Biotechnology

Introduction

The guidelines of the Biotechnology Program are research and development aiming to develop and manufacture products of pharmaceutical interest. This Program has two main research areas, namely Pituitary Hormones and Biopharmaceuticals.

The first one comprises a group with a long experience on Recombinant Human Pituitary Hormone synthesis, purification and characterization. Up to now they have worked mostly with human growth hormone (hGH), human prolactin (hPRL), human thyrotropin (hTSH) and more recently with human follicle stimulating hormone (hFSH) and human luteotropin (hLH). Besides research, they have also activities on the Biotechnological Production and Downstream Processing of the same recombinant hormones, which are produced in both *E. coli* and mammalian cells and in the development of joint-ventures with the National Industry. The biological effects of radiation on cells are also studied, especially concerning the administration of 131-I together with thyroid-stimulating hormone in thyroid cancer.

The Biopharmaceutical area is dedicated to the research of isolation, structural analysis and biological activities in different biological systems of macromolecules. These macromolecules are peptides or proteins, either native or recombinant with medical or pharmaceutical interest. During this period new proteins related to serine protease activity, breast cancer development and angiogenesis were described. The effects of ionizing radiation on macromolecules have also been investigated to detoxify animal venoms in order to improve antigens for anti-sera production, or even modify microorganisms for vaccination. Studies on the alterations caused by microgravity in excitable media in order to further characterize the physical and thermodynamical behavior of propagating waves, one of the major mechanisms of neural physiology, were also developed. These experiments were performed on a sounding rocket in collaboration with the Brazilian and German space agencies.

The Animal Laboratory Division of IPEN is responsible for the breeding and production of small laboratory animals. In this facility Specific Pathogen Free (SPF) animals are bred and maintained, under controlled sanitary conditions, to be used for testing of the radioisotopes production and research. This facility also produces different mutant mice, severely immunodeficient mice and their offspring, besides other mice lineages as well as normal rats.

Recently, Quality Assurance actions have been started for all the quality control system in order to certify the center for pre-clinical assays to be performed in this facility, assuring that clinical investigation using human volunteers will be done according to national and international guidelines.

It was given continuity to research and production activities related to the five human pituitary hormones, namely growth hormone (hGH), prolactin (hPRL), thyrotropin (hTSH), follitropin (hFSH) and luteotropin (hLH). The basic mission is the development of applied research, especially emphasizing the interaction between the Academic and the Industrial world that, although has been neglected in Brazil, led, in this case, to the introduction of some important facilities, as for example the CIETEC (a spin-off experience), at IPEN. In this 3-year period (2005-2007), in fact, it was experienced a joint-venture with an important Brazilian Pharmaceutical Company (Biolab-Sanus) that provided cospicuous achievements for the Institute. At the end of 2007 a new joint-venture was set up with a young and successful biotechnology company (FK Biotecnologia) that demonstrated great interest in hormone and antibody production for therapeutic and diagnostic applications. It can not be denied that this type of interaction is particularly challenging and sometimes difficult in this country. What can be done, still keeping this goal in mind, is to give at the same time great emphasis to scientific production and to collaboration with well-known national and international research groups. Thus, in this 3-year period, 15 scientific papers, practically all in international journals, whose impact factor was in general between 1 and 5, could be published.

Human growth hormone (hGH)

Human growth hormone (hGH) production and quality control has been already established at the laboratory level and, as stated several times, is only waiting for the "industrial decision", that unfortunately is sometimes independent from our will. However, an, important research in Gene Therapy has been carried out using the hGH gene. This approach represents indeed a way of administrating biopharmaceuticals via genetical modification of human cells that can be transplanted "ex vivo". The goal is the development of an animal model (immunodeficient dwarf mouse, lit/scid) in which, using transduced keratinocytes, could be possible to obtain useful and sustained circulatory levels of hGH and of mouse GH. Three important papers have been published in this period: an Invited Review on "Animal Models for Growth Hormone Gene Therapy" (Current Gene Therapy, 2005) and two works on the utilization of organotypic raft cultures either with hGH-secreting or with mGH-secreting keratinocytes (Molecular Biotechnology, 2006 and Journal of Gene Medicine, 2008).

