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ICGEB RESEARCH GRANTS PROGRAMME

RESEARCH GRANTS
COMPLETED
IN 2013

CRP - ICGEB RESEARCH GRANTS COMPLETED IN 2013



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PERU	Luisa Ines ECHEVARRIA CUREE	Hormonal and genetic characterization of ovarian follicular maturation in alpacas
RUSSIA	Aleksander G. GABIBOV	The role of B cell response in Multiple Sclerosis
RUSSIA	Nikolay Vasilevich GNUCHEV	Involvement of PGRP-S protein and its complex with Hsp70 in the cancer cells killing
SERBIA	Branka VASILJEVIC	Microbial diversity in the copper mine Bor
SLOVENIA	Elsa FABBRETTI	Pesticides and neuronal sensitization: a pilot study
URUGUAY	Marcos MONTESANO	Modulation and characterisation of potato defence

ARGENTINA

Title: Molecular and structural basis of NK cell receptors interaction with viral molecules

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ICGEB Contract No.: CRP/09/001

ICGEB Reference No.: CRP/ARG09-02

Abstract: The main goal of this project is to understand the structural basis for recognition by NK cell receptors. In mice, the inhibitory receptors that recognise MHC-I belong to the Ly49 family. NK cells also present activating Ly49 which ligands were not thoroughly analysed. Viruses encode decoy proteins that mimic the host's molecules to escape immunosurveillance. The murine cytomegalovirus (MCMV) encodes an MHC-I-like molecule, m157, which binds the inhibitory receptor Ly49I, but also the activating Ly49H. In addition, MCMV somehow modifies the MHC-I molecule H-2D^k in a manner that induces NK cells carrying the activating Ly49P receptor to kill the target cell. We analyse the interaction between activating Ly49s (H, P, D, L) and the MHC-I H2D^k through SPR employing a Biacore T100. We found that activating Ly49 react with H2D^k, despite the latter is not modified by m04. We next analysed m157 binding to immobilised Ly49H. The Ly49H-m157 K_D is one order bigger than the inhibitory couple Ly49I-m157. These results suggest that in spite Ly49H recognise m157 with a lower affinity than the inhibitory receptor Ly49I, it will be sufficient to allow NK cell to kill MCMV infected cells.

Objectives:

During evolution, viruses have developed an array of mechanisms to escape surveillance by the immune system, including production of decoy proteins that mimic the host's molecules for the purpose of avoiding nonself recognition. The murine cytomegalovirus (MCMV) genome codes for an MHC-I-like molecule, m157, which binds the inhibitory receptor Ly49I. The m157 protein is displayed on the surface of MCMV-infected cells to prevent NK cell attack. We will carry out biophysical and structural studies of the interaction of the inhibitory receptor Ly49I with the viral protein m157. The host immune system counterattacks with activating NK receptors, such as Ly49H, that activate NK cells once they encounter m157 decoy molecules on the surface of target cells. This interaction protects the host against viral infection by killing the infected cell. We will conduct research to understand the biophysical and structural basis for the interaction of the activating receptor Ly49H with the MCMV protein m157. In addition, MCMV somehow modifies the MHC-I molecule H-2D^k expressed on infected cell in a manner that induces NK cells carrying the activating Ly49P receptor to kill the target cell. To understand the nature of this mechanism, we will determine the crystal structures of the activating receptor Ly49P in complex with H-2D^k loaded with viral or self peptides.

Results Obtained:

We have cloned, expressed, refold and purified the NK receptors: Ly49I, Ly49H, Ly49P; the MHC-I molecules H-2D^d and H-2D^k and the MCMV proteins m157 and m04. Different version and mutants of these molecules including short version, full lengths and mutant with point mutation have been cloned and produced.

All the single molecules and interacting pairs were settled for crystallisation in several different conditions and low resolution crystals were obtained from some of them.

We analysed the rates of association and dissociation (k_{on} and k_{off}), the affinity constant (K_D) and thermodynamic parameters (ΔH, ΔS and ΔG) of the interaction between Ly49 and MHC-II molecules and the viral protein m157, by Surface Plasmon Resonance in a Biacore T100.

Publications:

Romasanta, P.N., Curto, L.M., Urtasun, N., Sarratea, M.B., Chiappini, S., Miranda, M.V., Delfino, J.M., Mariuzza, R.A., Fernández, M.M., Malchiodi, E.L. A positive cooperativity binding model between Ly49 natural killer cell receptors and the viral immunoevasin m157. Kinetic and thermodynamic studies. 2014. J. Biol. Chem. 289(8), 5083-5096

BOSNIA AND HERZEGOVINA

Title: TroutSNP chip based autochthonous populations detection characterisation and conservation

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ICGEB Contract No.: CRP/10/009

ICGEB Reference No.: CRP/BOS10-01

Abstract: The main rivers of the South-Western Balkans flow into the Black, Aegean and Adriatic seas. The Neretva River with its tributaries is the second largest river system in the east Adriatic watershed and very important habitat for ichthyofauna, harbouring considerable amount of genetic variation as well as morphology, ecology and behaviour. Salmonids from the Neretva basin are represented with several species identified in various studies (Heckel, 1852; Karaman, 1937). Brown trout (*Salmo trutta* Linnaeus), softmouth trout (*Salmo obtusirostris*, Heckel) and marble trout (*Salmo marmoratus* Cuvier) have never been disputed as species represented in the Neretva basin (Karaman, 1926; Vukovic, 1982; Kosoric et al., 1983), while *Salmo dentex* has questionable taxonomic position (Snoj, et al., 2010) due to uncertain origin or inadequate and insufficient original descriptions. Natural hybridisation between *Salmo obtusirostris* x *Salmo trutta* and *Salmo marmoratus* x *Salmo trutta* is observed in the Neretva basin, as well as introduction of non-native brown trout. The importance of preservation and conservation of indigenous freshwater fish resources is crucial, bearing not only biological but also very important economic value.

The main aim of this study was to detect intra-specific DNA sequence variations (SNPs) in the growth hormone 1 gene (GH1), as well as within different molecular-genetic markers (mtDNA control region, microsatellite regions) of brown trout (*Salmo trutta*), softmouth trout (*Salmo obtusirostris*, Heckel) and marble trout (*Salmo marmoratus*, Cuvier) inhabiting the Neretva River and its tributaries. PCR-RFLP analysis of mtDNA control region using AluI for *Salmo trutta* samples indicates that tributaries of the Neretva River, where the probability of stocking and admixing with non-native individuals is low, "autochthonous" Ad haplotype is dominant, while both "autochthonous and allochthonous" haplotypes were equally distributed within the main course of the Neretva River. In case of *Salmo obtusirostris*, 80% of sampled individuals clearly have obtusirostris haplotype and 20% At/Me/Da or Ad haplotypes indicating *Salmo obtusirostris* x *Salmo trutta* hybridisation. All observed *Salmo marmoratus* individuals have Ad haplotype, which is characteristic for the populations of this species in the Neretva River.

Estimation of genetic diversity for *Salmo trutta* samples based on total of 13 microsatellite loci revealed somewhat higher values in the group of individuals from the main stream of the Neretva River (0.7954) in comparison with individuals from the tributaries (0.6925). Tendency of Hardy-Weinberg equilibrium deviation was noticed for the most of observed loci. Also, genetic diversity is higher within non-native group (0.8103) in comparison with autochthonous one (0.6526). Overall genetic differentiation between tributaries of the Neretva River and the main stream is 5%, since between "autochthonous and non-autochthonous" is 13.68%. Statistical significant correlation was not detected ($r=0.297$, $P>0.05$) between geographic distance and pairwiseFst calculated among observed tributaries of Neretva River. Structure analysis (K=2) based on Bayesian model-based cluster procedure with admixture model indicates that 42 individuals (22.4%) from the tributaries have high probability ($qI >90\%$) to "belong" to the main stream of the Neretva River, since 70 individuals (44.7%) from the main stream have high probability to have originated from tributaries. The same analysis based on microsatellite loci shows that 19 individuals (8.85%) from autochthonous group (predefined by PCRRFLP of mtDNA control region haplotype detection) show high probability ($qI >90\%$) to be actually part of non-autochthonous group. Analysis of genetic diversity for *Salmo obtusirostris* samples based on total of 8 microsatellite loci indicates significantly higher value within non-autochthonous group (0.7564) in comparison with autochthonous one (0.5267). Tendency of Hardy-Weinberg equilibrium deviation was noticed for the most of observed loci. There is clear and underlined genetic differentiation between autochthonous and non-autochthonous groups (21.35%). Structure analysis (K=2) based on Bayesian model-based cluster procedure with admixture model designates that 63 individuals (28.2%) from autochthonous group (predefined by PCR-RFLP of mtDNA control region haplotype detection) have high probability ($qI >90\%$) to be actually part of non-autochthonous group. Opposite to that, 47 individuals (88%) from non-autochthonous group have probability ($qI >90\%$) to be of autochthonous background. The SsoSL417 locus is clear indicator of *Salmo obtusirostris* x *Salmo trutta* hybridisation since there was no amplification in *Salmo obtusirostris* samples which had obtusirostris haplotype. This locus was easily amplified within group of *Salmo trutta* individuals. From 54 non-autochthonous softmouth trouts, 49 had positive amplification of SsoSL417 locus. Genetic diversity of *Salmo marmoratus* based on 7 microsatellite loci is very low (0.2755), since tendency of Hardy-Weinberg equilibrium deviation was noticed for the most of

investigated loci.

Site and length variation within growth hormone 1 (GH1) gene fragment up to 600bp was observed. A certain number of site polymorphisms have been detected within GH1 in representative samples of brown trout populations. The highest variation score of investigated locus was four nucleotide differences between non-autochthonous and autochthonous samples of brown trout. In the most of the cases of autochthonous samples, absence of polymorphism was observed. No sequence polymorphism of GH1 locus was detected within samples of "pure genetic lineage" of *Salmo obtusirostris*. Variable nucleotide difference counts have been observed between *Salmo obtusirostris* and *Salmo obtusirostris* x *Salmo trutta* hybrids. Site variation was absent within samples of *Salmo marmoratus* as well as between *Salmo marmoratus* and hybrids (*Salmo marmoratus* x *Salmo trutta*). No length polymorphism within observed region of GH1 gene between samples of observed species was found.

However, polymorphisms observed in three indigenous samples of brown trout suggest that there is a potential "marker of authenticity" located in 5' UTR of GH1 to be explored in gene to function relationship in future experiments.

