsuzaki, K., Murase, O., Tokuda, H., Funakoshi, S., Fujii, N., and Miyajima, K., *Biochemistry* **33**, 3342–3349 (1994).
4. Matsuzaki, K., Sugishita, K., Fujii, N., and Miyajima, K., *Biochemistry* **34**, 3423–3429 (1995).
5. Matsuzaki, K., Murase, O., Fujii, N., and Miyajima, K., *Biochemistry* **34**, 6521–6526 (1995).
6. Matsuzaki, K., Murase, O., and Miyajima, K., *Biochemistry* **34**, 12553–12559 (1995).
7. Matsuzaki, K., Murase, O., Fujii, N. and Miyajima, K., *Biochemistry* **35**, 11361–11368 (1996).
8. Matsuzaki, K., Yoneyama, S., Murase, O., and Miyajima, K., *Biochemistry* **35**, 8450–8456 (1996).
9. Matsuzaki, K., Nakamura, A., Murase, O., Sugishita, K., Fujii, N., and Miyajima, *Biochemistry* **36**, 2104–2111 (1997).
10. Matsuzaki, K., Mitani, Y., Akada, K., Murase, O., Yoneyama, S., Zasloff, M., and Miyajima, K., *Biochemistry* **37**, 15144–15153 (1998).
11. Matsuzaki, K., Sugishita, K., Ishibe, N., Ueha, M., Nakata, S., Miyajima, K., and Epand, R. M., *Biochemistry* **37**, 11856–11863 (1998).

INTERFACIAL ACTIVITY AND ANTIPATHOGENIC PROPERTIES OF PULMONARY SURFACTANT PROTEINS.

J. Pérez-Gil[1], I. Plasencia[1], L. Rivas[2], C. Casals[1]

[1]Dept. Bioquímica, Fac. Biología, Universidad Complutense, 28040 Madrid (SPAIN)
[2]Centro de Investigaciones Biológicas, C.S.I.C., Madrid (SPAIN)

SP-C is an hydrophobic polypeptide of 35 residues which is specifically expressed by type II pneumocytes as a component of the pulmonary surfactant lipoprotein complex, essential to stabilize the alveolar epithelium of lungs during the respiratory mechanics. Although the presence of SP-C has been reported as essential to ensure rapid formation of a surface active phospholipid monolayer at the air-liquid interface, its precise role on surfactant dynamics is still under discussion. Structure of SP-C comprises a hydrophobic transmembrane α-helix and a 10-residue N-terminal tail, positively charged, which is dipalmitoylated through its two cysteine residues.

To analyze if the SP-C N-terminal segment has intrinsic properties to interact with phospholipid bilayers, we have analyzed the interaction of a 13-residue synthetic peptide, designed from canine SP-C sequence, with vesicles of the main surfactant phospholipids, phosphatidylcholine (PC) and phosphatidylglycerol (PG). Predictive studies and circular dichroism analysis of its secondary structure in organic solvents suggest that the conformation of this peptide could be dominated by a β-turn motif. The peptide, which is soluble in aqueous buffers, interacted with PC and PG vesicles causing instantaneous vesicle aggregation, and showed higher affinity for anionic than for zwitterionic bilayers as demonstrated by fluorescence spectroscopy. Furthermore, interaction of the peptide with DPPC bilayers caused strong effects on their thermotropic gel-to-fluid phase transition, as studied by differential scannig calorimetry. On the other hand, addition of SP-C N-terminal peptide to cultures of the protozoal parasite *Leishmania* caused dramatic effects on cell viability, at peptide concentrations around 10 μM. Membrane and pathogenicidal activities of this peptide suggest that SP-C N-terminal segment could show more complex interaction with surfactant bilayers or monolayers than expected before. The significance of the observed effects for the role of SP-C on surfactant biology at the alveolar spaces, needs still to be explored, which requires comparative study of this peptide with acylated versions and native sequences isolated from lung tissue.

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POSTERS

Snakins, a new antimicrobial peptide family from plants

Marta Berrocal Lobo, Francisco García Olmedo, and Antonio Molina
Laboratorio de Bioquímica y Biología Molecular
Departamento de Biotecnología- UPM, E.T.S.I. Agrónomos
E-28040 Madrid, Spain

A new type of antimicrobial peptide, snakin-1 (SN1), was isolated from potato tubers and found to be active, at concentrations <10 μM, against bacterial and fungal pathogens from potato and other plant species (Segura et al. 1998). The peptide has 63 amino acid residues (Mr 6,922), 12 of which are cysteines, and has some motifs in common with hemotoxic, desintegrin-like snake venoms. The SN1 amino acid sequence is not related to any previously purified plant protein, although it is homologous to some sequences deduced from cloned plant cDNAs of unknown function, including those of GAST1 from tomato (Shi et al. 1992), and GASA1 to GASA6 from *Arabidopsis thaliana* (Herzog et al. 1995), some of which are induced by gibberelic acid in mutants defficient in this hormone (Shi et al. 1992; Herzog et al. 1995; Ben-Nissan and Weiss 1996). Expression patterns of snakin genes from *A. thaliana* is quite diversified: some of them are seed-specific while others are more widely expressed in an overlapping fashion in different parts of the plant (Herzog et al. 1995; Aubert et al. 1998). Progress in the characterization of the expression patterns of Arabidopsis genes in response to pathogen infection, as well as the purification of snakin proteins, and isolation of T-DNA tagged mutants of snakin genes will be presented.

