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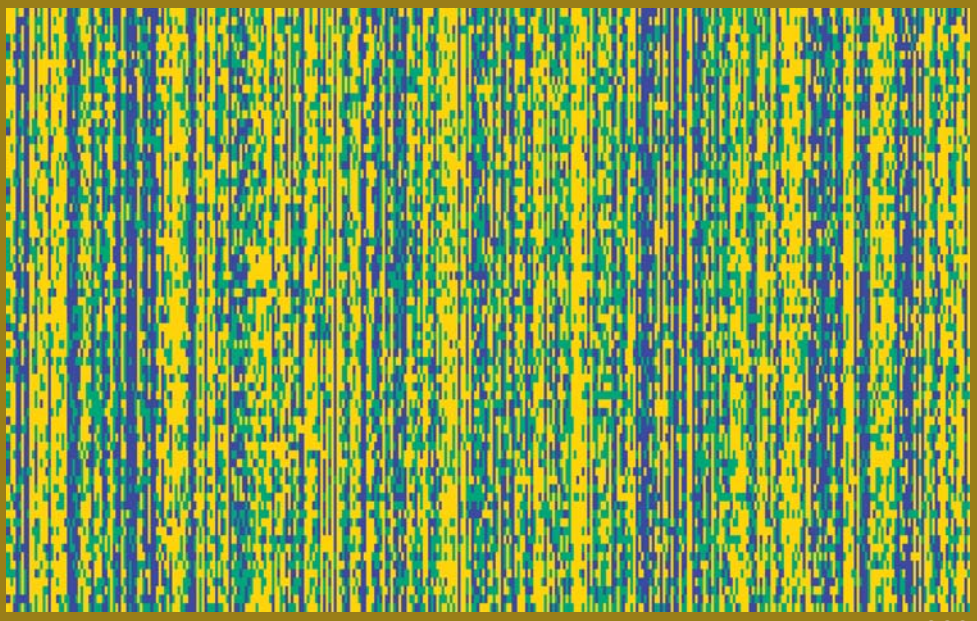

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editorial

Biotechnology and Puerto Rico: Promises that must be kept

The articles in this issue provide a sampling of the diverse efforts in the general area of biotechnology carried out in Puerto Rico. Appropriately, given the critical importance of the pharmaceutical industry in the island, I start by mentioning a meeting summary that presents the highlights of symposium entitled "Drug Discovery, Development and Clinical Research in Academia". This meeting was held on the 95th anniversary of the University of Puerto Rico School of Pharmacy. A major theme of the symposium was the increasing importance of academia in the process of drug discovery, which should lead us to ask some tough questions regarding this trend in Puerto Rico. Are we effectively transferring technology from our universities to industry? What are the appropriate parameters we should use to gauge our strides in this area – number of patents per faculty member or per dollar spent in research? How do we rate compared to others we would like to consider our peers? Are we supporting and remunerating faculty efforts to move in this direction? Whereas I do not have all the answers to these questions I venture that, if we could get the requisite data to answer them, we would conclude that there is much work to be done. Equally, I suspect we should be able to identify specific areas where Puerto Rico has a clear strategic advantage.

One such area may very well be the interface between medicine and engineering, which is addressed by two reviews in this issue. One on Magnetic Fluid Hyperthermia touches on the use of magnetic nanoparticles to specifically deliver anti-cancer drugs to tumors. The other deals with the use of nanoparticles to create imaging sensors of heart failure. Given the widespread and robust effort in medical devices in Puerto Rico, bioengineering represents an opportunity for the academic community.

Another unique strength of the community in Puerto Rico is a strong tradition of marine biology. The review entitled "Biotechnology and Biochemistry of Marine Natural Products" suggests a route to the production of important marine compounds by understanding the enzymes that synthesize them.

Finally, Puerto Rico sits at one of the crossroads between North and South America – a key location for the movement of goods and people. These positive aspects of globalization come hand in hand with a danger: the epidemic spread of disease. An excellent case in point is the rapid spread of the porcine H1N1 influenza of 2009 – the first declared pandemic in over 40 years. Two articles in this issue focus on infectious diseases. The first deals with the important question of pathogen discovery, in this case the authors use DNA sequencing to identify Mycobacterium mucogenicum in an autopsy specimen. Pathogen discovery technology and application should be a priority in Puerto Rico since the island due to its location, climate and socioeconomic structure could be an important partner in the global prevention of epidemic diseases. Following pathogen identification a

robust biotechnology industry should carry out investigation and intervention. The second article on infectious diseases highlights one of the more critical unmet needs in global health: the creation of a tetravalent vaccine for dengue. The vicissitudes of the immune response to the four dengue virus serotypes and the phenomenon of antibody dependent enhancement, which explains why subsequent infections with different serotypes can lead to more severe disease, make this a challenge. Are we ready to deal with a new pathogen reaching our shores or even for an old foe like yellow fever, which would have to travel only a few hundred miles to the north?

The sampling seen in this issue leads me to think of two promises that must be kept. Biotechnology promises to protect society from the ailments and threats described in these and many other articles – and we, the members of the biotechnology community, should continue to ask the difficult question: how well are we keeping this promise? Equally, society (represented by our government) promises to protect biotechnology. This can be justified because of the benefits in turn promised by biotechnology, but in the case of Puerto Rico the most important justification is that biotechnology provides us with one of the few viable avenues for economic sustainability.

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Message from the VP for Research and Technology

In the last decades, the powerful tools of biotechnology have revolutionized medicine, agriculture and environmental protection. Milestone discoveries and novel inventions have marked biotechnology's early history. These advances, boosted by public and private incentives, have increased attention and sustained research activity. According to a recent report from Ernst & Young, despite worldwide economic turmoil the global biotechnology industry delivered a solid financial performance in 2008, with revenues of publically traded companies growing to $89.7 billion. But biotechnology is about more than jobs and the economy; it provides the resources for an improving human longevity and health. Recent advances in understanding, preventing and treating diseases such as cancer, cystic fibrosis and Alzheimer's have been made possible by long-term investments in fundamental biomedical and biotechnology research. In addition to improving health, biotechnology has provided more abundant, appetizing and healthy foods produced with less dependence on pesticides and herbicides. Through biotechnology, microorganisms are used to remove oil spills and other hazardous wastes. In the future, many products and processes from plastic to energy will be cheaper and easier on the environment.

The full potential of biotechnology will only be realized if we continue to invest in a strong research agenda and extend our efforts into innovative application areas. To reach these goals, we must sustain education and training in disciplinary sciences while recognizing the need for communication and collaboration among disciplines. With well-recognized programs in health care, engineering, marine and environmental affairs, and agriculture, as well as research in cancer, neurological disorders, and infectious diseases, we stand to be a major player in many areas of biotechnology.

The University of Puerto Rico is committed to ensuring that Puerto Rico does not lose its competitive edge, not just because of the economic gains, but because of the many ways in which biotechnology contributes to the health of our people and the environment.

Emma Fernández-Repollet, Ph D
Vice President for Research and Technology

REVIEW ARTICLE

Antifungal Research Strategies Aiming for New Targets

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Yeasts and filamentous fungi can be true pathogens infecting healthy persons or they can be opportunistic pathogens that cause infections in humans with compromised immune systems due to cancer chemotherapy, organ transplantation treatment protocols, or HIV-1 infection, among other causes. Fungal infections can be characterized as systemic, indicating that the infection is deep, or localized cutaneous, meaning that the infection is superficial as it occurs on the skin, nails, hair or mucous membranes. Skin, nails, and hair infections by dermatophytes (*Trichophyton sp., Microsporum sp.*, *Epidermophyton sp.*) and skin, nails, mucous membranes and systemic infections by *Candida albicans* and other *Candida sp.* may occur. Also, deep fungal infections caused by *Histoplasma capsulatum* (Histoplasmosis), *Blastomyces dermatitidis* (Blastomycosis), and *Aspergillus fumigatus* (Aspergillosis) can affect the lungs and other internal organ systems. Over the years, the number of opportunistic fungal infections has increased dramatically due to the increase in immune-compromised patients, the use of broad-spectrum antibiotics, and the availability of more invasive medical procedures resulting in an increase in prescriptions of antifungal drugs and the emergence of drug-resistant pathogenic strains. Fungal infections are, therefore, an important modern medical problem causing morbidity and mortality among hospitalized patients. The increasing frequency of infections caused by *C. albicans*, *A. fumigatus* and *Cryptococcus neoformans* has also led to an expansion in the number of studies on the use of antifungal drugs in clinical practice (1-2). In the quest for new antifungal drugs, the budding yeast *Saccharomyces cerevisiae* has served as a valuable model system because many aspects of its genetics, cell wall construction, and stress signaling are also seen among other pathogenic fungal species, in particular *C. albicans*, a major opportunistic pathogen (2). Despite remarkable similarities between many fungal and mammalian metabolic and signal transduction pathways, promising antifungal targets are currently under study focusing on cell wall biosynthesis, lipid composition of the plasma membrane, and synthesis of DNA and protein.

Because of the limited success of traditional drug screening strategies and the mainstreaming of high throughput molecular genetic technologies, the search for more potent, specific, and non-toxic antifungal therapies has moved towards a wider use of applications such as gene expression profiling, insertional mutagenesis and RNA mediated gene silencing for identification of novel target proteins (3). The availability of DNA-microarrays for genome wide analysis of gene expression has provided a very important tool for research on host-pathogen interactions and the analysis of expression profiles of pathogens exposed to different physiological conditions (4-5). Gene expression profiling studies are currently being applied to the major pathogens *C. albicans*, *Cr. neoformans* and *A. fumigatus*, which can cause severe infections among the immune-compromised populations. These studies have been conducted using four main experimental strategies: 1) treatment of cells with an antifungal drug before profiling to obtain a drug-specific gene expression signature, 2) expression profiling of fungal strains containing gene deletions in suspected target genes or transcription factors as a strategy to identify cellular targets playing a central role in the control of growth and metabolism; 3) comparison of global expression profiles of phenotypic variants of the same pathogenic strain as a strategy to identify expression patterns of genes associated with drug resistance and virulence, and 4) RNA isolation for direct profiling of the pathogen while infecting the host (3). These methods have been applied in studies of differential gene expression in oral Candidiasis (6), identification of differentially expressed genes of the ergosterol pathway after treatment with itraconazole, and the role of transcription factors associated with virulence in *C. albicans* (3, 7).

Insertion mutagenesis by genetic recombination has been frequently employed to generate important information through genetic analysis of null mutant strains. In *C. albicans* the essentiality of the *RAM2* gene in the prenylation pathway was demonstrated using this technique (8) and transient loss of genes has enabled researchers to

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select essential gene targets required for cell viability (9). Using these many different variations of mutagenesis, more than 600 potentially essential genes have been identified for *A. fumigatus*. Though less widely used, gene silencing using antisense RNA or RNA interference followed by gene expression profiling, has also helped researchers identify critical genes required for growth in *C. albicans* (10) and the discovery of potential novel drug targets in *Cr. neoformans* and *Aspergillus* (11-12). Bioinformatic tools are of vital importance to these molecular genetic approaches for mining of fungal genome databases, analysis of gene expression, and sequence databases (3).

We will review areas of research that have shown promise for identification of new drug targets. For the purpose of this discussion we will organize these areas into four categories: i) Cell wall architecture, ii) Plasma membrane composition, iii) Cellular machinery, and iv) Signaling pathways. Where appropriate, we will describe our own recent efforts and contributions to this research being conducted in our laboratory.

i. Cell wall architecture

The cell wall is an essential structure for the fungal cell, which participates in multiple cellular processes such as nutrient transport, metabolism, and communication with the extracellular environment. It is the principal barrier to the surrounding environment because it controls cell turgor which maintains the normal osmolarity necessary for cellular biochemical reactions (2) and it sustains cell integrity that is essential for budding and cytokinesis (13-14). This structure is composed of glucans, chitin and mannoproteins. Since the cell wall is essential for fungal cell survival and because human cells do not have walls, this structure continues to be considered a principal target for the development of antifungal agents (2). Caspofungin, anidulafungin, and micafungin are echinocandins approved by the FDA in 2001 after several decades of intensive research (15-17). Echinocandins are natural cyclic lipopeptides that interfere with cell-wall synthesis by noncompetitive inhibition of β-1, 3-glucan synthase, an enzyme present in most pathogenic fungi (18). As a result, this inhibition leads to a depletion of cell wall glucan and its resultant osmotic instability. The echinocandin compounds LY 303366, MK 0991, and FK 463 are typically administered in combination with available standard antifungal agents because of their distinct mechanism of action (19). Another antifungal agent, nikkomycin Z (NZ), is a pyrimidine nucleoside linked to a peptide moiety that is a potent competitive inhibitor of chitin synthase III leading to inhibition of growth and osmotic fragility (19-20). Although the clinical importance of NZ is currently limited because high doses are required for effectiveness, we continue to explore its usefulness as a sensitizing agent for standard antifungal compounds. Our laboratory is currently analyzing the global expression profile of *S. cerevisiae* cells containing mutations that cause cell wall stress (21) in order to apply this knowledge in understanding the stress-response in clinically important fungi. Our approach involves genetic deletion of two components of the cytokinetic machinery, myosin II and chitin synthase II that cause cell wall stress and sensitize yeast cells to NZ (22). We have reported that sensitivity to NZ is also enhanced in a dose-dependent manner by co-treatment with the compound 2,3-Butanedione monoxime (BDM) (22), a disruptor of the cortical cytoskeleton in mammalian and fungal cells (23). As we will discuss later, the *PKC1* signaling pathway is essential for cell survival under these conditions (21). Inhibitors of *PKC1* or downstream effectors of this pathway may therefore have potential as sensitizing agents for standard antifungal agents.

ii. Plasma membrane composition

The plasma membrane is the permeability barrier of the cell composed mainly of sterols and phospholipids that determine its fluidity. Fungal plasma membranes are similar to mammalian plasma membranes in their structure except for having ergosterol rather than cholesterol as the principal sterol. Ergosterol is the site of interaction for polyene antibiotics while azoles interfere with ergosterol synthesis. The polyene antibiotics are produced by *Streptomyces* species and bind directly to ergosterol to form complexes that increase membrane permeability. As a result, there is a rapid leakage of cytoplasmic content including cellular potassium, other ions, and small molecules leading to the inhibition of glycolysis, respiration and cell death. Amphotericin B, nystatin, and pimaricin are polyene antibiotics widely used clinically because of their high affinity for ergosterol and relatively low affinity for its mammalian counterpart, thereby limiting their toxicity in mammalian cells (20). The azole antifungal agents are classified as imidazoles (ketoconazole and miconazole) or triazoles (itraconazole and fluconazole) as determined by the number of nitrogens (either two or three respectively) in the five-membered azole ring (19). Their mechanism of action consists in the inhibition of the ergosterol synthesis by interfering with the cytochrome P-450-dependent enzyme lanosterol demethylase (18-19). The resulting ergosterol depletion leads to plasma membrane permeability changes and inhibition of growth. A limitation of the azole antifungal drugs is their frequent interactions with co-administered drugs, which may result in adverse clinical consequences (24).

Besides targeting the ergosterol in the membrane, current efforts are also focusing on enzymes involved in the biosynthesis of phospholipids or sphingolipids that are exclusive of fungi (25). Fungal sphingolipids synthesized from phytoceramide are essential for growth and cell viability. Although the metabolism of sphingolipids is similar among fungi and animals, certain enzymes involved in sphingolipids synthesis are considered potential antifungal drug targets due to specific differences with their mammalian counterparts (1). Serine palmitoyltransferase is an enzyme that catalyzes the committed step in sphingolipid biosynthesis conserved from fungi to humans. Inhibition of this enzyme by the natural compounds myriosins, sphingofungins, lipoxamycins, viridiofungins, and the non-natural product cycloserine block the production of all sphingolipids resulting in fungal cell death (1). Inositol phosphorylceramide synthase (IPC-synthase) is the enzyme catalyzing the first fungus-specific step in sphingolipids synthesis and is therefore an ideal target of antifungal drugs (25). There are several natural compounds described that inhibit the activity of this enzyme, including aureobasidin A, khafrefungin, and rustmycin (1). Moreover, the *AUR1* gene of *S. cerevisiae* encodes a protein that is necessary for IPC synthase activity and confers resistance to the antifungal drug aureobasidin-A when mutated (26). Aur1 protein may represent a new target for screening for IPC synthase inhibitor compounds.

a. Novel fatty acids

Though not yet proven to interact with the plasma membrane, novel fatty acids derived from marine sponges have been found to be fungitoxic, either by plasma membrane disruption that eventually disintegrates the cell (27) or by inhibiting fatty acids biosynthesis (28). Acetylenic fatty acids (8-16 carbons) are one of the most promising groups of fatty acids to exert antifungal effects (29), while α-methoxylated fatty acids are less toxic (30). These represent a group of compounds with antifungal drug-sensitizing properties that will be investigated in order to elucidate their mechanism of action.

iii. Cellular machinery

Components of the cellular machinery have been proposed as potential drug targets in fungi. Specific examples are topoisomerases that belong to the nuclear DNA replication and RNA transcription machinery, where they are key components in DNA-RNA synthesis. Topoisomerases are enzymes that act in several biochemical processes such as chromosome replication, transcription, recombination, and chromosome segregation by controlling the topological state of DNA (31). Camptothecin, a topoisomerase-specific inhibitor, stabilizes topoisomerase/DNA complexes, leading to DNA damage and cell death (31). Several studies have demonstrated that Topoisomerase 1 (*TOP1*) is essential for life and is a virulence factor for some fungi (32). Its deletion in *C. albicans* induces slow cellular growth and aberrant cell morphology (32). The fungal *TOP1* gene has a considerable amount of coding sequence not present in human homologs (33) suggesting differences in their protein structure and presenting the possibility of exploring these topoisomerases as drug targets.

The elongation factors required for mRNA translation in eukaryotes are promising targets for antimycotic drugs. For example, the elongation factor III (EF3) is unique to fungi and is essential for protein synthesis (31). Sordarin and sordarin-like compounds act by blocking the protein elongation cycle at the initial steps of ribosomal translocation, prior to GTP hydrolysis. They have *in vitro* activity against a wide range of pathogenic fungi, including *Aspergillus* spp, *Candida* spp, *Cr. neoformans*, other filamentous fungi, (34-35) and *Saccharomyces cerevisiae*, making them promising antimycotic agents.

Pyrimidine analogs also have been used as antifungal agents. The fluoropyrimidine 5-fluorocytosine (5-FC) is an FDA-approved drug in this class although it has a limited spectrum of activity and significant potential for toxic effects (36-37). The mechanism of action of 5-FC involves intracellular deamination to 5-fluorouracil followed by its incorporation into RNA that causes miscoding and blocking of RNA and DNA biosynthesis (20).

The ubiquitin (Ub) system is involved in protein degradation in eukaryotic cells including fungi. It was reported that ubiquitins (Ubs) in the fungus *Aspergillus nidulans* were strongly expressed in the presence of antifungal drugs like amphotericin B and miconazole (38). Another study suggested that the expression of Ub genes in fungi might be enhanced by different kinds of antifungal agents, correlating with resistance to drugs (39). Investigators have suggested that Ub might be associated with different receptor-like components at the cell surface and these could be related to antifungal drug resistance (39-40). We have accumulated substantial experimental evidence to confirm that resistance to nikkomycin Z can be correlated to an elevated expression of the ubiquitin conjugating enzyme *UBC4* and the ubiquitin gene *UBI4* (41) while the actual mechanism for this resistance is not defined.

Sumoylation is a post-translational modification pathway composed of SUMO (Small Ubiquitin-like Modifiers) enzymes that regulate diverse cellular processes such as intracellular trafficking, cell cycle, DNA repair and replication, RNA metabolism, and cell signaling through

its reversible and covalent attachment to target proteins (42). Among the SUMO substrates are a large number of regulators of gene expression, particularly transcription factors, co-activators or repressors (43). An analysis of SUMO-mediated stress response in *S. cerevisiae* revealed that ethanol stress increases sumoylation while affecting cell growth (44). Further studies on the regulation of this pathway in pathogenic conditions can offer insights into sumoylation as a potential drug target.

Lindquist and colleagues have established that the molecular chaperone Hsp90 enables the emergence and maintenance of fungal drug resistance (45). For *Candida albicans*, Hsp90 mediates resistance to azoles while for *Aspergillus terreus*; Hsp90 is required for basal resistance to echinocandins. These investigators employed combination therapy with an Hsp90 inhibitor successfully as an experimental therapeutic strategy against these fungi in model systems. They have concluded from this work that the use of Hsp90 inhibitors "enhances the efficacy of existing antifungals, blocks the emergence of drug resistance, and exerts broad-spectrum activity against diverse fungal pathogens."

iv. Signaling pathways

a. PKC1-dependent cell wall integrity pathway (CWIP)

Integrity of the cell wall during fungal growth is essential for survival and adaptation to environmental stresses. Although several signaling pathways may contribute to the maintenance of cellular integrity, the CWIP is considered essential for sensing cell wall perturbations under many cell stress conditions. The signaling activity of this pathway is initiated at the cell surface through sensor-transducer proteins and is transmitted to downstream effectors through Rom2p-Rho1p complexes culminating with the activation of the MAPK/Slt2p. A family of sensors constituted by members of the Wsc-family, Mid2p, and its homologue Mtl1p, are localized upstream of the MAP kinase cascade (46). This signaling cascade is a linear pathway comprised of calcium-dependent protein kinase (*PKC1*), a MEKK (*BCK1*), a pair of redundant MEKs (*MKK1/2*) and a MAP kinase (*MPK1/SLT2*) (Figure 1) (2). The targets of Mpk1p include two transcription factors, Rlm1p and Swi4/6p, involved in the regulation of multiple cell wall components and cell cycle genes respectively.

Antifungal drugs such as caspofungin, block cell wall synthesis by inhibiting β-1, 3-glucan synthases (47). Synergistic effects are observed *in vivo* and *in vitro* when this echinocandin is combined with other antifungal drugs such as fluconazole or amphotericin B (48). Research has shown that caspofungin activates the *PKC1* pathway leading to Slt2p phosphorylation, and has identified Wsc1p cell surface protein as the sensor for the stress induced by caspofungin (46). Like the inactivation of β-1, 3-glucan synthases, a *PKC1* deletion mutant causes loss of osmotic integrity (49). The antifungal natural product cercosporamide is a highly selective and potent *PKC1* inhibitor (50) that in combination with caspofungin has a synergistic effect against the opportunistic pathogen *C. albicans*, as well as against budding yeast *S. cerevisiae*. In our own studies with *S. cerevisiae*, we have found that loss of myosin type II function by genetic deletion (*myo1*Δ) activates the *PKC1* pathway. Function of the *PKC1* pathway was shown to be essential because a null mutation in *SLT2*, the final MAP kinase effector of this pathway, exhibited a synthetic lethality phenotype in a *myo1*Δ strain (21). In this light, one of our current working hypotheses is that inhibition of myosin II function can sensitize yeast cells to antifungal compounds that interfere with cell wall biogenesis like caspofungin and nikkomycin Z.

b. TOR signaling pathway

The targets of rapamycin (TOR) proteins are members of a ubiquitous family of signaling proteins. These proteins have essential conserved roles in transducing signals in response to exogenous or endogenous cellular stimuli (1). TOR proteins were discovered by mutations that control cell growth by conferring resistance to the growth inhibitory effect of the potent antifungal agent rapamycin. Since this drug interferes with TOR signaling in eukaryotic cells, it has been used as a chemical probe to delineate TOR-dependent responses in these cells (51). Rapamycin is toxic to some pathogenic yeast and fungi, including *C. albicans*, *Cr. neoformans*, and *A. fumigatus* (52). In *S. cerevisiae*, rapamycin inhibits the TOR pathway by disrupting the stable complex formed between the phosphatase regulator Tap42p and the protein phosphatase Sit4p which results in cell cycle arrest and antiproliferative effects (53), downregulation of translation initiation (54), and repression of ribosome biogenesis (Figure 1) (55). Our ongoing studies have shown that *myo1Δ* cells are hypersensitive to the inhibitory effects of rapamycin suggesting that combined defects in cell wall biogenesis and cytokinesis can sensitize cells to this drug. The amino acid analog cispentacin that inhibits homoserine dehydrogenase (56) has been considered for use as an inhibitor of amino acid biosynthesis in fungi. A novel use of this compound may provide a strategy to inactivate the TOR pathway in fungal cells by inducing nutrient starvation while avoiding the immunosuppressive action of rapamycin observed in mammalian model organisms (Figure 1).

c. PKA signaling pathway

The signaling cascade mediated by cyclic AMP (cAMP)-dependent protein kinase A (PKA) is involved in the regulation of virulence, morphogenesis, and development in various fungi (57). This pathway is composed of Ras proteins (encoded by *RAS1* and *RAS2* GTPases) that consecutively activate adenylate cyclase (encoded by *CDC35* in *C. albicans* and *CYR1* in *S. cerevisiae*), which generates cAMP resulting in PKA activation (58). The regulatory subunit of PKA is encoded by BCY1 while the PKA catalytic subunit is encoded by two functionally redundant genes *TPK1* and *TPK2* (58). The PKA pathway was also implicated in fungal resistance to certain antibiotics, including polymyxin B, dicarboximide, and azoles (59). *CDC35* and *CYR1* mutations demonstrated a hyper-susceptibility to azole and other sterol biosynthesis inhibitors (59). Down regulation of the adenylate cyclase regulator *RAS1* was observed in myosin II-deficient (*myo1Δ*) cells (21). We are currently analyzing the correlation between hypersensitivity to nikkomycin Z and rapamycin, and the activation status of the RAS/PKA signaling pathway in this mutant.

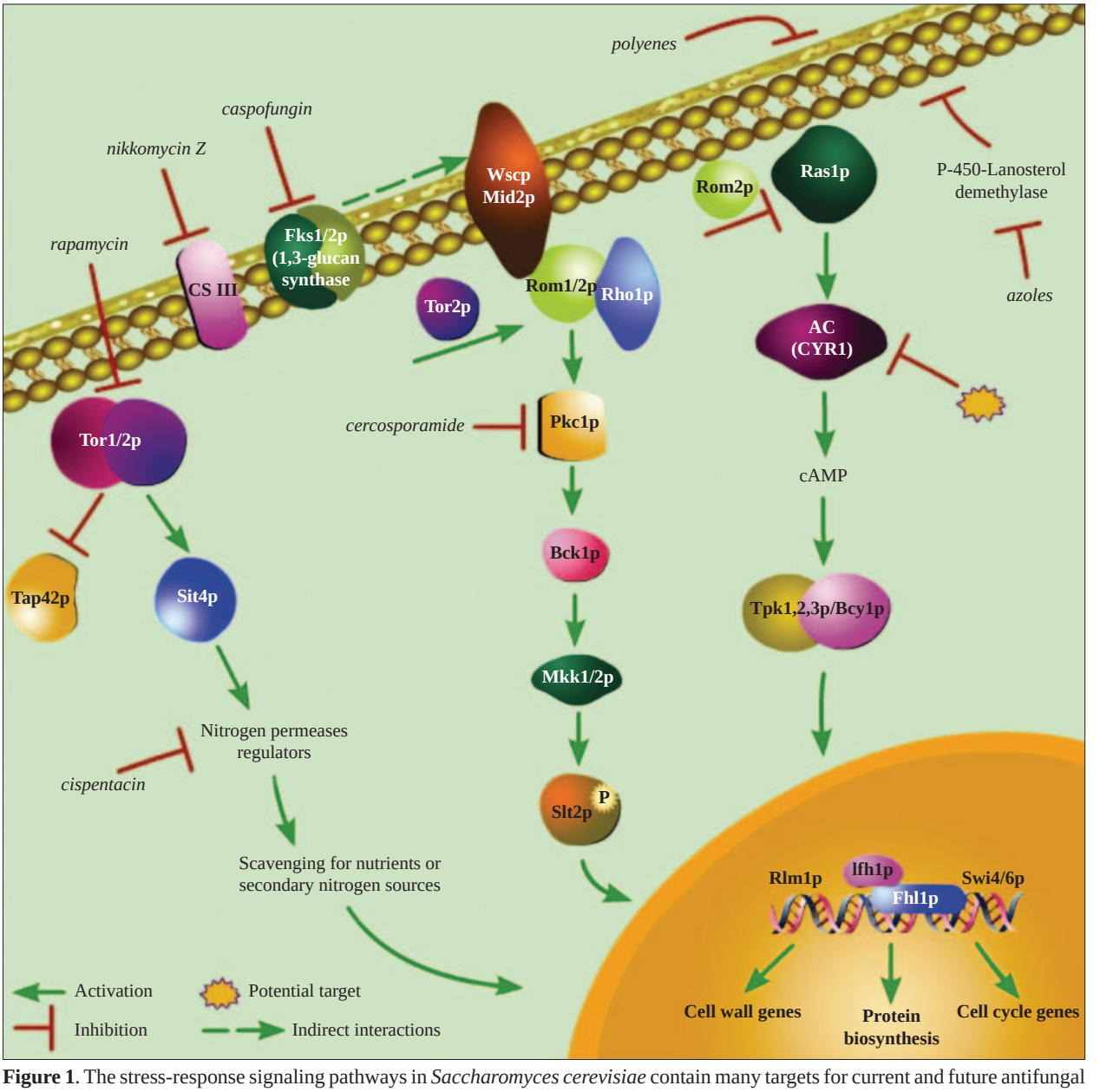

Figure 1. The stress-response signaling pathways in *Saccharomyces cerevisiae* contain many targets for current and future antifungal agents. Inhibitors for specific components of these signal transduction cascades are shown in italics.

