1st International and 2nd National Workshop on Vaccine Adjuvants. 5-8 December 2001, Havana, Cuba

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ABSTRACT

This workshop was organized by the Cuban Society for Immunology (CSI) with the cooperation of the Latin American Association for Immunology (ALAI), the Finlay Institute, the Center for Genetic Engineering and Biotechnology (CIGB), the American Society for Microbiology (ASM), and Alfa Universal Invest., Corp.

Adjuvants help antigens to elicit an early, high and long-lasting immune response with less antigens. In recent years, adjuvants received much attention because of the development of purified recombinant and synthetic peptide vaccine candidates, many of which are poor immunogens and require adjuvants to evoke the desired immune response. Besides, for many years, the only adjuvants available for general use in human vaccines were aluminium salts; but new adjuvants like proteoliposomes are already extensively used.

With the use of adjuvants the immune response can be selectively modulated to a Th1 or Th2 response which is very important for protection against diseases caused by intracellular and extracellular pathogens, respectively. To discuss updated aspects concerning the development of adjuvants of importance in human and veterinary vaccines, the Cuban Society for Immunology (CSI) organized from December 5-8 of 2001 The 1st International and 2nd National Workshop on Vaccine Adjuvants with the cooperation of the Finlay Institute, the Center for Genetic Engineering and Biotechnology (CIGB), The Latin American Association for Immunology (ALAI) and the American Society for Microbiology (ASM) at the Neptune-Triton Hotel in Havana, Cuba.

Dr. Gustavo Sierra (President of the CSI and ALAI, Vicepresident of the Finlay Institute and President of the Expert Vaccines Committee) chaired the meeting that he organized together with Dr. Oliver Pérez (General Secretary of CSI and ALAI). The meeting was opened by stating that the discussion and proceedings should involve the screening and application of new adjuvants for many different kinds of vaccines. The program emphasized vaccination for prophylaxis or therapy in infectious diseases and in therapy of cancer. It also focused on adjuvants as carrier proteins, immunomodulators, appropriate delivery systems, whole organism vectors, liposomes and proteoliposomes. Future meetings may also consider procedures for safetly testing of new adjuvants and regulatory aspects of their application in humans as well as emphasize in vaccine adjuvants already in, or very close to, entering clinical trials. Another interesting point suggested for future meetings was the use of cytolysins as a new approach for immune deviation and the induction of CTL response in vaccine development.

Topics of special interest were the role of dendritic cells as natural adjuvants, the ability of adjuvants to selectively modulate the immune response to Th1 or Th2, the advances in adjuvants for cancer vaccines, the rational design of new vectors as antigen-delivery systems, adjuvants for mucosal vaccine delivery, adjuvants that can be used in vaccines against viral or parasitic vaccines and the adjuvant capacity of liposomes and proteoliposomes.

Researchers representing twenty-three different academic institutions and three pharmaceutical companies involved in adjuvant research and development attended to the meeting. The most import aim of this workshop was to discuss updated results in the field of adjuvants and to strengthen relationships between researchers and companies dedicated to research and production of new vaccines.

The present report is structured in topics which include oral presentations and posters after a summarized explanation of each topic by the chairpersons.

Keywords: adjuvants, immunology, vaccines

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RESUMEN

1er Taller Internacional y 2do Taller Nacional Sobre Adyuvantes Vacunales. Diciembre 5-8 del 2001, La Habana, Cuba. Este taller fue organizado por la Sociedad Cubana de Inmunología (SCI) con la cooperación de la Asociación Latino Americana de Inmunología (ALAI), el Instituto Finlay, el Centro de Ingeniería Genética y Biotecnología (CIGB), la Sociedad Americana de Microbiología (ASM) y la compañía Alfa Universal Invest.

Los adyuvantes ayudan a inducir una respuesta inmune temprana, elevada y de larga duración contra el antígeno, siendo necesario incluso menores concentraciones del mismo. En los últimos años los adyuvantes han recibido mucha atención debido al desarrollo de péptidos sintéticos y recombinantes purificados como candidatos vacunales, muchos de los cuales son pobres inmunógenos y requieren adyuvantes para evocar la respuesta inmune deseada. Por muchos años, el único adyuvante disponible para el uso general en vacunas humanas fue las sales de aluminio, sin embargo, nuevos adyuvantes como los proteoliposomas son ahora extensivamente usados.