Aubert, D., Chevillard, M., Dorne, A-M, Arlaud, G., and Herzog, M. 1998. *Plant Mol.Biol.* 36:871-883.
Ben-Nissan, G., and Weiss, D. (1996). *Plant Mol. Biol.* 32:1067-1074.
Herzog, M., Dorne, A.-M., and Grellet, F. 1995. *Plant Mol. Biol.* 27:743-752.
Segura, A., Moreno, M., Madueño, F., Molina, A., and García-Olmedo, F. 1998. *Mol.Plant. Microb. Int.* (in press).
Shi, L., Gast, R.T., Gopalraj, M., and Olszewski, N.E. 1992. *Plant J.* 2:153-159.

SEARCH OF NEW ANTIBACTERIAL COMPOUNDS FROM A PSEUDOPEPTIDE LIBRARY.

Teresa Carbonell[1], Emilia Canton[2], Amparo Pastor[2], Miguel Gobernado[3] and Enrique Pérez-Payá[1].

[1] Dept. Bioquimica i Biologia Molecular, Universitat de València, E-46100 Burjassot, València. Spain.
[2] Unidad Bacteriológica Experimental.- Centro de Investigación. Hospital Universitario. Avda. Campanar, 21 - 46009 Valencia. Spain.
[3] Jefe de Servicio de Microbiologia. Hospital Universitario "La Fe". Avda. Campanar, 21 - 46009 Valencia. Spain.

Nowadays, antibiotic resistance is a mainful problem and it is widespreading from hospitals. A number of bacterial related diseases (tuberculosis, pneumonia, meningitis) that were thought to be eradicated, are now showing up with special virulence; thus bacteria that were successfully treated with well known antibiotics are now resistant to them (i.e., *Streptococcus pneumonia, Staphylococcus aureus, Haemophilus influenzae* and other Gram-negative bacteria). Unfortunately, the development of new antibiotic compounds is being slower than the bacteria's acquisition of new resistance mechanisms.

In this communication, progress on the search of new lead compounds showing antibacterial activity will be presented. As a source to develop such lead compounds we propose a positional scanning pseudopeptide library based on an N-alkylglycine framework, that would provide the chemical diversity needed to ensure an assay-directed synthetic evolution of molecules bearing the desired properties. The library has been synthesized on solid support using new chemical methodologies to allow the incorporation of selected pharmacophoric primary amines as building blocks.

Human lactoferricin induce permeability changes in liposomes and *Escherichia coli*

H. Ostolaza[a], O. Aguilera[b,c], L.M. Quirós[c] and J.F. Fierro[b,c,d]

[a]Department of Biochemistry. University of the Basque Country. Bilbao. [b]Laboratory of Oral Microbiology. School of Stomatology. [c]Department of Functional Biology (Microbiology). Faculty of Medicine. University of Oviedo. Oviedo. [d]Service of Microbiology. Central Hospital of Asturias. Spain.

The antimicrobial active *N*-terminal peptides derived from acid-pepsin hydrolysis of human lactoferrin (lactoferricin H) and bovine lactoferrin (lactoferricin B) have an enhanced activity that the native protein against a more wide range of microorganisms. Lactoferricin H corresponds to amino acid residues 1-47 at the *N*-terminus of human lactoferrin, and includes the antimicrobial sequence (residues 20-37) and the amino acids (residues 28-34) involved in the high-affinitity binding of the whole protein to LPS of *Escherichia coli*. The complete mechanism of antimicrobial action of the lactoferricin is unknown and has been suggested that the mode of action of this cationic peptide includes the disruption of the normal permeability functions of the cytoplasmic membrane.

We investigated the ability of a peptide (Lfpep) of lactoferricin H to interact with phospholipid vesicles and the bacterial cytoplasmic membrane of *Escherichia coli*. Determination of $\Delta\Psi$ and ΔpH in liposomes was performed by generation of a transmembrane K^+-diffusion potential (negative inside) in the phospholipid vesicles that had been pretreated with the dye 3,3´-dipropylthiacarbocyanine [diS-C_3-(5)] and valinomycin. The peptide induced depolarization of transmembrane potential of vesicles and a dissipation of the ΔpH in pyranine-containing liposomes. Interaction with the surface vesicle was assayed through the quenching of intrinsic tryptophan fluorescence, and the induction of dye (ANTS/DPX) release from phospholipid vesicles of different surface charge was determined, showing changes in the Try-fluorescence but the binding did not cause membrane leakage, suggesting that the peptide permeated phospholipid vesicles via pore formation rather that via membrane desintegration.

In order to define the antimicrobial mechanism of action of this peptide of lactoferricin H, the strain of *E. coli* 0111 was used as a bacterial model to compare the findings in liposomes with the activity of the peptide on the bacterial cytoplasmic membrane. In correlation with the observed results for collapse of the potential membrane in *E. coli* phospholipid vesicles, the peptide was able to induce a quick loss of the potential membrane in *E. coli* cells as measured by the isotope [^{3}H]TPP$^+$ distribution method. The ΔpH across the cytoplasmic membrane was determined with [^{14}C] acetic acid showing that the Lfpep-treated cells were unable to mantain the pH gradient, an energy-dependent process.