Conclusions

Development of new antifungal strategies requires detailed knowledge of the fundamental controls governing fungal cell division, intracellular signaling, and growth. We have discussed diverse approaches used to develop new compounds into viable drugs yet the mechanisms of action of many compounds with development potential remain undetermined. The use of non-pathogenic model systems such as *Saccharomyces cerevisiae* for such studies can contribute a great deal to our knowledge of basic fungal biology which can then be extrapolated to clinically relevant fungi and accelerate the development of translational applications.

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Applications of Magnetic Nanoparticles in Medicine: Magnetic Fluid Hyperthermia

MAGDA LATORRE, Ph D; CARLOS RINALDI, Ph D

Nanoparticle systems are an intense subject of research for various biomedical applications. Colloidal suspensions of magnetic nanoparticles are of special interest, particularly in bioimaging, and more recently, in Magnetic Fluid Hyperthermia (MFH). MFH promises to be a viable alternative in the treatment of localized cancerous tumors. The treatment consists of locally injecting magnetic nanoparticles in fluid suspension into the tumor site and exposing the site to an oscillating magnetic field, where nanoparticles dissipate energy in the form of heat, causing a localized rise in temperature and tumor cell death. Here we will review methods of magnetic nanoparticle synthesis, and the role of the nanoparticle surface coating in achieving colloidal stability, minimizing toxicity, and targeting. Finally, we review *in vitro* and in vivo MFH experiments, and clinical studies in the treatment of glioblastoma multiforme and prostate cancer.

Key words: Nanoparticles, Iron oxide, Magnetic fluid hyperthermia

1. Nanotechnology in Medicine

The term nanotechnology applies to the creation, manipulation, and application of materials at the molecular and atomic scale, that is 1-100 nm. The fundamental properties of many materials at the nanoscale are uniquely different than those of bulk materials (1). These differences are mainly due to the vastly increased ratio of surface area to volume. This in turn results in an increased number of surface atoms, quantum electromagnetic interactions, increased surface tension, and size confinement effects. For example, quantum effects become significant for structures under 50 nm, resulting in unusual optoelectronic and magnetic properties of nanostructured materials compared to bulk materials (2). Nanomaterials have become an intense subject of research in fields such as optimizing the use and harnessing of energy, waste management, electronics, information and communication, and medicine (3).

Because the nanoscale lies at the intersection between the largest biological molecules and the smallest manmade devices, nanomaterials are expected to have a major impact in the medical field. Already, there are a few examples of commercially available nanomedicines that are approved by the FDA. Abraxane™, an albumin-bound form of paclitaxel with a mean particle size of approximately 130 nanometers, is used to treat breast cancer. Doxil® is used for the treatment of refractory ovarian cancer and AIDS-related Kaposi's sarcoma and it consists of lipid nanoparticles with a polyethylene glycol (PEG) coating. This coating helps evade the potential impact of the immune system and provides a means for delivery of drugs to disease-specific areas of the body. Other examples are Feridex™ and Resovist™, iron oxide-nanoparticle based Magnetic Resonance Imaging (MRI) contrast agents (4).

1.1. Biomedical Applications of Nanoparticles.

Biomedical applications of nanoparticles can be divided into two broad categories: diagnostics and therapeutics. Nanotechnology promises to optimize technologies used for diagnostics as well as the development of numerous types of therapies, with potential uses in biosensing (5), imaging (6), drug delivery (7), and in cancer treatment (4). For general reviews on the development of nanoparticles for clinical and biomedical applications see (8-10).

The sensing of biological agents and diseases is an important goal for biomedical diagnosis (11). The unique physicochemical properties of nanoparticles make these systems promising candidates for sensing applications. As an example, Mirkin and colleagues have devised a system consisting of oligonucleotide- labeled nanoparticles (12). Upon recognition of a target DNA sequence, usually associated with some pathology, the oligonucleotide-nanoparticle complexes aggregate changing the color of the solution. This technique allows for recognition of

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target DNA without need of polymerase chain reaction (PCR), which is the method currently employed for screening of genetic diseases.

In the field of bioimaging, there are two types of nanoparticles that have played important roles: quantum dots are used for optical imaging (13) and magnetic nanoparticles are used for magnetic resonance imaging (MRI) (6). Quantum dots are characterized by stable, narrow fluorescence emissions, an advantage over organic dyes which suffer from rapid photo-bleaching. Magnetic nanoparticles on the other hand serve as MRI contrast agents which can be targeted to tissues with insufficient contrast for MRI.

Nanotechnology has also helped advance the design of novel drug delivery systems. Researchers exploit nanoparticle surface chemistry by encapsulating the drug within a polymeric layer that will allow release under desired conditions. As an example, Hong, et al. prepared cationic gold nanoparticles attached to a hydrophobic drug analog (14). The cationic surface of the nanoparticle allows it to penetrate into the cell. Drug release was triggered by the high intracellular concentrations of glutathione (GSH) relative to extracellular GSH concentrations.

1.2. Nanoparticle/Cell Interactions

The biodistribution of nanoparticles is primarily governed by their ability to negotiate biological barriers, such as the endothelial and epithelial barriers found in vessel walls, the placenta, and the intestines. Additional barriers include the cell membrane and cell organelles, enzymatic degradation, uptake by phagocytic cells, abnormal blood flow, abnormal hydrostatic pressure at target sites, and molecular and ionic efflux pumps that expel drugs form target cells (15).

In order for many of the biomedical applications mentioned above to be successful, the nanoparticle's physicochemical properties must be tailored to promote specific interactions between the cell and the nanoparticle. For example, for some of the imaging, sensing, and sorting applications it is required that nanoparticles selectively attach to a targeted cell type. On the other hand, for some therapeutic applications it is necessary that the nanoparticle be internalized into the targeted cell type, or even localized to a specific intracellular compartment (16). A major obstacle in the refinement of these tools is the lack of fundamental insight on the interaction of nanoparticles with biological systems. In order to achieve a desired effect, nanoparticles need to interact in precise ways with specific cell types, and a major setback has been a lack of research focused on studying such interactions. It is to be expected that the interaction between nanoparticles and cells should be governed by physicochemical properties such as particle size, shape, and surface chemistry (e.g., surface charge, hydrophillicity, chemical functional groups, etc.) (17). There is therefore a need for detailed studies that take into account how these physicochemical properties affect nanoparticle/cell interactions.

An essential component of a biocompatible nanoparticle is the shell and surface functional groups. Most targeting efforts have been directed towards attaching ligands that are selectively recognized by receptors that are expressed in the cells of interest. In antibody-directed cell targeting, antibodies against cell surface markers are attached to the nanoparticles. They allow precise selection of cells bearing an antigenic determinant (18, 19). However, antibodies are costly and even though the targeted cell population is recognized with high specificity, the fraction of targeted cells interacting with the antibody- decorated nanoparticle is relatively low. Therefore, other approaches besides antibody-directed cell targeting should be advantageous for applications where high fractions of nanoparticles interacting with cells are needed. There is much interest in studying nanoparticle/cell interactions where the nanoparticle coating is not specific, i.e., nanoparticles coated with various polymers or polysaccharides (16). There are various reports where "unspecific" nanoparticle coatings show preferential specifity for certain cell types (20-22). Elucidation of molecular mechanisms involved in nanoparticle uptake with non-specific polymeric coatings is pivotal for further studies aimed at regulation of molecular mechanisms of nanoparticle internalization. This will allow researchers to exploit these mechanisms to enhance total nanoparticle uptake and to manipulate intracellular sorting and trafficking of these nanoparticles for specific intracellular targeting.

1.3. Colloidal Stability

For nanoparticles to be useful in biomedical applications they must be colloidally stable in biological media. Colloidal stability refers to the capacity of a nanoparticle solution to resist agglomeration and subsequent precipitation. Colloidal nanoparticle systems for biomedical applications should exhibit low toxicity, resist sterilization techniques such as autoclaving, and possess a long shelf life (7).

Bare nanoparticles are inherently unstable under physiological conditions, thus they are usually coated with biocompatible polymers that improve stability. Colloidal stability will depend upon repulsive and attractive forces that exist between particles. Nanoparticles are attracted towards each other due to so-called dispersive van der Waals forces, which are very strong at close distances. In the case of magnetic nanoparticles, there is also an attractive magnetic interaction which is effective over

much longer range. Attraction between nanoparticles may also be the result of the presence of other solutes, such as high molecular weight molecules, in the medium in what is called depletion flocculation. On the other hand, common repulsive interactions include electrostatic repulsion of like-charged surfaces and steric and osmotic repulsion between surfaces coated with polymers. In many cases the attractive interactions are inevitable and the nanoparticle surface must be engineered to introduce sufficient repulsion to avoid aggregation and the undesirable changes in properties which ensue.

In order to be effective in biological applications, nanoparticles should remain colloidally stable when encountering physiological ionic strengths and the range of pH found in biological systems, and should resist absorption of proteins present in the bloodstream. For example, in oral drug delivery systems, the nanoparticle must withstand the acidic stomach environment until reaching its target, but release the drug in the more neutral pH of the intestines, where the drug can be more readily absorbed into the bloodstream (23). Figure 1 illustrates the colloidal stability of polyethylene glycol (PEG) -coated magnetite nanoparticles across a wide range of pH and ionic strengths. Monodisperse magnetite nanoparticles were synthesized and then modified with PEG using a silane functionalized PEG obtained by reacting 3-aminopropyl triethoxysilane with carboxylic acid methoxy PEG. Colloidal stability was studied by determining particle size in aqueous solutions with increasing pH and salt concentrations (24).

2. Magnetic Nanoparticles

Magnetic nanoparticles are the subject of intense research focusing on their synthesis, characterization, and functionalization. The growing interest in magnetic nanoparticles stems from the capability to induce particle motion and rotation using an external magnetic field, coupled with their small size, ease with which their surfaces may be functionalized with surfactants and polymers, and, in the case of iron oxides, their biocompatibility. They are attractive in various novel applications including: a) MRI contrast enhancement agents (25-27), b) magnetically targeted drug delivery (25-26), c) magnetic cell sorting schemes (28), d) nano-/bio-sensors (5, 29), and e) Magnetic Fluid Hyperthermia (30). Magnetite and maghemite are the most ubiquitous class of magnetic nanoparticles being studied, but other materials such as iron alloyed with cobalt, nickel, and platinum are being investigated.

In the field of diagnostics, Magnetic Resonance Imaging (MRI), based on the nuclear magnetic resonance phenomenon, provides the possibility of detecting early malignant tumors with the assistance of appropriate contrast agents. Researchers continue to develop novel magnetic materials to achieve this aim. While signal intensity of the contrast agent is a crucial feature for the detection of small tumors (31), the coating of the contrast agent must also be taken into consideration when engineering these nanoparticles. The surface coating will have important effects on nanoparticle half-life before excretion from the system, targeting specificity, and amount of nanoparticle internalization into cells (32). MRI detection of atherosclerotic plaques using magnetic nanoparticles is currently being investigated. Patients with substantial carotid narrowing caused by atherosclerotic plaques are at increased risk for major stroke. Magnetic nanoparticles show promise in identifying vulnerable plaque inflammation *in vivo* in humans, in which areas

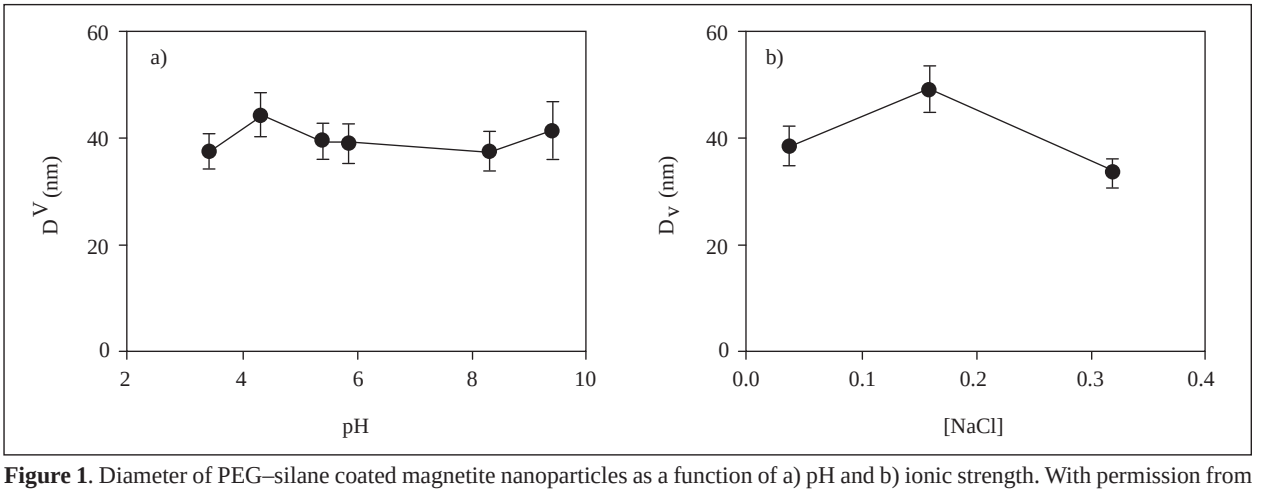

Figure 1. Diameter of PEG–silane coated magnetite nanoparticles as a function of a) pH and b) ionic strength. With permission from Barrera, et al. (24).

of focal signal loss on MR images have been shown to correspond to the accumulation of iron particles in plaque macrophages (33).

In the field of therapeutics, the use of magnetic nanoparticles for the treatment of cancer and other diseases is still at the proof-of-concept stage. There are currently many nanoparticle-based methods being investigated for the purposes of specifically targeting drugs and other molecules to diseased cells and tissues. One example is how magnetic cell sorting techniques exploit interactions between specific cells and nanoparticles specifically engineered to target these cells. These techniques have proven to be effective in such applications as embryonic stem cell purification (34) and in the enrichment of plasma cells from murine bone marrow (35).

One of the most recent and promising applications for the use of magnetic nanoparticles is the magnetically actuated delivery of drugs (36-40). Targeting is typically achieved, as mentioned previously, by tailoring the nanoparticle surface to recognize the cell of interest. Various researchers have successfully shown this can be achieved. Magnetically actuated drug delivery has the features of most other nanoparticle-based drug delivery systems, but it has the advantage that the release of the drug or molecule of interest can be controlled by a magnetic field. This application takes advantage of the temperature increase generated by the magnetic nanoparticles in the presence of an oscillating magnetic field. This temperature increase is then utilized to stimulate a thermoresponsive polymer which is surface grafted onto the nanoparticle. Another advantage of such a system is that the magnetic field can be applied after the particles reach the desired tissue, as determined by Magnetic Resonance Imaging. With proper design one can envision these particles as both therapeutic and diagnostic agents, so-called theranostics.

The rest of this review is focused on the application of magnetic nanoparticles in Magnetic Fluid Hyperthermia (MFH). MFH consists of targeting magnetic nanoparticles to cancerous tumors and applying an oscillating magnetic field to the tumor region. This will cause the magnetic particles to dissipate energy in the form of heat. The associated rise in temperature causes cancerous cells to undergo apoptotic cell death. Various groups have shown this treatment to be successful in *in vitro* and *in vivo* experiments, which will be discussed below.

2.2. Synthesis Methods

Superparamagnetic Iron Oxide Nanoparticles (SPION's) are typically monocrystalline and composed of magnetite (Fe_3O_4) or maghemite (γ-Fe_2O_4). Iron oxide nanoparticles vary in their size and types of surface coating, factors which significantly affect their blood half-life, biodistribution, and extent of uptake. *In vivo*, large iron nanoparticles tend to have a short blood half-life and are quickly removed by macrophages of the liver and spleen (26).

The synthesis method utilized to produce SPIONs determines the size and polydispersity of the particle population (10, 41). A commonly used method for magnetite synthesis is the coprecipitation of iron salts in aqueous media at room temperature under basic, inert conditions (42). The size, shape, and composition of the particles produced are highly dependant on reaction temperature, pH, and ionic strength (43). This method has the advantages of being facile, producing large amounts of particles in a single batch, and access to a substantial literature on surface modification of the particles. As an example, our group has produced superparamagnetic magnetite nanoparticles through the co-precipitation method (44). An aqueous solution of 0.36 M ferric chloride hexahydrate is mixed with an aqueous solution of with a solution of 0.18 ferrous chloride. This mixture is taken to pH 8.0 in the presence of a nitrogen stream and vigorously stirred for 1 hour. Magnetite nanoparticles are then precipitated from the aqueous solution. This relatively straightforward method results in the formation of large amounts of magnetic core clusters of about 36 nm composed of single particles around 10 with the drawback that the clusters generated are very polydisperse. Difficult control of aggregation and particle size distribution are the disadvantages of the co-precipitation method.

Attractive alternatives to co-precipitation are the thermal decomposition methods reported in recent years (45-50). In the method due to Park, et al. (47) one prepares an iron oleate precursor which is then decomposed into an iron oxide at high temperature in an organic solvent. The resulting nanoparticles have narrow size distributions but are unfortunately coated with a hydrophobic layer of oleic acid. In order to obtain stable aqueous dispersions of these particles in water, OA on the surface of the particles is exchanged for another ligand (51), which not only stabilizes the particle in suspension but can also serve to covalently attach other molecules to the surface of the particle (24).

Figure 2 shows TEM images of magnetite nanoparticles synthesized through the coprecipitation and thermodecomposition methods. Clearly the former method produces particles with a wider size distribution, but more importantly, the latter method produces particles that are separated from each other, i.e. that are singly dispersed in solution. Retaining this state of individual dispersion while modifying the particle surface to make the particles hydrophilic is an important step toward applying these very uniform particles in medicine and biology.

2.3. Nanoparticle Coatings

While manipulation of the magnetic core is a very important challenge in the engineering of SPIONs, additional factors need to be addressed. Colloidal stability, cytotoxicity, and target specificity of the nanopaticle system need to be considered (9, 41, 52). These challenges have motivated efforts to modify the surface of nanoparticles to improve their colloidal stability by introducing coatings that provide steric and/or electrostatic repulsive interactions. In addition to providing stability, the nanoparticle coating must also be non-toxic. Various groups have reported on the effect of nanoparticle coating on cellular toxicity. Goodman and colleagues demonstrated that cationic nanoparticles were moderately toxic, whereas anionic nanoparticles were nontoxic (53). They found that nanoparticles functionalized with quaternary ammonium had mild effects on cell viability in COS-2 and red blood cells, while carboxy-functionalized nanoparticles did not. Pisanic, et al. found that magnetic nanoparticles coated with dimercaptosuccinic acid (DMSA) were toxic to neurons in a dose-dependent manner (54). On the other hand Wilhelm and colleagues have shown that DMSA coated nanoparticles are non-toxic to HeLa cells or RAW macrophages (20). These examples serve to illustrate the importance and complexity of choosing an appropriate surface coating for the desired application.

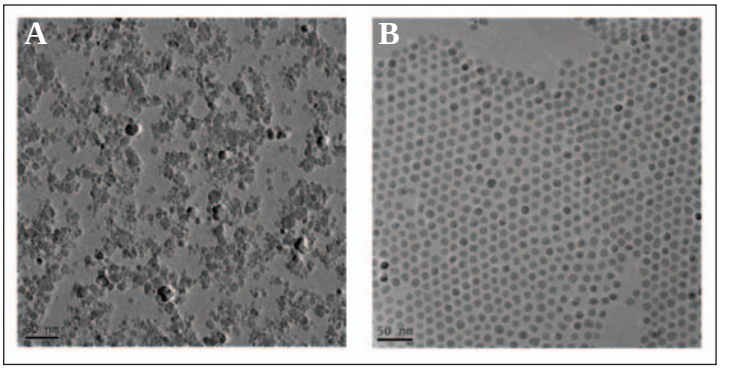

Figure 2. TEM images of magnetite nanoparticles pre-pared by a) coprecipitation and b) thermal decomposition.

Poly-ethylene glycol (PEG) (55), poly-vinyl alcohol (PVA) (56), and the polysaccharides chitosan (57), dextran (58), and carboxymethyl dextran (CMDx) (59) are commonly used polymers for coating magnetic nanoparticles. The stabilizing coating can also be used as a platform to anchor other molecules that give the original nanoparticle an extra degree of functionalization or specificity. Proteins and antibodies can be attached to the nanoparticle coating and these may be recognized by specific receptors in targeted cell types (60).

The commercially available liver contrast agent Feridex™ is composed of magnetic nanoparticles coated with cross-linked dextran. Commercially available carboxymethyl dextran coated nanoparticles (Magneticfluids, Berlin, Germany) were shown by Schwalbe and colleagues to efficiently separate tumor cells from leukocyte/cell suspensions (21). Bhattari, et al. have demonstrated that N-hexanoyl chitosan-coated magnetite nanoparticles are efficiently taken up by RAW macrophages, showing potential use in applications such as MRI and cellular labeling (57). Yoo and colleagues created magnetic nanoparticles for use as liver MRI contrast agents by successfully targeting rat hepatocytes *in vivo* (61). Nanoparticles were coated with polyvin ylbenzyl-O-B-D-galactopyranosyl-D-gluconamide (PVLA) containing galactose moieties. The galactose group is recognizable to the hepatic asialoglycoprotein receptors.

3. Magnetic Fluid Hyperthermia

Current cancer treatments generally fall into a few general categories: chemotherapy, radiotherapy, and tumor extirpation. Although these approaches have saved countless lives, they are not always enough to eradicate the disease. In addition, chemo- and radiotherapy produce such debilitating side effects that the patient's quality of life is so poor that they often refuse further treatment.

There is a need for localized, efficient treatments that allow the patient a better quality of life. Efforts are being made in locally treating tumors with high temperatures. The idea behind this approach is that due to poor oxygenation, tumor cells are more susceptible to damage from heat. Healthy cells, but not cancer cells, can survive temperatures of up to 42°C. According to the National Cancer Institute, hyperthermia treatment kills cancerous cells by elevating their temperatures to the therapeutic temperature range of 42-45°C. This approach can destroy tumors with minimal damage to healthy tissues and, therefore, limit negative side effects. There are various methods used to apply hyperthermia cancer treatment. Laser therapy uses high-intensity light to shrink and destroy tumors (62). In microwave therapy, body tissue is exposed to high temperatures to damage and kill cancer cells or to make cancer cells more sensitive to the effects of radiation and certain anticancer drugs (63). However, these treatments are also limited by the ability of laser and microwave energy to penetrate body tissues.

Magnetic fluid hyperthermia (MFH) cancer treatment involves injecting a fluid containing magnetic

nanoparticles directly into tumors. When placed in an alternating magnetic field, the nanoparticles dissipate heat and destroy the tumors (Figure 3). This minimally invasive procedure, unlike the alternatives of laser and microwave hyperthermia, prevents unnecessary heating in healthy tissues because only the magnetic nanoparticles absorb the magnetic field energy (64). Cancerous cells typically have diameters of 10 to 100 micrometers and have been shown to absorb magnetic particles. One way of targeting magnetic nanoparticles to tumors is through passive targeting by the Enhanced Permeation and Retention (EPR). Effect, originally described by Maeda and colleagues (65). The defective vasculature surrounding tumors have increased endothelial fenestrations and defective architecture, resulting in preferential lodging of injected nanoparticles into the tumor area (Figure 4). Another approach to targeting magnetic nanoparticles to the tumor site is through active targeting. This consists of attaching specific ligands to the nanoparticle surface that recognize specific receptors in the cancerous cells. Both these targeting mechanisms increase the effectiveness of hyperthermia by delivering therapeutic heat directly to cancerous cells. Nanoparticles can also effectively cross the blood-brain barrier (66), an essential step in treating brain tumors.

Efforts are being made in order to optimize the magnetic properties of nanoparticles for use in MFH. Doping the magnetic core with other metals can change properties such as specific absorption rate (SAR) and Curie temperature. SAR can be viewed as the heating capacity of a specific material (67). By developing materials that have higher heating capacity one can reduce the dosage required for MFH, in this way reducing potential toxicity and side effects. One alternative is using thermally blocked materials, such as cobalt ferrite, which have higher SAR compared to magnetite (Figure 5).

On the other hand, an important challenge in realizing MFH as a viable cancer treatment is monitoring and controlling the temperature during treatment. Left unchecked, magnetic nanoparticles embedded in a tumor and under the action of a magnetic field may dissipate enough energy to raise the local temperature well above the target of 42-45°C. This underscores the need to either monitor temperature and control the magnetic field conditions or develop magnetic materials which somehow stop dissipating heat once the target temperature range has been reached. The Curie point of a ferromagnetic material is the temperature above which it loses its characteristic ferromagnetic properties. At temperatures below the Curie point the magnetic moments are partially aligned within magnetic domains in ferromagnetic materials. As the temperature is increased towards the Curie point, the alignment within each domain decreases. Above the Curie point, there are no magnetized domains of aligned moments (68). If a material were designed so that its Curie temperature is just above the target temperature range in MFH such a material would stop dissipating heat at the target temperature. Various candidate materials have been suggested, such as manganese/zinc ferrites, iron/platinum alloys, and more recently, substituted manganese oxides (69-70).

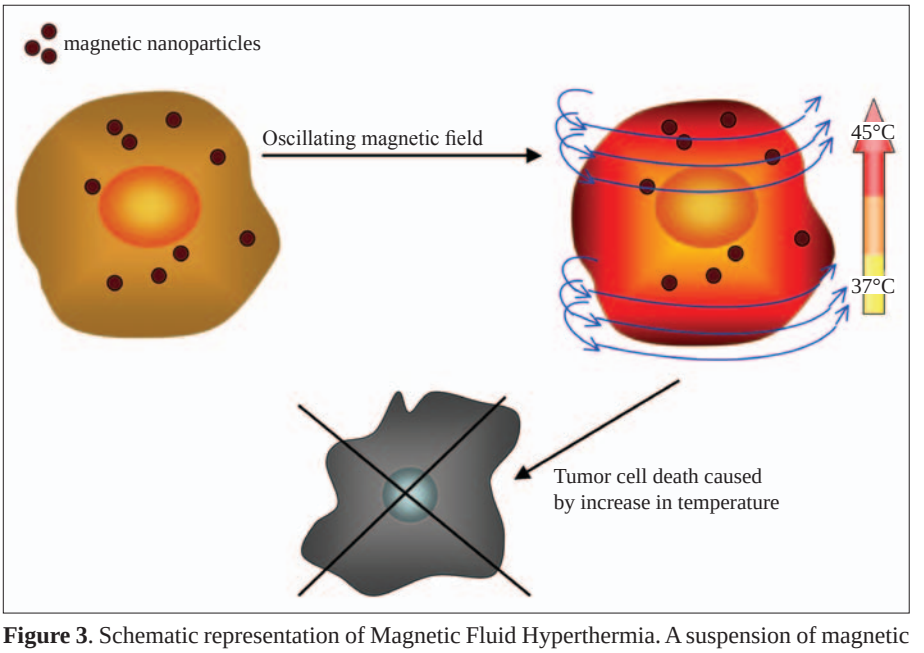

Figure 3. Schematic representation of Magnetic Fluid Hyperthermia. A suspension of magnetic nanoparticles is introduced into a tumor. When placed in an alternating magnetic field, the nanoparticles dissipate energy in the form of heat, causing a rise in temperature that leads to cell death.