Publications:

Pojskic, N., Kalamujic, B., Silajdzic, E., Lasic, L., Radosavljevic, G., Stanic, M., Kapur-Pojskic, L., Durmic-Pasic, A., Skrijelj, R. Genetic variation of *Salmo trutta* populations in northern tributaries to the Neretva River. 2013. (in preparation)

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CHILE

Title: Exploring light and circadian regulation in the plant pathogen *Botrytis cinerea*

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ICGEB Contract No.: CRP/09/003

ICGEB Reference No.: CRP/CHI09-02

Abstract: *Botrytis cinerea* is a necrotrophic fungus that infects over 200 plant species. In Chile, and worldwide, it produces significant damage in agribusiness infecting pre and post harvest fruits. It has been suggested that the interaction between a pathogen and its host is a process modulated by light and the circadian clock. Nevertheless, nothing is known about the molecular mechanisms behind the aforementioned interaction for necrotrophic fungi. In order to determine this phenomenon, we have started the characterisation of the circadian clock and the transcriptional responses mediated by the blue light photoreceptor White Collar 1 (WC-1) in *B. cinerea*. In *Neurospora crassa*, the circadian clock, which allows synchronising fungal physiology with the outside world, is composed by the FRQ protein and a transcriptional complex formed by WC-1 and WC-2. *In silico* analysis have shown that these genes are present in the *Botrytis* genome. Using a translational luciferase reporter, we have observed oscillatory levels of the BcFRQ protein, while RT-qPCR experiments have confirmed these daily oscillations in Bcfrq expression under constant dark conditions as well as light-inducible expression of several WCC target genes.

Objectives:

Our main objective was that to assess the existence of a molecular circadian clock and light responses in *B. cinerea* and to determine their effect in pathogenicity.

Results Obtained:

We confirmed the presence of light-inducible gene expression, mediated by the WC-1 transcription factor, as well as a functional circadian clock in *B. cinerea*. Importantly, both transcriptional mechanisms impact pathogenicity as determined by analysing the characteristics of $\Delta bcfq$ and $\Delta bcwc-1$ strains, generated by us.

Results Unforeseen in the Original Project:

We described the existence of additional rhythmic phenotypes (observed under cyclical environmental conditions) that do not seem to depend on a canonical FRQ-WC based oscillator. Our results also indicate that in *Botrytis* light responses are not exclusively mediated by WCC, as originally described in *Neurospora*. This suggests that the *Botrytis* photoreceptor system shares key characteristics with *Aspergillus*, and opens up an interesting model system where to study integration of environmental signals and fungal physiology.

Publications:

Canessa, P., Larrondo, L.F. Environmental responses and the control of iron homeostasis in fungal systems. 2012. Appl. Microbiol. Biotechnol. (in press)

Canessa, P., Schumacher, J., Hevia, M., Tudzynski, P., Larrondo L.F. Phenotypic and transcriptional responses to light in *Botrytis cinerea*: evaluating the role of the photoreceptor/GATA-type transcription factor White Collar-1 (in preparation)

CHINA

Title: Studies on the regulatory mechanisms of human proteins with important biological functions

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ICGEB Contract No.: CRP/09/004

ICGEB Reference No.: CRP/CHN09-01

Abstract: Dysfunction of proteins with important biological functions will cause diseases such as inflammation and cancer. Our previous studies have determined the crystal structure of a human protein HSCARG, hCINAP and SI-CLP. However, the precise working mechanisms of these proteins remained to be answered. During this project, we made some significant progress as follows: we identified HSCARG as a novel NF- κ B inhibitor and elucidated the regulatory mechanism of HSCARG in regulating NF- κ B activity, ROS production and cell cycle; we examined the effect of SI-CLP on rheumatoid arthritis by using *SI-CLP* knockout mice. We also investigated the function of hCINAP in Cajal body formation, histone transcription, and cell viability, and demonstrated this protein as a novel regulator of ribosomal protein-HDM2-p53 pathway. Besides, we determined the crystal structures of human proteins including PACSINs, FAIM and UDP-glucose pyrophosphorylase, and influenza virus PB2cap with or without a pre-mRNA cap analog m7GTP.

Objectives:

The purpose of this study is to elucidate the regulatory mechanisms of the proteins mentioned above, to further understand the biological function of these proteins and their relationships to diseases.

Results Obtained:

The regulatory mechanisms of HSCARG in cells. We delineate a pathway by which HSCARG negatively regulates NF- κ B activation: HSCARG interacts with IKK β and NEMO, functions together with USP7 in inhibiting NEMO polyubiquitination, which further blocks the degradation of I κ B α and suppresses NF- κ B activity. We identify a CRM-1-mediated NES in HSCARG. Nuclear accumulation of HSCARG in response to oxidative stress attenuates the inhibition of NF- κ B by HSCARG. Besides, HSCARG inhibits NADPH oxidase activity through regulation of the expression of p47phox. These results indicate that HSCARG plays broad regulatory roles in cells.

The mechanism of hCINAP in regulation of Cajal body (CB) formation, histone transcription, and p53 activity. We demonstrate that hCINAP is essential for the CB formation. Depletion of hCINAP leads to marked reduction of histone transcription and cell viability. We identify hCINAP associates with RPS14, a key protein of human 5q- syndrome. hCINAP plays important functions in regulating cell proliferation, cell cycle and apoptosis. We demonstrate hCINAP as a novel regulator of RPS14-HDM2-p53 pathway, which regulates HDM2-p53 pathway through controlling RPS14's NEDDylation. SI-CLP functions as a feedback negative regulator of inflammatory response by macrophage. We identified SI-CLP in the synovial fluid of patients with rheumatoid arthritis, but not in healthy individuals. Administration of SI-CLP to collagen-induced arthritis rats increased the severity of inflammation. And severer arthritis observed in *SI-CLP* knockout mice. Further investigations show that SI-CLP is specifically attached to surface protein(s) of macrophages. This specific association led to the up-regulation of IL-6, IL-12 and IL-10 expression in macrophages with an enhanced stimulation of inflammation in the collagen-induced arthritis rat model. Our results suggest that SI-CLP functions as a regulator of the inflammatory response by macrophages, which may play important roles in autoimmune diseases.

Structure determination of other disease related proteins. Beyond the original plan, we also determined the crystal structures of human FAIM, a Fas apoptosis inhibitory molecule; human UDP-glucose pyrophosphorylase, a marker of breast cancer; human PACSIN proteins that involve in membrane remodeling. Besides, we identify a K339T substitution in the PB2 cap-binding pocket of influenza A virus, which reduces cap binding, polymerase activity, RNA synthesis activity, and murine mortality.

Results Unforeseen in the Original Project:

We haven't obtained TDP-43 crystals that diffract well and unfortunately could not determine the crystal structure as expected.

Networking:

We collaborated with Dr. Francisco E. Baralle (ICGEB Trieste, Italy) on TDP-43, and Dr. Ming Luo (University of Alabama at Birmingham) on influenza virus protein.

Publications:

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- Meng, G., Zhao, Y., Green, T., Luo, M., Zheng, X.F.** Structure of human stabilin-1 interacting chitinase-like protein (SI-CLP) reveals a saccharide-binding cleft with lower sugar-binding selectivity. 2010. *J Biol. Chem.* **285**, 39898-39904
- Zhang, J., Zhang, F., Zheng, X.F.** Depletion of hCINAP by RNA interference causes defects in Cajal body formation, histone transcription, and cell viability. 2010. *Cell. Mol. Life Sci.* **67**, 1907-1918
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- Bai, X., Meng, G., Zheng, X.F.** Cloning, purification, crystallization and preliminary X-ray diffraction analysis of mouse PACSIN 3 protein. 2012. *Acta crystallogr. F.* **68**, 159-162
- Yu, Q., Zheng, X.F.** The crystal structure of human UDP-glucose pyrophosphorylase reveals a latch effect that influences enzymatic activity. 2012. *Biochemical J.* **442**, 283-291
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- Xiao, W., Peng, Y., Liu, Y., Li, Z., Li, S., Zheng, X.** HSCARG inhibits NADPH oxidase activity through regulation of the expression of p47phox. 2013. *PLoS One* **8(3)**, e59301
- Zhang, J., Bai, D., Ma, X., Guan, J., Zheng, X.F.** hCINAP is a novel regulator of ribosomal protein-HDM2-p53 pathway by controlling NEDDylation of ribosomal protein S14. 2012. *Oncogene* (in press)

COSTA RICA

Title: Molecular and cellular basis of skeletal muscle necrosis caused by snakebites

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ICGEB Contract No.: CRP/08/021

ICGEB Reference No.: CRP/COS08-03

Abstract: Snakebite envenomings are a relevant, although neglected, public health burden in vast areas of the world. A dramatic consequence of such envenomings is myonecrosis, which may lead to permanent tissue loss or amputations. Myonecrosis is mainly caused by phospholipase A₂ (PLA₂) toxins, whose mechanisms of action are only partially understood. In this project, we focused on the acute cellular and molecular events induced in myogenic cells or muscle tissue exposed to myotoxic PLA₂s purified from snake venoms. Both enzymatically-active (D49) and -inactive (K49) myotoxin variants were studied, mainly using the myogenic cell line C2C12 as an *in vitro* model. Important details of the intracellular calcium alterations and the release of cell markers induced by the myotoxins were obtained. A synergism between the two toxin types was observed for the first time, and a novel spreading mechanism of cell damage mediated by ATP release and PX2 purinergic receptors was demonstrated. Structural determinants of K49 myotoxins involved in toxicity were mapped, and two novel non-myotoxic PLA₂s were purified and sequenced, to gain a deeper understanding of structure-function relationships and mechanisms of muscle damage utilised by the myotoxins. Altogether, our findings open unforeseen possibilities for pharmacological interventions, aiming to minimise tissue damage in snakebite envenomings.

Objectives:

To characterise the molecular and cellular events occurring when muscle cells are exposed to myotoxic PLA₂s purified from snake venoms.

Results Obtained:

A more detailed knowledge of the immediate alterations induced by snake PLA₂ myotoxins in both cultured myogenic cells and muscle tissue has been obtained, including intracellular calcium level changes and release of diverse cell markers. A novel "spreading mechanism" of muscle fiber damage involving purinergic receptors was demonstrated, as well as the release of molecules that are relevant to the pathophysiology of muscle damage. Insights into the structural determinants of toxicity of these proteins were also gathered. Overall, results of this project increase current understanding of the mechanisms by which myotoxins induce skeletal muscle necrosis in snakebite envenomings, and open some novel possibilities for their treatment to be explored.

Results Unforeseen in the Original Project:

Immunodominant structural sites of a K49 myotoxin were identified.