These results suggest that the main target of lactoferricin is the cytoplasmic membrane of *E. coli*, and that the subsequent induced collapse of the potential membrane is the cause of its lethal effect.

INTERACTION OF α-THIONIN WITH LIPIDIC VESICLES

J.M.M. Caaveiro, I.G. Aguirre, F.M. Goñi and J.M. González-Mañas.
Department of Biochemistry and Molecular Biology, Faculty of Sciences, University of the Basque Country, P.O.Box 644, 48080 Bilbao, Spain.

Thionins are cystine-rich, basic polypeptides with a molecular mass of about 5000, which are quite abundante in the endosperms of many Graminae. They were the first plant peptides for which an antimicrobial activity had been reported [1], and have been shown to alter model and cell membrane permeability [2,3].

In our work, we have used large unilamellar vesicles with different lipid compositions and a set of fluorescent probes to study in detail diverse aspects of the interaction of wheat α-thionin with model membranes

Our results indicate that α-thionin interacts preferently with negatively charged liposomes producing several effects (i.e. leakage, lipid mixing and aggregation of vesicles). We failed to observe vesicle fusion. The binding of the protein is governed by the net charge of the membrane and the ionic strength, showing that the interaction is mainly electrostatic. A minimum number of 700 molecules of peptide per vesicle are necessary to obtain complete release of the contents. The small size of α-thionin and the fact that high molecular weight dextrans are released from the vesicles argues against the formation of discrete channels.

In the presence of calcium ions, the vesicles aggregate without loosing their contents, indicating that leakage and aggregation are independent phenomena. The presence within the membrane of lipids with a bulky polar head group which impose a steric hindrance (PEG-PE or DGDG) affect the interacion in a different way. Whereas PEG-PE inhibits aggregation without affecting the release of the contents, DGDG inhibits the release without affecting the aggregation of the vesicles.

We propose a "carpet-like" model to explain these results: (1) the α-thionin molecules bind to the outer surface of the membrane via electrostatic interactions, (2) protein-protein and lipid-protein interactions might result in the disorganization of the bilayer architecture, and (3) the membrane can no longer function as a permeability barrier and the vesicles aggregate. The leakage of entrapped solutes and the aggregation of the vesicles are two processes which take place simoultaneously. However, by choosing the adequate experimental conditions, they can be separated.

References

[1] Stuart, L. S. and Harris, T. H. "Bactericidal and fungicidal properties of a crystalline protein isolated from unbleached wheat flour". *Cereal Chem.* **19** (1942): 288-300.

[2] Carrasco, L., Vázquez, D., Hernández-Lucas, C., Carbonero, P. and García-Olmedo, F. "Thinins: plant peptides that modify membrane permeability in cultured mammalian cells". *Eur. J. Biochem.* **116** (1981): 185-189.

[3] Caaveiro, J.M.M., Molina, A., González-Mañas, J.M., Rodríguez-Palenzuela, P. García-Olmedo, F. and Goñi, F.M. "Differential effects of five antipathogenic plant peptides on model membranes". *FEBS Lett.* **410** (1997): 338-342.

CATIONIC ANTIMICROBIAL PEPTIDES: ACTIVITY IN FOOD MATRICES

Ellen van Kan[1,2], Arie van der Bent[1], Aart van Amerongen[1], Ben de Kruijff[2].

1) Dept. Industrial Proteins, ATO-DLO, PO Box 17, 6700 AA Wageningen, The Netherlands.
2) Dept. Biochemistry of Membranes, University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands.

Cationic peptides are important components of the natural defence against microbial infection of most living organisms. After infection these antimicrobial peptides can be rapidly induced in cells that are involved in immediate defence against microbes. High concentrations of the peptides are found on damaged mucosal surfaces or in specialized cells of which neutrophils are the most important.

Due to their potency and broad activity spectra, these peptides represent a class of antimicrobial agents having interesting characteristics for use in various applications. Within FAIR-project CT97-3135, the application of these antimicrobial components as food preservatives is studied.

For the preservation of food products, it is important that these antimicrobial peptides can act in complex food matrices. In addition to the pH and processing factors, food components like salts, proteolytic enzymes, proteins and many others may interfere with antimicrobial action. As many antimicrobial peptides lose their inhibiting action at high salt concentrations, salt tolerant peptides (showing activity at 100mM NaCl) were selected for structure-activity studies. Clavanins, protegrins and various PR-rich peptides are being investigated for these studies.

These antimicrobial peptides are studied in order to obtain good knowledge of the inhibition potency, mechanisms of action, the selectivity for prokaryotic organisms, as well as the behaviour in more complex media. Food-like conditions are simulated to study the effect of individual food components on the peptides' action.