3.1. *In vitro* studies

In order for an emerging biomedical technology to be accepted, an understanding of what happens at the cellular level is needed to safely assess possible safety issues and side- effects associated with the technology. *In vitro* studies are extremely useful

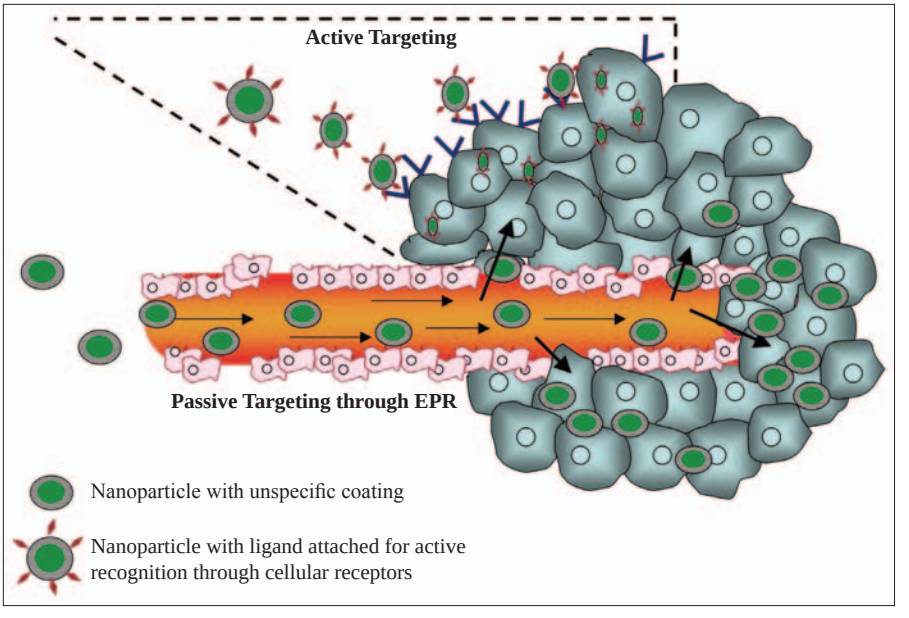

Figure 4. Passive versus active targeting of tumors. Nanoparticles can be passively delivered to tumor site by the Enhanced Permeation and Retention (EPR) effect. Defective tumor vascularization and increased vascular fenestrations permit lodging of the nanoparticles into the tumor. Active targeting is achieved by attaching a ligand that can be recognized by a surface receptor in the cell of interest.

for these purposes. In 1996, Jordan and colleagues found that dextran-coated magnetite nanoparticles were non-toxic to human colonic adenocarcinoma cells at concentrations of up to 5mg/mL. They demonstrated that these cells can internalize up to 1.0 pg of magnetic material per cell (71). In subsequent publications Jordan and colleagues went on to show time-dependent uptake of magnetic nanoparticles into tumor cells and were among the first to point out that the magnetite nanoparticle surface coating affects cellular internalization kinetics (72). They also demonstrated cell death by MFH at temperatures between 43-45°C. Prasad, et al. showed that inducing MFH in HeLa cells with manganese-doped iron oxides caused apoptosis and disrupted the actin and tubulin cytoskeleton (Figure 6) (73). Bergey, et al. labeled magnetite nanoparticles with luteinizing hormone releasing hormone (LHRH) and were able to selectively lyse cells that overexpressed the LHRH receptor when MFH was applied (74). Yan, et al. conducted MFH experiments with maghemite nanoparticles on human hepatocarcinoma (SMMC-7721) cells (30). They showed that 60 minutes of magnetic field application was capable of controlling cell proliferation and increasing chromatin condensation, features of apoptotic cell death. The decrease of cell proliferation and increase in apoptosis correlated with nanoparticle concentration. From these and other *in vitro* studies, the basic science behind MFH is being elucidated.

3.2. *In vivo* studies

To date, various groups have studied the principles of Magnetic Fluid Hyperthermia (MFH) in rodents and rabbits. The typical methods of application consist of injecting a superparamagetic ferrofluid into the tumor

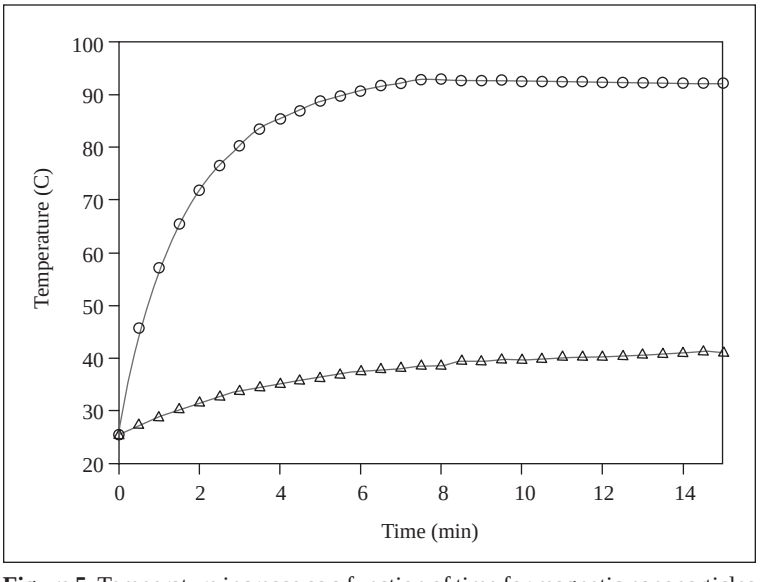

Figure 5. Temperature increase as a function of time for magnetic nanoparticles suspended in heptane at 2.5 %w-ferrite/v upon the application of a magnetic field (6.6 kA/m and 233 kHz). Circles represent magnetite-oleic acid nanoparticles. Triangles illustrate cobalt ferrite-oleic acid nanoparticles.

site, or through the portal vein in the case of hepatic tumors, and applying oscillating magnetic fields to the animal. Promising results have been obtained in these experiments. In a study by Minamimura, et al., dextran-magnetite ferrofluid was applied arterially for the treatment of liver tumors in rats (75). Animals were exposed to a 500 kHz alternating magnetic field in order to achieve intratumoral temperatures of 43°C for 30-40 minutes. Field amplitude was not reported. After 3 days, animals in the control group showed a 385% increase in tumor size, whereas MFH treated animals only had a 28% increase in tumor size. Hilger and colleagues applied a magnetic field with frequency of 400 kHz and amplitude of 6.5 kA/m for a period of 4 minutes to human breast adenocarcinomas implanted into immunodeficient SCID mice, using iron oxide nanoparticles. Intratumoral temperatures ranged between 45°C and 84°C by the end of treatment (76). They found early stages of coagulation necrosis in treated as compared to untreated tumors.

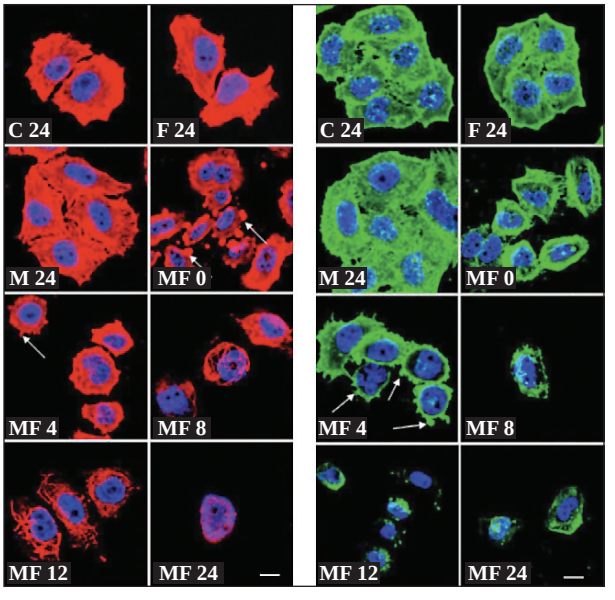

Figure 6. (a) Effect of MHT on microtubules (red) and nucleus (blue). Cells without field and without magnetic particles incubated for 24 h (C24), cells with field for 30 min but no particles incubated for 24 h (F24), cells with particles but without field incubated for 24 h (M24), cells with particles and field for 30 min and immediately fixed (MF0), cells with particles and field for 30 min and incubated for 4 h (MF4), cells with particles and field for 30 min and incubated for 8 h (MF8), cells with particles and field for 30 min and incubated for 12 h (MF12) and cells with particles and field for 30 min and incubated for 24 h (MF24). Arrows indicate blebbing. Bar, 10 mm. (b) Effect of MHT on actin (green) and nucleus (blue). Cells without field and without magnetic particles incubated for 24 h (C24), cells with field for 30 min but no particles incubated for 24 h (F24), cells with particles but without field incubated for 24 h (M24), cells with particles and field for 30 min and immediately fixed (MF0), cells with particles and field for 30 min and incubated for 4 h (MF4), cells with particles and field for 30 min and incubated for 8 h (MF8), cells with particles and field for 30 min and incubated for 12 h (MF12) and cells with particles and field for 30 min and incubated for 24 h (MF24). Arrows indicate blebbing. Bar, 10 mm. Figure reproduced from the article by Prasad et al., "Mechanism of cell death induced by magnetic hyperthermia with nanoparticles of γ-$Mn_xFe_{2-x}O_3$ synthesized by a single step process," Journal of Materials Chemistry, vol. 17, pp. 5042-51, 2007. Reproduced by permission of The Royal Society of Chemistry. (http://dx.doi.org/10.1039/b708156a).

When treating hepatic tumors, ferrofluid is usually injected intravenously through hepatic arterial infusion. Jones and colleagues were able to achieve MFH in experimental rabbit liver tumors (77). After infusing ferromagnetic microspheres through the hepatic artery, the animals were treated in sessions involving a single 20-min exposure to a 53 kHz alternating field with an amplitude of 43 kA/m. Mean intratumoral temperatures reached 42°C. This resulted in total suppression of tumor growth at 14 days compared to controls, in which tumor sizes increased dramatically over the same period.

In 2005, Johanssen, et al. applied MFH to Dunning R3327 rats suffering from prostate cancer (78). In order to avoid heat shock protein induced resistance to heat, animals received therapy at 48 hour intervals. Animals were subjected to field strength of 12.6 kA/m, and intratumoral temperatures between 41.2°C and 54.8°C were achieved. Compared to animals in the control group, animals who received MFH displayed a tumor growth inhibition of 50.9%. In 2006, Jordan and colleagues treated rats suffering from intracerebral glioblastomas by applying MFH twice in 48 hours after a single ferrofluid injection into the tumoral site (79). MFH was carried out by placing the animals in an alternating magnetic field applicator system for small animals operating at a frequency of 100 kHz and variable field strength of 0-18 kA/m. They found a correlation between animals where intratumoral temperatures of 43-47°C were achieved and a 4.5-fold prolongation in survival. In 2008, Kawai and colleagues used magnetic nanoparticles conjugated with cationic liposomes to treat prostate cancer tissue near the bone microenvironment into F344 rats (80). Field strength and amplitude were 360 kHz and 30.6 kA/m, respectively. Rats were irradiated for around 30 minutes and temperatures inside the tumor where maintained at 45°C. They found that compared to the control group, rats subjected to MFH had reduced tumor proliferation and reduced necrosis of the bone tissue associated to the prostate tissues.

There have also been promising results when using a synergistic approach where MFH along with

chemotherapeutic drugs or other molecules are utilized for the treatment of tumors. The high temperatures make the cancerous cells more susceptible to the agent designed to destroy the tumor cell. One example is the work of Ito and colleagues, who utilized tumor necrosis factor (TNF) alpha gene therapy along with MFH to successfully inhibit the growth of tumors in mice (81). TNF alpha is a factor that starts a signaling cascade that leads to cell death. TNF alpha expression was driven by the heat- and stress-inducible promoter, gadd 153, with MFH using magnetite cationic liposomes (MCLs) being responsible for the rise in temperature. Magnetic field and amplitude were not reported. In tumor bearing athymic mice, MCLs induced cell death throughout much of the tumor area on heating under an alternating magnetic field. This heat stress also resulted in a 3-fold increase in TNF-alpha gene expression driven by the gadd 153 promoter as compared with that of non-heated tumor. Over a 30-day period, the combined treatment strongly arrested tumor growth in nude mice, results being more dramatic than with MFH alone.

3.3. Pre-Clinical Studies

Although positive results have been reported *in vitro* and *in vivo* using MFH as treatment for cancer in various animal models, MFH has not yet been established in the clinical setting. This is most likely due to limitations in current techniques, where problems are encountered in selectively targeting the tumor and in homogenously and controllably distributing the heat within tumor tissues. To date, all SPIONs used in the pre-clinical setting are composed of the iron oxides magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3). This is due to the fact that these materials have been proven to show low toxicity and their metabolic pathway is known.

MagForce Nanotechnologies AG, a German company, has designed an alternating magnetic field applicator (MFH 300FTM). This magnetic field applicator generates alternating fields of 100kHz at variable field strengths of 0-18 kA/m. It can be used to treat malignancies at almost every location of the human body (82).

The first clinical feasibility study was carried out in March 2003, on 14 terminally ill patients suffering from glioblastoma multiforme (83). All patients received intratumoral injections and received 4-10 MFH sessions of 1-hour duration. Intratumoral temperatures reached were between 42.4 - 45.5°C. Treatment was well tolerated, and patient follow-up is ongoing. In 2008 a follow-up report was published about three patients that had died during the study due to progression of the disease (84). When brain autopsies were performed, the group found the installed magnetic nanoparticles were dispersed or distributed as aggregates within geographic tumor necroses, restricted in distribution to the sites of instillation.

In 2005, Johanssen and colleagues started a feasibility trial in ten patients suffering from recurrent prostate cancer. The ferrofluid was injected transperineally. The patients received six 60-minute MFH treatments at weekly intervals, at field strengths of 4 to 5 kA/m. Temperatures between 38.8°C and 43.4°C where achieved in 90% of the prostates. In a follow-up study performed 17.5 months earlier, nanoparticle deposits were still detectable in the prostate, and no systemic toxicity was observed (64).

4. Challenges and Conclusions

An important factor responsible for the slow adoption of hyperthermia as an available resource for physicians and patients is the limited targeting capabilities of currently available nanoparticles. This limitation hinders the ability to concentrate the nanoparticles within the targeted tumor site, negatively affecting the homogeneous distribution of heat. In order to overcome this constraint, the direct injection of the nanoparticles into the tumor sites has been presented as a viable solution for targeted delivery of superparamagnetic nanoparticles. However, more information needs to be uncovered in order to maximize nanoparticle concentration and energy dissipation within and in the vicinity of cancer tumors.

Magnetic Fluid Hyperthermia seems to be a viable approach in the treatment of localized tumors. Tumor size regression has been achieved in *in vivo* experiments in animal models such as rodents and rabbits. Human feasibility studies are under way, treatment seems to be well tolerated, and to date there is no toxicity associated with the treatments given. It remains to be seen if results in human patients will be as promising as those achieved in animal studies.

Resumen

Los sistemas de nanopartículas están siendo intensamente investigados para varias aplicaciones biomédicas. Suspensiones coloidales de nanopartículas magnéticas son de interés especial, particularmente como una herramienta para la capturación de bioimágenes, y más recientemente, por su utilidad en Hipertermia causada por Fluidos Magnéticos, o "MFH" (Magnetic Fluid Hyperthermia) por sus siglas en inglés. MFH promete ser una alternativa viable en el tratamiento de tumores cancerosos localizados. El tratamiento consiste en inyectar una suspensión fluida de nanopartículas magnéticas en el lugar del tumor, y luego exponer el

area del tumor a un campo magnético oscilante. Esto causa que las nanopartículas disipen energía en forma de calor, causando un aumento localizado en temperatura y eventualmente muerte celular. Aquí presentamos una revisión de métodos de síntesis de partículas magnéticas, el papel que juega la cubierta de la nanopartícula en alcanzar estabilidad coloidal, minimizar toxicidad, y su reconocimiento por parte de la célula cancerosa. Finalmente, discutiremos experimentos *in vivo* e *in vitro* de MFH, y estudios clínicos en el tratamiento de pacientes con glioblastoma multiforme y cáncer de la próstata.

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Induction of Neutralization Antibodies in Mice by Dengue-2 Envelope DNA Vaccines

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***Background*: Dengue (DEN) viruses have become a public health problem that affects approximately 100 million people worldwide each year. Prevention measures rely on vector control programs, which are inefficient. Therefore, a vaccine is urgently needed.**

***Methods*: The main goal of our laboratory is to develop an efficient tetravalent DEN DNA vaccine. In this study, we constructed four DEN-2 DNA vaccines expressing *prM/env* genes, using the homologous leader sequence (VecD2, VRD2E) or the tissue plasminogen activator (tPA) secretory signal (VecD2tpa, VRD2tpa). *In vitro* expression was tested by transient transfections and Western blot. The immunogenicity and protective efficacy of the vaccine candidates was evaluated in BALB/c mice, using intramuscular (IM) and intradermal (ID) vaccination routes.**

***Results*: Envelope (E) protein expression was detected in transfected COS-7 or 293T cells. We found statistical differences in the antibody responses induced by these vaccine candidates. In addition, the strongest antibody responses and protection were observed when the vaccines were delivered intramuscularly. Moreover, the tPA leader sequence did not significantly improve the vaccine immunogenicity since VecD2 and VecD2tpa induced similar antibody responses.**

***Conclusions*: We demonstrated that most of our DNA vaccine candidates could induce antibody responses and partial protection against DEN-2 virus in mice. These results provide valuable information for the design and construction of a tetravalent DEN DNA vaccine.**

Key words: Dengue-2 virus, DNA vaccines, BALB/c mice

Dengue (DEN) viruses belong to the Flavivirus genus, and consist of four antigenically different serotypes (DEN-1, DEN-2, DEN-3, DEN-4). These viruses are transmitted mainly by the mosquito *Aedes aegypti* and are a major cause of epidemics in the tropical and subtropical regions of the world (1). Infection with these viruses induces diseases ranging from dengue fever (DF) to dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) (1-2). There is no specific treatment available for these diseases and the prevention of epidemics is based on vector population control programs and community education. Thus, the development of a vaccine will be essential in controlling the 100 million new cases of DF distributed worldwide and the up to 500,000 cases of DHF that arise every year (2).

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Prototype DEN vaccines have been developed and evaluated in different laboratories for more than 30 years. Diverse vaccine strategies have been employed including: live attenuated virus (3-4), inactivated virus particles (5-6), recombinant subunit proteins (6-7), and chimeric viruses (8-9). From these, live attenuated and chimeric virus vaccines are the most advanced in clinical studies (10-12). Although these vaccines are capable of inducing antibody and cellular immune responses against DEN viruses, some problems have arisen in adjusting the virus dose of each serotype in the tetravalent formulas in order to obtain a balanced response (4, 13). In addition, there is a potential for reversion to pathogenic stage in the attenuated vaccines (14) or to cause adverse reactions in immunocompromised subjects due to their replication ability (15). Thus, a strategy that can stimulate cellular and humoral immune responses without imposing the risks associated with virus replication may be an excellent alternative for the development of an effective DEN vaccine.

DNA vaccines have been applied as an alternative approach for the development of DEN vaccines (16-20). DNA vaccines have several potential advantages over traditional immunization strategies. First, since the antigen is expressed intracellularly, it can be presented by MHC class I molecules to stimulate cytotoxic T lymphocytes (CTL). Second, vaccination with plasmid DNA may lead

to long-lasting immunity due to the continual priming or re-stimulation of the immune system. Studies using reporter genes and PCR have shown that the injected plasmid DNA remains in the muscle tissue several months after injection (21). Finally, DNA vaccines are easy to prepare and are not reactogenic (22-23).

Despite the potential of DNA vaccines in inducing anti-viral immunity, occasionally the immune responses they elicit are too weak to provide protection against viral pathogens. Thus, several approaches have been used to increase the immunogenicity induced by the expression vectors, such as using heterologous leader sequences (24) or different immunization routes (18, 25). Likewise, DNA-based vaccines have been immunologically assessed in the context of a prime-boost regimen, using pathogen-specific proteins to improve the humoral response elicited by DNA alone (26). These strategies for DNA vaccine improvement were employed in this study. Hence, the aim of this study was to compare the humoral immune responses and protection elicited in mice by four different DEN-2 envelope (E) protein expression vectors.

Materials and Methods

Cells

C6/36 and Vero cells, provided by Dr. Vance Vorndam (CDC Dengue Branch, San Juan, PR), were maintained in MEM and M199 media, respectively, supplemented with 10% FBS and 1% gentamycin (Invitrogen, Carlsbad, CA). COS-7 cells were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA) and were maintained in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA). 293T cells were obtained from Dr. Edmundo Kraiselburd's laboratory and were maintained in DMEM supplemented with 10% FBS (Invitrogen, Carlsbad, CA).

Plasmids

A molecular clone of DEN-2 virus (New Guinea C [NGC] strain) that was provided by Dr. Radma Padmanabhan (University of Kansas) (27), was used to amplify the *prM/env* genes. The DEN-2 viral genome was divided into two clones; the 5' clone containing nucleotides (nt) 1--2203 and the 3' clone, which contained the rest of the DEN-2 genomic sequences (nt 2203--10723). The eukaryotic expression vectors pJW4304 (provided by Dr. Jim Mullins from University of Washington) (28) and pVR1020 (provided by Vical, Inc., San Diego, CA) (29) were used for the construction of DEN-2 DNA vaccines. Both vectors contain the cytomegalovirus (CMV) immediate early promoter, including the intron A, the bovine growth hormone polyadenylation signal, and the tPA leader sequence. The advantage of pVR1020 over pJW4304 is that, according to FDA regulations (30), the former does not contain the SV40 origin of replication or the ampicillin resistance gene, making it suitable for further use in clinical trials.

Construction of DEN-2 E protein expression vectors

Four expression vectors containing the DEN-2 *prM/env* genes were constructed using the parental plasmids pJW4304 and pVR1020 (Table 1). The differences among these vectors are mainly in the leader sequence that precedes the viral genes. Vectors VecD2 and VRD2E use the homologous leader sequence, which consists of the last 39 nucleotides of the capsid gene. This leader sequence was replaced for the secretory signal of the tissue plasminogen activator (tPA) protein to construct vectors VecD2tpa and VRD2tpa. The resistance gene is also different, where the pJW4304-derived vectors contain ampicillin and those derived from pVR1020 have kanamycin.

Table 1. Description of the E protein expression vectors.

Vector Name	Parental Vector	Resistance Gene	Leader Sequence
VecD2	pJW4304	Ampicillin	Homologous
VecD2tpa	pJW4304	Ampicillin	Heterologous (tPA)
VRD2tpa	pVR1020	Kanamycin	Heterologous (tPA)
VRD2-E	pVR1020	Kanamycin	Homologous

A molecular clone of DEN-2 was used to construct VecD2. To obtain the full-length sequence, clone 5' was digested with ClaI and BamHI and was ligated to clone 3' digested with BamHI and XbaI. The full-length construct was used to PCR-amplify the *prM/env* region. Primers D2E.F (cccaagcttatggcaggcatgatcattatgctgattcc) and D2E.R (cgtctagatcacttcagttctttgtttttccagc) were used to construct VecD2, which uses the last 39 nucleotides of capsid as leader sequence for the *prM/env* genes. The PCR reaction was performed using Platinum® Taq DNA polymerase High Fidelity (Invitrogen, Carlsbad, CA) and the product obtained (2059 bp long) was cloned into pJW4304 using the HindIII and NheI sites. To construct VRD2E, the DEN-2 *prM/env* sequences were amplified from VecD2 and cloned into the SalI and BglII sites of pVR1020. Two modified versions of these vectors were constructed containing the tPA signal already present in either pJW4304 or pVR1020. For this, the *prM/env* genes were PCR-amplified from VecD2 using primers D2E5' (ttccatttaaccacaagtaacg) and D2E3' (tcacttcagttctttgtttttccagc). The PCR product (2022 bp long) was cloned into the unique NheI and MluI sites of pJW4304, or the unique BamHI site of pVR1020,

in-frame with the tPA leader to create VecD2tpa or VRD2tpa, respectively. All vectors were sequenced at Lark Technologies Inc. (Dallas, TX) and the results were compared to the GenBank-published sequences of DEN-2 NGC strain (accession number M29095) using the DNAsis version 2.1 program (Chicago, IL).

***In vitro* expression**

The E protein expression vectors (VecD2, VRD2E, VecD2tpa and VRD2tpa) and their respective parental vectors (pJW4304 and pVR1020) were individually transfected (5 µg each) into COS-7 or 293T cells using lipofectamine (Invitrogen, Carlsbad, CA). Transfected cells were cultured for 48 hrs. and cells were either fixed on poly-lysine-treated slides for immunofluorescence assays or lysed (1.8 mM NaH_2PO_4, 8.4 mM Na_2HPO_4, 5% Triton-X, 0.1% SDS, 0.1 M NaCl, 0.5% sodium deoxycholate, and 1 mM PMSF) for Western blot analysis. Cell supernatants were ultracentrifuged at 20,000 rpm for 2 hrs. in a sucrose cushion to test for the presence of virus-like particles (VLPs).

Western blot

Proteins were solubilized at room temperature in 10 µL of sample buffer (3% SDS, 33% Glycerol, 0.2 M Tris, 2.5 mM PMSF, and 0.02% bromophenol blue) for 30 minutes. Known protein concentrations were electrophorased on 15% polyacrylamide gels. Resolved proteins were transferred electrophoretically to nitrocellulose membrane sheets (Bio Rad, Hercules, CA). E protein was detected using monoclonal antibody (MAb) 3H5 and the anti-mouse IgG alkaline phosphatase Western blot kit from Vector Laboratories (Burlingame, CA).

Mice

BALB/c mice, age 6-8 weeks, were purchased from Harlan (Indianapolis, IN). Mice were housed in a temperature controlled, light cycled room. Bleedings were performed by retro-orbital puncture using sterile, glass capillary tubes. Mice were euthanized by cervical dislocation of the spinal cord. All procedures were performed under ketamine (Ketaset, Fort Dogde, Iowa) and xylazine anesthesia. Animal care complied with the National Institutes of Health guidelines for the humane use of laboratory animals. Animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC).

Immunization and bleeding schedule

DNA immunizations were performed at 4-6 or 3-weeks intervals by needle injection using 100 µg of plasmid DNA diluted in 50 µl of endotoxin-free H_2O. IM immunizations were performed at the quadriceps while ID inoculations were performed at the upper back portion. Boost was performed subcutaneously with formalin-inactivated DEN-2 virus adjuvanted in alum. For challenge, DNA injections were performed intramuscularly on weeks 0 and 2, followed by the virus inoculation on week 3. Bleedings were performed every 2 or 3 weeks after each immunization. Individual serum samples were used for antibody detection.

Indirect immunofluorescence assay (IFA) and plaque reduction neutralization test (PRNT)

Serum samples from mice were assayed for DEN-2 specific antibodies by IFA and for virus neutralizing antibodies by PRNT. For IFA, HTC slides coated with DEN-2-infected C6/36 cells were used. Serum samples from vaccinated mice were serially diluted (five-fold, starting at a 1:5 dilution) with PBS containing 10% goat serum. Ten µL of each dilution was added to the wells in duplicate and incubated for 30 minutes at 37°C. Slides were washed and incubated with a goat anti-mouse FITC-conjugated IgG. Slides were covered with glycerol and observed under a fluorescence microscope. PRNT were performed as previously described (5). Briefly, two fold dilutions (starting at a 1:20 dilution) were prepared in 0.3 mL of PBS supplemented with 30% FBS. An equal volume of the DEN-2 virus sample (300 500 pfu/mL) was added to the serially diluted sera preparations. Individual samples were mixed and incubated at 25ºC for 2 hours. Duplicate Vero cell monolayers were infected with 0.2 mL of each sample in 6-wells plate. Viral plaque counting was performed after 5 days of inoculation at 37°C, 10% CO_2. The number of plaques observed in the sample incubated with serum obtained from unvaccinated mice were taken to represent 100%. $PRNT_{50}$ (serum dilution that caused 50% reduction in plaque formation) was calculated using Probit regression from the SPSS program (version 11.5, Chicago, IL).

Intracranial challenge

Challenge was performed as previously described (5). Briefly, six week old vaccinated mice were inoculated intracranially with 100 pfu of live, mouse adapted DEN-2 virus (NGC strain, provided by Dr. Robert Putnak, WRAIR) in 25 µL Hanks balanced salt solution. After challenge, the animals were monitored for 16--18 days until they regained normal mobility. Morbidity status was scored as follows: 0 = healthy; 1 = ruffled coat, lethargic; 2 = partial hind limb paralysis; 3 = complete hind limb paralysis, wasting.

Statistical analysis

All statistical analyses were performed using the SPSS version 11.5 Program (Chicago, IL). Fisher's exact test

(using Crosstabs) was used to compare the percentage of seroconversion among the vaccinated groups. Geometric mean titers (GMT) were calculated after log transformation of reciprocal titers. Multiple comparisons of GMT were performed by analysis of variance (One-way ANOVA) using Games Howell. Survival times were estimated by the Kaplan-Meier method and compared with Fisher's exact test (using Crosstabs). All p values were two-tailed.

Results

In vitro analysis of the E protein expression vectors

All four DEN-2 vectors were constructed by PCR and therefore, were sequenced to confirm the fidelity of the amplification (data not shown). Sequencing results were compared to the published sequences of *prM/env* of DEN-2 NGC strain. We found three point mutations in VecD2 and VRD2E but only two can cause amino acid (aa) substitutions. The changes at nucleotide (nt) 608 (A to G) and at nt 1075 (G to A) can cause a Lys to Arg substitution at aa 57 of *prM*, and a Glu to Lys mutation at aa 49 of the E protein, respectively. A silent mutation at aa 257 of E protein was identified as a C to A change at nt 1701. A fourth mutation was identified in VecD2tpa and VRD2tpa at nt 2337, which was also a silent mutation at aa 469 of the E protein. VRD2tpa also contained four additional amino acids at the 3' end of the tPA leader that resulted from cloning into the BamHI site of pVR1020.

In vitro expression of the DEN-2 E vectors was determined by direct immunofluorescence assays and Western blot analysis of transfected cells. We found that FITC-conjugated anti-DEN immune serum reacted to VecD2-transfected cells (Figure 1A). Similar results were obtained with the other three vectors (data not shown). No fluorescence was detected in COS-7 cells transfected with either of the parental vectors, pJW4304 (Figure 1A) and pVR1020 (data not shown). Western blot analysis was performed under non-denaturing conditions using the conformation-dependent MAb 3H5. The E protein (54 kDa) was detected in lysates of COS-7 cells transfected with VRD2tpa, VecD2tpa or VecD2 (Figure 1B, lanes 1, 3 and 4, respectively), but not from lysates of pVR1020- or pJW4304-transfected cells (Figure 1B, lanes 2 and 5, respectively). In addition, the molecular weight of the recombinant E protein is similar to that of the native E protein obtained from DEN-2-infected C6/36 cells (Figure 1B, lane 7). These results suggest that the DEN-2 vectors expressed the E protein in its native conformation. This protein was also detected in ultracentrifuged cell-free supernatants from VRD2tpa-, VecD2tpa-, and VecD2-transfected COS-7 cells (Figure 1C, lanes 1, 3 and 4, respectively), suggesting that the E protein may be associated with VLPs. A densitometry analysis showed that expression of the E protein in lysates and supernatants of VecD2tpa-transfected cells was 5-fold higher than that of cells transfected with VecD2 (Figure 1B and C, lanes 3 and 4). However, only a 2-fold increase in envelope protein expression was detected in the lysates but not in the supernatants of cells transfected with VRD2tpa when compared to VecD2-transfected cells (Figure 1B and C, lanes 1 and 4). This data suggests that the tPA leader was capable of enhancing the intracellular expression of the DEN-2 E protein.