Publications:

Cintra-Francischinelli, M., Pizzo, P., Rodrigues-Simioni, L., Ponce-Soto, L., Rossetto, O., Lomonte, B., Gutiérrez, J.M., Pozzan, T., Montecucco, C. Calcium imaging of muscle cells treated with snake myotoxins reveals toxin synergism and presence of receptors. 2009. *Cell. Mol. Life Sci.* **66**, 1718-1728

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Fernández, J., Gutiérrez, J.M., Angulo, Y., Sanz, L., Juárez, P., Calvete, J.J., Lomonte, B. Isolation of an acidic phospholipase A₂ from the venom of the snake *Bothrops asper* of Costa Rica: biochemical and toxicological characterisation. 2010. *Biochimie* **92**, 273-283

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Lomonte, B. Identification of linear B-cell epitopes on myotoxin-II, a Lys49 phospholipase A₂ homologue from *Bothrops asper* snake venom. 2012. *Toxicon* **60**, 782-790

Lomonte, B., Rangel, J. Snake venom Lys49 myotoxins: from phospholipases A₂ to non-enzymatic membrane disruptors. 2012. *Toxicon* **60**, 520-530

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COSTA RICA

Title: Role of cell-mediated immune response in the natural history of HPV infection

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ICGEB Contract No.: CRP/10/001

ICGEB Reference No.: CRP/COS09-01

Abstract: Infection with an oncogenic type of human papillomavirus (HPV) is a necessary step for the development of cervical cancer and its precursors (Cervical Intraepithelial Neoplasia, CIN2). Most HPV infections are cleared by the host immune system within twelve months, and persistence of infection is the clinically relevant risk factor. For this reason, the immune response appears to be a determinant of risk for this disease. However, little is known about the immunological determinants of HPV persistence. In previous work among women of 45 of age and older, we found an association between markers of T-cell phenotype and persistent HPV infection. These results are in agreement with a determinant role for the immune system in disease persistence and progression.

However, it is not known if our finding applies to other age groups, specifically to women in the 18-25-age range. This group is of particular interest because the highest prevalence of HPV infection is observed shortly after sexual debut. For this reason, a prospective study was conducted to investigate in more detail the correlation of immune cell phenotype with resolution of HPV infection.

An analysis of results stratifying participants according to duration of HPV infection did not confirm an association with levels for the selected cell markers. A more detailed analysis of results is currently being carried out.

Objectives:

(i) To determine the proportions of cell populations, as well as the basic lymphocyte populations in a sample of participants of the HPV vaccine trial in Guanacaste, Costa Rica. These markers were measured using flow cytometry.

(ii) To evaluate prospectively the impact of different lymphocyte markers on the time to clearance and final outcome of prevalent HPV infections.

Results Obtained:

Study samples: 165 study participants were selected according to selection criteria, and blood sample availability. Blood samples were kept at a repository at the National Cancer Institute. A requisition was made to repatriate the samples to our local repository at the Guanacaste Epidemiological Project in Liberia, Guanacaste. This process required more time than previously expected. Samples were finally transported to the laboratory at the University of Costa Rica. Ten of the received blood samples were lost after a liquid nitrogen freezer at CIBCM accidentally thawed. A total of 155 participant samples were therefore processed for this study.

Method Validation: In order to ensure the robustness of our results, we stained the complete panel for normal samples, and measured them in two different flow cytometers. Stained cell samples were injected in two different flow cytometers, to assess instrument-to-instrument variability. To assess the reproducibility of results among instruments, Pearson's linear correlation coefficient was calculated. The correlation between the measured values for replicate samples was good, which indicates that the obtained results do not depend on the instrument used.

Data acquisition and processing: The 165 samples were randomly assigned to 38 batches of five samples each. Data were acquired on a FACScalibur (Becton-Dickinson) flow cytometer, as specified in the experimental section. Lymphocytes were selected based on CD45 staining (tubes 3-7) or light scattering properties (tubes 1,2,8-13).

A total of 10,000 events in the lymphocyte gate (tubes 1-10), in the CD8⁺-lymphocyte gate (tube 11) or in the CD4⁺-lymphocyte gate (tubes 12,13) were acquired. We flagged results and excluded them from calculations if the total number of acquired events for a particular tube was below 5,000.

Results were extracted from the analysis file using the FCS Express 4 Flow Cytometry (De Novo Software, Inc.). Before statistical analysis, data were pre-checked to assess tube-to-tube reproducibility. For this, repeated measurements among tubes for the same subject were compared, needed to lie within 5% of each other to be accepted.

Once all quality control checks were performed, a central file containing all flow cytometry results was created, and data were unblinded by adding the demographic and HPV status data. This file contains flow cytometric results for a total of 67 unique variables for 155 participants.

Prospective evaluation of the association of different lymphocyte markers on the time to clearance and final outcome of prevalent HPV infections.

According to selection criteria, all women who participated were sexually active and were found positive for at least one type of oncogenic HPV. Median age at recruitment was 21 years (range: 18-25 years), and median number of sex partners at recruitment was 2 (range: 1-30 partners). Of the total of eight follow-up visits that were scheduled for this study, women attended to a median of 6 (range: 2-8 visits). 55% of participants had an infection with a single HPV type, whereas two types were detected on 26%, three types on 12%, four types on 4% and five types on 3% of women.

Regarding the duration of HPV infection, a high variability was observed among women: 26 participants were positive only at their first visit, and negative at all subsequent visits; 24 participants were positive at all visits, and the remaining 105 women were positive at more than one visit. The latter group is, however, very heterogeneous, as HPV infection could either be detected at the first few consecutive visits, followed by negative visits; or only positive visits followed by a negative test at the final visits, or positive visits alternating with negative visits. A more detailed analysis for these results will take into account the types of HPV that were detected at every visit.

In order to obtain evidence for the association of the measured markers and persistence of viral infection, participants were subdivided into three groups according to duration of HPV infection: women that resolved infection after the first visit (only recruitment); those who did not resolve infection (all visits); and those who had both positive and negative visits (several visits). The rationale for this is that those participants who rapidly resolved infection are more likely to have a stronger immune response than those who required a longer time period to resolve infection, and that subjects who could not resolve infection in a 48-month time period would have the weakest immune system.

When the median values for each of the main seven cell populations that were previously reported to vary in our previous study, none of the markers showed a significant difference among the three groups. However, additional analysis is required to rule out the presence of an association between these markers and the persistence of HPV infection. Such analysis will determine the length of infection among those participants who were HPV positive at several visits, and will take into account the type of HPV that was detected at each visit. This approach is much more sensitive than the one presented in this work, and might be able to detect an association between these markers and duration of infection.

Final comment: Even though the work summarised here did not find an association between phenotype markers and time to clearance, the quality of the data that we generated is very high, as judged by the low number of flagged tubes, and high agreement in internal quality controls. This outcome is very satisfactory for the investigators, as this project was the first of its kind in Costa Rica. For this reason, we aim to take on new challenges to increase our research capabilities and contribute to the much-needed research in public health in our country.

EGYPT

Title: Development of novel insecticides against *Spodoptera littoralis* using RNAi approach

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ICGEB Contract No.: CRP/09/006

ICGEB Reference No.: CRP/EGY09-04RG

Abstract: RNA interference is an effective technology for gene regulation and its expression both *in vitro* and *in vivo*. P450 monooxygenase enzymes play a vital role in adaptation to cotton defense compounds such as gossypol, which is very toxic to Lepidopteran pests. These insects groups have machinery (monooxygenase enzymes) that is able to degrade gossypol through digestion in their guts. This action is a vital factor for such insects to feed and survive on cotton plants. In the current CRP project we therefore investigated the possibility of blocking those enzymes using RNAi approach. The results revealed the effect of siRNAi on cotton leafworm, *Spodoptera littoralis*, by selective targeting of cytochrome P450 gene. This gene is highly expressed in insect midgut and its expression correlates with larval growth when gossypol is included in diet, in both cases feed or injection larvae with double-stranded RNA (dsRNA) specific to P450 gene. In order to study the effect on dsRNAi on insect development and growth, two different bioassay methods were used, Diet mixing and microinjection bioassays were performed. The LC50 was calculated and the larval mortality percentage was also determined. The mortality % was ranged from 3.3 to 60% with 20ng to 300ng dsRNAi/g diet. The bioassay results showed that the esterase activity values were varied between the untreated and treated larvae (0.19 - 0.08). The levels of transcription of P450 gene in *S. littoralis* midgut increased when gossypol is present. The level of esterase was decreased when the larvae were injected with 300ng dsRNAi/g diet. The larval mortality percentage was less in microinjection assay compared with diet mixing assay. The larval body weight was severely affected when the larvae exposed to various doses of dsRNAi. Overall, the siRNAi sequence (unpublished) tested in the current study is an effective one and could be cloned and inserted into cotton plant with other genes such as BtCry1A cotton modified plant (first or second generation) in order to control *S. littoralis* under field condition.

Objectives:

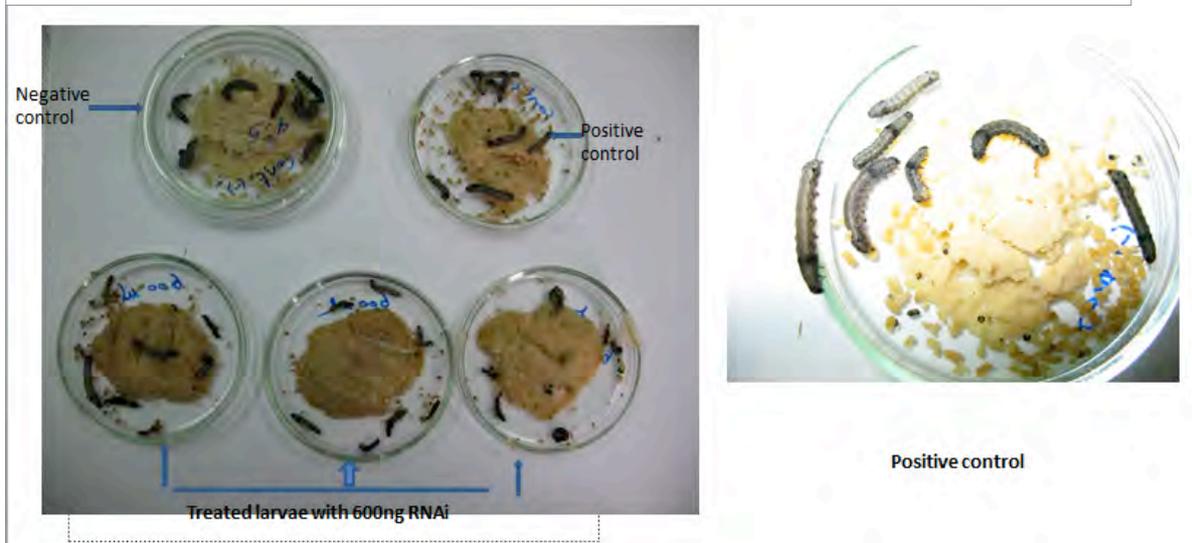
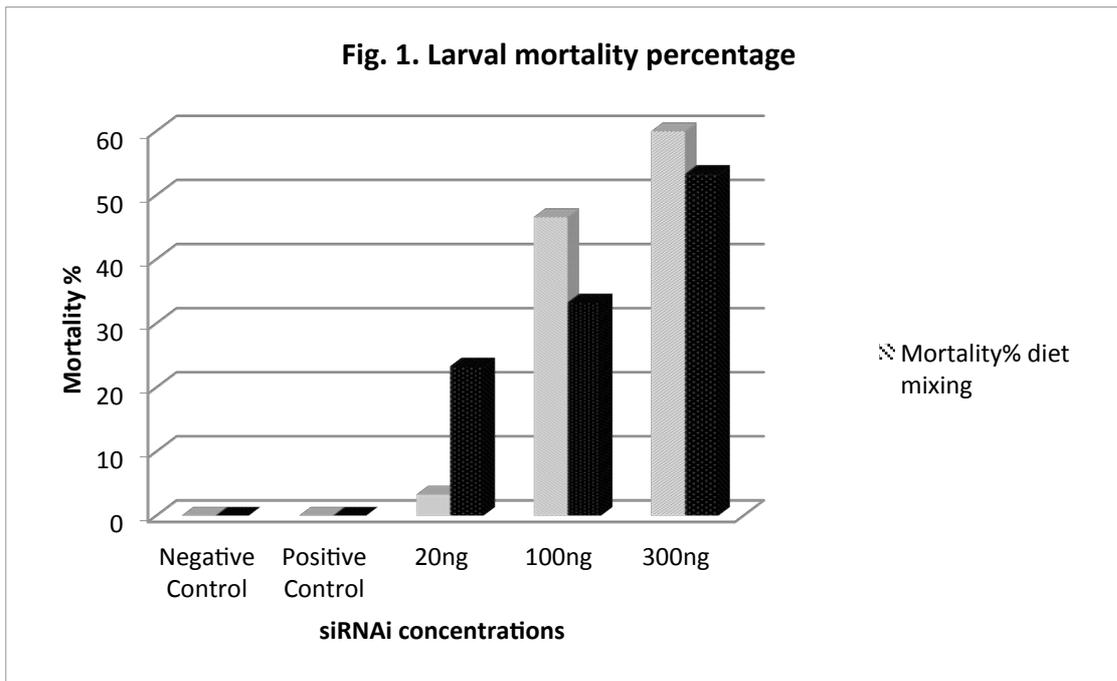
Main objective was that silencing a *Spodoptera littoralis* cytochrome P450 inhibits the metabolism of gossypol using RNAi technique.

Results obtained:

siRNAi sequence of (GCGAAGTGGTAGAGGATTA) is blocking monooxygenase enzyme.

Results Unforeseen in the Original Project:

Cloning the above sequence in a suitable vector.



Negative control larvae: Treated with dH2O only.
 Positive control: treated with cotton oil contains *gospool* only.
 Treated larvae: Larvae treated with cotton oil along with 600ng RNAi
 One day old of the fourth instar larvae were used in all the exp.
 The larval body weight of the survival larvae was measured as well.
 In the positive and negative control assay the larvae were all survived and the body weight was bigger in size compared with the treated ones, see the right plate.
 More than 75% mortality was found in the treated concentrations.

FYR MACEDONIA

Title: Molecular profiling of infertile men

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ICGEB Contract No.: CRP/09/007

ICGEB Reference No.: CRP/MAC09-01

Abstract: Infertility is a major health problem today, affecting about 15% of couples trying to have a child. Impaired fertility of the male partner is causative or contributory in up to two-thirds of all couples unable to conceive spontaneously. Despite enormous progress in the understanding of human reproductive physiology, the underlying cause of male infertility remains undefined in about 50% of cases. The main aim of this project was an extensive molecular profiling of infertile men in order to increase the knowledge of genetic causes and genetic risk factors contributing to male infertility and to enable better management of infertile men. Study population of this project consisted of almost 1,400 male individuals, including ~600 infertile men with different spermatogenic failure and ~600 control fertile men. A wide range of DNA and RNA analysis, such as PCR, multiplex QF-PCR, RT-real-time PCR, multiplex SNaPshot analysis, RFLP, SSCP, direct DNA sequencing, arrayCGH, miRNA microarray expression analysis, 2D-DIGE electrophoresis etc. were used. The results obtained within this project enabled better insights of the common genetic causes responsible for male infertility among men from the R. Macedonia, clarified the role of some potential risk factors and pointed to some new genetic risk factors and molecular biomarkers for male infertility.

Objectives: The main aim of this project was an extensive molecular profiling of infertile men, including screening for the presence of the most common genetic causes of male infertility. Additional objectives were: (i) clarification of the role of partial AZFc deletions and duplications on male fertility; (ii) screening for the presence of mutations in *CFTR* and *AR* genes; (iii) investigation of the possible association of polymorphisms in different genes with male infertility; (iv) determination of spermatozoal RNA profiles; and (v) proteomic profiling of seminal plasma and sperm cells.

Results Obtained:

We have developed a 13-plex QF-PCR method and have shown that it is a rapid, simple, reliable and inexpensive method, that can be used as a first-step genetic analysis in infertile/subfertile men to detect the most common genetic causes of male infertility (sex chromosomal aneuploidies and AZF deletions) and to study some potential risk factors (AZFc partial deletions and duplications and AR CAG repeats). Among the 340 infertile patients from Macedonia, we have detected 8 patients with Klinefelter (XXY) syndrome, one XX male, one patient with XY/X0, one patient with a Y deletion including all three AZF regions (AZFa, AZFb and AZFc) and 5 patients with AZFc deletion. Our results confirmed our previous findings that Klinefelter syndrome and Y chromosome deletions are the most common genetic causes of male infertility with a frequency of 7.7% and 4.6% in patients with idiopathic azoospermia. The most frequent Y microdeletion is the AZFc deletion that represents 81,25% of all deletions detected among infertile men from Macedonia thus far.

The incidence of both gr/gr and b2/b3 deletions and b2/b4 (or gr/gr) and b2/b3 duplications did not differ significantly among infertile men with different spermatogenic impairment and control males, both among men from R. Macedonia and Slovenia. However, a trend of higher incidence of b2/b4 (or gr/gr) duplication in men with higher sperm counts was noticed. We expect that our ongoing study for determination of the incidence of partial AZFc deletions and duplications in relation to the ethnic origin of the studied men and Y haplogroups will unravel the real contribution of these rearrangements to the male infertility.

Our results of the *CFTR* mutations screening among infertile men with different spermatogenic defects showed that the frequency of *CFTR* heterozygosity was slightly higher in all groups of infertile males in comparison to the fertile controls. The assessment of the TG repeat adjacent to the 5T allele in patients with DF508 (or G542X)/intron 8 5T genotype showed that only 5T alleles with 12 and 13TG repeats are fully penetrant and if inherited in trans with a severe CF mutation cause obstructive azoospermia.

The association studies performed during the work on this project suggested that there is no association between the *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G and *MTRR* A66G polymorphisms and male infertility among the men from our country. The association study of nine SNPs in eight different genes (*FASLG*, *JMJDIA*, *LOC203413*, *TEX15*, *BRDT*, *OR2W3*, *INSR* and *TAS2R38*) in a total of 644 infertile men with azoospermia and oligozoospermia from three different ethnic populations (Macedonian, Albanian and Slovenian) showed that men with *LOC203413* minor T allele have an increased risk for impaired spermatogenesis. The study of three SNPs in *SLC6A14* gene, that lies 200kb upstream of SNP rs5911500 in *LOC203413* suggested that SNP 22510 C/G

located in the 3'-UTR of the *SLC6A14* gene might be associated with male infertility among Macedonian men, however this was not confirmed among Slovenian men. Some differences in the distribution of Y haplogroups were observed among infertile and fertile males with same AZFc rearrangements.

We have found a homozygous deletion of *DPY19L2* gene as a cause of globozoospermia in two infertile men from our country, thus confirming that this deletion represents a major cause of this rare form of male infertility.

Our preliminary results using array CGH analysis in 20 infertile men with azoospermia and oligozoospermia and 7 normozoospermic men revealed a total of 157 CNVs (85 gains and 72 losses), of which 55 were present only among infertile men. Some of these CNVs involve genes that might represent a risk factor for impaired spermatogenesis and male infertility (*UGT2B17*, *STEAP2*, *TPTE*, *H2BFWT*, *RBM22*, *RBM1*, *PIWIL1*).

The results of the study investigating relative *TSPY1* copy number in infertile men with azoospermia and oligozoospermia showed an association of *TSPY1* copy number with oligozoospermia. It also showed that the *TSPY* copy number differs between different Y chromosome lineages.

The PCR Array expression analysis of 84 gene transcripts in paraffin tissues of 3 men with hypospermatogenesis in comparison to 3 men with normal spermatogenesis showed that there is higher number of down-regulated genes among the groups of genes involved in spermatogenesis and fertilisation compared to the groups of genes involved in male sex differentiation, cell motility, cell cycle and response to stress. The miRNA microarray analysis of 16 paraffin tissues from men with hypospermatogenesis (with AZFc deletions and with no AZF rearrangements) in comparison to those of men with normal spermatogenesis showed a number of differentially expressed miRNAs.

Our initial results from the study that aimed to investigate the quality and percentage of proteins isolated from FFPE compared to the matching fresh tissue by 2-D PAGE suggested that only very small portion of full-length proteins can be extracted from FFPE compared to fresh frozen tissues.

We applied a 2-D DIGE/MS/MS approach to detect differential protein expression of seminal plasma proteins between four distinct groups with normozoospermia, oligozoospermia, asthenozoospermia and azoospermia and detected 8 differentially expressed proteins: fibronectin, prostatic acid phosphatase, proteasome subunit alpha type-3, beta-2-microglobulin, galectin-3 binding protein, prolactin inducible protein and cytosolic non-specific dipeptidase.

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HUNGARY

Title: The role of Nonsense-mediated mRNA decay in plant antiviral response

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ICGEB Contract No.: CRP/09/008

ICGEB Reference No.: CRP/HUN09-01

Abstract: Breeding of resistant plants is the cheapest way to protect plants against viruses. Although RNA silencing cannot be used in traditional breeding, understanding of silencing led to design more efficient virus resistance constructs. Characterisation of other antiviral systems could accelerate breeding and help to design new transgenic resistance strategies.