The influence of mono- and divalent cations on the interaction of the positively charged peptides with bacteria is very diverse. Salt cations and peptides compete for the same binding positions on the membrane. Both the mono- and divalent cations influence the "long-range" electrostatic interactions between the peptide and negatively charged membrane. Compared to monovalent cations, the divalent counterparts increase the stability of the membrane upon binding to the membrane surface. By the addition of salts to the system an osmotic effect will be induced, which inhibits the bacterial growth. Furthermore, the peptide structure itself can be affected according to the theories for protein (de)stabilization (Hofmeister ions).
In general, the overall effect is a combination of several phenomena and it is often difficult to assess which phenomena play a role in a particular peptide-membrane interaction.

Optimisation of a β-sheet peptide with *Perla*, a software that engineers and designs peptides and proteins

Emmanuel Lacroix, Marina Ramírez-Alvarado, Michael Petukhov & Luis Serrano

Structural & Computational Biology Department, EMBL,
Meyerhofstrasse 1, 69117 Heidelberg, Germany

We developed *Perla*, a software that manipulates peptides and proteins, enabling the identification and sorting of amino acid sequences capable of folding into desired three-dimensional structures, and the engineering of sequences at the interfaces of protein multimers. *Perla* reconstructs amino acid sidechains onto the main chain of the molecules, with an accurate atomic description, and samples sequences with efficient optimisation routines for combinatorial problems. A sophisticated energy function scores the sequences, roughly evaluating the energy difference between the molecule folded conformation and a reference state.

Perla has been used to predict mutations to stabilise and destabilise Betanova, a peptide *de novo* designed in our group (Kortemme *et al.*, 1998). Chemical denaturation experiments monitored by fluorescence and NMR measurement of conformational shifts agree with the predicted ranking of the peptides stabilities. Most importantly, a favorable mutation confers to the three-stranded β-sheet a significantly higher stability than the initial design.

Reference:

Tanja Kortemme, Marina Ramírez-Alvarado & Luis Serrano (1998). Design of a 20-amino acid, three-stranded β-sheet protein. *Science 281*: 253-256

Gloverin, an antibacterial *Trichoplusia ni* protein

Gang Liu, Annika Lundström, Daiwu Kang, and Håkan Steiner

Department of Microbiology, Stockholm University, 10609 Stockholm, Sweden

Antibacterial peptides and proteins are important in combating pathogens in invertebrates. A gloverin cDNA was cloned from last instar larvae of cabbage looper, *Trichoplusia ni* by differential display PCR (DD-PCR). It codes for a 174 amino acid residue protein including a 19 residue signal sequence and a 24 residue prosegment. The expression of the gene was inducible and reached the highest level after 20 hr upon bacterial challenge. The mature protein showed 49% identity to *H. gloveri* gloverin.

T.ni gloverin cDNA with a six amino acid residue histag at the C-terminus was expressed in the baculovirus system. After affinity purification on a Ni-column, a 18 kDa protein was isolated. It was shown to contain the propart. This progloverin inhibits the growth of *E coli*. The minimal concentration for growth inhibition was 0.3 uM. A 14 kDa protein in immune larval hemolymph was detected by Western blot with an anti-gloverin antibody. The antibacterial activity of histagged proprotein and mature protein will be compared.

STUDY OF THE MEMBRANE PERTURBATION INDUCED BY THE HEMOLYTIC PROTEIN STICHOLYSIN II: POTENTIAL INVOLVEMENT OF TETRAMERIC PORE.

De los Ríos,V.(1); Manchefio, J.M.(1); Martin-Benito, J. (2) ; Oñaderra, M.(1) and Gavilanes, J.G (1)

(1) Dpto. Bioquímica y Biología Molecular I, Facultad de Ciencias químicas, UCM 28040. Madrid
(2) Centro Nacional de Biotecnología, CSIC, UAM 28049. Madrid

Sticholysin II (Stn II) is a hemolytic protein isolated from the sea anemone *Stichodactyla helianthus*. To analyze the mechanism of Stn II bilayer interaction we have studied the interaction of Stn II with model membranes. Stn II induces leakage of aqueous contents of vesicles composed of phosphatidylcholine (PC) or sphingomyelin (SM) only when cholesterol (Cho) is included in the bilayers, although cholesterol itself is not essential for membrane damage as binary systems (PC:SM) are also susceptible to the toxin action. Kinetic analysis of the leakage process suggsts the existence of the protein -protein interactions as a requirement for membrane perturbation what can be interpreted in terms of protein oligomerization. Moreover, the study of the dependence of the Stn II induced leakage on the molecular size of the encapsulated solutes permits to conclude the existence of membrane damage with a maximum overall diameter about 1 nm. Electron microscopy studies of two dimensional crystals of Stn II on lipid monolayers show the existence of a structure that clearly resembles a tetrameric pore. Finally, the existance of conformational states with properties typical from partly folded polypeptides has been deduced from the spectroscopic properties of Stn II at acid pH values

TRANSCRIPTIONAL REGULATION OF THE BOMBININ GENE EXPRESSION

Rossella Miele[1], Gunnel Bjorklund[2], Donatella Barra[1], Maurizio Simmaco[3] and Ylva Engström[2]

[1]Department of Biochemical Sciences, University La Sapienza, 00185 Roma, Italy; [2]Department of Molecular Biology, Arrhenius Laboratories for Natural Sciences, Stockholm University, S-106 91 Stockholm, Sweden; [3]Department of Biomedical Sciences, University G. D'Annunzio, 66100 Chieti, Italy.