Expression of the E protein by VRD2E was also tested by transient transfection in 293T cells and compared to that of VecD2. Both vectors expressed similar levels of the E protein in both, cell lysates (Figure 1D) and supernatants (data not shown), 24 and 48 after transfection.

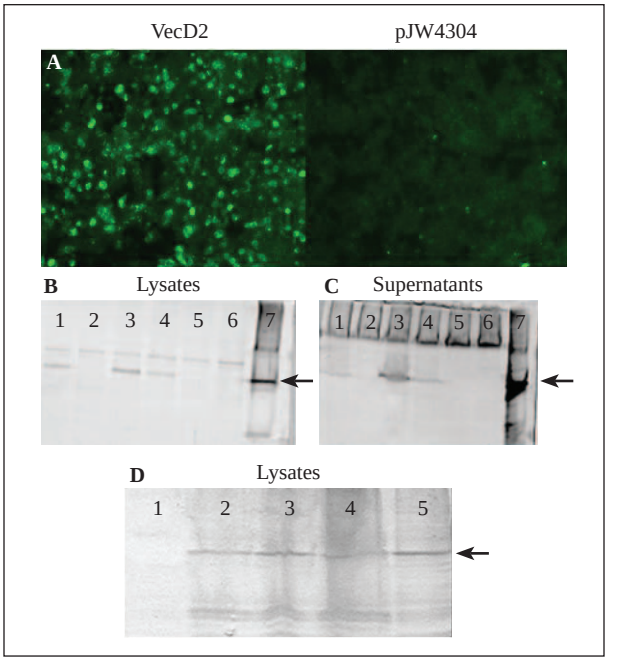

Figure 1. Detection of the DEN-2 E protein in transient transfected cells. FITC-conjugated anti-DEN-2 hyperimmune serum and the MAb 3H5 were used for detection of the DEN-2 E protein by direct immunofluorescence (A), and Western blot (B--D), respectively. Cell lysates (B) and ultracentrifuged cell-free supernatants (C) of non-transfected COS-7 cells or DEN-2 infected C6/36 cells were used as negative and positive controls, respectively for Western blot. Lane 1: VRD2tpa, Lane 2: pVR1020, Lane 3: VecD2tpa, Lane 4: VecD2, Lane 5: pJW4304, Lane 6: Control COS-7, Lane 7: D2 virus. Arrows indicates the position of DEN-2 E protein. (D) Lane 1: pVR1020, Lane 2: VecD2 48hrs, Lane 3: VecD2 24 hrs, Lane 4: VRD2E 48 hrs, Lane 5: VRD2E 24 hrs.

Immunogenicity of DEN-2 vaccine candidates

The first vector that was constructed and tested for its immunogenicity in mice was VecD2. Groups of seven 6-8 week-old BALB/c mice were intramuscularly inoculated with either VecD2 or pJW4304 on weeks 0, 4, 10, and 16. Serially diluted serum samples, obtained from inoculated mice, were tested for DEN-2 specific antibodies by IFA. Antibody responses were first detected on week 10 (6 weeks after the second DNA dose) but only in one animal inoculated with VecD2 (data not shown). Seroconversion was observed in 2/7 and 5/7 VecD2-vaccinated animals two weeks after the third and fourth DNA inoculations, respectively (data not shown). Although, 100% seroconversion was not reached after administration of four DNA doses, the difference in seroconversion between VecD2-vaccinated mice and the control animals was statistically significant on week 18 (5/7 vs. 0/7, $p < 0.05$). Neutralizing antibodies (NAb) were also measured by PRNT but were not detected in animals inoculated with VecD2, even after four DNA inoculations (data not shown). As expected, seroconversion was not found in control mice that were inoculated with pJW4304.

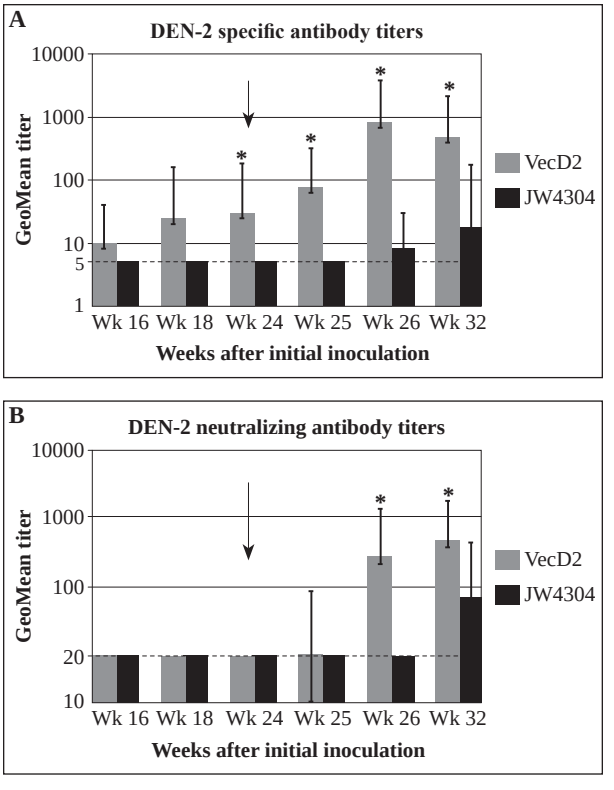

Figure 2A & 2B. Specific and neutralizing antibody titers induced by VecD2. All animals (experimental and control) received four DNA doses (on weeks 0, 4, 10, 16) and a boost with inactivated DEN-2 virus (on week 24). Geometric mean antibodies titers with their respective 95% confidence limits are shown. Comparison among groups were performed by analysis of variance (ANOVA) using Games Howell test (SPSS), and * indicates when the difference in antibody titers between Vec D2 and pJW4304 was statistically significant ($p < 0.05$). (A) Titers of DEN-2 specific antibodies were measured by IFA and negative results were given titer of 5 (limit of detection) (B) PRNT50 titers were obtained by probit analysis (SPSS), and negative results were given a titer of 20 (limit of detection).

Geometric mean titers (GMT) of DEN-2 specific antibodies (Ab, measured by IFA) and NAb are shown in Figure 2. Up to week 24, VecD2-vaccinated animals developed low Ab titers (GMT < 32) and non-detectable NAb responses (Figure 2A and B). Although IFA titers of mice inoculated with VecD2 were higher than those observed in pJW4304-inoculated animals, the difference in titers between these groups were statistically significant only on week 24 ($p < 0.05$).

To determine if VecD2-immunized mice were primed for the development of NAb responses, an inactivated virus boost was administered 8 weeks after the last DNA injection (week 24). All serum samples obtained after this boost were analyzed by IFA and PRNT. We found that Ab titers significantly increased after the inactivated virus boost in VecD2-inoculated mice, reaching their maximum on week 26 (GMT = 817) (Figure 2A). Ab titers induced by the virus boost were statistically higher in the DNA-primed animals than in the controls. NAb responses were also observed on week 26 (two weeks after the virus boost) in 5/6 animals inoculated with VecD2 but not in the control group (data not shown). In addition, six weeks after the virus boost (week 32), all VecD2-vaccinated animals and only one control developed NAb responses. These differences in seroconversion were statistically significant ($p < 0.05$). NAb titers also increased dramatically in the VecD2 group, reaching statistical significance, (GMT = 230 and 403 on weeks 26 and 32, respectively, Figure 2B) after the virus boost.

In an attempt to improve the immunogenicity of VecD2, we constructed two new vectors containing the tPA leader (VecD2tpa and VRD2tpa). In addition, we used two different routes of inoculation (IM and ID) and shorter intervals between inoculations (3 weeks instead of 4-6 weeks intervals). Groups of 8-10 BALB/c mice were inoculated with VecD2, VecD2tpa, VRD2tpa or the parental vectors (pJW4304 + pVR1020, control group) on weeks 0, 3, 6, and 9. As above, serum samples were tested by IFA and PRNT. Ab responses were first detected 3 weeks after the second DNA dose in mice vaccinated intramuscularly with VecD2 (3/9), VecD2tpa (5/8) or VRD2tpa (1/8) (data not shown). A statistically different seroconversion was observed on week 9 (3 weeks after the

third DNA injection) in mice vaccinated with either VecD2 (7/9) or VecD2tpa (7/8), in comparison to the control group (0/8) or the VRD2tpa group (2/8). These two groups (VecD2tpa and VecD2) reached 100% seroconversion on week 15 (six weeks after the fourth DNA dose) when the vaccine candidates were administered intramuscularly. Seroconversion was slower in the VRD2tpa group than in the other experimental groups and 100% seroconversion was never reached. Surprisingly, none of the vaccine candidates elicited Ab responses when delivered intradermally (data not shown).

NAb were first detected on week 6 (3 weeks after the second DNA dose) in the VecD2- and VecD2tpa-vaccinated groups (1/9 and 1/8 respectively, data not shown) but not in the VRD2tpa group. Interestingly, on week 9 (3 weeks after the third DNA dose) the proportion of VecD2tpa-inoculated mice that showed NAb responses was significantly higher than in the naked vector group (6/8 vs. 0/8, respectively, data not shown). However, it was not until week 15 (6 weeks after the fourth DNA inoculation) that the same was true for the VecD2 group (6/9 vs. 0/8, data not shown). Both groups, VecD2tpa and VecD2, reached 100% seroconversion for NAb on weeks 15 and 18, respectively. In VRD2tpa-vaccinated mice, NAb was first detected on week 12 but only in 2/8 animals and this response was transient (data not shown). Of importance, this vaccine group never reached 100% seroconversion and the NAb induction was statistically lower than in the other two experimental groups (VecD2 and VecD2tpa) towards the end of the experiment (on weeks 15 and 18). As with Ab responses, NAb were not detected in intradermally inoculated mice with any of the DEN-2 expression vectors.

Geometric mean titers (GMT) of specific antibody (measured by IFA) and NAb are shown in Figure 3. IFA titers were weak after three DNA doses (week 9) in all vaccinated mice (GMT $\leq$ 25, Figure 3A). However, those titers increased in VecD2- (GMT = 79) and VecD2tpa- (GMT = 152) vaccinated mice after the fourth DNA inoculation, reaching statistical significance ($p \leq 0.05$) when compared to titers from mice inoculated with naked vectors (GMT $\leq$ 5). These two groups produced GMT above 100 on weeks 12 (VecD2tpa) and 15 (VecD2). IFA titers were consistently lower in VRD2tpa-vaccinated mice than in the other experimental groups, but not statistically different (Figure 3A). VecD2-vaccinated mice developed weak NAb titers on weeks 6 and 9 (GMT~30, Figure 3B). This was also true in VecD2tpa group, except on week 9 when these mice developed a GMT of a 100. VecD2 and VecD2tpa elicited strong NAb responses (GMT > 100) by week 12 (3 weeks after the fourth DNA dose) and these titers were maintained during the entire experiment. NAb

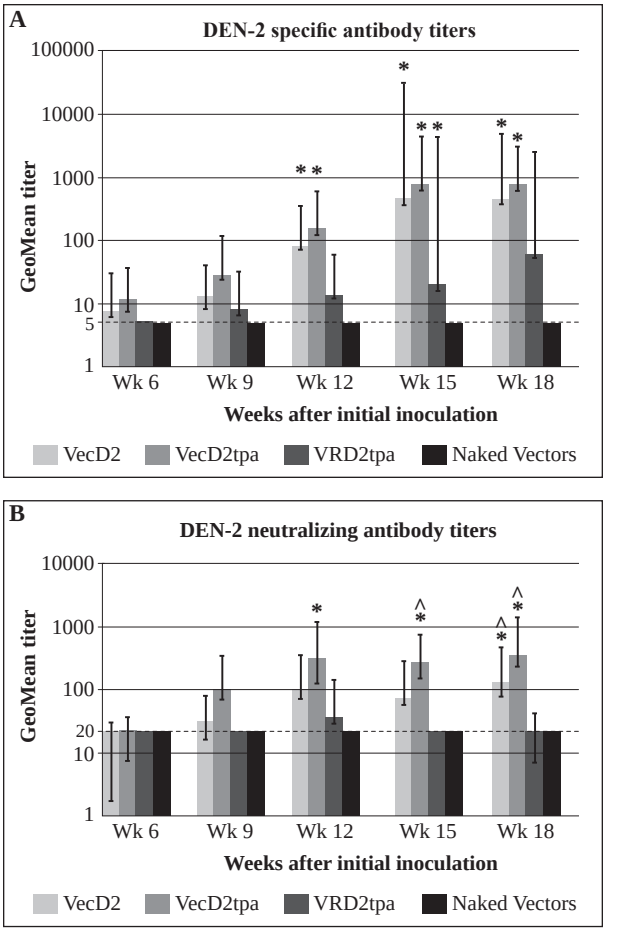

Figure 3. Specific and neutralizing antibody titers induced by the DEN-2 E protein expression vectors. Mice were vaccinated intramuscularly on week 0, 3, 6 and 9. Geometric mean antibodies titers with their respective 95% confidence limits are shown. Comparison among groups were performed by analysis of variance (ANOVA) using Games Howell test (SPSS), and * indicates when the difference in antibody titers between experimental and control (naked vectors) group was statistically significant ($p < 0.05$). ^ indicates when the difference in antibody titers between VecD2tpa or VecD2 and VRD2tpa group was statistically significant ($p < 0.05$). (A) Titers of DEN-2 specific antibodies were measured by IFA and negative results were given titer of 5 (limit of detection) (B) PRNT50 titers were obtained by probit analysis (SPSS), and negative results were given a titer of 20 (limit of detection).

responses in VecD2tpa group were significantly higher than in the negative control (GMT > 20) from week 12 to the end of the experiment. VecD2 group reached a significant level of NAb only on week 18. Although VecD2tpa developed stronger NAb titers than VecD2, the difference in titers was not statistically significant. On the other hand, VRD2tpa group only induced weak

NAb (GMT ≤ 50) during the entire experiment and those responses never reached statistical significance when compared to the control group. Moreover, NAb titers of mice inoculated with VecD2 and/or VecD2tpa were statistically higher ($p < 0.05$) than those of the VRD2tpa-vaccinated animals on week 15 (VecD2tpa only) and on week 18 (both).

Intracranial challenge

A challenge experiment was performed to evaluate if the DEN-2 vaccine candidates would induce immune responses that protect mice against intracranial inoculation of live virus. Groups of 8 animals were inoculated intramuscularly with 100 µg of each expression vector on weeks 0 and 2. All animals were challenged with 100 pfu of live, mouse adapted DEN-2 virus (NGC strain). Partial protection was observed in VecD2- and VecD2tpa-inoculated mice, where 6/8 and 7/8 animals, respectively survived challenge after 18 days of observation (Figure 4). These results were statistically significant when compared to the survival of the naked vectors group (0/8, $p < 0.05$). Survival of VRD2tpa-inoculated animals was also statistically higher (5/8, $p < 0.05$) than in the control group. IFA titers were detected pre-challenge especially in some mice immunized with VecD2tpa (4/8, GMT = 20), VecD2 (3/8, GMT = 11) and VRD2tpa (1/8, GMT < 10) (data not shown). Pre-challenge NAb responses were not observed in the vaccinated mice. However, anamnestic Ab (including NAb) responses were observed in all survivors (GMT > 100) after challenge, but the levels of these Ab did not reach a significant difference between the experimental and control groups (data not shown).

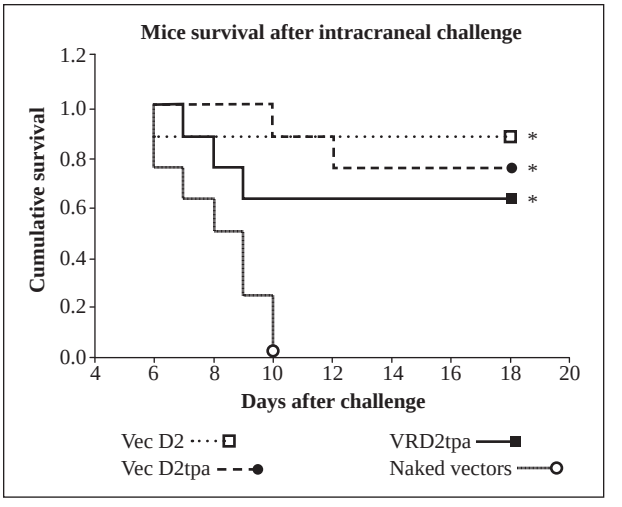

Figure 4. Mice survival after intracranial challenge. Animals were inoculated by needle on weeks 0 and 2, followed by the intracranial challenge on week 3 and were monitored for signs of paralysis for 18 days until they regained normal mobility. Survival analysis was performed using the Kaplan-Meier method (SPSS) and the proportion of animals that survived challenge was compared by Fisher exact test. * indicates when the difference in survival between experimental and control (naked vectors) group was statistically significant ($p < 0.05$).

Antibody responses induced by VRD2E

Despite the fact that VecD2 and VecD2tpa were more immunogenic and induced stronger protection against intracranial challenge in mice than VRD2tpa, these vectors cannot be used in human trials. Therefore, we constructed a modified version of VRD2tpa, called VRD2E, by using the natural DEN-2 leader sequence instead of the tPA signal. This new vector was also tested *in vivo*. Two groups of 13 BALB/c mice received four intramuscular inoculations (100 µg, on weeks 0, 3, 6, 9) of VRD2E or pVR1020 as control group. Mice were bled and serum was tested as described previously. Seroconversion for specific Ab was first observed 3 weeks after the initial DNA inoculation (week 3) in 9/13 inoculated with VRD2E (data not shown). This proportion was significantly higher than the seroconversion rate observed in the VecD2- (from previous experiment) or pVR1020- vaccinated animals (0/9 and 0/13; respectively). The VRD2E group reached 100% seroconversion on week 6 (3 weeks after the second DNA dose) while the VecD2 group required 4 DNA doses to reached similar levels of seroconversion. NAb responses were also observed in the majority of the inoculated animals (10/13) after the first DNA dose. However, those responses were only maintained in 2/13 and 7/13 of the VRD2E-vaccinated mice after the second and third DNA inoculation, respectively (data not shown). Nevertheless, seroconversion for NAb was faster in the VRD2E group than in those animals inoculated with VecD2. Indeed, three DNA doses of VRD2E were required to maintain a significantly higher seroconversion rate than the control group while four DNA doses of VecD2 were necessary to observe the same results.

For comparison purposes Ab responses (including NAb) elicited by VecD2 in the previous experiment are shown in Figure 5 together with the data obtained from VRD2E- and pVR1020-vaccinated mice. We observed that the VRD2E induced stronger IFA titers (GMT= 46) than VecD2 or pVR1020 (GMT≤5) after the first DNA inoculation. This response was statistically significant over the other two groups and the difference was maintained during the entire experiment. Specific Ab titers above 100 were observed in the VRD2E group on week 6 (3 weeks after the second DNA dose) and at the end of the experiment (week 15, Figure 5A) those titers increased to a GMT = 8,414. In contrast, VecD2 did not induce specific GMT

near 100 until week 12 (three weeks after the fourth DNA dose), when those titers were significantly higher than in the control group. VRD2E also induced strong NAb responses (GMT=58), which were significantly higher than those elicited by VecD2 or pVR1020 after the initial DNA inoculation (week 3, Figure 5B). However, those responses were not sustained and after the second DNA dose, NAb titers were similar in both experimental groups; VRD2E and VecD2. Four DNA doses of VRD2E and VecD2 were required to induce NAb titers ≥ 100 (week 12, Figure 5B). However, those responses were significantly higher than those of the control group in the VRD2E group, but not in the VecD2 group (up to week 15).

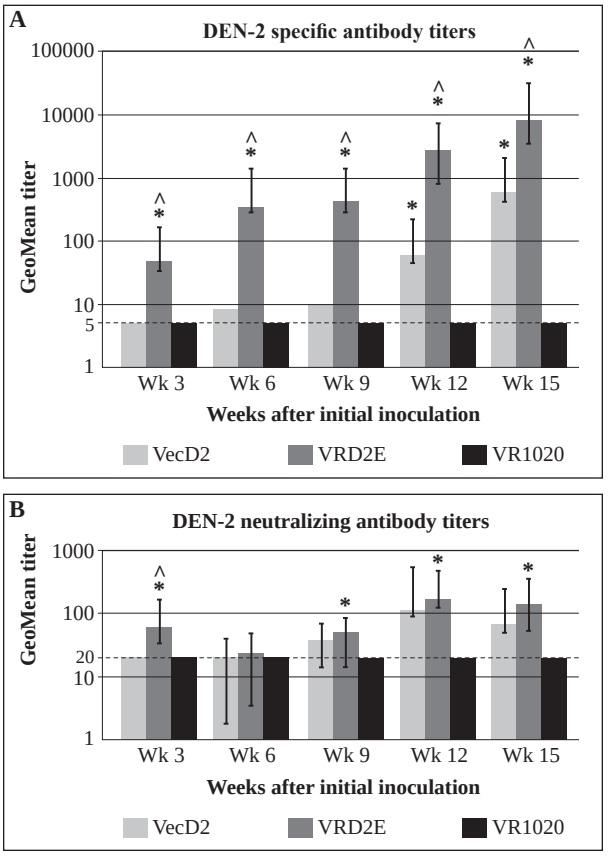

Figure 5. Specific and neutralizing antibody titers induced by VRD2E. Mice were vaccinated intramuscularly on week 0, 3, 6 and 9. Geometric mean antibodies titers with their respective 95% confidence limits are shown. Comparison among groups were performed by analysis of variance (ANOVA) using Games Howell test (SPSS), and * indicates when the difference in antibody titers between experimental and control (pVR1020) group was statistically significant ($p < 0.05$). ^ indicates when the difference in antibody titers between VecD2 and VRD2E group was statistically significant ($p < 0.05$). (A) Titers of DEN-2 specific antibodies were measured by IFA and negative results were given titer of 5 (limit of detection) (B) PRNT50 titers were obtained by probit analysis (SPSS), and negative results were given a titer of 20 (limit of detection).

A challenge experiment was also performed as previously described to evaluate if VRD2E could induce immune responses that protect mice against intracranial inoculation of mouse adapted DEN-2 virus. The survival rate for the VRD2E group was 9/10 but in the control group (pVR1020) high survival was also observed (6/10, data not shown). This result was unexpected and, as a consequence, significant differences in the survival rates between the experimental and the control groups could not be established. Therefore, we could not determine the level of protection against DEN-2 induced by VRD2E.

Discussion

The aim of this study was to evaluate the humoral immune responses elicited in mice by four different DEN-2 DNA vaccine candidates. Our strategy was focused on the DEN E protein because it mediates the receptor binding and the entry steps during virus infection. In addition, NAb against this protein were shown to be protective against pathogenic virus infection and disease (5). Two DEN-2 E protein expression vectors (VecD2 and VRD2E) were constructed using the last 39 nucleotides of capsid as the leader sequence plus *prM* and the full-length *env* sequences from DEN-2 NGC strain. The other two vectors constructed, VecD2tpa and VRD2tpa, also contained the *prM/env* genes but the homologous leader sequence was replaced with the tPA signal. The *prM* region was necessary to prevent conformational changes in the E protein that may occur when it is exposed to a mildly acidic pH in the Golgi apparatus and the secretory vesicles (31). The full-length *env* gene was selected to stimulate E protein secretion in the form of VLPs in order to expose conformational antigens that may be critical in eliciting protective immune responses. Studies with recombinant plasmids containing the *prM* and the full-length *env* of tick-borne encephalitis (TBE) showed that co-expression of these genes induce the secretion of VLPs from cells (32) and that these VLPs elicited protective NAb responses (33). In addition, Konishi and collaborators (34) developed a Japanese encephalitis (JE) DNA vaccine containing *prM/env* (100%), which also produced VLPs that were able to protect mice against lethal virus challenge. Moreover, comparative studies of truncated and full-length E protein of DEN-1 have demonstrated that plasmids expressing the entire E protein are better immunogens than those that express truncated forms of this protein (18). Thus, the published data supports the hypothesis that vectors

expressing the full-length E protein are able to induce better protective immune responses than those that express truncated forms of this protein.

Three antigenic domains have been identified on the E protein (I, II and III) by MAbs (35). Domain I includes aa 130 to 185 and its role has not yet been defined. Domain II spans from aa 50 to 130 and from aa 185 to 300 and contains the putative membrane fusion sequence (aa 98 to 110). Domain III extends over aa 300 to 400 and contains most of the conformation-dependent virus neutralizing epitopes. Thus, domain III may be the most important in terms of generating NAb responses. Four point mutations were introduced in the DEN-2 E protein expression vectors during PCR amplification of the *prM/env* genes. Only two of these mutations can cause substitutions at aa 57 of *prM* and at aa 49 of E protein. The last mutation is at the border of domain II and it is very likely that this mutation did not significantly affect the E protein conformation. Indeed, Western blot analysis performed with MAb 3H5, which recognize a conformation-dependent epitope (aa 383-397) (36) revealed that all vectors expressed a recombinant E protein of similar size as the native protein expressed in DEN-2-infected cells (Figure 1). Thus, the results obtained demonstrated that the epitopes expressed by the recombinant E protein were similar to those expressed by the native E protein of DEN-2 and that these vectors had the ability to produce VLPs.

The homologous *prM* leader sequence was replaced with the tPA signal in VecD2tpa and VRD2tpa to enhance the expression and secretion of the DEN-2 E protein. Our hypothesis was that enhanced expression and secretion of E protein could lead to an enhanced immunogenicity in mice. This secretory signal peptide was chosen because it could enhance expression and exportation of several viral proteins such as the Rotavirus VP4 protein (37) and the SIV envelope protein (38). In addition, tPA was more efficient for secretion of HIV-1 gp120 than other leader sequences (39). In accordance to the published information, intracellular expression of the DEN-2 E protein was 5-fold and 2-fold higher in cells transfected with VecD2tpa and VRD2tpa, respectively than in VecD2-transfected cells (Figure 1B). However, increased secretion of E protein was only detected in VecD2tpa-transfected cells and not in those transfected with VRD2tpa. Since the tPA leader in VRD2tpa contains four additional amino acids that resulted from cloning into the unique BamHI site of pVR1020, it is possible that this insertion had a negative impact in the secretion of the E protein from this vector.

There is no ideal animal model for the evaluation of DEN vaccine candidates. However, mice are routinely used for initial assessment of vaccine immunogenicity (40). In this study, we used BALB/c mice to test the potential immunogenicity of the DEN-2 E protein expression vectors. Three *in vivo* experiments were performed using four DNA doses that were administered either intradermally or intramuscularly by needle injection. Only in the first experiment was an inactivated virus boost included in the vaccination regimen. The rationale for this inclusion was to corroborate if VecD2 was capable of priming mice for the development of protective Ab responses (i.e. NAb). VecD2 induced weak specific Ab titers and non-detectable NAb responses after four DNA doses during the first experiment (Figure 2). However, vigorous anamnestic responses were observed in VecD2-vaccinated animals after administration of the inactivated DEN-2 virus boost. Furthermore, antibody titers (both, specific Ab and NAb) were significantly higher in animals inoculated with VecD2 than in the controls. Thus, the data obtained indicates that, despite the weak antibody responses elicited by DNA vaccination alone, the VecD2-inoculated animals were primed for the induction of NAb against DEN-2 virus. It is possible that the long resting periods (4-6 weeks) between DNA inoculations may have been partially responsible for the low immunogenicity of VecD2 in this experiment.

The second experiment described in this report was performed to enhance the immunogenicity of VecD2. Two strategies were followed. First, the replacement of the *prM* leader sequence with the tPA signal. Two of our vectors, VecD2tpa and VRD2tpa, contained this substitution because, as discussed above, this signal peptide has been shown to increase the expression of several viral proteins. Studies performed using the mouse intracranial virus challenge model revealed that a JE *prM/env* DNA vaccine containing tPA leader confers higher levels of protection in challenged mice than those with the JE *prM* leader sequence (41). Putnak, et al. (6) evaluated a DEN-2 DNA vaccine containing *prM/env* genes and the tPA leader sequences in monkeys and they observed an induction of anti-E NAb responses that partially protected these animals against challenge with live DEN-2 virus. Although VecD2tpa induced faster and stronger antibody responses (including NAb) than VecD2, these responses were not statistically different. Thus, the contribution of the tPA to the immunogenicity of the DEN-2 DNA vaccine candidates could not be clearly established.