Nonsense-mediated mRNA decay (NMD) is an RNA degradation system that identifies and degrades aberrant transcripts having unusually long 3'UTRs. Most plant viruses have polycistronic RNA genomes, therefore the 3'UTR of the first ORF is very long. As NMD degrades transcripts with long 3'UTR, we hypothesised that plant NMD targets RNA viruses and acts as an efficient antiviral mechanism.

During this program we studied the role of NMD in antiviral defence. We found that in plants, NMD does not play an efficient antiviral role. We show that translational readthrough (RT) is efficient NMD evasion strategy for plant viruses and that even low RT can effectively protect the viruses from NMD. During this program we generated several useful NMD mutant and reporter lines, elaborated new experimental tools and described important novel elements of plant NMD. New NMD regulatory circuits were also identified.

Objectives:

- (i) To clarify whether NMD plays an antiviral role in plants;
- (ii) To study how plant viruses can suppress or evade NMD.

Results Obtained:

To test whether NMD plays an antiviral role, wild-type and NMD mutant *Arabidopsis* and *N. benthamiana* lines were infected and comparatively studied. If NMD is an antiviral system, NMD-deficient lines should be more susceptible. Weak NMD mutant *Arabidopsis* (*upf3-1*, *upf1-5*) lines and an NMD-deficient transgenic *N. benthamiana* line, in which UPF2 NMD factor was silenced with an artificial miRNA were infected with several viruses but we could not find significant differences in the virus accumulation. Thus we concluded that NMD is not an efficient antiviral system.

If a plant virus suppresses NMD, the endogenous NMD target should be overexpressed in the infected plant. We identified and characterised two conserved endogenous NMD targets, SMG7 and Barentsz. We showed that both genes play a role in NMD and in NMD autoregulation. SMG7 is involved in both long 3'UTR-based and intron-based plant NMD, while Barentsz is only required for intron-based NMD. Relevantly SMG7 is regulated by both types of NMD, whereas Barentsz is controlled by intron-based NMD (Nyiko et. al., 2013, Nucl. Acids. Res.). To generate NMD reporter lines, GUS-based NMD reporter constructs were used to create transgenic plants. Wild-type and NMD reporter lines were infected with different viruses and then the expression of NMD targets were studied. As the NMD targets were not overexpressed we concluded that plant viruses do not suppress NMD. Consistently, we co-expressed several viral proteins with NMD reporters and none of them inhibited NMD. Interestingly, a recent report (Microbes Infect. 2013, doi: 10.1016/j.micinf.2013.03.006.) showed that NMD plays an important role in antiviral defense against a HTLV-1 human retrovirus and that the retrovirus expresses an NMD suppressor protein. Thus our hypothesis that NMD acts as an antiviral system might be true in certain host-virus interaction and specific plant viruses also suppress NMD.

Translational readthrough (RT) rescues NMD target transcripts in mammals (Hoff, 2010, Cell, 143(3), 379-389). As several plant viruses have RT stop, we postulated that RT plays a role in NMD evasion. To test this, the TMV RT stop context was incorporated into several NMD targets and their NMD sensitivity was tested. We found that TMV RT can efficiently protect transcripts from both types of NMD. We showed that low RT is sufficient to protect NMD target transcripts. We propose that RT is a frequent viral strategy to evade NMD.

We tried to generate plants with reduced RT activity. We proved that in plants, unlike in yeast, overexpression of eukaryote Release Factor 1 (eRF1) is sufficient to reduce RT. We also selected transgenic plants expressing eRF1 and an eRF1 mutant that suppressed RT very efficiently were generated and the transgenic lines were infected. We expected that the plants will be resistant against viruses that require RT for replication. Unfortunately the plants were susceptible to both TMV and CyMRSV. Moreover, the plants showed pleiotropic phenotype suggesting that normal eRF1 level is required for development. Thus eRF1 overexpression is not a feasible strategy to create virus resistant plants.

Results Unforeseen in the Original Project:

NMD does not affect on the accumulation of many plant viruses, which are strong potential NMD targets even though that these viruses do not express NMD suppressor proteins. Thus viruses evade NMD RNA degradation system. Notable, plant viruses overcome RNA silencing RNA degradation mechanism by expressing suppressor proteins.

Unexpectedly, we found that plant NMD is strictly regulated, complex autoregulatory circuits controls the intensity of NMD. More surprisingly we found that eRF1 level is regulated by a unique and highly complex homeostatic control mechanism.

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MOROCCO

Title: Molecular basis of hemoglobinopathies in Morocco

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ICGEB Contract No.: CRP/09/010

ICGEB Reference No.: CRP/MOR09-01

Abstract: The alpha-thalassemias are a group of recessively inherited disorders caused by reduced or absent synthesis of the α -globin chain. The present work was carried out to evaluate the spectrum and the allelic frequency of alpha-thalassemia (alpha-thal) defects in Morocco. This first study conducted on 1787 newborns cord blood samples revealed an allele frequency of 1.23%. Molecular screening was conducted by Gap-PCR and Multiplex Ligation-dependent Probe Amplification (MLPA). Our data revealed five molecular defects responsible for a-thalassaemia: $-a^{3.7}$, $-a^{4.2}$, $-^{MED}$, *HS-40 deletion* and the *African Polymorphism* (G→TCGGCCC at position 7238 and T→G at 7174). Overall, the $-a^{3.7}$ deletion was the most prevalent allele (0.83%). This work is the first in Morocco to determine the molecular basis of a-thalassemia and had permitted an overview on this pathology in the country.

Objectives: Unlike the other haemoglobinopathies, little is known of both epidemiological distribution and spectrum of a-thalassemia mutations in Morocco. The aim of the present work is to acquire further data concerning a-thalassaemia prevalence and molecular defects spectrum in Morocco.

Objectives of this project are doubles: first, the major objective of this study is to delineate the allelic frequency of a-thalassemia mutations in the Northern and the North-West of Morocco. The second objective is to expand the knowledge on the spectrum of a-globin gene defects in the Mediterranean basin, providing further insight into the molecular basis of this pathological condition.

Results Obtained: 1787 newborns cord blood samples were collected over a period of three years from different hospitals in the North of Morocco including Tangier, Tetouan and Larache cities. Phenotypic analysis revealed 244 suspected alpha-thalassaemic cases with microcytic hypochromic hemolytic anemia and normal serum iron and ferritin levels. Among these relevant samples, seven newborns carrying Hb Bart's were detected, out of which five cases carried in addition an HbS fraction. Molecular analyses using Gap-PCR and MLPA were performed on all the relevant cases. Our results showed five molecular defects responsible for a-thalassaemia detected in 22 newborns (Table 1): 15 cases carried the $-a^{3.7}$ deletion, nine in the heterozygous state ($-a^{3.7}/aa$) and six in the homozygous state ($-a^{3.7}/-a^{3.7}$). The deletion $-a^{4.2}$ was observed in two cases: one in the heterozygous state ($-a^{4.2}/aa$) and the other in the homozygous state ($-a^{4.2}/-a^{4.2}$). Two other cases revealed a triplicated α -globin gene ($aaa^{anti-3.7}/aa$). Three molecular alterations were found for the first time in the Moroccan population in three newborns, one with $-^{MED}$ deletion, the second showed a *deletion* limited to a region containing the upstream regulatory element *HS-40* and the *third revealed the African Polymorphism* (G→TCGGCCC at position 7238 and T→G at 7174). This first study revealed an allele frequency of 1.23% with a predominance of the $-a^{3.7}$ deletion (0.83%). These results outline the heterogeneity of the alpha-thal alleles in Morocco. The majority of cases are originating from suburbs in Larache city. We think that this has to do with a probable ancient malaria infection in the region.

This work is the first in Morocco to determine the molecular basis of a-thalassemia. Further researches covering all the Moroccan population are needed for a better patient management and for a much better targeted strategy of prevention.

Publications:

Publications:

Laghmich, A., Alaoui Ismaili, F.Z., Barakat, A., Ghailani Nourouti, N., Khattab, M., Bennani Mechita, M. Alpha-Thalassemia in north Morocco: Prevalence and molecular spectrum. 2019. Biomed. Res. Int. **2019**, 2080352

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Table 1: Haematological and Molecular analysis data of the 22 cases with alpha-thalassaemia

Cases	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	
Gender	M	M	F	M	F	F	F	M	M	M	M	M	F	M	M	F	M	F	F	F	F	M	M
RBC (10 ⁶ /mm ³) (RF: 4.92 ± 0.6)	4.8	4.63	4.98	5.43	4.73	5.26	5.32	5.61	4.99	4.87	4.49	4.65	5.04	5.33	4.74	4.43	5.50	4.88	5.00	4.76	4.63	4.98	
Hb (g/l) (PF: 17.6 ± 2)	9.7	9	7.80	13.60	9.20	9.60	9.50	11.70	8.90	11.50	11.30	10.30	8.60	10.70	12.00	9.00	10.90	7.50	12.00	6.8	9	7.8	
MCV (fl) (PF: 104.4 ± 4.8)	69	66.1	77.6	61.3	59.3	66.5	65	67.8	68.2	73.7	83.8	63.1	62.2	68.9	58	88	55	67.2	75	77	67	65	
MCH (pg) (PF: 35.7 ± 1.7)	20.21	19.44	15.66	25.05	19.45	18.25	17.86	20.86	17.84	23.61	25.17	22.15	17.06	20.08	25.32	20.32	19.82	15.37	24.00	14.29	19.44	15.66	
Hb profile	FAB	FSA	FSAB	FA	FAS	FA	FA	FA	FA	FA	FA	FAS	FA	FA	FA	FAB	FA						
Molecular analysis*	-α ¹⁷ Homo	-α ¹⁷ Hetero																					

S = samples; RBC = red blood cells; RF = reference values; Hb = hemoglobin; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; Homo = homozygous; Hetero = heterozygous; RF = reference values; M = males; F = females; F = Hemoglobin F; A = Hemoglobin A; B = Hemoglobin Bart's; S = Hemoglobin S.
* by Multiplex gap-PCR and/or Multiplex Ligation-independent Probe Amplification (MLPA).

NIGERIA

Title: Surveillance for enteroviruses in Nigeria

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ICGEB Contract No.: CRP/09/018

ICGEB Reference No.: CRP/NIG09-02

Abstract: To determine the prevalence of enteroviruses in kids with febrile illnesses in Nigeria, stool samples were collected from 2009 to 2012. After RNA extractions, 53 of 240 stool samples (24.7%) were positive for RT-PCR with Pan-entero primers. Nucleotide sequence comparison and phylogenetic analysis of the sequence from the 151-bp 5'-UTR products revealed 98.1% similarity and clustering with the recently proposed new serotype of HEV-D enterovirus 94 (EV94) isolates from sewage in Egypt and acute flaccid paralysis patients in the Democratic Republic of Congo. The finding of this strain in Nigeria, which may represent a new subgroup, has great public health implications.