Many immunity related genes are under the dual control of the Rel-containing transcription factor NF-κB and different inhibitors called IκBs. In mammalian cell cultures, it has been demonstrated that glucocorticoids induce a dramatic synthesis of IκBα, thus blocking the transcription of genes with promoter sites for NF-κB binding. We have demonstrated that the *de novo* synthesis of antibacterial peptides present in the skin secretion of two different amphibian species (*Rana esculenta* and *Bombina orientalis*) is inhibited by treatment of the frog with glucocorticoids [1-3]. A concomitant increase of IκBα levels was also observed. So far, all mammalian genes encoding antibacterial peptides have been found to contain promoter motifs that potentially bind NF-κB-like factors.

We have determined the structure of two genes encoding bombinins from *B. orientalis* [3]. Upstream sequences of these genes were found to contain motifs related to NF-κB and NF-IL6 binding sites. We have also demonstrated that the expression of genes coding for antimicrobial peptides in *B. orientalis* is induced after contact with bacteria [3].

In order to study the regulation of the synthesis of antimicrobial peptides upon bacterial infection, we used an insect cell line as a model system. The Drosophila hemocyte cell line mbn-2 was transfected with the promoter of a bombinin gene fused to a bacterial gene for β-galactosidase. LPS was used to induce an immune response in these cells. We could demonstrate that LPS induces the expression of the reporter gene. The level of expression was proportional to the amount of construct added, until a saturation level was reached. Expression could be increased further by co-transfection with the Dif gene, the Drosophila Rel factor. These results indicate that the κB sites in the bombinin promoter are functional and that Dif has a strong transactivation capacity even on a heterologous promoter.

1. Simmaco, M., Boman, A., Mangoni, M.L., Mignogna, G., Miele, R., Barra, D., Boman, H.G. (1997) *FEBS Lett.* 416, 273-275.
2. Barra, D., Simmaco, M., Boman, H.G. (1998) *FEBS Lett.* 430, 130-134.
3. Miele, R., Ponti, D., Boman, H.G., Barra D., Simmaco, M. (1998) *FEBS Lett.* 431, 23-28.

Preliminary evaluation of the ophthalmic application of cecropin A - melittin hybrid peptides as antimicrobial agents.

Authors: A. Morilla-Grasa, PhD (1); J.L. Ordóñez, BSc (1); M. Portolés, PhD.(2); D. Andreu, PhD (3) & S. Nos-Barberá, PhD (1,4).
(1) Universidad de Valladolid, Valladolid, Spain.
(2) present address Channing Laboratory, Brigham & Women's Hospital, Harvard Medical School, Boston, MA, USA.
(3) Universitat de Barcelona, Barcelona, Spain.
(4) University of Louisville, KY, USA.

Introduction.
The ophthalmic application of cecropins and related peptides has been claimed based on: in vitro activity studies against corneal ulcer pathogenic isolates, growth factor activity studies in rabbit corneal epithelium cells and corneal preservation studies during storage before transplantation.
We used "hybrid peptides" from cecropin A and melittin in order to obtain shortened size peptides maintaining the high activity of natural cecropin A but lower toxicity levels. A preliminary evaluation of their efficacy and safety for a potential ophtnalmic use as antimicrobial agents was carried out based on the following pattern.

Methodology.
A group of cecropin A-melittin hybrid peptides was tested for *in vitro* activity, using a microdilution test; Minimal Inhibitory Concentration (MIC) values were obtained against a broad taxonomical spectrum of collection and clinical strains (*S. aureus, B. subtilis, E. coli* and *P. aeruginosa*).

Selected peptides were then studied for *in vivo* activity, in an experimental model of pseudomonal keratitis in albino rabbits under different experimental conditions. Variables were related to: peptide, source of bacterial strain, inoculum size and treatment (application and regimen). Clinical evaluation of the anterior segment inflammatory response was recorded at different times.

In parallel studies *in vitro* toxicity of these peptides was determined by measuring mitochondrial succinate dehydrogenase activity (MTT test) of SIRC cells (rabbit corneal epithelium cells).

Results.
Two cecropin A-melittin hybrid peptides of 18 (cecropin 1-8 and melittin 1-10) and 15 (cecropin 1-7 and melittin 5-12) residues were identified as "promising agents" based on: a) MIC values $\leq$ 8 µg/ml, b) No statistical differences between peptide, gentamicin and ciprofloxacin treatments but differences from control group ($p \leq 0.05$) in a keratitis model and c) Concentrations about those of MIC values showed no toxicity against SIRC cells.

Conclusion.
As a preliminary evaluation of efficacy and safety at least two peptides showed a promising profile to be considered potential candidates for a new topical antimicrobial treatment in certain types of corneal infection.

Effect of a fragment of HNP (human neutrophil peptide) on bacterial attachment to corneal epithelium: Is it a "preliminary step" for a new therapeutical hipothesis alternative to the use of conventional antimicrobials?

Authors: S. Nos Barberá, PhD; A. Morilla, PhD, J.L. Ordóñez, B.Sc. & M.Portolés, PhD.
University of Valladolid, Valladolid, Spain.