Con el uso de los adyuvantes la respuesta inmune puede ser selectivamente modulada a Th1 o Th2 lo cual es muy importante para la protección contra enfermedades causadas por patógenos intracelulares y extracelulares

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respectivamente. Con el objetivo de discutir los más actuales aspectos concernientes al desarrollo de adyuvantes de importancia para vacunas humanas y veterinarias la SCI organizó del 5-8 de Diciembre de 2001 el 1er Taller Internacional y 2do Taller Nacional Sobre Adyuvantes Vacunales que tuvo lugar en el Hotel Neptuno-Tritón en Ciudad de La Habana, Cuba.

El Dr. Gustavo Sierra (Presidente de la SCI y de la ALAI, Vicepresidente del Instituto Finlay y Presidente del Comité de Expertos en Vacunas) presidió el taller junto al Dr. Oliver Pérez (Secretario General de la SCI y de la ALAI). La reunión fue inaugurada sobre la base de propiciar que las discusiones y procedimientos involucraran principalmente la selección y aplicación de nuevos adyuvantes para diferentes tipos de vacunas. Tópicos de especial interés fueron el papel de las células dendríticas como adyuvantes naturales, la habilidad de los adyuvantes para modular selectivamente una respuesta inmune Th1 o Th2, los avances en adyuvantes para vacunas contra cáncer, el diseño racional de nuevos vectores como sistemas liberadores de antígenos, adyuvantes para vacunas mucosales, adyuvantes que pueden ser usados en vacunas contra enfermedades virales o parasíticas y la capacidad adyuvante de liposomas y proteoliposomas.

Se propusieron como temas a discutir en futuras reuniones los procedimientos para evaluar de forma segura nuevos adyuvantes y los aspectos regulatorios para su aplicación en humanos, así como enfatizar en los adyuvantes vacunales que ya están en estudios clínicos. Otro punto interesante sugerido para reuniones futuras fue discutir el uso de citolisinas en el desarrollo de nuevas vacunas como una novedosa aproximación para desviar al sistema inmune hacia la inducción de respuesta CTL

Investigadores de veintitrés instituciones académicas y tres compañías farmacéuticas asistieron a la reunión, siendo el objetivo más importante de este taller discutir los resultados más actuales en el campo de los adyuvantes y estrechar las relaciones entre los investigadores y compañías dedicadas a la investigación y producción de vacunas. El presente reporte está estructurado por temas los cuales incluyen una explicación resumida de cada uno elaborada por los presidentes de cada sesión seguida de los resúmenes de las presentaciones orales y en carteles.

Palabras claves: adyuvantes, inmunología, vacunas

Workshop on Dendritic Cells as Natural Adjuvants

Chairpersons: José Alejandro López,[1] Circe Mesa[2]

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ABSTRACT

The fast growing field of research on dendritic cell (DC) biology is the focus of attention by vaccinologists and scientists developing optimal adjuvants for currently utilized/developed vaccines. This workshop was aimed to discuss basic aspects of the biology of DC for its application in the practice of current adjuvant technology in the development of more efficient vaccines. The overall take-home message transmitted during the meeting can be summarized in that DC are the main targets of a vaccination program and that it can be achieved in two main forms. First, the delivery of "danger signals" to DC generates their activation and, therefore, their more efficient performance in the initiation of the immune response. Products derived from bacteria, like proteoliposomes, from virus-like particles (VLP), or from DNA contain elements that prompt DC to be activated and therefore to present antigens to both naïve and cognate lymphocytes. The second element that is crucial in the optimal utilization of DC is the choice of DC aimed vehicles that may directly target one (or more) of the functions of DC. Specific receptors have been now identified and may play a major role in the re-direction of the immune response. Specifically, the performance of G protein-coupled receptors and their integrative role as neurotransmitter and cytokine is an exciting field of research that may be conducive to the best dissection of the Th1/Th2 dichotomy in the generation of an immune response to a vaccine.

*I*ntroduction

Five talks within the programmed workshop plus an additional talk on the subject delivered in the second day of the meeting constituted the core of the presentations dealing with DC. However, the issue of the function of DC was brought up in most of the papers presented at the meeting, alluding to their potential as the initiators of the immune response elicited by adjuvants/vaccines. Although there was no a poster session explicitly devoted to the topic, discussions that took place around the poster presentation in other sections often included DC as a subject. The speakers presenting in the workshop were:

Johannes Hoebeke from the C.N.R.S. "Immunologie et Chimie Thérapeutiques", I.B.M.C., 15, rue Descartes, F-67084 Strasbourg (France) with the title: "G protein coupled receptors: from dendritic cells to lymphocytes".

José Alejandro López from the Mater Medical Research Institute, South Brisbane, 4101, (Australia) with the titles: "A human blood dendritic cell purification platform for clinical applications" and "Dendritic cell therapy of melanoma" (Presented on behalf of Dr. Chris Schmidt from the Queensland Institute of Medical Research, Brisbane, Australia).