Objectives:

There are three different genetic lineage lineages described of EV71, designated A, B and C. Within lineage B and C, five subgroups have been identified as B1-B5 and C1-C5. No definitions have been established for lineages and subgroups. However, generally speaking, there is a nucleotide variation of between 16 and 20% between lineages and approximately 6-12% between the subgroups within each lineage.

EV71 may cause large outbreaks as in Europe and in the USA in the Seventies. Large outbreaks have, since then, been confined to Asia. The latest outbreaks occurred in China in 2008, with hundreds of fatalities among young children. Many of the infected children have a broad range of symptoms as hand, foot and mouth disease, encephalitis, meningitis, paralysis, respiratory disease, pulmonary edema, haemorrhagic disease or myocarditis. The last outbreaks in Asia were mainly caused by subgroup B strains. EV71 subgroup C is the circulating strain in Europe. This lineage does not give rise to outbreaks, and most infections are subclinical. Nothing is known of EV1 in Nigeria, nor has enterovirus surveillance been performed for the identification and typing of non-polio strains. The finding of this divergent EV71 strain may indicate a spread of this type in Nigeria, which may represent a new subgroup. It, therefore, has great public health implications to identify the spread of EV71 strain in Nigeria.

Accordingly, we plan to collect samples from patients with diverse symptoms. The samples will be typed by PCR amplification and sequencing of the structural VP1 gene. The non-structural P3 region will be sequenced in divergent strains, and complete genomes will be sequenced in suspected new subgroups or recombinant strains.

Results Obtained:

(i) Using the Pan-enteroviral primer pair MD91F and MD90R, a 155-bp fragment was amplified from 47 of the 220 specimens (21.4%) obtained during the 2010/2011 sampling at the Lagos and Lokoja sites;

(ii) Using EV71-specific primer pair VP1F2 and EV71R2 on the positive samples from (i), RT-PCR generated expected bands of 341-bp (corresponding to partial VP1 region from EV71 strains isolated from various geographic locations (S. Singh et al., 2000) in 10 of the 48 samples that were initially positive for the general enteroviral primers;

(iii) Amplifications with EV71-specific primer pair in (2) are not reproducible;

(iv) Sequencing of five Pan-enteroviral primer pair 155-bp PCR products in (i) produced the sequence: designated as EID/N/001;

(v) Sequence Similarity Search with NCBI BLAST of the 155nt EID/N/001 sequence aligns to nucleotide 601 to 447 of the Human Enterovirus 94 sequence;

(vi) The sequence also matches sequences from enteroviruses including Enterovirus, Poliovirus and Coxsackievirus;

(vii) EID/N/001 cluster with EV94 isolate E210.

Results Unforeseen in the Original Project:

The earlier report (unpublished) of an EV71 in a sample dictated the subsequent work on Pan-entero primers-positive samples with EV-71 specific primers for a partial VP1 region from the samples.

PERU

Title: Hormonal and genetic characterisation of ovarian follicular maturation in alpacas

Principal Investigator: Luisa Inés Echevarría Curee, Laboratorio de Reproducción Animal, Facultad de Veterinaria y Zootecnia, Universidad Peruana Cayetano Heredia, Av. Honorio Delgado 430, San Martín de Porras, Lima, Peru. Tel: +51-1-3190059, 3002, E-mail: luisa.echevarria@upch.pe

ICGEB Contract No.: CRP/09/011

ICGEB Reference No.: CRP/PER09-02

Abstract: To understand reproductive physiology in alpaca it is important to increase its natality and to promote ART techniques to genetically improve the species for the production of fine fibers. Fertility of the species is in relation to oocyte quality that depends on the type of the follicle that contains it. In this study we proposed to define some maturation follicular indicators, using spontaneous follicular waves of alpacas. Hormonal levels of progesterone, estradiol and testosterone of follicular fluid were determined to establish normal concentrations and rates between E/P and E/T, in different phases of the follicular wave: Growing, Selection, Dominance and Atresia. At the same time, the granulosa layer from follicles was analysed for aromatase gene expression. Using these results we implemented a superovulation protocol in order to produce embryos to use in future phases in embryo transfer.

Dominant follicles were characterised as follows: high intra-follicular estradiol levels and E2/T, presence of progesterone, low testosterone levels, and low gene expression of aromatase after mating. Additionally, these follicles have more chances to ovulate with HCG injection and lower probabilities to differentiate into follicular cysts. We validated a Buserline-based protocol to induce arrest follicular in alpacas, and we obtained promising results with an HMG-based superovulation protocol in alpacas, paving the road to embryo transfer in this important specie.

Objectives:

- (i) Our main objective is to define follicular maturation indicators in alpacas;
- (ii) To characterise ovarian follicles by defining the real follicular development phases during the spontaneous wave: Growing, dominance and atresia: according to genetic (aromatase) and hormone factors in follicular fluid,.
- (iii) To characterise ovarian follicles, to define the real follicular development phases after endogenous LH stimulus after mating: dominance and atresia: according to genetic (aromatase) and hormone factors in follicular fluid.
- (iv) To evaluate superovulation protocols (Busereline, HMG) by determining blastocysts production according to a specific follicular phase.

Results Obtained:

Intrafollicular steroids levels are reported in alpaca. Steroids levels were different ($P < 0.05$) among follicular phases, and considering different sizes of follicles. When spontaneous follicular wave was studied, dominant and selection follicles have higher levels of estradiol than atretic and growing ones ($P < 0.05$). Statistical analysis showed a relationship between estradiol levels with follicle diameter, considering only growing and dominant follicles and E2/P4 ratio was different in atretic follicles ($P < 0.05$) vs. dominant, selection and growing follicles. In reference to testosterone, detectable levels were determined from 5-6 mm of selection follicles and atretic follicles had higher testosterone levels than dominant and growing ones ($P < 0.05$). Progesterone levels were found only in dominant and atretic follicles. Rate E2/P and E2/P in atretic follicles were lower ($P < 0.05$) than in the other follicular phases.

Alpacas with dominant and atretic follicles were mated. Dominant and atretic follicles had detectable levels of estradiol, progesterone and testosterone, but not differences were found among groups. Statistical analysis showed a relationship between estradiol levels and progesterone levels, and between estradiol/progesterone with follicle diameter, considering only dominant follicles. There was a statistically significant difference between progesterone content and the E2/P rate, between dominant and atretic follicles ($P < 0.05$).

When follicles did not receive the endogenous LH stimulus, aromatase expression was 15.3, 12.8, 21.4, 29.8 and 1.6 folds higher in follicles with diameter of 5-6mm, 7-8mm 9-10mm, >10mm and atresia respectively, compared to follicles of 2-4mm of diameter, respectively. On the contrary, when follicles were under the stimulus of endogenous LH, aromatase expression was down-regulated in follicles with diameter of 7-8mm, 9-10mm and in atresia, but the expression was 2.3 folds higher compared to follicles of 2-4mm of diameter.

Validation of super stimulation protocols using Busereline to produce follicular arrest, and HMG to promote multiple follicular growing, had the following results: follicular development did not resume after ablation and during 10 days of Buserelin treatment, in 75% ($n=6$) of 8 animals. The first day of total arrest in 6 animals was found on the sixth day of treatment.

After follicular arrest, and using HMG, animals showed follicular development from day 3 of the beginning of the treatment in three doses groups (75 – 75, 150 and 300 UI HMG). There was not statistically significant difference in the number of follicles of 7–10 mm produced at days 5 and 6 after beginning of treatment with HMG, although there was a tendency to have more total follicles in 300 HMG group. A protocol was used with 2 doses of HMG, to give an augmented stimulus necessary for follicle development, and with the criterion of monitoring follicle development in each animal until verifying the presence of follicles of 7–10 mm, as it is recommend for Vaughan (2006), instead of inducing the ovulation in a fixed day for all of them. This protocol had more success as 68% of follicles ovulated and finished as CL and with a Recovery rate of 44%.

Results Unforeseen in the Original Project:

In spite of finishing the treatments of HMG, follicles continued growing in alpaca ovaries, in 3 experimental groups, so it was proposed an alternative protocol to be evaluated in the project.

RUSSIA

Title: The role of B cell response in Multiple Sclerosis

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ICGEB Contract No.: CRP/09/012

ICGEB Reference No.: CRP/RUS09-01

Abstract: Multiple sclerosis (MS) is a severe inflammatory and neurodegenerative disease with an autoimmune background. Despite the variety of therapeutics available against MS, the development of novel approaches to its treatment is of high importance in modern pharmaceuticals. Previously we succeeded to show the cross reactivity of antibodies toward main neuroantigen, Myelin basic protein (MBP) and LMP1, EBV antigen. The virus as triggering machinery of MS was re-recognized. According to the research plan of the project, we aimed to design new therapeutics of autoimmune neurodegeneration, taking into account MS as a main pathology. The B-cell depletion specific therapeutics was designed during the first and second year of this project. The next approach was dealt with the development of specific peptide therapy toward MS. During the last year we succeeded to show that experimental autoimmune encephalomyelitis (EAE) in Dark Agouti rats has been treated with immunodominant peptides of MBP encapsulated in mannosylated small unilamellar vesicles. The results show that liposome-encapsulated MBP 46-62 is the most effective in reducing maximal disease score during the first attack, while MBP124-139 and MBP147-170 can completely prevent the development of the exacerbation stage. Both mannosylation of liposomes and encapsulation of peptides are critical for the therapeutic effect, since neither naked peptides nor non-mannosylated liposomes, loaded or empty, have proved effective. The liposome-mediated synergistic effect of the mixture of 3 MBP peptides significantly suppresses the progression of protracted EAE, with the median cumulative disease score being reduced from 22 to 14 points, compared to the placebo group; prevents the production of circulating autoantibodies; down-regulates the synthesis of Th1 cytokines; and induces the production of brain-derived neurotrophic factor in the central nervous system. Thus, the proposed formulation ameliorates EAE, providing for a less severe first attack and rapid recovery from exacerbation, and offers a promising therapeutic modality in MS treatment.