Introduction.
Adherence is considered an initial step during the instauration of the infection process.
Pseudomonas aeruginosa is a common causal agent of keratitis and keratoconjunctivitis with devasting consequences in vision if the treatment is not applied quickly enough and effectively.
The inhibitory effect of two synthetic peptide, not related to bacterial adhesins, is now described on the adherence of Pseudomonas aeruginosa to the corneal epithelium.
These two peptides have 16 and 21 residues with a common sequence of 9 residues (IAGERRYGT), three R residues and one disulphonide bond between the two C terminal residues. These sequences are part of certain HNP sequences (Human Neutrophil Peptides).

Methodology.
The inhibitory effect of these peptides was tested, at different concentrations and time of contact, in three different experimental systems: a) In vitro in primary cell cultures of NZ rabbit corneal epithelium, b) Ex vivo in an organ culture of NZ rabbit cornea and c) In vivo in an experimental model in pigmented rabbits.
Estimation of adhered bacteria and calculation of the adherence inhibition was obtained by three different methods: a) agar plate counting, scanning electron microscopy and radiolabelled counts.

Results.
The effect on adherence is peptide concentration dependent and not inoculum size dependent (under certain limits). Concentration of 10mcg/ml inhibit around 90% of bacterial attachment in the three experimental conditions.
This effect has no relation with real antimicrobial activity or citotoxicity as both peptides show no antimicrobial or citolitic activity.

Conclusion.
These preliminary results show a first approach for a potential application of certain peptides as novel "antibacterial attachment agents". This new therapeutical hypothesis alternative to the use of conventional antimicrobials might be useful in practice for some specific procedures (surgery) and high risk patients.

LIST OF INVITED SPEAKERS

David Andreu — Department of Organic Chemistry, University of Barcelona, Marti i Franquès 1-11, 08028 Barcelona. (Spain). Tel. / Fax: 34 93 402 1260. E-mail: andreu@admin.qo.ub.es

Donatella Barra — Dipt. di Scienze Biochimiche, Universitá La Sapienza, Piazzale Aldo Moro 5, 00185 Roma (Italy). Tel.: 39 6 445 6663. Fax: 39 6 444 0062. E-mail: barra@axrma.uniromal.it

Charles L. Bevins — Dpt. of Immunology, Research Institute/NN10, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH. 44195 (USA). Tel.: 1 216 444 9107. Fax: 1 216 444 9329. E-mail: bevinsc@cesmtp.ccf.org

Hans G. Boman — Microbiology and Tumor Biology Center, Karolinska Institutet, Box 280, SE-171 77 Stockholm (Sweden). Tel.: 46 8 728 6209. Fax: 46 8 342 651. E-mail: Hans.G.Boman@mtc.ki.se

Bruno P.A. Cammue — F. A. Janssens Laboratory of Genetics, Catholic University of Leuven, Kardinaal Mercierlaan 92, B-3001 Leuven (Belgium). Tel.: 32 16 329 682. Fax: 32 16 321 966. E-mail: bruno.cammue @agr.kuleuven.ac.be

Tomas Ganz — UCLA School of Medicine, Dpt. of Medicine, University of California, Los Angeles, CA. 90095-1736 (USA). Tel.: 1 310 825 6112. Fax: 1 310 206 8766. E-mail: tganz@mednet.ucla.edu

Francisco García-Olmedo — Laboratorio de Bioquímica y Biología Molecular, Departamento de Biotecnología, E. T. S. de Ingenieros Agrónomos, Avda. Complutense s/n., 28040 Madrid (Spain). Tel.: 34 91 336 5706. Fax: 34 91 336 5757. E-mail: olmedo@bit.etsia.upm.es

Renato Gennaro — Dipt. di Biochimica, Biofisica e Chimica delle Macromolecole, Universitá degli Studi di Trieste, Via L. Giorgieri 1, I-34127 Trieste (Italy). Tel.: 39 40 676 3696. Fax: 39 40 676 3691. E-mail: gennaro@bbcm.univ.trieste.it

Félix M. Goñi — Grupo Biomembranas, Dpto. de Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad del País Vasco, Campus de Leioa, Apdo. 644, 48080 Bilbao (Spain). Tel.: 34 94 464 7700. Fax: 34 94 464 8500.