The second approach we used to enhance the immunogenicity of VecD2 included some modifications in the vaccination regimen such as shorter resting periods between immunizations (3 weeks instead of 4-6 weeks intervals) and using an alternative inoculation route, ID. Changing the vaccination route from IM to ID can influence the efficacy of DNA vaccines because the skin has more

antigen presenting cells (APC) than the muscle. APC can be transfected with the plasmid DNA and move to the lymph node where they can be potent activators of naive T cells (42). Thus, we hypothesized that better antigen presentation could result in better induction of protective immune responses. However, in our second experiment, Ab responses were weak or non-detectable when the vaccine candidates were delivered intradermally. This result was unexpected since previous reports describing the evaluation of DEN DNA vaccines demonstrated induction of both, specific Ab and NAb responses after ID immunization (16, 18). A possible explanation for this result is that we used a different inoculation site (upper back portion) than in the published studies (base of the tail) and it is possible that injections in some anatomical areas are more immunogenic than others.

The immunogenicity of VecD2 and VecD2tpa was improved when delivered intramuscularly at 3-weeks intervals. A statistically significant seroconversion was observed in mice vaccinated with these two vectors after the third DNA inoculation. The proportion of mice that elicited NAb responses was also significant after the third and fourth inoculation with VecD2tpa and VecD2, respectively. Antibody titers (both, specific and NAb) were weak after three DNA doses, except in the VecD2tpa group, which produced strong NAb (Figure 3B). However, after four DNA inoculations, specific Ab and NAb responses were strong in the VecD2 and VecD2tpa groups (GMT > 100 in most cases, Figure 3). Indeed, these titers were higher than those elicited by VecD2 before the virus boost in the first experiment (Figure 2). Thus, it is possible that shorter intervals between inoculations might have improved the boosting effect of subsequent DNA doses, leading to a better immune response.

The Ab titers induced by VRD2tpa were consistently lower than those induced by the other DEN-2 E protein expression vectors. Although specific Ab titers were not statistically different among the three DEN-2 vaccine candidates, NAb responses were statistically higher in the VecD2tpa and VecD2 groups in weeks 15 and 18, respectively, than VRD2tpa group (Figure 3B). Moreover, NAb responses in VRD2tpa group never reached statistical difference when compared to the control group (naked vectors). Therefore, VRD2tpa only induced short-lived, weak protective Ab responses against DEN-2 virus. This may be due to the modified tPA leader of VRD2tpa, which contained 4 additional aa that might have caused a negative impact in the immunogenicity of this vector.

Challenge studies are performed to test the efficacy of DEN vaccines to protect mice against live virus inoculation. These studies require the mice to be inoculated intracranially with DEN viruses on or before 6-weeks of age (43). This gives enough time for only two DNA inoculations at 2 week intervals. Since the challenge is one week after the second DNA inoculation, it is possible that 21 days is not enough time to produce a mature immune response in these young animals. This was evident in our studies, since DEN-2-specific antibody responses were absent in the pre-challenge serum of most vaccinated mice. Thus, this might explain why the DEN-2 vaccine candidates only provided partial protection against intracranial challenge (Figure 4).

Although two of the DEN-2 vaccine candidates (VecD2 and VecD2tpa) induced Ab responses, including NAb, both vectors were derived from a plasmid (pJW4304) that cannot be used in human trials (30). Therefore, we constructed and evaluated a new DEN-2 E protein expression vector called VRD2E. The results obtained with VRD2E in the immunogenicity studies were compared with those obtained with VecD2 because this vector contains the same viral sequences present in VRD2E. This comparison was not extended to the other two DEN-2 expression vectors (VRD2tpa and VecD2tpa) because in these vectors the homologous leader sequence was replaced by the tPA secretory signal (Table 1).

Our data demonstrated that mice immunized with the VRD2E vaccine candidate seroconverted earlier for both specific and NAb responses, than those immunized with VecD2. In addition, VRD2E elicited statistically higher levels of DEN-2 specific Ab than those generated by VecD2, during the entire experiment (Figure 5A). However, NAb titers were similar in the two experimental groups and were not statistically different except on week 3 (Figure 5B). On the other hand, NAb titers in the VRD2E group were statistically higher than the control at most time points tested (except week 6) but the same was not true for the VecD2 group. These results demonstrated that VRD2E induced faster and stronger DEN-2 specific Ab responses than VecD2, although NAb titers did not increase accordingly.

To determine if the immune enhancement of VRD2E was due to a better expression and secretion of the DEN-2 E protein, transient transfection studies were performed to compare the E protein expression between VRD2E and VecD2. Results obtained by Western blot analysis demonstrated that both expression vectors produced similar levels of the E protein both intracellularly (Figure 1D) and extracellularly (data not shown). Therefore, the level of expression is not responsible for the enhancement in the immune response observed in VRD2E-vaccinated mice.

Our DEN-2 DNA vaccines elicited responses that are comparable to those elicited by DNA vaccines developed by other groups, despite the differences in the experimental protocols (6, 16, 18-20). The DNA vaccine

candidate developed by Konishi and collaborators (17) closely resembles our vaccine candidates since it includes the *prM* leader, *prM* and the full-length *env* sequences. In their experiments, they performed three intramuscular inoculations (at intervals of two weeks) with 100 µg of the vaccine candidate and only detected NAb (1:10, at 90% plaque reduction) in one out five vaccinated mice. However, strong anamnestic responses (titers > 1:40, at 90% plaque reduction) were observed after a virus boost. These results are similar to those obtained in our first experiment where NAb were not elicited by VecD2 alone, but we demonstrated that the immunized animals were primed for protective immune responses.

In summary, data collected in this study demonstrates the ability of four DNA vaccine candidates, VecD2, VecD2tpa, VRD2tpa and VRD2E, to express DEN-2 E protein in vitro. In addition, we found that two of our DNA vaccine candidates (VecD2 and VecD2tpa) induced strong protective humoral responses when delivered intramuscularly. Since these two vectors cannot be used in human trials (30), we constructed and evaluated a new vector, VRD2E, which elicited stronger Ab responses than VecD2. Thus, VRD2E merits further evaluation as a DEN-2 vaccine candidate. The findings described in this report are relevant to the design and development of an effective, tetravalent DNA vaccine against DEN virus.

Resumen

Trasfondo: El virus del dengue (DEN) se ha convertido en un problema de salud pública que afecta mundialmente a aproximádamente 100 millones de personas cada año. Las medidas preventivas están enfocadas en programas de control del véctor, los cuales son infructuosos. Por lo tanto, se necesita urgentemente una vacuna. Métodos: El objetivo principal de nuestro grupo de trabajo es desarrollar una vacuna de ADN tetravalente que sea eficaz contra el DEN. En este estudio, construímos cuatro vacunas de ADN contra DEN-2 que expresan los genes *prM/env*, usando la secuencia líder homóloga (VecD2, VRD2E) o la señal secretora del activador del plasminógeno tisular (tPA, por sus siglas en inglés) (VecD2tpa, VRD2tpa). La expresión in vitro fue evaluada mediante transfecciones transitorias y "Western blot". La inmunogenicidad y eficacia protectora de los candidatos a vacuna se evaluaron en ratones BALB/c, empleando dos rutas de vacunación: intramuscular (IM) e intradermal (ID). Resultados: La expresión de la proteína de la envoltura (E) fue detectado en las células transfectadas COS-7 o 293T. Encontramos diferencias estadísticas en la respuesta de anticuerpos estimulada por estos candidatos a vacuna. Además, se observó respuesta de anticuerpos y protección más potente cuando las vacunas se administraron intramuscularmente. Por otra parte, la secuencia líder del tPA no mejoró significativamente la inmunogenicidad de las vacunas dado que VecD2 y VecD2tpa estimularon respuesta similar de anticuerpos. Conclusiones: En este estudio demostramos que la mayoría de nuestras potenciales vacunas de ADN pueden inducir respuesta de anticuerpos y protección parcial contra el virus de DEN-2 en ratones. Estos resultados proveen información valiosa para el diseño y construcción de una vacuna tetravalente de ADN contra el virus del dengue.

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Biotechnology and Biochemistry of Marine Natural Products

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Marine ecosystems are a source of biologically active compounds, many of which are currently in clinical use. With the goal of increasing the availability and the chemical diversity of these important compounds, more researchers are applying the tools of biotechnology to the discovery and production of marine natural products. This review summarizes the recent efforts made towards the characterization of the biochemical pathways that result in the production of marine natural products, with an emphasis on the work aimed at understanding the enzymatic activity involved in the biosynthesis of marine natural products.

Key words: Marine biotechnology, Natural products, Polyketides, Diterpenes, Polyunsaturated fatty acids

Natural products isolated from marine samples have a wide spectrum of biological activities and numerous therapeutic applications. Many compounds with antiviral, antibiotic and antitumor activity have been isolated from marine samples and tested in cell culture for their ability to specifically target and kill pathogenic or cancerous cells by disrupting diverse cellular processes (1-2). A number of these compounds, or synthetic analogues based on natural compounds, have entered clinical trials and some are currently administered as therapeutics (3).

Despite the great promise and the wide spectrum of biological activities of marine natural products, their subsequent development as therapeutic products has been slowed down due to several factors including the low availability of biologically active compounds (4), their high chemical complexity (5) and, in some cases, their high toxicity at therapeutic doses. Some compounds are produced by organisms which comprise only a minority component within the diverse population in the sample. In fact, many promising natural products associated with marine sponges and corals have been found not to be made by the invertebrate itself, but rather by symbiotic bacteria which are difficult to isolate, characterize and cultivate.

Several commercial ventures have been created precisely to apply the ethos of biotechnology to the development of marine drugs by devising ways to overcome some of the aforementioned difficulties in order to bring some of the more promising compounds closer to the clinic and to the market (6). Companies such as PharmaMar (Madrid, Spain) and Nereus Pharmaceuticals (San Diego, USA) have developed strategies for identifying cultivable organisms, optimizing growth, maximizing compound production, and increasing chemical diversity through synthetic modification (7) (Figure 1). This approach has yielded the first generation of biotechnology-derived marine natural products. PharmaMar currently makes and sells the natural anticancer compound trabectedin (Yondelis ®), which is produced by a semi-synthetic method in which a natural fermentation product from *Pseudomonas fluorescens* is chemically modified to form the final product (8). Another biotech company developing marine drugs is Nereus Pharmaceuticals, which is currently conducting Phase I clinical trials for proteasome inhibitor, salinosporamide A, for the treatment of solid tumors, lymphomas and multiple myeloma (9). This active compound is isolated directly from the fermentation of marine actinomycete, *Salinispora tropica*, a species first identified and characterized at the Scripps Institution of Oceanography by Paul Jensen and William Fenical (10).

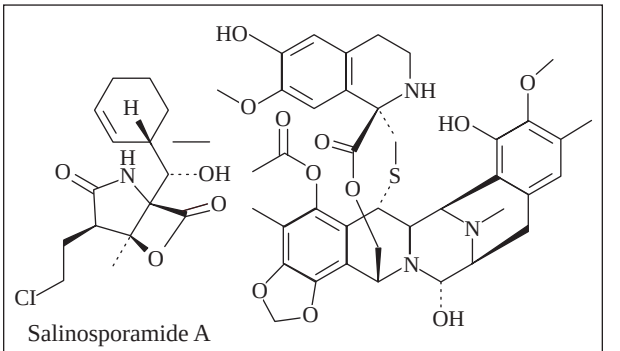

Figure 1. Salinosporamide A and Trabectedin are two compounds derived from marine biotechnology. Salinosporamide is currently in Phase I clinical trials, while Trabectedin has received orphan drug status for the treatment of soft tissue sarcoma.

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The successful application of biotechnology to the development of marine-derived pharmaceuticals has been met with an increased drive towards elucidating the enzyme pathways that lead to the biosynthesis of such complex and biologically active molecules. These recent efforts involve the cloning and characterization of multienzyme complexes, such as polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS), terpene cyclases and halogenases (11-12). Additional efforts involve the mechanistic and structural characterization of some of these biosynthetic enzymes as well as their expression and activity in heterologous hosts. This review attempts to provide a general view of the recent work aimed at understanding the enzyme pathways involved in the biosynthesis of marine natural products.

Marine actinobacteria and salinosporamide A

Actinobacteria are a group of gram-positive, GC-rich bacteria which are well-known producers of biologically active compounds. Many antibiotics, immunosuppressants and anticancer drugs are made from the cultivation of actinobacteria, many of them originally identified in tropical soils. The antibiotics erythromycin and rifamycin are still produced commercially from the fermentation cultures of soil actinobacteria.

Actinobacteria have also been found in marine sediments (10). Both *Salinispora arenicola* and *Salinispora tropica* are marine actinobacteria which were initially identified in sea sediments from the Bahamas. They both require seawater for cultivation and produce a number of natural products. In fact, analysis of the genome of *S tropica* reveals that approximately 9.9% of the DNA sequence is dedicated to the assembly of secondary metabolites (13). One of these secondary metabolites is Salinosporamide A, a potent inhibitor of the proteasome which is currently in Phase I clinical trials for the treatment of solid tumors, lymphomas and multiple myeloma.

The biosynthetic route to Salinosporamide A was proposed on the basis of feeding experiments in which *S tropica* in cell culture was fed labeled precursors, in this case acetate and shikimate (14). From the labeling pattern of the isolated final product the authors deduced the enzyme-catalyzed reactions that lead to its biosynthesis from three building blocks (Figure 2). It was proposed that a polyketide synthase (PKS) catalyzes the condensation of a chlorobutanoyl intermediate with an acetate unit, and subsequently with the clyclohexenyl-alanine amino acid arising from the shikimate pathway which is incorporated into the product by the activity of a non-ribosomal peptide synthetase (NRPS).

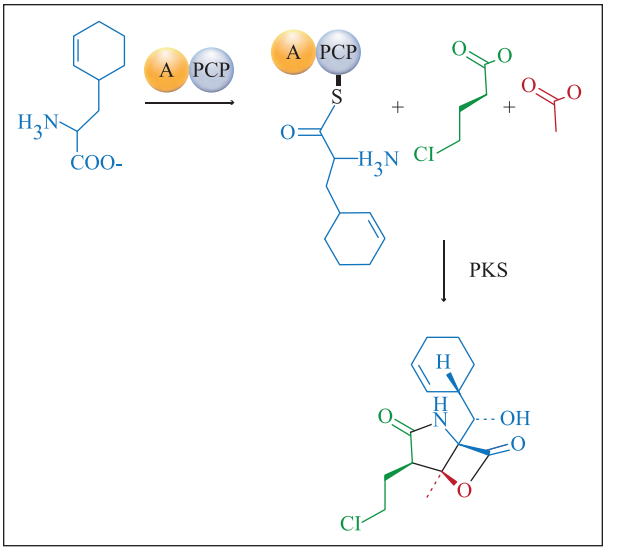

Figure 2. Salinosporamide A is made from three building blocks (14): an amino acid containing a cyclohexene side chain (blue) from the shikimate pathway, a chlorobutyl from S-adenosyl-methionine (11) (green) which is condensed with an acetate unit (red) by a polyketide synthase.

Based on this proposed biosynthetic pathway, a number of analogues of salinosporamide A have been generated using the tools of biotechnology (Figure 3). The inactivation of the Sal X gene, which is required for the biosynthesis of the amino acid,cyclohexenyl-alanine, resulted in the production of antiprotealide, a novel biologically active compound in which the amino acid leucine occupies the place of the original cyclohexenyl amino acid (15). Additionally, chemical complementation experiments in which other non-proteinogenic amino acids were fed to the SalX$^-$ strain, resulted in the incorporation of the exogenous amino acids into the final product, thus demonstrating the capacity of a "mutasynthetic" approach for the discovery of new compounds not easily accessible by conventional chemical methods.

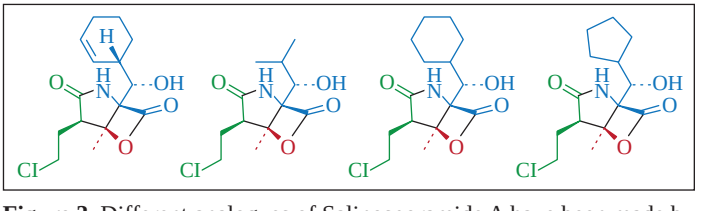

Figure 3. Different analogues of Salinosporamide A have been made by mutasynthesis (15). The approach consists of feeding different amino acids to a strain of *S tropica* that cannot produce the amino acid that gives rise to the cyclohexenyl moiety (blue). Leucine, cyclohexyl alanine and cyclopentylalanine have been incorporated into the final product in the absence of the preferred amino acid.

Enterocin and the reconstitution of biosynthetic pathways

The marine actinobacteria *Streptomyces maritimus* produces a diverse family of antibacterial compounds known as the enterocins and wailupemycins. These compounds are made by a Type II polyketide synthase (PKS) which catalyzes successive condesations of a benzoyl-CoA starter unit with seven malonyl-CoA building blocks by the proteins encA, encB, encC and encD (16). In an attempt to generate novel polyketides, the biosynthetic genes for the benzoyl-CoA starter unit in *S maritimus* were introduced into a streptomyces strain that produces truncated versions of erythromycin. The resulting Streptomyces strain produced novel PKS-derived triketides, containing the benzoate starter unit prescribed by the *S maritimus* genes (17). This result not only confirmed the tolerance of terrestrial Type I PKS toward alternative starter units, but also highlighted the enormous potential of using genes from a marine organism to modify known polyketides.

More recently, the entire biosynthetic pathway for enterocin was reconstituted in vitro from recombinantly expressed enzymes (18). While most enzymes were expressed in *Escherichia coli*, some enzymes were insoluble or inactive when expressed in that host. In those cases, the enzymes were overexpressed in well characterized hosts for the expression of streptomycete proteins, Streptomyces lividans or Streptomyces coelicolor. Efforts to reprogram the biosynthesis of enterocins and wailupemycins include the mutasynthesis of analogues of enterocin with non-natural starter units in which the authors fed aryl acids to a strain of *S maritimus* that was incapable of producing benzoate-primed polyketides. The result was a number of enterocin analogues with a different aryl starter unit (19). This general strategy has been taken a step further with the in vitro reconstituted enterocin enzyme complex (20). In this paper, Kalaitzis, et al. directly prime the corresponding biosynthetic enzyme with an alternative priming unit, other than the natural benzoate. The result is a number of novel enterocin and wailupemycin analogues obtained by means of recombinant technology.

Curacin biosynthesis in cyanobacteria

Cyanobacteria are another source of biologically active marine natural products many of which result from the activity of hybrid PKS:NRPS enzyme complexes (21). This is the case for hybrid molecules barbamide and curacin, both of which result from a biosynthetic route that includes both PKS domains and NRPS domains (22-23). The biosynthesis of barbamide, a molluscicidal compound, involves the condensation of three amino acids (trichloroleucine, phenylalanine and cysteine) with a malonate unit (Figure 4). The ability of the barbamide enzyme complex to specifically incorporate those amino acids has been confirmed in vitro using purified adenylation domains from the barbamide gene cluster over-expressed in *E coli* (22).

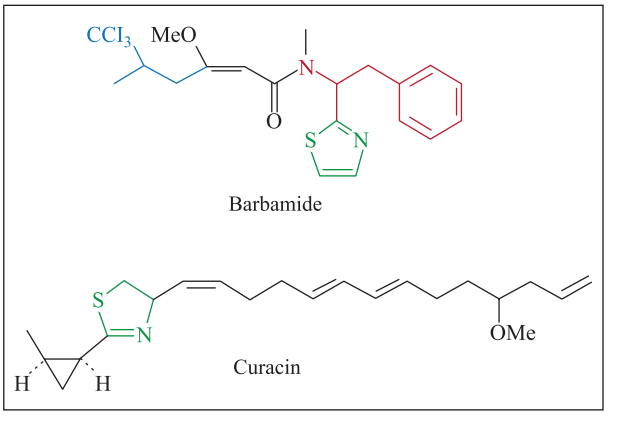

Figure 4. Barbamide and curacin are made by the activity of hybrid PKS-NRPS enzymes. The precursor for the barbamide starter unit is trichloroleucine (blue), followed by malonate condensation and the sequential incorporation of Phenylalanine (red) and cysteine (green). The backbone of curacin is mostly of polyketide origin, except for the thiazole ring (green) which comes from cysteine.

The biosynthesis of curacin, a potent anticancer compound from the cyanobacterium *Lyngbya majuscula*, involves the incorporation of a rare cyclopropyl moiety into the polyketide chain. The initial steps of this enzymatic transformation have been explored using two purified enzymes from the curacin gene cluster, CurE and CurF (24). Both sequences were found to align with the enoyl-CoA hydratase (ECH) family of enzymes which typically catalyze the hydration of double bonded intermediates during the beta-oxidation of fatty acids. Assays using purified enzymes showed that CurE catalyzes the dehydration of HMG-ACP while CurF catalyzes the subsequent decarboxylation to form the 3-methyl intermediate that gives rise to the cyclopropyl moiety. This work opens the possibility of using this CurE-CurF enzyme pair to engineer priming units for other PKS systems as a way to introduce cyclopropanes into known polyketide compounds.

Bryostatin in a symbiont of Bugula neritina

Marine bacteria are not the only source of biologically active natural products. There are many reports of natural products isolated from marine invertebrates. However, many of these natural products have been since found to be made not by the invertebrate animal but rather by a

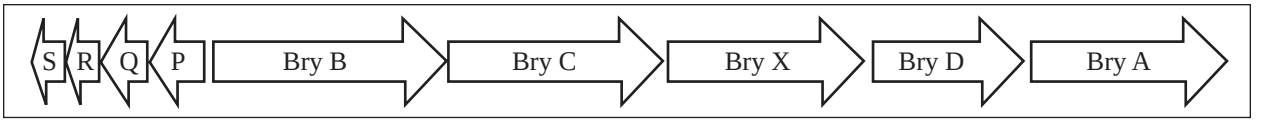

Figure 5. The gene cluster responsible for the production of bryostatin is composed of five Type I PKS multienzymes. According to the proposed biosynthetic route, only four of these multimodular proteins are used. BryX is comprised of enzymatic domains not arranged in the conventional order for Type I PKS.

symbiotic microorganism. This is the case of bryostatin 1, a potent anticancer compound found in the organism *Bugula neritina*, a common and abundant member of the fouling community in harbors and bays. Studies to characterize the genes required for the biosynthesis of bryostatin 1 in *B neritina* revealed that the PKS genes required for the production of bryostatin 1 were located not in the bryozoan but rather in a symbiont *Endobugula sertula* (25).

The benefit of using DNA technology to enhance the production of marine drugs is particularly important in the case of a compound like bryostatin. Bryostatin is an inhibitor of protein kinase C and is currently in clinical trials for the treatment of various cancers, Alzheimers disease and strokes. Yet, the abundance of this polyketide in the producing organism is fairly low. In order to obtain 1 g of bryostatin, over 1500 pounds of *B neritina* are required. For this reason, finding the gene cluster responsible for the production of bryostatin in *E sertula* will be essential in the development of a sustainable source of this important compound.

A gene cluster for a Type I PKS has been cloned and sequenced in *E sertula* strains collected at both deep and shallow waters (26). The genes, named BryA,B,C,D and X, are the only large open reading frames in an otherwise degenerate genome and the only genes for Type I PKS found in *E sertula*, suggesting that they are, in all likelihood, responsible for the production of bryostatin (27). According to the proposed biosynthetic route for this compound, only BryA,B,C and D are necessary for building the polyketide backbone, while BryX is an inactive or seemingly superfluous gene. However, the fact that BryX is present in both deep and shallow strains together with the fact that RT-PCR data shows transcription of the BryX gene, both suggest that the BryX gene is utilized by the organism.

Recently, the first biochemical evidence of biochemical activity in the bry gene cluster came from work with the bryP protein (28). BryP is a discrete acyltransferase domain which loads malonate units onto the ACP domains located in the elongation modules. This activity is required in this type of cluster because there is no acyltransferase present in the modular PKS. Results show BryP to be active against other ACP domains in "AT-less" PKS modules, as well as against excised ACP domains from other Type I PKS.

Pseudopterosins in corals

Pseudopterosins are diterpene molecules isolated from corals with anti-inflammatory and analgesic properties. These compounds have been found to significantly inhibit phorbol myristate acetate-induced topical inflammation in mice and the methyl ether of this natural product has shown promise as a treatment for contact dermatitis (29-30). They are made by the cyclization of geranylgeranyl diphosphate (GGPP) to form the intermediate elisabethatriene, which is in turn converted to a variety of final products (Figure 6). The enzyme that catalyzes such cyclization has been purified directly from the source organism and assayed for cyclase activity (31). The purified enzyme, the first diterpene cyclase from a marine organism ever characterized, has similar properties and kinetic parameters as other diterepene cyclases, such as pI, pH optimum, and Km.

In more recent work, the purified elisabethatriene synthase was challenged with alternative shorter substrates, geranyl diphosphate and farnesyl diphosphate,

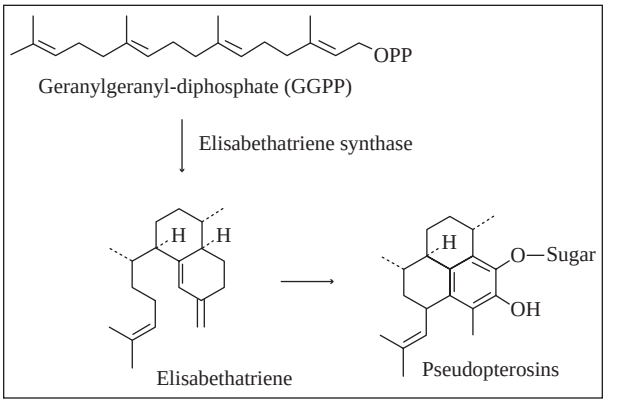

Figure 6. The biosynthesis of pseudopterosins starts with the conversion of GGPP to intermediate elisabethatriene which can be further converted to different pseudepterosins which differ in the nature of the glycosyl (R-group) moiety attached, presumably by a glycosyl transferase.

resulting in the production of alternative diterpenes (32). This result highlights the potential application of this family of enzymes in the generation of novel compounds derived from known diterpene precursors.

Omega-3 Fatty Acids from deep-sea bacteria

Long chain omega-3 fatty acids are essential nutrients in the human diet. They are involved in brain and eye development as well as in the maintenance of good cardiovascular health (33-34). Most of the long chain omega-3 in human diet comes either directly from the consumption of oily fish or indirectly from the relatively inefficient elongation of the medium-chain omega-3 present in some vegetable oils. Fish, in turn, also have an inefficient mechanism for producing their own long-chain omega-3 and must obtain it from a diet rich in microalgae and bacteria, both major sources of these important nutrients.

The biosynthesis of omega-3 in deep-sea bacteria takes place not by the desaturation and elongation of saturated fatty acids, but rather by a polyunsaturated fatty acid (PUFA) synthase system which resembles a PKS in that it builds the fatty acid one acyl unit at a time, introducing the double bonds as the chain grows attached to an acyl carrier protein (35). The PUFA synthase genes have been identified in different organisms and the organization of genes within the clusters show some commonalities (35-36) (Figure 7). For instance, all gene clusters for PUFA synthase sequenced so far have a number of between 4-6 ACP domains in tandem. Also, all PUFA synthase clusters dehydratase (DH) domains, presumed to be responsible for the formation of double bonds in the final product, are organized in pairs. This organization of DH domains is unlike that of DH domains normally found in modular PKS.

The PUFA synthase genes have been introduced into *E coli*, resulting in the production of omega-3 fatty acids in a heterologous host (37-39). These results indicated that PUFA synthase genes alone were all that was required for the biosynthesis of long-chain omega-3 in deep sea bacteria and that no elongase-desaturase enzyme was required. These results also highlight the potential of using *E coli* as a heterologous hosts for the production and for the expression of PUFA synthase proteins.

The biochemical activity of some of the PUFA synthase enzymes has also been reported. In order to determine how many of the tandem ACP domains are required for product assembly, the ACP domains from *Shewanella japonica* were systematically inactivated and expressed in *E coli* with the other components of the PUFA gene cluster (40). Results confirmed the presence of ACP domains that were functional as revealed by the fact that

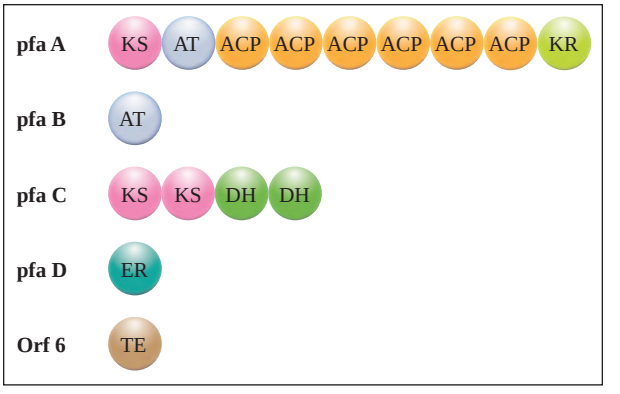

Figure 7. The gene cluster for the EPA-producing PKS in *Photobacterium profundum* consists of 5 open reading frames36. This domain organization is conserved among marine bacteria that produce polyunsaturated fatty acids.

they are recognized as substrates for phosphopantetheinyl transferases (PPTases). More importantly, results showed fatty acids can still be produced if all but one ACP domain has been inactivated by mutagenesis. These results answer the question of how many ACP domains are required for product assembly, but it does not explain the prevalence of tandem ACP domains in all known PUFA clusters, while virtually absent in other modular PKS systems.