Publications:

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RUSSIA

Title: Involvement of PGRP-S protein and its complex with Hsp70 in the cancer cells killing

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ICGEB Contract No.: CRP/09/013

ICGEB Reference No.: CRP/RUS09-02

Abstract: We consider the novel means of attack and defence in the host versus cancer combat that involve interactions between widespread multifunctional proteins, focusing on the aspects that may seem paradoxical in the framework of established notions. Particularly, we show that a protein broadly known for its protective functions such as Hsp70 can make a tumouricidal "binary weapon" with another nontoxic protein Tag7 (PGRP-S); that the same Hsp70, a ubiquitous intracellular chaperone, when expressed on the MHC-negative tumour cell surface, can itself be the hallmark of immune evasion rather than a primordial MHC substitute; that a device functionally equivalent to the T-cell receptor (Tag7-Centered Recogniser) can be assembled of components in no way related to the classical pathways of T-cell-mediated immunity, and operate where the orthodox immunosurveillance fails; and that one and the same protein Mts1 (S100A4) under different circumstances may work as "reactive armour" of a tumour cell against humoral agents and as a vital part of the T-cell machinery aimed against immuno-evasive cells, i.e., perform both prometastatic and antimetastatic functions.

Objectives:

- (i) Investigation a role of co-chaperons in the lymphocytes and in the human serum in the PGRP-S/Hsp70 dependent cytotoxicity;
- (ii) Study the mechanism of secretion of PGRP-S/Hsp70 complex and regulation of its activity;
- (iii) Characterisation of the subpopulation of the lymphocytes, carrying PGRP-S molecule as a recognition receptor for cancer cells;
- (iv) Involvement of several cofactors including Mts1 protein in cancer cell recognition via PGRP-S/Hsp70 interaction;
- (v) Study of cancer cells resistance mechanisms to PGRP-S/Hsp70 cytotoxic action for mouse anticancer studies.

Results Obtained:

2010 year (objectives i and ii)

Heat shock binding protein HspBP1 is a member of the Hsp70 co-chaperone family. The interaction between HspBP1 and the ATPase domain of the major heat shock protein Hsp70 up-regulates nucleotide exchange and reduces the affinity between Hsp70 and the peptide in its peptide-binding site. Previously we have shown that Tag7 (also known as peptidoglycan recognition protein PGRP-S), an innate immunity protein, interacts with Hsp70 to form a stable Tag7-Hsp70 complex with cytotoxic activity against some tumour cell lines. This complex can be produced in cytotoxic lymphocytes and released during interaction with tumour cells. Here the effect of HspBP1 on the cytotoxic activity of the Tag7-Hsp70 complex was examined. HspBP1 could bind not only to Hsp70, but also to Tag7. This interaction eliminated the cytotoxic activity of Tag7-Hsp70 complex and decreased the ATP concentration required to dissociate Tag7 from the peptide-binding site of Hsp70. Moreover, HspBP1 inhibited the cytotoxic activity of the Tag7-Hsp70 complex secreted by lymphocytes. HspBP1 was detected in cytotoxic CD8+ lymphocytes. This protein was released simultaneously with Tag7-Hsp70 during interaction of these lymphocytes with tumour cells. The simultaneous secretion of the cytotoxic complex with its inhibitor could be a mechanism protecting normal cells from the cytotoxic effect of this complex.

2011 year (objective iii)

Within the broad problem of host immune surveillance versus tumour immune evasion, a most intriguing question is how the cellular immunity can cope with cancerous cells that have got rid of the classical antigen-presenting machinery. One such option stems from (i) the fact that HLA loss is often attended with expression of Hsp70 on the tumour cell surface, and (ii) our findings that human lymphocytes express a protein PGRP-S capable of tight and specific interaction with cognate Hsp70. Here we show that a subpopulation of human CD4+CD25+ lymphocytes, obtained either in culture as lymphokine-activated killers or directly from healthy donors, carry PGRP-S and FasL on their surface and can indeed kill HLA-negative tumour-derived cells K562 and MOLT-4 that expose Hsp70 and Fas. The primary binding of lymphocyte PGRP-S to target-cell Hsp70 is very specific (e.g., it is blocked by preincubating either cell with minimal peptides from the "partner" protein) and secures cell contact indispensable for subsequent FasL/Fas-triggered apoptosis. Unrelated to NK action or the putative role of Hsp as an antigen-presenting substitute, this novel mechanism is rather a backup analog of orthodox (CD8+) target recognition (PGRP-S acting as built-in TCR and Hsp70 itself as ligand).

2012 year (objectives iv and v)

We compare the physical and functional interactions between three widespread multifunctional proteins [metastasin (Mts1/S100A4), innate immunity-related Tag7/PGRP-S, and Hsp70] in two experimental models relevant to host-tumour relationships on humoral and cellular levels. (i) Tag7 and Hsp70 in solution or in a lymphocyte make a stable binary complex that is highly cytotoxic for some tumour cells. Here, we show that Mts1 prevents Tag7 Hsp70 assembly in solution, and an excess of Mts1 disrupts the existing Tag7 Hsp70 complex; accordingly, Tag7 Hsp70 cytotoxicity (exemplified with L929 cells) is diminished in the presence of excess Mts1. (ii) Tag7 exposed on a specialised subset of lymphokine-activated killer cells makes specific contact with Hsp70 exposed on some HLA-negative tumour cells, thus enabling FasL/Fas-mediated induction of apoptosis. Here, we show that some CD4 CD25 cells co-expose Mts1 with Tag7 and FasL, that Mts1 and Tag7 closely contact the same Hsp70 molecule on the target K562 cell (as evidenced by cross-linking), and that killing of such targets is abolished by Mts1-specific antibodies (or selective removal of Mts1-exposing lymphocytes). Thus, this phenotype active against immune-evasive cancerous cells is defined as CD4 CD25, FasL, Tag7 Mts1 (0.5% of total lymphocytes in culture). Remarkably, similar effectors with at least the same activity are often found in fresh donor blood samples (104 effectors/mL). Thus, our models suggest that interactions between the three proteins in different situations may have opposite functional outcomes as regards antitumor defence, immune escape, and metastasis.

Publications:

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SERBIA

Title: Microbial diversity in the copper mine Bor

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ICGEB Contract No.: CRP/09/014

ICGEB Reference No.: CRP/YUG09-03

Abstract: Copper mining in Serbia is situated at the Bor mining district (eastern Serbia). The Bor basin represents a type of deep surface mining. There are several copper deposits in this metallogenic zone and some of the mines have been known since the Roman times. Bor is also a potential regional risk spot, since the failure of its tailing dams would release high amounts of toxic materials that would reach the Danube River through the Borska River and other effluents. Since microorganisms play a crucial role both in the bioleaching and bioremediation, the goal of this project was to study microbial diversity in the copper mine Bor as prerequisite for downstream applications, such as bioleaching and bioremediation. Microbial diversity was analysed through culture-dependent and culture-independent approaches i.e., metagenomic approach. In culture-dependent approach the strains with increased level of resistance and ability to accumulate copper were characterised taxonomically. Their abilities to produce exopolysaccharides, form biofilms in flow cell reactor and the presence of known genetic resistance markers for the metal tolerance, were analysed. Bioleaching experiments were done with natural and artificial consortia. Microbial community dynamics during bioleaching experiments were followed by T-RFLP analysis and it was shown that in artificial consortium *At. ferrooxidans* represents 75% of all microorganisms, while natural consortium consisted of 55% *L. ferrooxidans*, 42% *At. ferrooxidans*, and 3% unknown bacteria.

Objectives:

Since the analysis of microbial community is prerequisite for downstream applications, such as bioleaching and bioremediation, the main goal of the proposed project was the study of microbial diversity in the copper mine Bor.

Microbial diversity in the copper mine Bor was analysed through:

- 1) culture-dependent (isolation and characterisation of the strains);
- 2) culture-independent approaches (metagenomics).

Results Obtained:

The culture independent (metagenomic) and culture dependent methods were applied in assessing microbial diversity of the surface and deep sediment of the Bor mining and smelting complex, in order to obtain more complete overview of microbial community structures of these two sites. Two clone libraries were generated and after sequencing it was found that in library generated from surface sample predominant phylum was *Proteobacteria* (34.8 %) and in deep sediment library the most abundant phylum was *Nitrospirae* (92%). Overall, greater microbial diversity was in the library generated from the mine surface sediment with members of 7 different phylogenetic groups detected, while in the library from the deeper mine sediment representatives of only 3 different groups were detected.

Thirty-two strains isolated from mine surface sediment (MSI isolates) and 30 strains isolated from mine underground (MUI isolates) were identified by 16S rDNA sequencing. With respect to the bacterial community composition, bacterial groups detected in both samples were affiliated to the *Actinobacteria*, *Firmicutes* and *Proteobacteria*. While identified MSI isolates mostly belong to phylum *Actinobacteria*, which was 5 and 2.5 times more represented than *Firmicutes* and *Proteobacteria* respectively, among MUI members of *Firmicutes* were 3 times more represented than *Actinobacteria* and *Proteobacteria*. The higher degree of diversity within the analysed mine samples was obtained using the metagenomic approach. On the other hand, analysis of the cultivated bacteria revealed species that were not obtained using metagenomic method.

One of the isolates identified as *Staphylococcus* sp. MSI08, was further characterised and investigated for the ability to tolerate exceptionally high concentrations of metal ions when grown in minimal medium with lactate as carbon source. Although MSI08 isolate, identified as *Staphylococcus* sp., had the highest sequence identity with *Staphylococcus haemolyticus* strain CCGE3068 of 95% coupled with 99% coverage, it differed from the known *S. haemolyticus* strains in the ability to hydrolyse esculin, and not being able to utilise arginine and glycerol. Thus, it was identified as possibly novel species of *Staphylococcus* genus. The isolate MSI08 could tolerate Cd²⁺ (535 µM), Ni²⁺(17 mM) and Cr⁶⁺ (38.5 mM), and as potentially useful strain for the bioremediation efforts it was investigated for the ability to produce exopolysaccharides, form biofilms in flow cell reactor and for the presence of known genetic resistance markers for the metal tolerance.

Results Unforeseen in the Original Project:

Although not proposed at the time of submission we have conducted bioleaching experiments. For these experiments two types of ore concentrate were used, chalcopyrite "Majdanpek" with 17% Cu and "Srebri" with 24% Cu and 17g/t Ag. In one set of experiments natural consortium from "Lake Robule" was used, while for the other set artificial consortium was designed (*At. ferrooxidans* (several strains), *L. ferrooxidans*, *L. feriphilum*, *Sb. thermosulfidooxidans*, *Am. ferrooxidans*, *At. Ferrivorans*). Artificial consortium was more efficient in bioleaching of "Majdanpek" ore concentrate, while natural consortium performed better with "Srebri" ore concentrate. At the end of this experiments each sample was analysed by T-RFLP and it was shown that in artificial consortium *At. ferrooxidans* represents 75% of all microorganisms, while natural consortium consisted of 55% *L. ferrooxidans*, 42% *At. ferrooxidans*, and 3% unknown bacteria. Bacterial strains and access to specialised data base for analyses of T-RFPL fragments were obtained by the courtesy of Prof. D.B. Johnson, Bangor Acidophile Research Team, School of Biological Sciences, University of Wales, Bangor UK.