Charles Hetru	Institute of Molecular and Cellular Biology (IBMC) CNRS and University Louis Pasteur, Strasbourg, (France). Tel.: 33 3 88 417 037. Fax: 33 3 88 606 922. E-mail: hetru@ibmc.u-strasbg.fr
Jules A. Hoffmann	Institute of Molecular and Cellular Biology, (IBMC) CNRS, and University Louis Pasteur, Strasbourg, (France). Tel.: 33 3 88 417 077. Fax: 33 3 88 606 922. E-mail: jhoff@ibmc.u-strasbg.fr
Fotis C. Kafatos	European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117-Heidelberg (Germany). Tel.: 49 62 21 387 201. Fax: 49 62 21 387 211. E-mail: kafatos@embl-heidelberg.de
Katsumi Matsuzaki	Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501 (Japan). Tel.: 81 75 753 4574. Fax: 81 75 761 2698. E-mail: Katsumim@pharm.kyoto-u.ac.jp
Shunji Natori	Graduate School of Pharmaceutical Sciences, University of Tokyo, Bunkyo-ku Tokyo 113-003 (Japan). Tel.: 81 3 5684 2973. Fax: 81 3 5684 2973. E-mail: fnatori@hongo.ecc.u-tokyo.ac.jp
Enrique Pérez-Payá	Dpto. de Bioquímica y Biología Molecular, Universidad de Valencia, Doctor Moliner 50, 46100 Burjassot, Valencia (Spain). Tel.: 34 96 386 4385, Fax: 34 96 386 4635. Email: paya@uv.es
Luis Rivas	Centro de Investigaciones Biológicas, CSIC, Velázquez 144, 28006 Madrid (Spain). Tel.: 34 91 561 18 00. Fax: 34 91 562 75 18. E-mail: cibli@fresno.csic.es
Pablo Rodríguez-Palenzuela	Dpto. de Biotecnología, E.T.S. Ingenieros Agrónomos, UPM, Avda. Complutense s/n, 28040 Madrid (Spain). Tel.: 34 91 336 5705. Fax: 34 91 336 5757. E-mail: PABLO@BIT.ETSIA.UPM.ES
Michael E. Selsted	Dpt. of Pathology, College of Medicine, University of California, Irvine, CA. 92612 (USA). Tel.: 1 949 824 2350. Fax: 1 949 824 2346. E-mail: meselste@uci.edu
Yechiel Shai	Department of Biological Chemistry, The Weizmann Institute of Science, Rehovot 76100 (Israel). Tel.: 972 89 342 711. Fax: 972 89 344 112. E-mail: bmshai@Weizmann. Weizmann.ac.il
Jeffrey J. Smith	Department of Pediatrics, University of Iowa College of Medicine, 200 Hawkins Drive, Iowa City, IA 52242 (USA). Tel.: 1 319 356 7265. Fax: 1 319 356 7171. E-mail: smithjj@blue.weeg.uiowa.edu

LIST OF PARTICIPANTS

Teresa Alarcón — Hospital de la Princesa, Servicio de Microbiología, c/Diego de León 62, 28006 Madrid (Spain). Fax: 34 91 309 00 47.

Marta Berrocal — Lab. de Bioquímica y Biología Molecular, Dpto. de Biotecnología, UPM. E.T.S.I. Agrónomos, 28040 Madrid (Spain). Tel.: 34 91 336 57 09. Fax: 34 91 336 57 57. E-mail: mberrocal@bit.etsia.upm.es

Anita Boman — Microbiology and Tumorbiology Center, Karolinska Institutet, Box 280, SE-171 77 Stockholm (Sweden). Tel.: 46 8 728 6209. Fax: 46 8 342 651.

Emilia Cantón — Unidad Bacteriológica Experimental, Centro de Investigación, Hospital Universitario La Fé, Avda. Campanar 21, 46009 Valencia (Spain). Tel.: 34 96 386 27 97. Fax: 34 96 386 87 18.

Teresa Carbonell — Dept. de Bioquímica i Biología Molecular, Universitat de València, Dr. Moliner 50, 46100 Burjassot, Valencia (Spain). Tel.: 34 96 386 43 85. Fax: 34 96 386 46 35. E-mail: Teresa.Carbonell@uv.es

Cristina Casals — Dpto. de Bioquímica y Biología Molecular I, Facultad de Biología, Universidad Complutense, 28040 Madrid (Spain). Tel.:34 91 394 4261. Fax: 34 91 394 4672. E-mail: casals@solea. quim.ucm.es

David Derry — Division of Infectious Diseases, St. George's Hospital Medical School, Cranmer Terrace, London SW17 0RE (U.K.). Tel.: 44 181 725 5330. Fax: 44 181 725 3487.

J. Fernando Fierro — Department of Functional Biology (Microbiology), Faculty of Medicine, University of Oviedo, c/ Julián Clavería, s/n, 33006 Oviedo (Spain). Tel.: 34 985 10 35 55. Fax: 34 985 10 35 34. E-mail: ffr@sauron. quimica.uniovi.es

Juan M. González Mañas — Dept. of Biochemistry and Molecular Biology, Faculty of Sciences, University of the Basque Country, P.O. Box 644, 48080 Bilbao (Spain). Tel.: 34 94 464 77 00. Fax: 34 94 464 85 00. Gbpgomaj@lg. ehu.es

Ion Gutiérrez-Aguirre — Dpt. of Biochemistry and Molecular Biology, Faculty of Sciences, University of the Basque Country, P.O. Box 644, 48080 Bilbao (Spain). Tel.: 34 94 464 77 00. Fax: 34 94 464 85 00. E-mail: gbbguagj @lg.ehu.es