A new approach is also being carried out with basis on naked hGH or mGH DNA administration followed by electroporation in either muscle or skin of the same animal. This technique, that avoids the use of heterologous cells, is already showing some positive results.

Figure 1. Muscle electroporation of lit/lit mice after administration of DNA plasmid containing the GH gene

Considering that hGH is positively influencing muscular dystrophy, a collaboration with Dr. Mayana Zatz from the "Centro de Estudos do Genoma Humano" - IBUSP was set up. Dystrophic mice of four distinct mutated strains obtained from the Jackson Laboratory (Maine, USA) were maintained in the animal house of the Biotechnology Center. A series of mating were carried out and techniques for the determination of dystrophy and growth hormone deficiency by DNA sequencing were established.

Human pituitary glycoprotein hormones

Human pituitary glycoprotein hormones include thyrotropin (hTSH), follitropin (hFSH) and luteotropin (hLH), all heterodimers formed by an alpha and a beta subunit. Human TSH is related with thyroid function and metabolism, and is used in the diagnosis and therapy of thyroid cancer, while hFSH and hLH are mostly used for the treatment of human infertility. These recombinant products are among those with the highest aggregate value, its purified forms reaching prices up to US$ 12.000/mg! Considering their carbohydrate moiety, which is strictly related to their in vivo bioactivity, these proteins must be synthesized in mammalian cells, the most commonly utilized for their industrial production being CHO cells. The laboratory has synthesized and characterized hTSH, having also the know-how for synthesizing

hFSH and hLH. In this period, though, three main objectives were focused: (i) HPLC (size-exclusion and reversed-phase) set up for analytical and preparative purposes; (ii) MALDI-TOF mass spectrometry determination of these heterodimeric glycoproteins and of their alpha and beta subunits; (iii) characterization of their carbohydrate moiety, essentially composed of complex N-glycan structures, whose determination was carried out in collaboration with the University of Vienna, Austria. Four major publications were concerned with these aspects. An invited Review described the state-of-art concerning HPLC analysis of human pituitary hormones for pharmaceutical applications, obviously including also hGH and hPRL (Current Pharmaceutical Analysis, 2006). An original research article has set up a reversed-phase HPLC methodology, specific for hFSH preparations, including MALDI-TOF-MS conditions that, for the first time, could provide, in a single assay, the molecular masses of heterodimer, alpha- and beta-subunit (Journal of Chromatography A, 2006). A semi-preparative reversed-phase HPLC column has also been used for setting up a practical laboratory-scale purification of hTSH (Journal of Chromatography A, 2007). Finally, still concerning hTSH, we studied a reduced CO_2 environment that led to a higher productivity, starting for the first time to characterize the N-glycan structures of our recombinant preparations in comparison with the only commercial preparation available (Thyrogen from Genzyme, USA). It should be emphasized that for the case of recombinant glycoproteins while the protein moiety is identical to the natural, the carbohydrate moiety is necessarily different. This difference must be studied and evaluated, especially considering the repeated parenteral use of these biopharmaceuticals. More work is in progress concerning this aspect, especially considering the comparison of the N-glycans structures of the recombinant proteins with those of the native ones: some of these studies are being carried out in collaboration with the University of Oslo (Norway). Also more work is in progress, related to hLH characterization and to the synthesis of a human-like form of hTSH.

Prolactin (hPRL)

Prolactin (hPRL) is the second (after hGH) unmodified protein hormone secreted by the anterior pituitary. Its therapeutic use is still quite limited, while it is important for diagnostic applications. A different importance has been attributed to their analogs/antagonists - an anti-proliferation activity, especially concerning breast and prostate cancer. Two hPRL antagonists (S179D-hPRL and G129R-hPRL), discovered by two leading laboratories in the US and in France, have been synthesized for the first time in our laboratory, in their authentic forms. One published paper (Protein Expression and Purification in 2006) carried out a complete physico chemical and biological characterization and comparison of the two antagonists, synthesized by CHO (Chinese Hamster Ovary) cells. Moreover one student working at the University of California at Riverside, where S179D-hPRL had been discovered, carried out advanced studies showing that this antagonist is a potent anti-angiogenic hormone (Endocrine Related Cancer, 2006), uses particular signaling pathways capable of inducing apoptosis (Endocrinology, 2006) and has an opposing effect in comparison with hPRL on the expression of cell cycle regulatory proteins (Oncology Research, 2006). A complete study, comparing hGH and hPRL behavior when synthesized in bacterial periplasmic space and setting up specific fermentation conditions for hPRL-secreting E. coli (Patent N. PI 070 1082-6, 2007) was also published in the Journal of Biotechnology (2008). Finally, in collaboration with the Neuroendocrine Unit of the “Hospital das Clínicas” of the FMUSP, a study on human macroprolactin and its biological activity in different groups of patients has been carried out and published in the Journal of Clinical Endocrinology and Metabolism (2006).