The activity of the enoyl reductase enzyme in the PUFA gene cluster, pfaD, has also been measured in vitro (41). In this first measurement of enzymatic activity of a purified PUFA synthase component, the pfaD was expressed, purified and assayed for its ability to catalyze the reduction of the 2-butenoyl-ACP, a predicted authentic intermediate of the reaction. High-resolution mass spectrometric measurements showed a 2 Da increase in the molecular weight of the holo ACP in the presence of NADPH, demonstrating that pfaD is indeed an enoyl reductase as had been predicted by sequence alignments.

Summary

Biotechnology is being applied successfully to the study of marine natural products and to their subsequent development as therapeutics. In the examples discussed in this review, the biosynthetic routes for some of the more interesting and promising marine compounds are studied by a combination of approaches which include genetics, bioorganic chemistry and enzyme biochemistry. Two underlying goals can be discerned from these efforts: 1) finding more sustainable and economically viable ways to produce marine compounds and 2) increasing

the biochemical diversity of compound families. Biotechnology is driving the steady advancement of both of these goals as it promises to open doors in the quest for new medicines and new treatments.

Resumen

El ecosistema marino tropical es una fuente de compuestos con actividad biológica de un valor incalculable. Son varias las iniciativas que ya están en pie con el propósito de explorar tanto la diversidad biológica y química de los océanos como el uso de compuestos de origen marino en el tratamiento de enfermedades mortales. Algunos de estos esfuerzos recientes se centran en el uso de las herramientas de la biotecnología y la bioquímica para el descubrimiento de nuevos compuestos. Otras iniciativas buscan emplear la biotecnología con el propósito de generar diversidad química alrededor de compuestos prometedores así como para generar fuentes renovables para la producción industrial de estos compuestos. Es evidente que el alcanzar estas metas ambiciosas requerirá un conocimiento más completo de los procesos enzimáticos y moleculares que dan pie a la producción de compuestos activos en el ambiente marino. En este artículo se resumen los hallazgos más recientes en la identificación y caracterización de rutas enzimáticas de biosíntesis de compuestos marinos con énfasis particular en el trabajo realizado con enzimas purificadas.

Abbreviations and acronyms

PKS = Polyketide Synthases
NRPS = Non-ribosomal peptide synthetase
ACP = Acyl carrier protein
AT = Acyltransferase
PUFA = Polyunsaturated fatty acids
ECH = Enoyl CoA hydratase
GGPP = geranylgeranyl diphosphate
DH = dehydratase
PPTase = phosphopantetheinyl transferase

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Initial Study on Fibers and Coatings for the Fabrication of Bioscaffolds

VICTOR M. PANTOJAS, Ph D; ERICKA VELEZ; DARIMAR HERNÁNDEZ; WILFREDO OTAÑO, Ph D

Scaffolds composed of a mixture of poly (L-lactic) acid (PLLA) and polyethylene glycol (PEG) biodegradable polymers were prepared by electrospinning. Three-dimensional scaffolds of highly porous non-woven fibers were produced for biomedical applications and coated with calcium phosphate for bone tissue engineering. A mixture (80/20) of PLLA/PEG was dissolved at 5.7%, 7%, 8% and 9% blend solution concentrations. The structure and morphology of the scaffolds were investigated by scanning electron microscopy. Average fiber diameters ranging from 600 nm to 800 nm were obtained as result of the change in viscosity. The low polymer concentration fibers were found to be flat with fused junctions between fibers. For high polymer concentrations fibers they were cylindrical with fibers overlaying each other. For samples deposited at 9% concentration, individual fibers contained pores on their surface with nanometric dimensions. In addition, thin films of calcium deficient hydroxyapatite were prepared by rf magnetron sputtering on silicon substrates heated to temperatures between 300-600°C. These results suggest that it is feasible to fabricate biopolymer scaffolds using methods combining electrospinning and sputtering techniques.

Key words: Biodegradable polymers, Scaffolds, Electrospinning, Hydroxyapatite, Sputtering

The natural scaffold for most tissues is the extracellular matrix (ECM), whose structure and morphology contribute greatly to the properties and function of each organ. The EMC contributes to the rigidity and tensile strength of bone, the resilience of cartilage, the flexibility and hydrostatic strength of blood vessels, and the elasticity of skin (1). ECM is composed of two main groups of macromolecules, proteoglycans and collagen that together form a composite-like structure. Fibrous collagens embedded in proteoglycans maintain structural and mechanical properties. The collagen is organized in a three dimensional network composed of collagen fibers that form hierarchical structures from nanometer scale multifibrils to macroscopic tissue architecture (2-3). The ideal dimensions of the building blocks of the scaffold should be on a similar scale than of those of natural ECM. Hence, a nano-structured porous scaffold with interconnective pores, a wide pore size distribution and large surface area is needed for cell in-growth in a three dimensional fashion. The structure of these scaffolds is expected to promote cell adhesion, maintain cell functions and organize their growth. For bone generation, for example, pore sizes between 100 and 300 μm and porosities of more than 90% are preferred (4). The ideal scaffold should also have a sufficient mass of seeded cells, and these cells should be uniformly distributed throughout the entire scaffold. Other transport issues, including nutrient delivery, waste removal, and exclusion of material or cells, and protein transport are also affected by the pore size distribution and hydrophobicity of the scaffold. In addition, scaffolds should have the mechanical supportive properties while at the same time guiding cell differentiation and function.

A simple method to produce scaffolds formed by nanostructures in the shape of nano-micro fibers that are exceptionally long in length and uniform in diameter is electrospinning. This technique is based on the uniaxial stretching of a viscoelastic polymer solution through the action of electrostatic forces acting on charges in the solution when exposed to an external electric field. Under the action of this external electric field, the polymer-based jet continuously stretches and reduces its diameter due to the repulsion between the charges and the evaporation of the solvent. A fiber is formed with long length, and uniform diameter. Attracted to a grounded collector the charged fibers are usually deposited as a randomly oriented, non-woven mat. The morphology and diameter of the electrospun fibers are dependent on a number of

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parameters such as the properties of the polymer and solvent, the strength of the applied electric field, the distance between the spinneret and the collector, and the feeding rate of the polymer solution (5). Scaffolds produced by electrospinning of polymer solutions also allow for the control of specific cell binding interactions, control of degradation rate, the release of cell growth factors included in the solution and the use of polymers that can respond to environmental cues (6).

A number of natural and synthetic polymers are currently being employed as tissue scaffolds. Most synthetic biodegradable material such as polyglycolides (PGA) and polylactides (PLLA) can provide the necessary strength for structural stability. PLLA is one of the most widely used biomedical polymers owing to its biodegradability and biocompatibility. However, its application has been limited because of its stiffness and hydrophobicity. One method to improve the hydrophilicity of PLLA is to modify its bulk properties by adding Poly (ethylene glycol) (PEG) to the electrospinning solution. In a recent work, Bhattarai et.al., was able to demonstrate that adding a small fraction of low molecular weight PEG to the PLLA electrospinning solution improves the hydrophilicity of the scaffolds. A mixture of PLLA/PEG (80/20) was the best candidate amongst different ratios tested (7).

In the case of scaffolds for bone growth, coating the polymers with hydroxyapatite (HA) is expected to improve the scaffold's osteoconductive or bone formation ability. Hydroxyapatite (HA), the main constituent of bone and teeth, is a calcium phosphate (CaP) material in the apatite crystalline phase and shows the best bioactivity among the CaP ceramics (8). The crystalline phase hydroxyapatite shows excellent biointegration with no fibrous connective tissue formation, low tendency to evoke cytotoxic responses, and good osteoconductivity (9-10). The most important biomedical applications of HA are its use as a bone graft substitute in dental and orthopedic applications and as a coating of biomedical implants (11). HA coatings have been prepared using different techniques including plasma spray, laser ablation, sol-gel methods, dual ion beam, and sputtering, amongst others. Plasma spray is the most common commercial technique used to coat medical implants with HA (12). Unfortunately, the coatings have some drawbacks that limit their use (13). Problems associated with this technique are the mechanical weakness of HA-substrate interface resulting in delamination and debris formation, non-uniformity of the coating requiring use of thicker films, several crystalline calcium phosphates phases present simultaneously resulting in poor temporal retention of coating characteristics, occlusion of the porous substrate surface, and resorption of the HA coating due to poor crystalline properties (14). Sputtering techniques have the advantage of producing high coating density and the ability to coat difficult geometries (15). Wolke and co-workers deposited HA using magnetron sputtering producing thin films with good uniformity and density and found that the coatings had crystallographic texture (001). In vitro tests showed that the dissolution of the coating was determined by its crystallinity (13). The effect of substrate temperature on the crystallinity of sputter deposited HA films was studied by Nelea V, et al. (16). For substrate temperatures below 300ºC the films were mostly amorphous while samples deposited at 550ºC contained mostly the crystalline HA phase. Coating by sputtering the micro-nano fibers also has the advantage of producing several morphological structures from ribbons to hollow tubes as demonstrated by Pantojas, et al. in a recent work (17).

Osteoblasts are anchorage-dependent cells and it is expected that the high surface area and porosity of electrospun fibers might aid attachment and migration of cells inside the scaffold. There have been recent efforts to combine biodegradable polymers with hydroxyapatite in scaffold constructs. Deng, et al., for example, used a mixture of PLLA and HA nanoparticles in the electrospinning solution for the formation of composite scaffolds (18). They observed a slower degradation rate as compared to pure PLLA as well as improved cell attachment and growth. Natural fibers such as collagen have also been electrospun with HA nanocrystals in solution resulting in the formation of uniform nanoparticle-filled fibers with the needle-like crystals oriented along the fibers (19).

In this work, scaffolds of PLLA/PEG (80/20) mixtures were prepared by electrospinning with the aim of producing a dense mat with excellent pore size distribution and mechanical properties to be used in biological implants. The characteristics/properties of the scaffolds are dependent on the process used for their synthesis. A clear understanding is necessary of the parameters controlling the electrospinning process, and how they affect fiber diameter, pore size distribution and microstructure. It is also expected that controlling the individual fiber nanoscale features will result in the formation of materials with superior physical and chemical properties. Fiber mats prepared at different solvent concentrations were characterized in terms of fiber diameter and surface microstructure. Variations in solvent concentration produce changes in the viscosity of the mixture and affect the resulting fiber diameter and morphology in the electrospinning process.

Present efforts in the deposition of HA on silicon substrates using the sputtering technique are also reported. The properties of the film will depend on the deposition

parameters which have to be optimized (20). The long term goal is to combine electrospinning and sputtering techniques with the aim of producing biopolymer scaffolds covered with HA nanocrystals.

Materials and Methods

Scaffolds of the 80/20 PLLA/PEG polymer mixture were fabricated by electrospinning under optimum conditions. Polymer solutions were prepared by dissolving 0.2162 g of PLLA (Mw = 152,000 g/mol) and 0.0547 g of PEG (Mw = 10,000 g/mol) onto dichloromethane/chloroform (50:50) at concentrations of 5.7%, 7%, 8%, 9% w/w. From each concentration 0.2 ml was fed into a 10 ml plastic syringe, which was controlled by a pump at a feeding rate of 0.6 ml/hr. A vertical electrospinning system was used since it improves the deposition rate and minimizes the loss of material. The distance between the syringe needle and collector was 18 cm. A high voltage of 16 kV was applied by a voltage regulated DC power supply (Gamma Research) to generate the polymer jet. The resulting fibers were collected on a previously cleaned undoped silicon wafer.

Calcium phosphate thin films were deposited by rf magnetron sputtering. The deposition systems used cryo or turbomolecular pumps to maintain background pressures below 6.6 x 10-5 Pa. A commercial hydroxyapatite target was mounted on the water cooled magnetron gun and sputtered at a power of 100 W and in a 100% argon gas atmosphere. The substrate holder was facing the target at a distance of 10 cm. The substrate holder also had the capability to heat the substrate to temperatures over 1,000°C if desired. The temperature at the substrate position was calibrated using two thermocouples, one inside the heater casing and the other at the substrate position.

A JEOL JSM-6360 scanning electron microscope (SEM) was used to analyze the as-spun and coated fibers. Micrographs were taken at random areas at low, medium and high magnifications and at different locations to verify that they represent the morphology of each sample. The diameters of the fibers were measured from the micrographs using commercial image analysis software. An energy dispersive spectrometer (EDS) attached to the SEM was used to obtain information about the sample composition. A voltage over 7 kV was used to minimize the substrate interference as described elsewhere (21). X-ray diffraction (XRD) was performed on a Bruker Hi-star System with a General Area Detector. The sample angle was set at $\omega=20°$ while the detector angle was set at $2\theta=45°$. The two dimensional diffraction frames were integrated to obtain the one-dimensional diffractogram.

Results

Electrospinning

Figure 1 represents a typical electrospun mat prepared by electrospinning. Fibers are deposited in a non-woven dense mat with voids between fibers producing the porosity of the scaffold. Fiber mats can be deposited over large areas with a thickness that can be controlled by the amount of deposited material. Fibers are long, continuous and without the formation of beads which are typical of solutions with very low polymer concentration. The diameters of the fibers obtained at different polymer concentrations are given in Table 1. Average fiber diameters range from 650 to 830 nm with a slight increase as a function of polymer concentration.

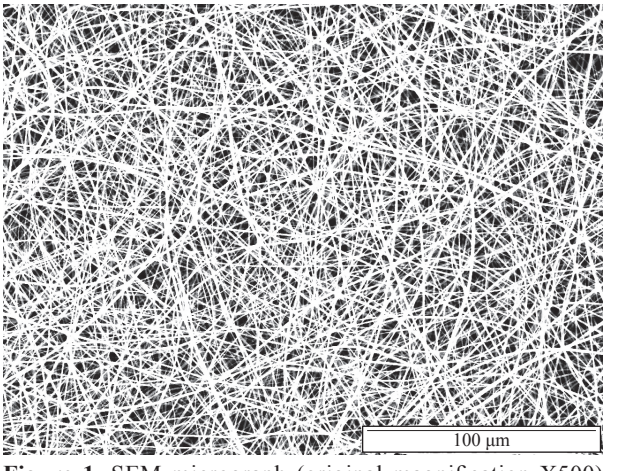

Figure 1. SEM micrograph (original magnification X500) of a typical electrospun scaffold composed of an 80/20 mixture of PLLA/PEG showing the randomly oriented fibers composition.

Table 1.

Blend Solution Concentration (%w/w)	Fiber Diameter (nm)
5.7	680 ± 200
7.0	650 ± 300
8.0	740 ± 360
9.0	830 ± 220

Figure 2 shows a SEM micrograph of the fiber mats produced at different polymer concentrations in the electrospinning solution. For low polymer concentration (5.7% w/w) fibers were flat with a curved and bent structure (Figure 2A). Relatively large voids with a range of sizes are observed between fibers. Also, individual fibers appear to fuse at their bonding points with other fibers indicating

that the fibers were still wet when they reached the collector, thus producing fused junctions and acquiring the flat shape of the substrate. This fused junction morphology can be beneficial to the mechanical integrity of the scaffold as it can provide reinforcement to individual fibers. For slightly higher polymer concentration, 7% w/w, fibers are straighter but the flat shape and fused junction morphology are maintained (Figure 2B). Mats produced at 8% w/w, Figure 2C, also show straight fibers but individual fibers overlap each other instead of fusing at their junction. In addition, some degree of fiber alignment is present.

Finally, for high polymer concentration (9%), figure 2D, cylindrical shaped fibers with a porous surface are obtained. At this polymer concentration the fibers solidify before reaching the substrate and maintain their cylindrical shape. Fibers produced by electrospinning usually have a smooth surface but under certain deposition conditions surface features may appear. The formation of pores in this system has been related to the rapid phase separation during electrospinning where the solvent rich regions apparently transformed into surface pores after evaporation (22). The average pore size along the fiber axis is 5.44 ±0.82 μm while perpendicular to the fiber axis is 2.59±0.82 μm.

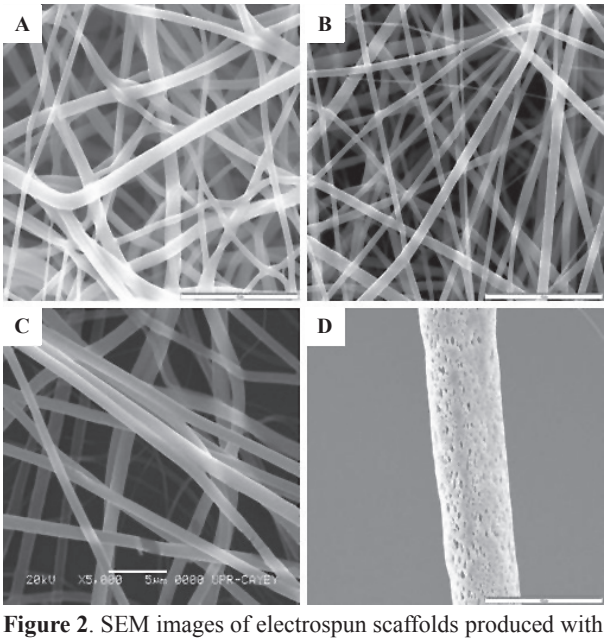

Figure 2. SEM images of electrospun scaffolds produced with different polymer concentration; A: 5.7% (x5000, 10μm bar), B: 7% (x5000, 10μm bar), C: 8% (x2000, 10μm bar), D: 9% (x500, 100μm bar). The produced morphologies included flat curved fibers with fused bonding points (A), flat straight fibers with fused bonding points (B), flat straight fibers without fused bonding points (C) and cylindrically shaped fibers without fused bonding points at intersections (D).

Sputtering

Thin films composed of CaP were deposited by sputtering on top of silicon in order to analyze its composition, crystallinity and morphology. Previous results already reported in literature showed that it is possible to deposit HA films by sputtering without the necessity of an external heat treatment to induce the growth of the apatite crystals (21). The apatite phase was obtained by in-situ heating during the deposition of the film at 600°C but the crystallinity was poor as observed by XRD. A magnetron gun with high strength permanent magnets was added to the system to increase the plasma ion density and, consequently, the deposition rate. Several samples were deposited at a pressure of 1.33 Pa, for two hours, and at a substrate temperature ranging from room temperature to 600°C. The diffractogram for samples prepared with substrate temperatures below 400°C did not show sharp peaks indicating that the films were amorphous. Figure 3 shows the diffractogram for samples deposited at substrate temperatures of 400, 500 and 600°C. For the sample deposited at 400°C the high intensity peaks of the HA crystalline phase are present and the peak intensities indicate partial (002) texture, typical of hexagonal structures. The small number of counts and peak broadening suggests nanocrystalline sizes that are typically formed in low mobility processes such as sputtering. Deposition at higher temperatures and direct observation of crystals by Transmission Electron Microscopy are needed to confirm these results.

Figure 4 shows the EDS spectrum typical of CaP samples prepared by sputtering. The silicon peak is produced by the substrate. The ratio of calcium to phosphorous is calculated from the peak intensities and plotted as a function of substrate temperature as shown in Figure 5. Excess calcium is observed when compared to the Ca/P ratio of 1.67 for the stoichiometric crystalline HA. This excess could be the result of different sputtering rates for Ca and P at the target and, consequently, can be modified by changing plasma process parameters such as deposition pressure and sputtering power. It could also depend on the history of target processing that alters the target stoichiometry if sputtering rates are not identical for the different components.

Discussion

Electrospinning is a technique which produces extremely fine fibers and is easy to implement in a laboratory. This technique can be used to fabricate scaffolds with fiber diameters ranging from several microns down to several hundred nanometers. Pores in the structure are formed by the space between differently oriented fibers lying on top

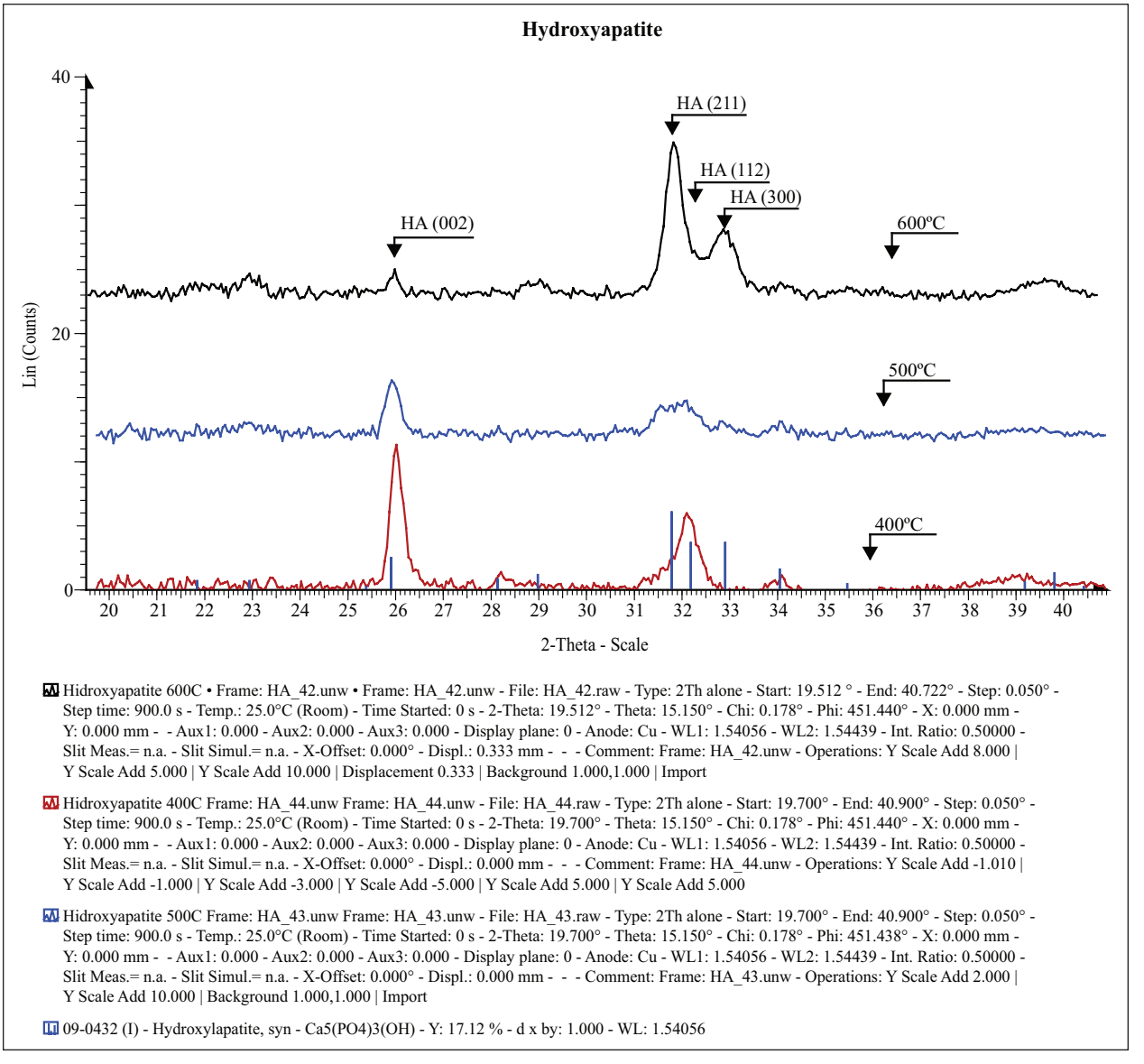

Figure 3. XRD of samples deposited at substrate temperatures of 400°C, 500°C and 600°C. The markers on top of the 2-Theta-Scale correspond to the peak positions and relative intensities from HA 09-0432 standard card.

of each other. For cell ingrowth the range of required pores sizes will depend on the type of cell but, for the majority, the pore diameter is in the range of 25 to100 μm. Pore sizes for the scaffold prepared at 7%, for example, has pore sizes ranging from 12 μm to 800 nm. An advantage of using scaffolds with cm long fibers for cell growth is that, even if the pores are too small for cell migration, the fiber structure allows the cells to push the surrounding fibers aside to expand the holes since the small fibers offer little resistance to cell movement.

The viscosity of the solution, as controlled by changing the polymer concentration, has been found to be one of the biggest determiners of fiber size and morphology. Only a small variation in fiber diameter is observed in our samples suggesting that, to better control size another deposition parameter, such as the feeding rate or the type of solvent has to be explored. In general, fibers produced by electrospinning solutions with low polymer concentration or low viscosity are observed to be flat with fused junction between fibers. This fused junction morphology can improve the mechanical stability and structural integrity of the scaffolds. This is an important factor in its design since it is expected to provide biomechanical support during the process of tissue regeneration and structure degradation.

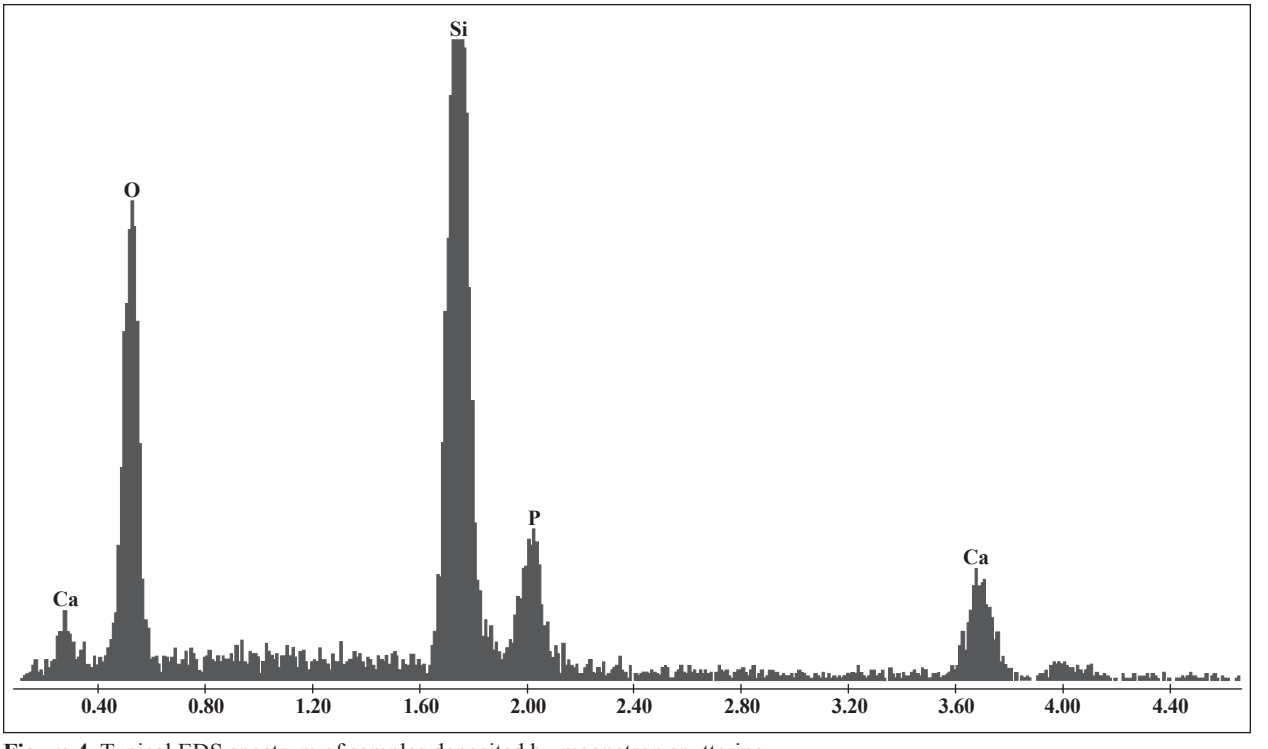

Figure 4. Typical EDS spectrum of samples deposited by magnetron sputtering.

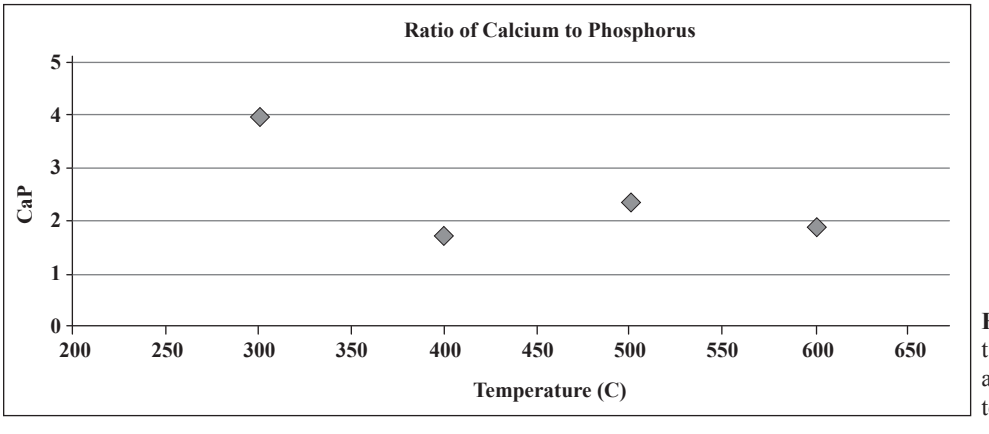

Figure 5. Plot of calcium to phosphorous ratio as a function of substrate temperature.