Ellen J. M. van Kan	Dept. Industrial Proteins, (ATO-DLO), Bornsesteeg 59, P.O. Box 17, 6700 AA Wageningen (The Netherlands). Tel.: 31 317 47 50 00. Fax: 31 317 47 53 47. E-mail: e.j.m.vankan@ato. dlo.nl
Valle Lacadena	Dpto. de Bioquímica y Biología Molecular I, Facultad de Química, Universidad Complutense, 28040 Madrid (Spain). Tel.: 34 91 394 41 58. Fax: 34 91 394 41 59.
Emmanuel Lacroix	Structural & Computational Biology Department, EMBL, Meyerhofstrasse 1, 69117 Heidelberg (Germany). Tel.: 49 6221 387 402. Fax: 49 6221 387 306. E-mail: lacroix@embl-heidelberg. de
Gang Liu	Department of Microbiology, Stockholm University, 10609 Stockholm (Sweden). Tel.: 46 8 16 41 59. Fax: 46 8 61 29 552. E-mail: Gang.Liu@mibi.su.se
Emilia López-Solanilla	Dpto. de Biotecnología, E.T.S. Ingenieros Agrónomos, Ciudad Universitaria, 28040 Madrid (Spain). Tel.: 34 91 336 57 09. Fax: 34 91 336 57 57. E-mail: emilia@bit.etsia.upm.es
José Miguel Mancheño	Dpto. de Bioquímica y Biología Molecular I, Facultad de Ciencias Químicas, UCM, 28040 Madrid (Spain). Tel.: 34 91 394 42 58. Fax: 34 91 394 41 59. E-mail: jmmg @solea.quim.ucm.es
José Manuel Martínez-Caaveiro	Department of Biochemistry and Molecular Biology, Faculty of Sciences, University of the Basque Country, P.O. Box 644, 48080 Bilbao (Spain). Tel.: 34 94 464 77 00. Fax: 34 94 464 85 00. E-mail: gbbmacaj@lg.ehu.es
Alvaro Martínez del Pozo	Dpto. de Bioquímica y Biología Molecular I, Facultad de Química, Universidad Complutense, 28040 Madrid (Spain). Tel.: 34 91 394 41 58. Fax: 34 91 394 41 59.
Rossella Miele	Department of Biochemical Sciences, University La Sapienza, Piazzale Aldo Moro 5, 00185 Roma (Italy). Tel.: 39 06 445 66 63. Fax: 39 06 444 00 62. E-mail: rmiele@axrma.uniroma1.it
Antonio Molina	Laboratorio de Bioquímica y Biología Molecular. E.T.S. Ingenieros Agrónomos, Dpto. de Biotecnología, UPM, 28040 Madrid (Spain). Tel.: 34 91 336 57 09. Fax: 34 91 336 57 57. E-mail: molina@bit. etsia.upm.es
Antonio Morilla	IOBA, Facultad de Medicina, Universidad de Valladolid, Av. Ramón y Cajal 7, (Planta 3ª), 47005 Valladolid (Spain). Tel.: 34 983 42 35 34. Fax: 34 983 42 32 74. E-mail: amorilla@ioba.med. uva.es

Josefa Muñoz Alamillo — Dpto. de Biotecnología, E.T.S. Ingenieros Agrónomos, Avda. Complutense s/n°, Ciudad Universitaria, 28040 Madrid (Spain). Fax: 34 91 336 57 57.

Salvador Nos Barberá — IOBA, Facultad de Medicina, Universidad de Valladolid, Av. Ramón y Cajal 7, (Planta 3ª), 47005 Valladolid (Spain). Tel.: / Fax: 34 983 42 32 74. E-mail: nos@ ioba.med.uva.es

Jesús Pérez-Gil — Dpto. de Bioquímica, Facultad de Biología, Universidad Complutense, 28040 Madrid (Spain). Tel.: 34 91 394 42 61. Fax: 34 91 394 46 72. E-mail: perejil@ solea.quim.ucm.es

Anthony G. Quinn — Centre for Cutaneous Research, St. Bartholomew's & The Royal London Hospital, School of Medicine & Dentistry, 2 Newark St. Whitechapel, London E1 2AT (U.K.). Tel.: 44 171 295 7166. Fax: 44 171 295 7171. E-mail: quinn@icrf.icnet.uk

Vivian de los Ríos — Dpto. de Bioquímica y Biología Molecular I, Facultad de Ciencias Químicas, Universidad Complutense, 28040 Madrid (Spain). Tel.: 34 91 394 41 58. Fax: 34 91 394 41 59. E-mail: vivian@solea. quim.ucm.es

Blanca San Segundo — Departamento de genética Molecular, CID-CSIC, Jordi Girona 18-26, 08034 Barcelona (Spain). Tel.: 34 93 400 61 00. Fax: 34 93 204 59 04. E-mail: bssgmb @cid.csic.es

Monisha G. Scott — Dept. of Microbiology & Immunology, University of British Columbia, #300 - 6174 University Boulevard, Vancouver, BC. V6T 1Z3 (Canada). Tel.: 1 604 822 33 08. Fax: 1 604 822 60 41. E-mail: gough@interchange.ubc.ca

Laura Vila — Dpto. de Genética Molecular, CID-CSIC, Jordi Girona, 18-26, 08034 Barcelona (Spain). Tel.: 34 93 400 61 00. Fax: 34 93 204 59 04. E-mail: lvugmb@cid.csic. es

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Instituto Juan March de Estudios e Investigaciones
Castelló, 77 • 28006 Madrid (España)
Tel. 34 91 435 42 40 • Fax 34 91 576 34 20
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