Cellular response to ionizing radiation

Ionizing radiation is a very-known genotoxic agent, but on the other hand it is a therapeutic modality used in medicine. Therefore, a better comprehension of the cellular response to ionizing radiation is of great value from the radiobiological as well as from oncological viewpoints. The present project has been developed in three interlinked aspects. In the first one (cytogenetic aspect) a comparative study of the effects of different radiation types and radionuclides in human and rodents cells has been carried out. Parameters such as survival curve, proportion of affected cells, distribution of lesions, kinetics of cell proliferation, cell viability and apoptose were studied. So, cytogenetic (chromosome aberrations and micronuclei) and biochemical (comet assay and viability test) techniques were developed and standardized in the laboratory. A study on the cytogenetic effects of neutrons produced in the Reactor R1 of IPEN-CNEN/SP (in collaboration with the “Centro de Engenharia Nuclear”) in human blood lymphocytes is being developed. Following this, dose-response curves for different types of radiation (γ, β, n) will be constructed for biological dosimetry (dosimetric aspect) purposes. Cytogenetic techniques will be applied for the quantitative estimate of absorbed dose, according to the criteria adopted by IAEA (2001). Another study that has been done is about radiosensitivity of tumoral (T-47D and MCF-7) and non tumoral (MCF-10) human breast cell lines exposed to various doses of γ-radiation, analyzing radioinduced damage, DNA repair capacity and cell viability. The data obtained showed that the tumor cell lines responded to the genotoxic action

of ionizing radiation with more intensity than non tumoral cell line. The analysis of the influence of the radioprotetor (melatonin and propolis) in the induction of cytogenetic damage caused by γ-radiation of 60Co in CHO cells has been another goal of our study. Preliminary data showed a promising reduction of the frequency of micronucleus induced by γ-radiation. Another aspect of the study (therapeutic approach) has been the analysis of the cytogenetic effects on blood lymphocytes of radiopharmaceuticals used in nuclear medicine, as 131I administered to patients with thyroid diseases such as differentiated thyroid carcinoma with or without administration of recombinant thyrotropin (Thyrogen or rhTSH produced at IPEN). The data obtained in an animal model (rats) showed that the treatment did not cause any major damage in peripheral blood lymphocytes. Thyrogen, an imported product, and rhTSH produced at IPEN behave similarly.

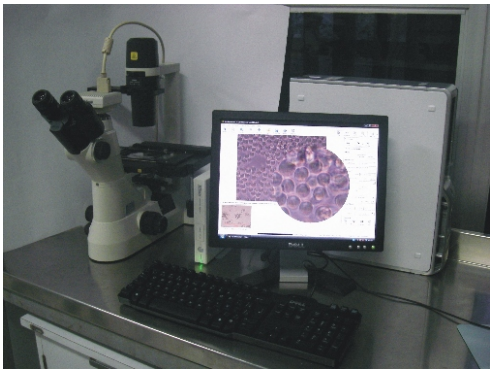

Figure 2. Inverted microscope showing blood lymphocytes

Identification, isolation and characterization of new compounds of clinical interest, from plants and animal molecules have been developed. Additionally, studies involving ionizing radiation have been done in order to detoxify or even modify molecules mainly to improve antiserum production and vaccines. Many of pre-clinical assays, the main tool, employed to characterize the candidate to a new medicine have now been standardized to get an accreditation.