Fibers from solutions with high polymer concentration or high viscosity have cylindrical shapes and overlapped with each other instead of fusing at their junctions. Surface features can also be controlled by the selection of polymer concentration as porous fibers are formed when phase separation occurs during the electrospinning process. For tissue engineering it may advantageous if the surface is porous instead of smooth. Surface pores, for example, can function as anchoring points for cells, modifying the wetting properties, increasing surface area, or influencing the kinetics of biodegradation of the scaffold. It is possible to generate pores during the electrospinning process by choosing particular solvents or solvent mixtures, by varying the humidity, or by using polymer mixtures (23).

Electrospinning also allows for the alignment of the scaffold's fibers through a number of possible modifications to the system including the use of a rotating drum as collector and the application of designed voltages to avoid the fiber whipping effect. Fiber alignment is desirable in applications where cell growth guidance is desired as in the case of neural cells. As cells move across

a surface, physical and chemical cues can be used to cause the cells to preferentially adhere in a given direction. Physical cues, e.g. microchannels, are a standard technique for aligning cells in the field of tissue engineering. Fabrication of these surface microchannels by techniques such as electron beam lithography are time consuming, requires expensive equipment and the surface coverage is small. Electrospinning may provide a vast improvement over the current microscale alignment methods by moving tissue engineering into the nanoscale domain.

Meanwhile, deposition of calcium phosphate by sputtering appears to be a promising process for coating biomedical implants. The preliminary results demonstrated in this article point to the formation of a HA coating on top of a suitable implant material, e.g. titanium, by in-situ heating. The methodology for coating the biopolymer scaffolds requires sputtering the calcium phosphate without substrate heating to prevent the thermal destruction of the scaffold. After deposition, the coated scaffold can be heated in air to promote the growth of HA nanocrystals.

Resumen

Matrices extracelulares compuestas de una mezcla de dos polímeros biodegradables, ácido poli láctico (PLLA) y polietileno-glicol (PEG), fueron preparados por "electrospinning". Matrices tridimensionales de fibras entrelazadas para aplicaciones en biomedicina fueron formadas. Una mezcla (80/20) de PLLA/PEG fue disuelta a concentraciones de 5.7%, 7%, 8% y 9 % en solución para electrospinning. La estructura y morfología de las matrices fue investigada con microscopia electrónica de barrido. Matrices compuestas de fibras con diámetros desde 600 nm hasta 800 nm fueron obtenidas al variar la viscosidad de la solución. Para bajas concentración de polímero, las fibras son planas con las intersecciones entre fibras fusionadas. Para altas concentraciones de polímero, las fibras son cilíndricas con las fibras sobrepuestas. Para las matrices formadas a una concentración de 9% las fibras individuales contienen poros nanométricos en su superficie. En el caso de aplicaciones en la formación de huesos, películas finas de hidroxiapatita fueron preparadas por "magnetron sputtering" de radiofrecuencias sobre substratos de Silicio calentados a temperaturas entre 300-600 °C. Estos resultados sugieren que es posible fabricar matrices de biopolímeros mediante la combinación de las técnicas de "electrospinning" y "sputtering".

Abbreviations and acronyms

PLLA = poly(L-lactic) acid
PEG = polyethylene glycol
ECM = extracellular matrix
PGA = polyglycolides
HA = hydroxyapatite
CaP = calcium phosphate
SEM = scanning electron microscope
EDS = energy dispersive spectrometer
XRD = X-ray diffraction

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COMMENTARY

Bringing DNA-Guided Medicine to the Hispanic Population

DNA-guided medicine poses great advantages for the highly heterogeneous Hispanic population. Similar to others in Latin America, the Puerto Rican population originated as a result of admixture between Amerindians, whose ancestors had migrated from the Amazon Basin and arrived in Puerto Rico 2200 years before present, and Spaniard and West-African individuals. The island of Puerto Rico thus is endowed with a distinctive population in terms of its gene flow. There are growing numbers of Puerto Ricans in the USA. Currently, Puerto Ricans represent 1.2% of the USA population and 9.6% of the Hispanic population in the USA. Admixture studies in Puerto Ricans, either in the island or the continental USA, have been scarce. The history of migration and admixture can be reconstructed and interpreted using genetic markers. Our results demonstrated that population analysis can be performed with functionally important genes instead of conventional ancestry informational markers to increase the relevance of population genetic studies for clinical epidemiology and personalized medicine. In this commentary, we examine the various implications of these findings and suggest an interpretation of the data utilizing a collage technique.

Physiogenomic Profile of the Puerto Rican Population

We performed physiological genomic analysis on 196 important cardiovascular, neuroendocrine and metabolic genes (including key pharmaceutical targets) in 100 anonymous, geographically representative DNA samples from the Newborn Screening Program at the University of Puerto Rico Pediatric Hospital (1). Population structure was examined using gene polymorphisms for clustering. The Puerto Rican sample is found to be broadly heterogeneous. We observed 3 main clusters in the population, which we hypothesize to reflect the historical origin of the Puerto Rican population as Amerindian with relatively recent European and African admixture. We present evidence for this interpretation by comparing inferred allele frequencies for the 3 clusters with the allele frequencies found for the same gene polymorphisms by the Human Genome Project in European, African and Asian populations. Each individual in the cohort was a 'genetic mosaic', with contributions from each of the clusters, but in widely different proportions. The maximal contribution from a single cluster observed in this sample is ~85% for African, ~85% for European and ~70% for Amerindian ancestries. The range of possible genetic combinations in the Puerto Rican populations is considerable, and certain to exceed that in populations without admixture.

Implications for healthcare and clinical research

In highly heterogenous populations, existing "one-size-fits-all" medications and prescription recommendations are likely to be ineffective, or worse, cause harm through side effects. With history and ancestry spanning African, Amerindian, and European origins, the typical Puerto Rican person defies conventional ethno-geographic definitions. Clinical research should be undertaken in diseases demonstrating higher prevalence in the Hispanic population to improve the existing treatments and re-adjust dosing regimens in targeted recipients from such a highly heterogeneous population. Heterogeneity in how people respond to medications has confounded the prescription of modern medicines, with detrimental consequences for the safety, efficacy and patient compliance of potent drugs. A major advance in healthcare would be a transition from the current empirical approaches and trial employed in drug therapy to a genetically predictive framework for determining the individual patient's response to medicines.

What are the barriers to getting these technologies used by healthcare system? How could we overcome these barriers? The first step in developing the genetic rules for personalized medicine is clinical research to "train the algorithms" supporting medical decision making. For example, a range of responses to a drug (efficacy, side effects, therapeutic ratios) in a target populations can be modeled by additive effects of DNA polymorphisms when a drug is given and the recipients have agreed to have their DNA and clinical response analyzed. This research can be fundamentally observational, and not pose any risk to subjects beyond that of standard medical therapy.

Implications for economic development

Genome diversity provides an opportunity to leapfrog health standards in heterogeneous Hispanic populations to areas of medical need with potential disparities of care including diabetes, heart disease, mental health, and cancer. Genomic heterogeneity also poses a great resource for translational clinical science in Latin America and innovation in healthcare. The paradigm of drug development in homogeneous populations for questionable extrapolation to real world heterogeneous populations can thus be supplanted with one of intentional diversity for broader export applicability. The determination of

individualized treatment most suitable to each patient, using the personal genome for clinical decision support, and the implementation of drug prescription safeguards, are integral to personalized health. Such DNA-guided personalization provides a new venue to assure advances in medical technology improve the health of the Hispanic population.

Within the broad personalization paradigm, we have an enormous opportunity to turn our genetic variability into a clinical asset rather than a complication. Similar to the Global Positioning Systems used in vehicles today to help us reach our intended destinations, a 'genetic prescription system' could be configured from correlations of gene variation and clinical outcomes. This other GPS will help lead physicians and patients to their desired treatment goal, resulting in more effective healthcare.

Implications for education

Often new concepts require unconventional ways of depicting them in order to enhance understanding. The fusion of art and science is relevant to this quest, and we had utilized a digital montage technique in a previous illustration, *DNA Collage I*, published in 2008 (2). In order to construct an image relevant to the Puerto Rican physiogenomic data, we turned to the illustrious painter Francisco Oller, who in his iconic *El Velorio* (1893) had encompassed a mosaic of Puerto Rican society where the characters in effect demonstrate the range of diversity in the population. He was one of the first painters to bring the techniques of the French impressionists to interpret the tropical landscape. In *Hacienda Aurora* (1898) he captured the essence of this landscape with generous blue space for the sky, lush greenery and strokes of yellow for the countryside brightly illuminated by the tropical sun. These works provided inspiration to the *Mosaico Genético Boricua* (DNA Collage PR), which is shown in the cover of this issue of the *Puerto Rico Health Sciences Journal*.

For this collage, we utilized the genotype data for single nucleotide polymorphisms (SNPs) in cardiometabolic and neuroendocrine genes, shown vertically, one per column, and Puerto Rican people aligned horizontally, one person per row. Each SNP is a variable site in the DNA with either of two sequences. In total, the cover represents an array of 25,489 DNA markers for 359 variable gene sequences (columns) from each of 71 individuals (rows). The colors represent the genotype a person has inherited from both parents at every SNP site: two copies of either sequence (homozygous) or one of each (heterozygous). Each genotype was colorized as green if heterozygous, and randomly as blue or yellow if homozygous. The genotypes ensembles, while unique for each person, blend harmoniously in colorful patterns with the pointillist touches of variability preventing any column or row from being homogeneous. This image reflects the admixture in the Puerto Rican population, and subtly suggests the unique societal aspects that led to the fusion of people in Puerto Rico, "la isla del encanto" (the island of enchantment).

In explaining the genetic characteristics of the Puerto Rican people, the image in the *Mosaico Genético Boricua* contextualizes the diversity and admixture as an innate asset. It would have been the case that such characteristics pose a drawback in a world where one medication is supposed to fit all. But in the era of DNA-guided medicine and personalized health, such diversity is precisely the enabling platform of opportunity. The delivery of DNA-guided healthcare for each person is good medicine for all people.

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About the Cover

Mosaico Genético Boricua, Digital Montage on vinyl board of 25,489 pixels colorized algorithmically from DNA variability by Gualberto Ruaño, MD, Ph D and Mohan Kocherla.

SPECIAL ARTICLE

Symposium Review: Drug Discovery, Development and Clinical Research in Academia

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The development of new drugs for the pharmacological treatment of diseases is a costly and time-consuming process. The pharmaceutical industry has traditionally played the main role in the discovery, development, manufacturing and marketing of new drugs. However, in recent years, the role of academia in the process of drug discovery and development, and its eventual translation into clinical applications has steadily increased. At the occasion of their 95th anniversary, the School of Pharmacy at the University of Puerto Rico organized a symposium titled "Drug Discovery, Development and Clinical Research in Academia". This article presents a summary of the symposium presentations and the potential role of academic drug discovery and development as a complement to the pharmaceutical manufacturing industry in Puerto Rico.

The pharmaceutical industry has had a longtime presence in Puerto Rico. Currently, it contributes to one-third of the Gross Domestic Product of Puerto Rico and provides employment to 120,000 highly trained and qualified workers. However, its main focus thus far has been in the area of manufacturing of drug substances (the active ingredients of a drug) and drug products (the vehicle, i.e. tablets, creams, injectable solutions). The patent-expiration of high-volume drugs, the decline of the introduction of new products in the drug development pipeline, combined with recent competition with countries with lower production costs, provide a challenge for Puerto Rico to maintain its attractiveness for new investments. However, its highly trained workforce and experience in the pharmaceutical industry remains an important asset, especially with the introduction of new, advanced manufacturing technologies. Even though in recent years considerable investments have been made in the Biotechnology manufacturing industry in Puerto Rico, an area of opportunity still remains in another aspect of the "Biotech" industry that include the development of both new large biological as well as new small chemical drugs. An important complementary opportunity to the pharmaceutical manufacturing industry will arise when researchers in academia are able to translate their current research into clinical applications, and eventually into commercial applications. Academic investigators at Puerto Rican universities have been successful in attracting federal and private funding in order to support the advancement of their research projects. However, few projects have led to products that directly benefit patients, and thus, that eventually could be brought to market. New discoveries in the area of drug development could lead to such products and, combined with appropriate patent protection, attract new investments in start-up "Biotech" companies. In addition, the local government currently has a major interest in promoting Puerto Rico as a "Bio-Island" and in stimulating investment in "Research and Development". A review of the factors that determine success in the area of drug development, therefore, warranted the organization of a symposium in this area.

On September 18-19, 2008, the School of Pharmacy of the University of Puerto Rico, on the occasion of its 95th anniversary, held a symposium titled "Drug Discovery, Development and Clinical Research in Academia". As later described in this symposium review, the discovery and development of new drug therapies for the treatment of diseases, requires a multidisciplinary effort from investigators in basic, applied and clinical sciences. Therefore, one of the main goals of the symposium was to foster relationships between investigators, present examples of successful drug development projects and provide investigators with opportunities to establish new collaborations. An integrative approach towards drug development, its clinical applications, and eventual commercialization was followed throughout the symposium. Topics included the description of the relevance of traditional medicine, natural products, chemical synthesis, activity screening, biochemical and

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pharmacological implications, animal studies, clinical research and relevance for patients, patent opportunities and venture capital investments, all of which, eventually, could lead to the introduction of new drugs to the market for the benefit of patients.

The drug discovery process

The path towards the discovery, development, and eventual marketing of a new drug is generally represented as in Figure 1 (1) and has been further detailed in the "critical path" initiative as outlined by the U.S. Food and Drug Administration (FDA) (2).

Target identification: In target identification, a biological process is identified that is associated with the disease of interest. Traditional biochemical methodology, as well as modern proteomics and genomics approaches can determine if a certain target is implicated in the progression of disease. Selective interference of this target with biological or small chemical molecules can potentially be developed into novel drugs, and eventually can lead to improved health outcomes.

Assay development: In order to identify these novel molecules, an assay procedure needs to be developed, which determines the extent of interference of these molecules of interest with the target.

Screening: "Lead" compounds that selectively bind to the target can be identified via high-throughput screening procedures, (in some cases more than 100,000 compounds are tested).

Lead development: In subsequent lead development, the compounds are modified in order to optimize the binding for better activity, and their effects in animal models of the disease are investigated.

Preclinical studies: In preclinical studies, required before submission of an Investigational New Drug (IND) application to regulatory agencies, the pharmacokinetic and pharmacodynamic parameters of the new compound, as well as the potential for metabolism and toxicity are determined in suitable animal systems. Furthermore, a drug dosage form is developed for administration of the drug to patients. During the above-described process preceding a Phase I clinical study, there are multiple factors that determine whether further development can be successful, i.e. the compounds does not demonstrate the expected efficacy, the compound is not reaching its in vivo target, the compound shows toxicity, etcetera. Opportunities exist for academic institutions and its researchers to play a role in all steps of the development process described so far. Due to the exponentially increasing costs, later stage clinical trials are prohibitive for academic research. However, after the academic institution licenses patents related to the drug, venture capitalists interested in starting a small biotech company, or major pharmaceutical companies, may be attracted in sponsoring further development of the drug. Nevertheless, these clinical trials are frequently carried out in academic medical centers via collaboration with academic physicians interested in clinical investigations.

Phase I clinical trials: In general, in a phase I clinical trial, a few dozen healthy volunteers are administered the new drug in order to assist in the determination of the optimal dosing amount, and to study potential side effects. Exceptions are terminal diseases such as cancer, in which patients for whom no other medications have proven effective are enrolled.

Phase II clinical trials: In a phase II clinical trial, several hundred patients with the disease to be treated, are administered the novel drug. The efficacy of the drug in treating the disease is monitored, as well as side effects.

Phase III clinical trials: When the results of these investigations are successful and correlate with the expected outcomes, a larger phase III clinical trial with thousands of patients is carried out. Upon successful demonstration that the drug is useful for the treatment of the disease, a New Drug Application (NDA) is submitted to regulatory agencies requesting approval of the drug for marketing. Disappointingly, despite considerable investments of time and resources, the success-rate of compounds entering clinical trials is only about 11% (3).

Marketing-Phase IV: In phase IV studies, the safety of the drug in the larger general population is monitored, as well as the potential of the drug in the treatment of diseases not included in the original approval.

The pharmaceutical industry in Puerto Rico

In the first presentation, Dr. Sigfredo García, vice-president of Pfizer Puerto Rico and president of INDUNIV (Industry and University collaboration with Government support) emphasized the importance of the symposium as a contribution to the development

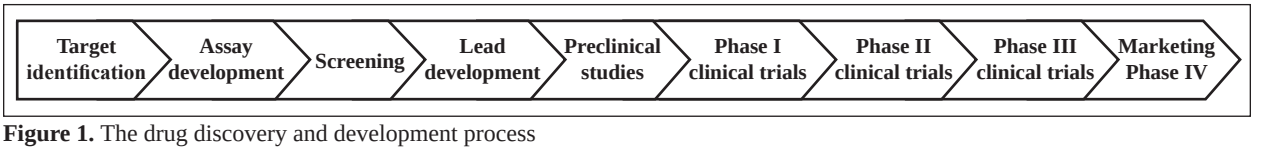

Figure 1. The drug discovery and development process

of an "innovation eco-system". Currently, with 89 manufacturing plants from 29 different companies in the biopharmaceutical and pharmaceutical industry, the importance for further development of this industry in Puerto Rico cannot be underestimated. Not only do 19 of the 33 largest pharmaceutical companies have a presence in Puerto Rico, but also 13 of the 20 top-selling drugs are manufactured here. However, as Dr. Garcia indicated, the industry is under pressure to bring new drugs to market, even though estimated costs for the development of a new drug are $800,000,000 to $1,000,000,000 (4). At the same time, the amount of new drugs with different mechanisms of action entering the market has shown a significant reduction. Therefore, "Big Pharma" is interested in new ventures with academia, and several collaborations between Pfizer and academic institutions in the US in this area of drug discovery and development were explained. He also indicated that similar collaborations with academic institutions in Puerto Rico could be advantageous due to the beneficial tax law for Research & Development investments and the fact that many pharmaceutical companies already have a presence on the island.

Nature as a source or as an inspiration for drugs

Nature has been an important resource for the discovery of new drugs. Of the drugs brought to market in the last twenty-five years, 34% are natural products or have been directly derived from natural products (5). A particular field in natural product drug discovery is ethnobotany, in which the use of plants in traditional medicine is investigated. Dr. Michael Balick, vice-president for Botanical Science, Research and Training, and Director and Philecological Curator of the Institute of Economic Botany of the New York Botanical Garden, presented several aspects of his research in the area of ethnobotany. From investigations into the use of medicinal plants in traditional use by healers or by the general population in Belize (6-7), Micronesia and in the Dominican Republic and the Dominican population in Manhattan (8-9), several interesting applications of plants or plant extracts for disease treatments have been identified. Potentially, these plants could lead to novel drug therapies to be implemented in modern Western medicine. Moreover, Dr. Balick emphasized that in ethnobotany it is important to consider the comprehensive aspects of 'Plants – People – Tradition'. Unfortunately, modernization leads to the disappearance of ancient cultures, and as a consequence, their extremely valuable knowledge of traditional medicine is on the verge of extinction. An example of the importance of studying traditional methods can be seen in the use of Cinnamomum carolinense bark shavings as a medicinal tea on the island of Pohnpei in Micronesia (10). While ethanolic extracts of the bark contain saffrole, a known carcinogenic compound, a special preparation procedure utilized by the native residents eliminates saffrole from the tea. It should be mentioned that Puerto Rico, with its tropical climate, has a large diversity of plant species, some of which have been used traditionally for treatment of a variety of ailments or diseases. A newly remodeled Medicinal Plants Garden is located within the University of Puerto Rico's Botanical Garden. Therefore, the opportunity exists for an ethnobotanical investigation in the island which, together with identification of the active substances in specific plants, could eventually lead to novel drugs.

Chemistry-inspired drug discovery

An example of a successful account of drug discovery in academia was presented by Dr. Waldemar Priebe, Professor of Medicinal Chemistry, MD Anderson Cancer Center. Dr. Priebe presented his research based on chemistry-inspired drug discovery and development of novel anti-cancer drugs. His approach to drug discovery is to prepare novel chemical entities, based on molecular "LEGO®" blocks. Each Lego-block represents a small specific chemical structure designed for its ability to interact with biologically relevant macromolecules. By connecting and combining the Lego-blocks with each other, a large variety of combinations of drug-like molecules can be prepared. The newly prepared compounds are then tested for their biological activity. Depending on the target for the (potential) drug, this could be the extent to which the new molecules interact with DNA, or their ability to inhibit enzymes known to be involved in disease processes. Several of the compounds synthesized in his laboratory have been successfully brought into Phase I clinical trials for 'first-in-man' testing. One example of a potential new drug that is currently in clinical trials is identified as WP744 (also known as RTA744) (11). This doxorubicin-derivative was designed to be active against multidrug resistant tumor cell lines and, even more interestingly, was shown to be able to cross the blood brain barrier (12). This new molecule, therefore, offers promise as a new drug in the treatment of glioblastoma multiforme, a highly malignant form of brain cancer for which treatment options are very limited. However, in order to comply with regulatory requirements of an IND-submission for clinical testing, an extensive evaluation of its pharmacological properties and potential toxicity had to be carried out. As described below, the "Pharmaceutical Development Center" at M.D. Anderson has proven to be invaluable in providing the required support and pharmacological data necessary in this process.

Preclinical studies

Before a compound can enter clinical trials, pre-clinical studies are required since these ultimately determine whether the compound is safe for administration to humans. Via pharmacokinetics (PK), pharmacodynamics (PD) and ADME-Tox (Absorption-Distribution-Metabolism-Excretion-Toxicology) studies, a safe starting dose for clinical trials can be established. At MD Anderson, these studies are carried out in the "Pharmaceutical Development Center" (PDC), which provides these services to its investigators with promising drug candidates in development. One of the compounds that was studied in the PDC and was able to advance into phase I clinical trials was the above-described WP744.

The Director of the PDC, Dr. Timothy Madden, MD Anderson Cancer Center, described his experiences during the five years the center has been in existence. The goal of this center is to facilitate the availability and testing of novel, promising therapies for patients. The center supports pre-clinical to Phase I studies, including safety testing. The testing procedures for qualified projects, as determined by an internal advisory board, provides researchers all needed data required by government regulations in order to commence clinical trials. These testing procedures include analytical assay validation, mechanism based experiments, proof of concept studies and in vitro metabolism studies. In addition, pharmacokinetic studies for ADME-Tox data are performed. Also, support is provided in the area of dosage form development for pharmaceutical production, including drug solubility and stability studies. During the last five years, more than 40 projects have utilized the services of the center, leading to 23 patent filings, 37 publications and 11 projects for which an IND application (Investigational New Drug), required by the FDA to start a clinical trial have been submitted. At present, clinical trials are in progress for nine projects supported by the PDC.

Advancing to Phase I clinical trials

Dr. Charles Conrad, MD Anderson Cancer Center, presented his clinical research in the area of brain cancer treatments. As one of the examples, he described his successful collaboration with Dr. Walter Priebe, in which WP744, synthesized in Dr. Priebe's laboratory was brought into clinical trials as a potential treatment for glioblastoma multiforme. Because surgery and radiation are frequently not an option for the treatment of glial tumors, it was decided to investigate this novel compound as a potential therapeutic alternative for this disease.. It was observed, from clinical studies under the direction of Dr. Conrad, that WP744 showed clinical activity in patients with brain tumor; the activity ranged from stabilization of the disease to a complete response, thus providing the promise of a novel treatment for this devastating disease. Other compounds, several of which were also developed in collaboration with Dr. Priebe are in different stages of development (13-14). As was emphasized by Dr. Conrad, the success in developing new drugs and the development of other novel clinical candidates would not be possible without the collaboration of researchers from various disciplines. As exemplified in Figure 2, the interdisciplinary collaboration of scientists from various disciplines is extremely important in order to translate discoveries made in the laboratory to clinically relevant applications.

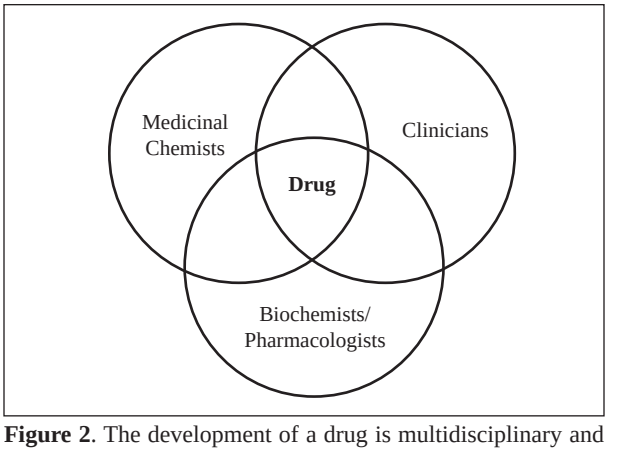

Figure 2. The development of a drug is multidisciplinary and interdisciplinary effort

Neglected diseases

An especially relevant opportunity for drug discovery and development in academia is in the area of diseases with unmet needs (15-16). Typically, diseases that only affect a relatively small part of the population, or diseases primarily occurring in developing countries such as malaria or tuberculosis, are not of primary interest to large pharmaceutical companies. Dr. Scott Franzblau, Director Institute for Tuberculosis Research, College of Pharmacy, University of Illinois, presented his work on the establishment of a research center dedicated to the development of novel anti-tubercular therapies. The center has developed methodology that allows the high-throughput screening of compounds for their ability to inhibit the growth of Mycobacterium tuberculosis, the causative microbe of tuberculosis (17). Thus far, 50,000 chemical compounds and 60,000 natural product extracts from investigators worldwide have been tested for activity. From these experiments, several dozen "hits" have been obtained that potentially could be further developed into clinically useful drugs.

Drug discovery and development at the University of Puerto Rico

The symposium provided a forum for a dozen University of Puerto Rico faculty researchers to present their work in the area of drug discovery and development. Even though many more researchers on the island are active in this area, the presentations varied from topics of the pharmacologic effects of plant extracts to methods for quantification of active plant constituents, the development and evaluation of new targets, the chemical or biochemical synthesis of new compounds that can interfere with disease-related enzymes or DNA, nanotechnological approaches via self-assembly as well as the development of methodology for drug delivery of siRNA's to the brain. A remaining challenge, one of the reasons in organizing this symposium, is to increase the potential to translate the ongoing research projects into clinical applications via multidisciplinary collaborations.

Commercialization and intellectual property of innovations

The industry, academic and government perspectives of the value of intellectual property based on inventions related to novel drugs and their potential for commercialization were discussed in a round-table discussion. Ms. Véronique Riethuisen, Director Worldwide Business Development of Pfizer, indicated that, on average, it takes approximately thirteen years for an idea to develop into a drug. This leaves only a limited amount of time for a compound to be marketed with patent protection before generic manufacturers can market the drug without the high investments in research and development that the originator company has committed. Importantly, in order for the pharmaceutical industry to become interested in potentially licensing patents based on inventions originated in academic institutions or in other types of industry-academic partnerships (18-19) the intellectual property rights must be well-defined. Dr. Emma Fernández, vice-President of Research of the University of Puerto Rico, described the potential role of the recently established "Fideicomiso de Ciencia, Tecnología e Investigación" (Science, Technology and Research Trust), which supports the promotion and financing of projects that strengthen research and development of science and technology in Puerto Rico, and promotes the commercialization of products resulting from local inventions. Dr. Roberto Rodríguez from the Puerto Rico Industrial Development Company (PRIDCO), a government-owned corporation responsible for attracting investors, highlighted examples of infrastructural investments made with this aim such as a new Molecular Sciences Complex with eight floors and 120,000 square feet of research space currently under construction in Río Piedras, as well as the Bioprocess Development & Training Center, recently inaugurated in Mayagüez.

Funding opportunities

As was mentioned above, the discovery and development of new drugs is a costly enterprise. In a second round-table discussion, several potential sources for financial support for drug discovery and development projects were presented. Besides investments by academic institutions, frequently via their own trust funds, the US National Institute of Health (NIH), with its recent focus on translating laboratory discoveries into clinical applications via the NIH roadmap initiative, could provide a potential resource for funding (20-21). As was further explained by Dr. Franzblau, more recently, non-profit "public-private partnerships" have been established such as the "Global Alliance for TB Drug Development" (22) and the "Medicines for Malaria Venture" (MMV) (23). These partnerships support research in their respective disease areas. Ms. Kate Carr, president and chief executive officer of Accelerate Brain Cancer Cure (Washington), presented her experiences as representative of various "venture philanthropy" organizations. These organizations are interested in sponsoring research in a very specific interest area as determined by the organization and could be a valuable option for obtaining financial resources for investigators. Finally, Dr. Maribelis Ruiz, Venture Capital Group Advent-Morro, pointed out the existing interest of venture capital groups in investing in promising research projects. However, the presence of intellectual property and the potential for commercialization are still the main considerations before venture capitalist funds finance possible projects.

Clinical studies and translational research

In a last round-table, Dr. José Carlo, Chancellor Medical Sciences Campus UPR, described the establishment of the Comprehensive Cancer Center at the campus, a shared effort between the government and MD Anderson Cancer Center. The facility is soon to be opened and will have 20,000 sq. ft. of research laboratories dedicated to basic and translational cancer research. A related new clinical building for cancer research and treatment is in the design phase. In addition, the Medical Sciences Campus houses the Clinical Research Center, in which various clinical trials, some in collaboration with major pharmaceutical companies, are currently ongoing. Dr. Carlo stated the strong commitment of the Medical Sciences Campus to advancing the progress of translational research, in harmony with the priorities of the NIH roadmap.