Networking: The project enabled and promoted collaboration with the groups of Prof. Vladica Cvetkovic at the Faculty of Mining and Geology, University of Belgrade, and Prof. D.B. Johnson, Bangor Acidophile Research Team, School of Biological Sciences, University of Wales, Bangor UK.

Publications:

Bajkić, S., Narančić, T., Đokić, L., Đorđević, D., Nikodinović-Runić, J., Morić, I., Vasiljević, B. Microbial diversity and isolation of multiple metal-tolerant bacteria from surface and underground pits within the copper mining and smelting complex Bor. 2013. Arch. Biol. Sci. **65**, 375-386

SLOVENIA

Title: Pesticides and neuronal sensitisation: a pilot study

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ICGEB Contract No.: CRP/09/015

ICGEB Reference No.: CRP/SLO09-03

Abstract & Objectives:

The study of chronic effects of chemicals on biological systems is a hot field for the constant generation of new compounds, limited knowledge of their transformation products and unknown reactivity with other molecules. Imidacloprid is a new generation neonicotinoid insecticide widely applied in agriculture treatment. In ecological perspective, the use of imidacloprid is highly debated for its negative impact on bee populations, and its long-term effects on human health are little known, therefore increasing the pressure to improve its testing and regulation for environmental and health protection.

Results Obtained:

With an interdisciplinary team, we studied chemical toxicity on different biological models, including peripheral sensory neurons highly subjected to neuropathies after chronic exposure to environmental stressors. We therefore focus our attention on the study of ATP release, as a key molecule involved in peripheral neuron hypersensitisation, neuroinflammation and oxidative stress. Our study contributed to provide an integrated approach for both health and environmental exposure monitoring on chemical stimuli.

Results Unforeseen in the Original Project:

This project has open new possibilities of collaboration and research especially in the field of testing innovative materials for biomedical engineering and drug delivery application.

Publications:

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Malev, O., Chen, K., Žabar, R., Trebše, P., Fabbretti, E. Characterisation of imidacloprid toxicity on peripheral sensory neurons. Neurotoxicology (submitted)

URUGUAY

Title: Modulation and characterisation of potato defence

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ICGEB Contract No.: CRP/07/023

ICGEB Reference No.: CRP/URU07-03

Abstract: Potato is the third most important crop as nutrition source for humans. The potato susceptibility to pests and diseases causes important economic losses and makes this crop an important consumer of agricultural pesticides worldwide. Therefore, other environmentally less harmful approaches for controlling plant diseases are needed. Plant genetic engineering and conventional breeding for disease resistance seem to be good strategies for approaching these problems. However, as part of developing such strategies, a better knowledge of the molecular events occurring during plant-pathogen interactions is needed. This project aimed to characterise plant defence components identified by our research team, specifically potato kinases and a novel UDP-Glycosyl transferase. We approach such characterisation by genetic engineering aiming to modulate the expression of such specific potato components. The data obtained during the performance of this project suggest that potato kinases such as the PRK family of receptors could be important for the regulation of plant defence responses. However, the role of the UDP-glycosyl transferase studied is less clear and more research is needed. Taken together, the results suggest that genetic engineering of specific potato key components could be a useful strategy to generate plants with enhanced resistance to biotic stress.

Objectives:

- (i) Characterisation of potato defence components; generation of polyclonal antibodies raised against PRK-2 and DRU-13; analysis of the subcellular localisation of PRK-2 and DRU-13; characterisation of protein expression patterns for PRK-2 and DRU-13 in plant tissues.
- (ii) Functional modulation of potato defence mechanisms; generation of plants overexpressing the potato components mentioned above as well as generation of plants silencing them; molecular characterisation of the corresponding generated lines and their plant defence responses to biotic stress.

Results Obtained:

i) We have previously characterised the expression of potato transcripts for the PRK family of receptors and for DRU-13. Such components are induced in potato plants after biotic stress caused by *Pectobacterium carotovorum* subsp. *carotovorum* (ex *Erwinia carotovora* subsp. *carotovora*). PRK-2 and DRU-13 proteins were expressed in *E. coli* and used to produce antibodies in rabbits. The polyclonal antibodies recognised such proteins expressed in *E. coli*. However, when the antibodies were used in western blot for testing plant tissues the results were not clear; suggesting that optimisation of the technique is required. For that purpose we planned subcellular fractionation of proteins. The analysis of DRU-13 suggests that this UDP-Glycosyl transferase is probably located at the apoplast, although further studies need to be performed to confirm it. In the case of the PRK family of receptors, they seem to be located at the plasma membrane. Subcellular localisation of PRK-2 was studied by laser confocal microscopy in plant protoplasts transformed with vectors expressing PRK-2-GFP fusion proteins. PRK-2 transcripts expression and subcellular localisation studies suggest that PRK-2 is located at the plasma membrane of tubers, stems, and leaves of the potato plant.

ii) We have generated several lines of genetically engineered potatoes with the expression of its own specific defence components modulated. Specifically, we have generated potato plants that overexpress PRK-2, and plants that silence the whole family of PRK receptors. Similarly, we have also generated potato lines that overexpress DRU-13. The analysis of the DRU-13 overexpressing lines suggests that such modulation of the potato affects the defence mechanisms against *P.c. carotovorum* and more studies are needed. On the other hand, the genetic modulation generated on PRK-2 overexpressing lines also affects the potato defence mechanisms triggered by *P.c. carotovorum* and more studies are needed. However, when such PRK-2 modulated plants are challenged by fungal pathogens they exhibited an enhanced plant resistance. Taken together, the results suggest that the genetic engineering of specific potato key components could be a useful strategy to generate plants with enhanced resistance to biotic stress.

Results Unforeseen in the Original Project:

The results basically meet the hypothesis we have proposed. The modulation of the expression of key plant defence components may be a good strategy to generate potato plants with enhanced

resistance to biotic stress. It is important that some of the engineered plants clearly exhibited enhanced resistance to biotic stress. Novel studies are now on the future research of such plants as well as new challenges for young and future scientists.

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URUGUAY

Title: Nitroarachidonic acid: A novel anti-inflammatory nitrated lipid

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ICGEB Contract No.: CRP/09/017

ICGEB Reference No.: CRP/URU09-01

Abstract: Nitrated lipids represent novel endogenous signalling molecules displaying pleiotropic activities, including modulation of macrophage activation, prevention of leukocyte and platelet activation and promotion of blood vessel relaxation. However, their biological roles, mechanisms of formation and levels reached in inflammatory milieu are poorly characterised. Nitration of arachidonic acid (AA), which serves as a major cell mediator through its conversion into eicosanoids, may lead to the formation of novel products that could divert AA from the straight reactions during inflammation. The proposed research plan utilised a chemical, molecular, cellular and *in vivo* experimental framework to detect, quantify and characterise the biological chemistry and therapeutical potential of nitroarachidonic acid (AANO₂). Thus, the research efforts proposed concentrated on mechanisms and consequences of AA nitration as novel footprint of inflammation. We hypothesised that a) during prostaglandin H synthase (PGHS-1) turnover AA-derived radicals could be "sequestered" by reactive nitrogen species (RNS) to form AANO₂ which in turn modulates enzyme activity; b) PGHS-1 in the presence of RNS will serve as sources of regio- and stereo-specific-derived AANO₂ and c) AANO₂ represent a novel signalling marker of inflammatory processes, facilitating the resolution of inflammation. Accordingly, we tested the capacity of AANO₂ to limit the formation of atheroma lesions in an animal model of atherosclerosis.

Objectives:

(i) Synthesis of AANO₂ via radical pathways. This includes the synthesis of AANO₂ isomers from biologically-relevant nitrating agents (peroxynitrite, nitrogen dioxide) plus the enzyme involved in AA metabolism: prostaglandin synthase-1 (PGHS-1).

(ii) Quantify AANO₂ during macrophage activation in inflammatory conditions both in culture and *in vivo* (atherosclerotic mice).

(iii) Determine the ability of AANO₂ to modulate cell activation/differentiation, with emphasis in monocytes/macrophages and platelets. These effects will be characterised in terms of cell properties (synthesis of cytokines and inducible enzymes) as well as the involved signalling pathways.

(iv) Analyse AANO₂ as novel pharmacological strategy to prevent the development of lesions in a well-established animal model of atherosclerosis: low-density lipoprotein receptor-deficient mice (LDLR^{-/-}) fed with high fat diet.

Results Obtained:

(i) Novel isomers of AANO₂ were synthesised via radical pathways, using peroxynitrite plus prostaglandin synthase-1 (PGHS-1).

(ii) We determined the ability of AANO₂ to interfere with the activation of macrophages, demonstrating that AANO₂ prevents NADPH oxidase (NOX2) assembly and superoxide radical production. We also evaluated AANO₂ effects on human platelet activation. From a metabolic prospective, AANO₂ increased 12-HETE synthesis, thus diverting AA from PGHS to the lipoxygenase (LOX) pathway. Platelet aggregation was potently inhibited in the presence of low micromolar AANO₂ in a cGMP-independent mechanism. While Ca²⁺ store-dependent mobilisation was unaffected by AANO₂, P-selectin exposure on platelets membrane was strongly inhibited. Moreover, AANO₂ inhibited protein kinase C (PKC)-dependent aggregation in agreement with immunofluorescence confocal microscopy data showing AANO₂-mediated inhibition of PKCα translocation to the membrane during platelet activation. Taken together, our results demonstrate that AANO₂ inhibits platelet activation modulating at least two metabolic pathways, PGHS-1 and PKC, providing a possible novel mechanism for platelet regulation *in vivo*.

(iii) We investigated the potential of our synthesised nitrated lipids as novel antiatherosclerotic compounds in LDLR^{-/-} mice. Feeding with HC diet caused a ~3-fold increase in plasma levels of cholesterol. AANO₂- treatment provoked a reduction in the area of aortic atheroma plaques compared with AA vehicle. Furthermore, plasma from AANO₂-treated mice showed a decrease in lipid and protein nitration suggesting lower nitro oxidative stress. All together these data support that AANO₂ represent a unique anti-atherogenic mediator down-regulating oxidative and nitrative stress pathways linked with an inflammatory disorder.

Results Unforeseen in the Original Project:

We could not quantify AANO₂ during macrophage activation (specific aim 2). Both positive and negative ion mass spectra were recorded and MS/MS was utilised to generate daughter ion spectra for structural determinations. For quantitation, calibration curves were constructed with

appropriated standards using the MRM scan mode. Under our experimental conditions, AANO₂ was not detected in plasma. This is not surprise, since nitroalkenes are fastest metabolised in plasma to exert signalling effect in cell targets.

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