Biological screening of snake venoms and toxins

Snake venoms are an extremely rich source of biologically active substances modulating several aspects of the regulation of homeostasis. The investigation of potential new drugs in biological samples has been a major field of research in many laboratories. This activity has focused part of its efforts in screening and isolating toxins with potential therapeutic uses. Amongst those, toxins from the Brazilian rattlesnake which seem to be involved in the down regulation of the sodium/potassium pump have been investigated. Such molecules have a potential for further understanding the fine physiology of many cell types and for guiding the rationale design of cellular metabolism modulators. During investigation of the action mechanism of snake neurotoxins, we developed a model of excitable medium, the Belousov-Zabotinsky reaction, was developed. It allows understanding many aspects of neuronal transmission and membrane physiology. Among several experiments a set of reactions to microgravity, using a sounding rocket provided by a collaboration between Brazilian and German Space Agencies was done, and it was possible to observe that, indeed gravity seems to be able to act at molecular levels, modulating reactions which can be compared to those ruling neuronal transmission, onset of migraine and epileptic crises and regulation of heart beating.

Figure 3. Training the insertion of the experiment in the rocket payload

Also toxins with modulatory activity on the blood clotting system (Snake Venom Serine Protease-SVSP) are under investigation. Such toxins might be employed for the treatment of coagulation disorders and as auxiliary drugs in major surgeries where unexpected activation of the blood clotting cascade could put the patient at risk. These proteases are also key players in a wide range of biological processes; for example, in regulating the cell cycle, cell growth and differentiation, affect the haemostatic system, antigen processing and angiogenesis. In addition, it is becoming apparent that the aberrant functioning of certain proteases may be involved in several disease states, including Alzheimer's disease, in cancer metastasis and in inflammation (over-reactive inflammatory reactions in CNS often cause irreversible neuronal damage). In this period it was reported the molecular cloning of five new nucleotide sequences of SVSP (GenBank accession number AY954040 EU360951; EU360952; EU360953; EU360954) that were retrieved from a cDNA library constructed with the venom gland of a single specimen of Brazilian rattlesnake Crotalus durissus terrificus. These sequences have been analyzed in silico with respect to their cDNA organization, similarity in relation to others SVSPs, their probable biological functions and the overall particularities of these nucleotide sequences. The functional dendrogram was generated to group the serine protease activities in relation to others snake venom thrombin-like enzymes. Moreover, a rapid and efficient method for screening vectors for mammalian cell expression was developed. It is based on the fact that SVSPs are difficult-to-express toxins since they contain several disulfide bounds and are glycosylated. The biochemical properties and the molecular weight of recombinant toxin were compared to native gyroxin purified from the venom and are essentially identical.

Biological evaluation of new products for health

This activity is mainly based on the biological evaluation of substances and biomaterials performing in vitro and in vivo tests. These tests are carried out in compliance with the rules of ISO-10.993 and some other international directives. Such tests include: cytotoxicity, genotoxicity, hemocompatibility etc and some others tests of systemic toxicity and implants. Synthesizes of polymeric biomaterials were also done, resulting in 3 (three) patents submitted to INPI in 2007. A biomaterial can be defined as a substance (with the exception of drugs) or a combination of substances (either synthetic or natural), employed in the treatment, improvement or substitution of organism tissues, organs or function. Since interaction with the biological system is involved, biocompatibility implies the capability of the material to exhibit in the host the

appropriate functional and "biomimetic" qualifications. In recent years, interest in biomedical applications of natural and synthetic polymers has grown steadily, with a substantial contribution to the quality and duration of human life. Presently, novel porous biologically active composites based on hydroxyapatite (HA) and poly(caprolactone) (PCL) have been developed and tested, with potential for use in scaffolds for bone tissue engineering. The experiments are focused on the synthesis and biological response of bone to the PCL/HA composite. Such work resulted in a partnership with the Biosintesis Company which received a financial support from FAPESP (PIPE project). Another approved FAPESP project about lyophilization process of biological tissues to make cardiac valves, includes IPEN, INCOR, UNICAMP and the Pharmaceutical Sciences Faculty of USP. The biofunctionality of the bovine pericardium with endothelial cells has been tested.