Conclusions

The "Drug Discovery, Development and Clinical Research in Academia" symposium highlighted the importance of integrative and interdisciplinary research collaborations between basic research scientists and clinical scientists in the process of drug discovery and development. The increasing availability of freely accessible resources such as the Protein Database, PubChem and others, increase the access of academic researchers to valuable information (24-25). Discoveries of novel compounds for the treatment of diseases in academia can lead to intellectual property, which could attract new investments, either via venture capital funds for small biotech (start-up) companies, or via collaborative agreements with big pharma. The development and investments in Research and Development facilities in Puerto Rico are an important step in complementing the current emphasis on pharmaceutical manufacturing on the island. An expanded academic research base that connects the biomolecular, pharmaceutical and clinical sciences will not only provide the foundation to attract new investments, but it will also provide newly trained graduates a multidisciplinary education, allowing a smooth transitioning into employment in the pharmaceutical industry. Most importantly however, is that the development of new drugs and pharmacological treatments will extend the lives and improve the quality of life of those suffering from one of the many diseases prevalent worldwide.

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HISTORICAL NOTES

Does it Work? Assessing Technology in Health Care

Health care in the United States is caught in a self-reinforcing cycle of escalating costs, unaffordable care, rising numbers of uninsured, and greater reliance on late and intensive care, which in turn propels the inflationary spiral (1). The result is that calls for universal health coverage on the part of policy-makers are accompanied by measures aimed at getting a handle on costs.

Aware that rising costs are being driven by expensive care of dubious efficacy, there is a growing consensus that the US has to do a better job of monitoring technology and innovative practices to determine which services actually work and which therapies are more effective than others in addressing a given condition. The expectation is that this process, known as comparative effectiveness research, would identify best practices and inform decisions on medical coverage and payment. As a result, any health plan would pay only for those treatments, drugs, or devices found to be more effective for a given patient vis-à-vis other options. The logic behind this is so clear that the obvious question is: Why has this not been done before?

The short answer is: It has. Between 1972 and 1995 the US had an Office of Technology Assessment (OTA) whose mandate was to "provide early indications of the probable beneficial and adverse impacts of the applications of technology" (2). Reporting to Congress, the agency was designed to give the legislative branch the tools it needed for the independent evaluation of national policy in a wide number of areas, including health, agriculture, transportation, energy, and the environment, among others. Once enacted, OTA's mandate broadened and became increasingly problem-oriented (3). Its agenda was driven by Congressional priorities; its reports clearly reflected the issues that legislators were addressing at any given time.

The agency began operating in 1974 and quickly established itself as a valuable adjunct to the decision-making process. Each assessment included the convening of an advisory panel, an in-house research team, workshops with experts and stakeholders, extensive peer review of drafts, and delivery of reports through congressional hearings, briefings, and public releases (3). Models of clear thinking and clean writing, OTA reports gathered, summarized, and translated technical issues in ways that were intelligible to the public. Because of the political environment in which it operated, OTA did not draw conclusions; rather, it presented the facts, distilled problems and alternatives, and discussed the pros and cons of different courses of action (4). OTA reports thus framed issues so that debates could proceed from a consistent knowledge base and set of facts. The agency therefore earned high marks from the press. The Washington Post characterized it as "a dispassionate, nonpartisan player in the legislative process." The Washington Times described it as "the voice of authority in a city inundated with statistics and technical gobbledygook" (4).

While health was only a fraction of its complete portfolio, the agency tackled a number of technological issues related to the efficacy of services. These ranged from artificial insemination to wheelchairs, and included cost-benefit analyses of cholesterol screening, risks and benefits of artificial hearts, and the effectiveness of AIDS prevention strategies, among many others. The result was a vast literature that addressed many aspects of health care and created both better-informed policy-makers and a more aware public.

OTA's usefulness, however, did not insure its survival. While it had some important allies in Congress and had gained an international reputation, OTA was ultimately trampled in a "political stampede on the Hill to downsize and streamline" (5). Its relatively small size, which made it efficient and nimble, facilitated its downfall. In addition, some of OTA's key supporters failed to show up or file proxies when the agency's survival was at stake. In the words of M. Granger Morgan, professor and head of the Department of Engineering and Public Policy at Carnegie Mellon, "Through a comedy of errors, oversight, and political machismo, Congress [chose] ignorance and ended the 23-year history of its best and smallest agency" (4). More simply, Chris Mooney described Congress as having performed "a stunning act of self-lobotomy" (5).

The end of OTA ushered in an era in which Congress often operated with unreliable data and great uncertainty when passing legislation related to science and technology. This in turn fostered the politicizing of scientific facts, and the promotion of what has been called "faith-based science" on issues ranging from individual contraception to global warming. Reversing this trend will require recapturing OTA's analytical capabilities, modus operandi, and significant legacy. But this is essential if health decisions are to be informed as to best practices, and if technology assessment and effectiveness

research are once again to be part of the craft of health care policy-making.

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Fatal Granulomatous Meningoencephalitis associated to Mycobacterium Mucogenicum-like Microorganism: a Case Report

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Mycobacterium mucogenicum is rarely associated to human infections. However, in the last year, a few reports of sepsis and fatal cases of central nervous systems have been documented. Here we report a fatal case of granulomatous meningoencephalitis of three weeks of evolution where DNA from a M. mucogenicum-like microorganism was identified post-mortem in samples of brain tissue.

Keywords: M. mucogenicum, CNS, immunocompetent, Meningoencephalitis

Since the early 1980's there has been an increase in diseases caused by nontuberculous mycobacteria (NTM); mycobacteria other than Mycobacterium tuberculosis and M. leprae. Mycobacterium mucogenicum is a common environmental mycobacterium found in water and soil with a worldwide distribution. Until its identification as a new taxon, it was known as Mycobacterium chelonae-like organisms (1). It was first identified as causing diseases in humans in 1982, during two outbreaks of peritonitis associated with peritoneal dialysis in the United States (2). Recent reports have identified this mycobacterium in immunocompetent patients. Multiple infections were described in a patient with the diagnosis of Münchausen syndrome most likely due to self-inoculations (3). The Central Nervous System is rarely involved in M. mucogenicum symptoms, however, two unrelated fatal cases, one of lymphocytic meningitis and other of a cerebral thrombophlebitis in immunocompetent patients have been described (4). Molecular techniques to identify NTM include nucleotide amplification followed by restriction fragment length polymorphism (RFLP) or phylogenetic reconstructions. The ß subunit of RNA polymerase (rpoB gene) (nucleotide sequence 2573 to 3337) showed the highest genetic heterogeneity within the clinical isolates of M. mucogenicum and M. abscessus (5-6).

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Case Report

Case of a 42 year-old male who presented himself at the emergency room on Day 0 with history of headache, fever, chills, dizziness, myalgias and prostration of three weeks duration. In the previous three weeks he sought ambulatory treatment and was treated with analgesics for the headaches. On the day of admission he complained of fever, headache, blurred vision and feeling ataxic. Physical examination was remarkable for neck tenderness. Babinski, Brudzinski and Kernig signs were negative. He also brought results of a head CT scan performed five days before which revealed changes suggestive of old lacunar infarcts and a hypodense cystic area on posterior aspect of right internal capsule. He was admitted with a diagnostic (vs. clinical) impression of meningoencephalitis. On day 1, neurologist noted mild sixth cranial nerve palsy. There was no photophobia. Spinal tap revealed clear yellow fluid with increased protein 457 (15-45 mg/dL), reduced glucose 27 (40-80 mg/dL), diminished chloride 109 (119-129 meq/l), increased RBC's 140.8 (0-2 cmm), and leukocytosis with monocytic predominance 84%. That same day a brain MRI showed tiny areas of hyperintensity in basal ganglia and right frontal location but no evidence of encephalitis. Patient was started on Acyclovir, Ampicillin and Azithromycin IV. On day 2, serology and microbiology results were reported as negative. On day 3 Herpes B serology (Cercopithecine herpesvirus 1) was ordered due to occupational exposure to non-human primates. That same day, spiking fevers were noted by attending physician. On day 4, the patient became disoriented with respect to time and place. On day 6, Babinski reflex was elicited. That same afternoon, patient's speech became unintelligible; he was unable to

be fully aroused by painful stimuli and sixth nerve palsy worsened as per physical examination. A head CT scan revealed sudden dilatation of the lateral and third ventricles and patient was transferred to the Intensive Care Unit due to increased aggressiveness, disorientation, and abrupt onset of diplopia, blurred vision and vertigo. Because of an anecdotic report of exposure to Mycobacterium tuberculosis more than 14 years ago, he was also started on Isoniazid, Rifampin, Ethambutol, and Pyrazinamide. On day 7, full neurological examination disclosed no spontaneous respiration, no response to painful stimuli, no decerebrate nor decorticate posturing; fixed, dilated pupils unresponsive to light, no response to ice water irrigation, no corneal or gag reflexes and no deep tendon reflexes. An electroencephalogram was subsequently performed yielding an isoelectric line, a finding compatible with brain death. Patient's condition continued to deteriorate and death ensued on the ninth day after his admission.

Results

Negative microbiology results included Herpes simplex PCR, Cryptococcus antigen, spinal fluid bacterial culture (Gram and Ziehl-Neelsen acid-fast staining), HIV 1 and 2 serology, blood culture, spinal fluid fungi culture, urine culture, anti-streptolysin O titer, VDRL-RPR and Cytomegalovirus IgG. Immunoglobulin quantification assays revealed low IgG and IgM values (595.00 mg/dL and 60.5 mg/dL, respectively). A leukocytosis with neutrophilic predominance of 80.3-88 % was present in all complete blood counts.

At the autopsy the most significant findings were located in the brain, heart, lungs and liver. Examination of the heart revealed cardiomegaly, left ventricular hypertrophy, subendocardial necrosis, early fibrosis and increased lipofuscin pigment , consistent with hypertensive vascular disease despite negative history. Lungs were heavy and edematous but there was no evidence of pulmonary tuberculosis or granulomatous disease. Liver showed mild macrovacuolar steatosis, cholestasis and scattered areas of hepatic necrosis. Examination of the brain demonstrated extensive, diffuse lymphoplasmatic leptomeningeal infiltrates accompanied by multinucleated giant cells, non caseating granulomas and necrosis (Figure 1). There were perivascular inflammatory infiltrates with occasional hemosiderin laden macrophages extending into the Virchow-Robin spaces. There were microglial nodules present on the sections of the hippocampus, pons and medulla. No viral cytopathic changes were seen. Autopsy results were confirmed by the Armed Forces Institute of Pathology staff.

In order to conduct molecular biology studies, several parts of the brain were processed for DNA extraction. Samples were obtained both from paraffin embedded and formalin fixed samples. DNA was subject to PCR amplification for herpes B virus, amoebas and mycobacteria. This patient worked with non-human primates during 15 years as veterinary supervisor technician. Because of that, special emphasis was placed in the diagnosis of herpes B virus. Both serological and PCR studies were negative when tested in our Virology Laboratory and at the National Reference B virus Laboratory, Atlanta, GA. PCR for Acanthamoebas, Balamuthia and Naegleria were negative when tested in our laboratory (7-9). Stained slides were examined at the Division of Parasitic Diseases, National Center for Infectious Diseases, CDC, Atlanta GA, for Acanthamoebas, Balamuthia, Naegleria and Sappinia and structures compatible with these organisms were not found. Immunofluorescence conducted on unstained slides using anti-amoebic sera (Acanthamoebas, Balamutia and Naegleria) was also negative. Patient serum was tested for antibodies to Acanthamoebas and Balamuthia and titers (1:32 and 1:16 respectively) were not indicative of infection with these amoebas.

After amplifying DNA extracted from 35 different parts of the encephalon, a robust PCR band at the expected size (360 bp) was obtained from two samples representing the temporal brain lobe. To characterize the mycobacterium a RFLP was done essentially according to Lee, et al (10). The restriction pattern produces five MspI bands (157, 95, 57, 45, 21 bp) and four Hae III bands (197, 107, 32, 63 bp) (results not shown). Because RFLP pattern from other M. mucogenicum strains were not available for comparison, nucleotide sequencing was performed to clarify the origin

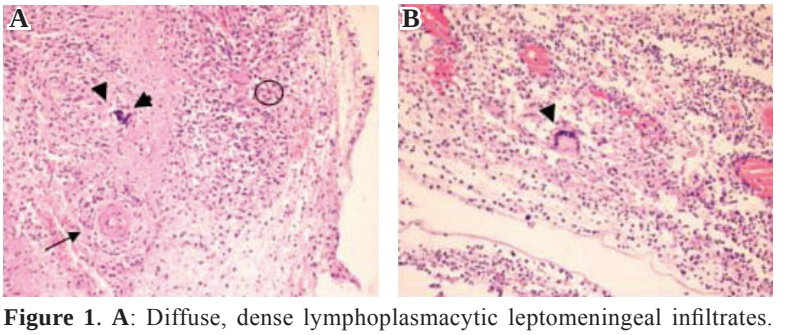

Figure 1. **A**: Diffuse, dense lymphoplasmacytic leptomeningeal infiltrates. Note meningeal congestion (right) and a non caseating granuloma (arrow) with associated multinucleated giant cell (upper left, arrow head). There are also occasional hemosiderin laden macrophages (inside circle) and perivascular inflammatory infiltrates, also seen in B. **B**: Multinucleated giant cell (arrow head) within a dense lymphoplasmacytic infiltrate (20 X).

of this PCR band. The amplified sequence showed the maximal homology of 86% percent with M. mucogenicum (data not shown).

Then we amplified a region of the rpoB gene that has been used for phylogenetic reconstruction and identification of interspecies strains. For the first and the nested PCR we used the pair of primers MycoF and MycoR and MycoseqF and MycoSeqR respectively (5). The PCR was sequenced using internal vector primers on an ABI Prism 3100 Genetic Analyzer (Perkin-Elmer Applied Biosystem). The resulting sequence was aligned with available sequences in the GeneBank using the program Clustal W (11). Phylogenetic reconstruction was performed using the PHYLIP (version 3.66) software package (12). The amplified sequence from this case clustered with M mucogenicum sequences as defined by Neighbor-joining (NJ) and Parsimony (PARS) methods with a 99.6% and a 73.7% of bootstrap values respectively (Figure 2).

Discussion and Conclusions

M. mucogenicum is considered a rapidly growing mycobacterium (RGM) and an environmental organism. However, like other species of this group it was initially isolated from a patient. In the present case, a potential contamination from tap water during the autopsy could be suggested as the original source of this agent. However, if that were the case, we could expect more than two positive tissue samples from those 35 tested. Additionally, the identification of this agent in tap or drinking water required a concentration process of 500 to 50 ml of water followed by subsequent bacterial cultures. (13-15). The count of nontuberculous mycobacteria in water samples have been determined to be 1 to 20 colonies in 500 ml (13). The running water from the autopsy room was only in contact with the encephalon surface while washing it. After that, water residues were displaced by the buffers used: embedding in paraffin or fixed by formalin. Since after this process, only few milligrams of formalin-fixed tissue or paraffin embedded tissues were used for the DNA extraction, it is unlikely we would get a positive PCR directly from the samples. In addition, the 1 ml water sample from the autopsy room used as control was negative.

The microorganism was not isolated because at the moment of the PCR identification, adequate samples for culture were not longer available. However, several facts support this microorganism as etiological agent. The clinical presentation of neurological symptoms without meningeal signs, as well as the fulminant evolution resemble the cases of the two previously reported patients (4). In addition, the cerebrospinal fluid profile of this patient is consistent with the presence of bacterial growth, while the negative standard bacterial culture supports the presence of an uncommon microorganism. The presence of a mycobacterium is reinforced by the exclusion of other potential agents like amoebas and the presence of a granulomatous reaction and a lymphoplasmacytic infiltrate in the brain sections.

There was no evidence of immunodeficiency in this patient. However, roommates reported daily alcohol intake for the last 14 years. Despite that, this person had no impaired social nor vocational functioning. It is known that the acute and chronic use of alcohol dampers the immune system. Impaired host defense after alcohol exposure appears to be linked to a combination of decreased inflammatory response, altered cytokine production, and abnormal reactive oxygen intermediate generation (16-17). The cellular immunity, which is essential for an effective immune response to intracellular pathogens like mycobacterium, is particularly affected (18).

The source of infection with this microorganism is quite difficult, if not impossible, to determine. Because of the generalized symptoms present and the febrile syndrome three weeks before the admission, it is unlikely that the infection was nosocomial.

In our opinion, after considering all circumstances including clinical presentation, the CSF profile of this patient, the anatomic-pathological findings and the phylogenetic reconstruction results, we conclude that a Mycobacterium mucogenicum-like organism is the most plausible causal agent involved in the clinical evolution (and subsequent demise) of this patient.

To our knowledge, this is the first report of a M. mucogenicum-related organism associated with a Granulomatous Meningoencephalitis.

Resumen

Desde los comienzos de los años 80 se ha reportado un incremento en las enfermedades causadas por micobacterias no-tuberculosas (MNT) y otras micobacterias distintas a M. leprae. Mycobacterium mucogenicum es una micobacteria ambiental que se encuentra fundamentalmente en los suelos y aguas alrededor del mundo. Hasta su identificación como un grupo taxonómico independiente, fue clasificado como microorganismo relacionado al Mycobacterium chelonae. Su primera asociación con una enfermedad en humanos fue en 1982, Estados Unidos, donde se identifico como agente causal en dos brotes de peritonitis durante diálisis peritoneal. Reportes mas reciente han encontrado este agente en pacientes inmunocompetentes. En un caso de infecciones múltiples en una paciente

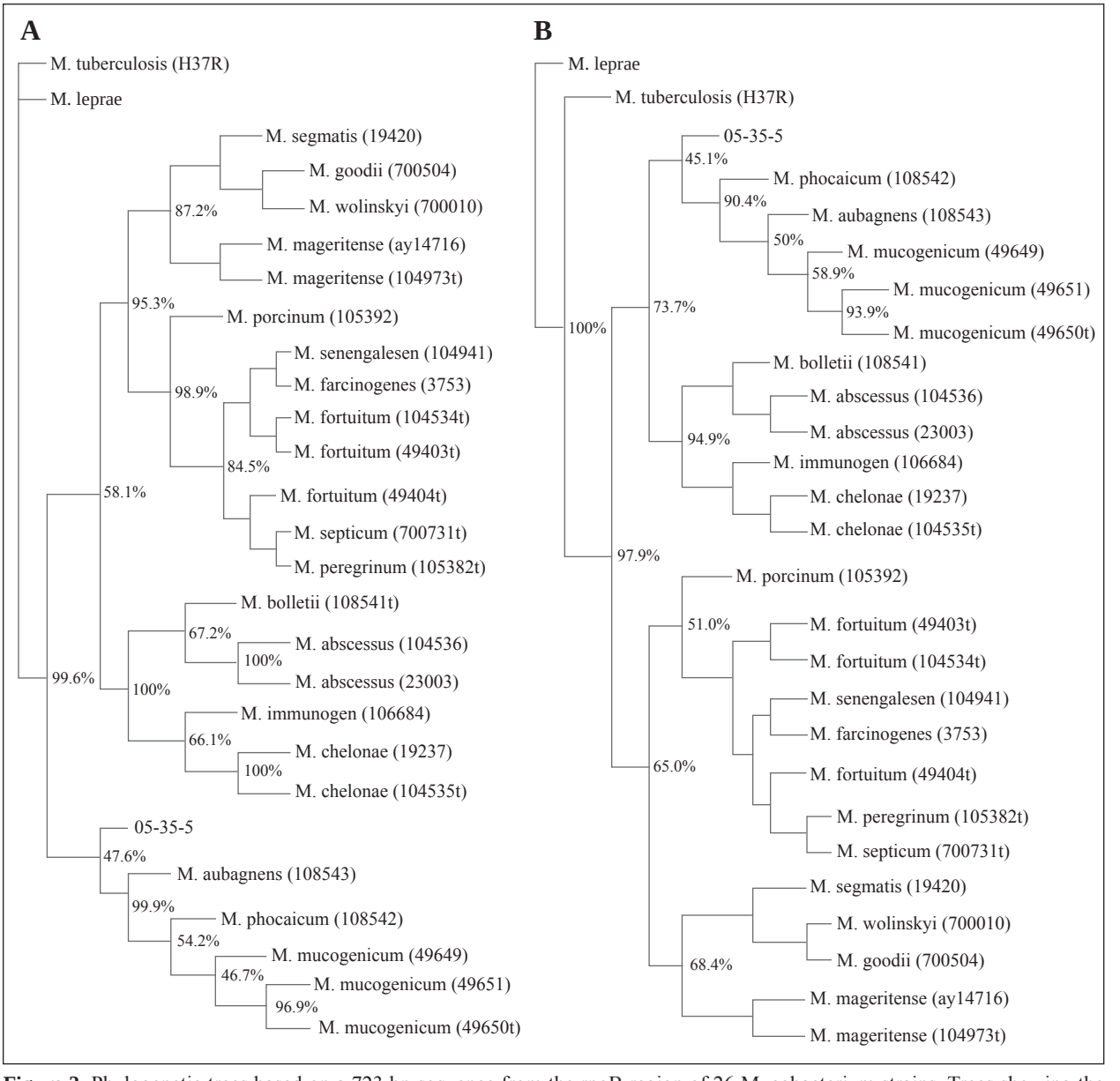

Figure 2. Phylogenetic trees based on a 723 bp sequence from the rpoB region of 26 Mycobacterium strains. Trees showing the segregation of the 05-35-5 sequence amplified in this work along other M mucogenicum sequences. Phylogeny was determined (A) by use of Kimura 2-parameters analysis for distance estimates or (B) by use of the DNAPARS program included in the PHYLIP software package. In A the tree was constructed by use of the Neighbor-joining method. A bootstrap analysis (1000 repeats) using M tuberculosis and M leprae as outgroups was performed to evaluate the topology of the phylogenetic trees. Bootstrap values (out of 1000) are given at each relevant node. Values above 70 % were considered significant. 05-35-5 is an arbitrary designation of the sequence amplified on this work. In parenthesis, EMBL's accession numbers of the other sequences used for this analysis are given. Gene Bank Accession number for MMQ-5 sequence: EU018141.

con síndrome de Münchausen, probablemente debido a lesiones auto inflingidas t aunque manifestaciones del sistema nerviosos central han sido asociada solo en raras ocasiones al Mycobacterium mucogenicum, al menos dos casos de fatales se han reportado también en pacientes inmunocompetentes. Uno de meningitis linfocítica y otro con una tromboflebitis cerebral. Entre las técnicas moleculares utilizadas para identificar a las MNT se encuentran la reacción en cadena de la polimerasa, el análisis de fragmentos de restricción y la reconstrucción

filogenética. El segmento geonómico mas utilizado, por presentar el mayor por ciento de heterogeneidad dentro de los aislados de M. mucogenicum y M. abscessus, es el correspondiente a la subunidad ß de la RNA polymerasa (gen rpo ß). En este trabajo, aplicando estas tecnicas, nosotros describimos un caso de meningoencefalitis granulomatosa con un desenlace fatal en tres semanas asociado a la detección de ADN de un microorganismo relacionado al M. mucogenicum en muestras de tejido cerebral tomadas post-mortem.

Acknowledgement

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CORRESPONDENCE

Fluorescence Response of CdS Nanoparticles to Serum of Cardiac Patients: towards the Development of a Real Time Sensor for Heart Failure Detection

Nanotechnology may represent a tool for the improvement in our quality of life by providing a platform for new sensors or sensing elements – components that can be integrated into functional sensors- to monitor chemicals closely related to health issues. A sensor is a device that receives and responds to a signal or stimulus indicative of a real-world condition. The aim of the work presented here is to evaluate the response of CdS semiconductor nanoparticles to the serum of patients that have been evaluated for cardiac troponin, a protein that has been the subject of previous works related to its value in the prognosis, risk stratification and management of patients with acute coronary syndromes and myocardial necrosis (1-5). These conditions are major health issues, costing consumers millions of dollars through high insurance premiums and health care. Development of a real time sensor that targets the detection of cardiac troponin in the blood is a major technological challenge that can reduce mortality and as well as health care costs.

In a typical semiconductor, electrons can be excited by light from the valence band into the conduction band. The minimum energy required to promote electrons from the valence band into the conduction band represents the band gap of the semiconductor, which is inversely proportional to the nanoparticle size (6). Photo-excited electrons in the conduction band are energetically relaxed to lower energy states or traps, which are states resulting from defects in the structure of the nanoparticle or from the adsorption of chemicals on their surface. Emission of light results from relaxation of electrons from trap states into the valence band. When the trap states arise from the adsorption of molecules on the particle surface, the resulting luminescence is very sensitive to the chemical environment of the semiconductor nanoparticle: a potential sensor element to the specific chemical that created the trap state.

The inset in Figure 1 shows the absorbance spectrum of the CdS nanoparticles in dimethylsulfoxide (DMSO) employed for the work described here. The absorbance decreases with wavelength until the band edge of the semiconductor nanoparticle reaches around 515 nm. Based on the absorbance cutoff it is possible to estimate a nanoparticle diameter of about 2.4 nm (6). Serum samples of patients obtained from the Perea Hospital's Clinical Laboratory were analyzed for cardiac troponin levels using the Abbott method. A typical emission spectrum of a solution prepared by adding 90 μL of the serum of a patient with no detectable cardiac troponin to a solution containing the CdS nanoparticles is labeled as Patient 1 on Figure 1. No new emission bands besides those associated with the emission of the CdS nanoparticle appear in the spectrum. On the other hand, the photoluminescence of a mixture containing CdS nanoparticles and 90 μL of serum of a patient with a troponin level of 1.8 mg/mL, labeled as Patient 2 on Figure 1, exhibits bands around 545 nm, a low wavelength shoulder between 500 and 540 nm, and a high wavelength peak around 580 nm. The light emission

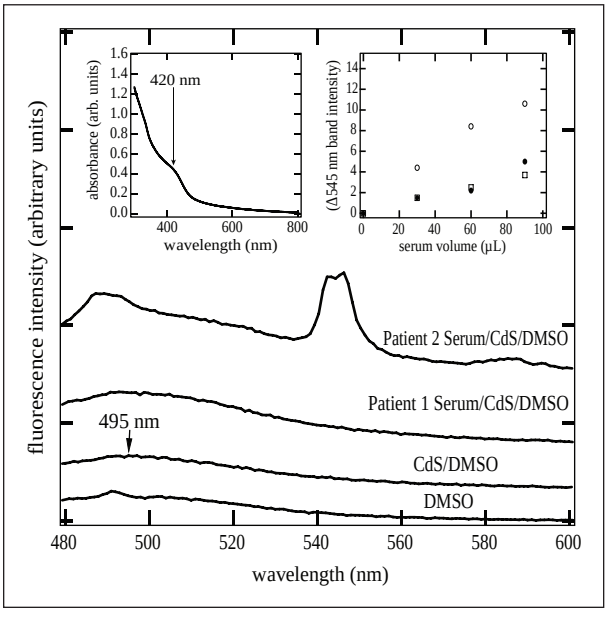

Figure 1. The inset at the upper left hand side shows a typical absorption spectrum of CdS nanoparticles. The fluorescence spectra of DMSO, the CdS nanoparticles in DMSO, the serum of patients with no detectable troponin (patient 1), and of a patient containing 1.8 mg/mL of troponin (patient 2). Excitation wavelength is 420 nm in all fluorescence measurements. The inset at the right hand side shows the change in fluorescence intensity at 545 nm as a function of the serum volume of patients with troponin levels of 15 (open circles), 12 (closed circles) and 2 (open squares) mg/mL.

intensity decreases linearly with dilution in this example as well as in four other serum samples containing troponin indicating that the emission is from the mixture and not an experimental artifact. Control experiments with troponin containing serum do not show these bands in the absence of the CdS particles. The number and chemical nature of other biomarkers that may be present in the serum samples limits the interpretation of the results in terms of specific troponin-CdS nanoparticle interaction (7). However, the results encourage pursuing further work focused on the use of CdS nanoparticles for the detection of cardiac troponin and other biomarkers related to coronary syndromes.

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E R R A T U M

The following correction should be noted for the article by Alberto de la Vega, MD, FACOG, RDMS and Ronald López-Cepero, BS, MS; entitled **Seasonal Variations in the Incidence of some Congenital Anomalies in Puerto Rico based on the Timing of Conception**, that appeared in the Puerto Rico Health Sciences Journal, Volume 28, Number 2, June 2009: Table 3 (Seasonal Variations in the Incidence of Open Neural Tube Defects in Puerto Rico from 1995 through 2005) should be as follows:

Table 3. Seasonal Variations in the Incidence of Open Neural Tube Defects in Puerto Rico from 1995 through 2005

Season	Conceptions resulting in live Births	Cases of Open Neural Tube Defects	Incidence/ 1000 live births	Relative Risk	95% confidence interval
Winter	116804	91	0.78	1(ref.)	
Spring	108363	86	0.79	1.02	0.76-1.37
Summer	102193	106	1.03	1.33	1.01-1.76
Autumn	113885	94	0.82	1.06	0.79-1.41
Total	441245	377	0.85		