Recombinant proteins - Refolding from inclusion bodies using high hydrostatic pressure

Escherichia coli is the most efficient and cost-effective host for transgenic protein production for therapeutic or for scientific purposes which does not require post-translational modification. However, *E. coli* often is unable to fully refold the foreign protein during overexpression of recombinant proteins. Often bacteria accumulates these misfolded intermediates in their cytoplasmic as insoluble and misfolded aggregates known as inclusion bodies. Aggregated proteins do not possess biological activities and in many instances, injections of this kind of proteins induce immunologic reactions. A novel and robust method to disaggregate and refold murine globular protein was described. It was demonstrated that high pressure can successfully convert insoluble globular protein expressed in E. coli (like endostatin or QM) to a preparation with native tertiary structure and full biological activity. Endostatin can specifically inhibit endothelial cell proliferation and thus potently inhibit angiogenesis and tumor growth. QM or ribosomal protein L10 is originally identified as a tumor suppressor protein. The QM gene encodes the ribosomal protein L10 that is necessary for joining the 60S and 40S subunits of ribosome. This study will analyze the structure and the function in the cell of this protein.

Biological effects of ionizing radiation

Ionizing radiation, in aqueous solution, produces several highly reactive species. The most important are hydroxyl radical (OH) and hydrated electron (e-aq.). Theses products interact with peptides and proteins causing several modifications such as fragmentation, aggregation or oxidation, which are responsible for detoxification or even few modifications on proteins. These properties of ionizing radiation make it a good tool to improve antiserum production and vaccination process. Additionally, some substances called scavenger can be used to modulate these effects. It was found that the irradiated protein could be selectively incorporated to the cells, due to specific receptor for oxidized protein, the scavenger receptors. This increased uptake could also result in better antigen presentation and high immune response, either humoral, as demonstrated with purified crotoxin or cellular, as recombinant M. leprae. Rp 18 heat shock protein.

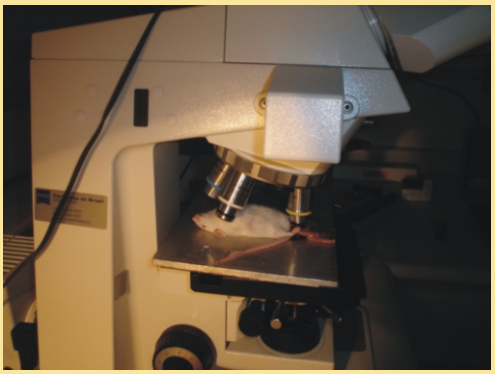

Figure 4. Facility used for Intravital microscopy

Ionizing radiation can also modify biological and structural properties of toxins as crotamine, used here as a model. Biological alterations occurred in irradiated crotamine were observed with intravital microscopy; native crotamine causes a time-dependent vasoconstriction that suffers inversion with irradiated toxin (2kGy) leading to the vasodilatation. Structural analysis suggested alteration in tertiary structure, keeping the primary structure unbroken.

The Animal Laboratory Division (DALI) is a facility having 840 m^2, distributed in production and stock areas of animal models for IPEN as well as for other institutions. Some of these models bred in this division are unique in Brazil, thus providing extremely useful tools for many investigators. The goal of this division is to act as an animal breeding and experimentation facility, sterilizing products and providing services to guarantee the genetic and sanitary quality of animals employed in investigations focusing mostly on the development of new drugs and radiopharmaceuticals.

Figure 5. Specific Pathogen Free animals kept under genetic, sanitary and environmental controlled conditions

Besides breeding animals for use in our institution, this facility also sells animals for other laboratories and offers housing of special care requiring mice and rats upon request. For further information contact nnascime@ipen.br.

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Honor Mention and Awards

2006 Best Poster presented in IV Congress of Latin American in Biomaterials and Artificial Organs RODAS, A.C.D.; HIGA, O.Z.; MAIZATO, M.J.S.; LEIRNER, A.A.; PITOMBO, R.N.M. "Cytotoxicity and genotoxicity of bovine pericardium preserved in glycerol", Caxambú, 2006

2007 Top Cited Article 200-2006 Award, Journal of Chromatography - UEDA, E.K.M.; GOUT, P.W.; MORGANTI, L. - "Current and prospective applications of metal ion-protein binding", Holanda, 2007