Claude Leclerc from the Unité de Biologie des Régulations Immunitaires, Institut Pasteur, Paris,

(France) with the title: "Cross-priming of CTL responses by exogenous virus-like particles".

Ann Hill from the Department of Microbiology and Immunology, L220 Oregon Health Sciences University, Portland, OR 97201 (USA) with the title: "Coupling apoptosis to antigen expression in a DNA vaccine: augmenting immunity or tolerance?".

Circe Mesa from the Vaccine Department, Centre of Molecular Immunology, PO Box 16040, Havana, Cuba, with the title: "VSSP: an ideal adjuvant for DC activation".

Main Body

The field of research on DC biology is currently in a fruitful period that resembles that of research on AIDS 10 years ago. Furthermore, since the discovery of a system to produce large quantities of DC-like cells derived from monocytes in 1994 [Romani *et al.*, 1994, *J. Exp. Medicine*, 180:83; Sallusto *et al.*, 1994, *J. Exp. Medicine*, 179:1109], the use of DC in immunotherapy both in animal models and, more recently, in human clinical trials has increased exponentially. In the Figure, the number of publications containing the words "DC" and "immunotherapy" in the last years has augmented substantially, in parallel to a significant decrease in the publications related to AIDS. This indirect indication of the success in this area of research reflects the importance of DC in various areas of the immune response.

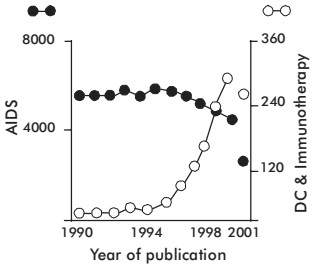
The evaluation of G-protein receptors, the type of Rhodopsin has been extended now to DC. Chemokine receptors belonging to the family of molecules with similar structure, have been identified in DC and their function in maturation and migration is being currently studied. Immature DC express α1b adrenergic receptors that may participate in the cross talk between the immune and the nervous systems. A number of neurotransmitters such as histamine, Substance P, or various chemokines, may be some of the small molecules interacting with these receptors on DC. The dimerization of the molecules upon activation triggers the NF-$\kappa\beta$ activation pathway leading to lymphocyte specific responses that may alter the Th1/Th2 balance of the response (Hoebeke, France).

A new approach for the generation of DC derived from blood (BDC) was presented and it utilizes magnetic beads and biotinylated monoclonal antibodies. Data presented during the workshop suggest that the physiology of DC derived by this mechanism differs from those obtained via cytokine meditated maturation of monocyte (or $CD34^+$) precursors. The induction of primary responses in the context of both class I and II MHC molecules appears to be more efficient by BDC; furthermore, the expression of costimulatory molecules [Hart *et al.*, 2000, 7th leukocyte differentiation antigen workshop DC section summary, *in press]* and antigen uptake [Ho *et al.*, 2002, Blood 99: *in press]* differ between the two DC types. Evidence shown here demonstrates that it is possible to generate sufficient number of BDC for immunotherapy using a combination of apheresis and a single step magnetic bead isolation (CliniMACS) in a closed, sterile, clinical grade protocol (López, Australia).

In studies in a mouse system, the presentation of antigen to CD8 lymphocytes (CTL) mediated by virus like particles (VLP) was shown to be mediated by DC; specifically, CD8+ in particular those from the CD11c subtype are the cells responsible for the powerful adjuvant capacity of VLP. Using the ovalbumin as an antigen model, the recognition of the famous K^b restricted SIINFEKL CTL epitope by CTL was achieved by injecting recombinant porcine parvovirus (PPV)-VLP-OVA; the construct, derived from the VP2 capsid protein of the virus formed 24 nm particles after their expression in insect cells that were used in the absence of any additional adjuvant. These findings highlight the importance of directing antigens to DC in order to optimize the adjuvant capacity of these cells (Leclerc, France).

DC can also induce immuno-regulatory responses that modulate the recognition of cognate antigens. A follow-up study of a DNA vaccination protocol including apoptosis-inducing genes and a model antigen showed induction of regulatory T-lymphocytes upon a second injection of the cognate antigen in the absence of specific CTL activity. In fact, the expected splenomegaly induced by the vaccinia infection was absent in animals previously immunized s.c. with a DNA construct containing the apoptosis-inducing gene in the presence of the specific cognate antigen. These results could be interpreted as the function of tissue DC that, lacking the expression of a second "danger" signal, induced regulatory responses to cognate antigens (Hill, USA).

The use of less toxic novel adjuvant formulations is currently a subject of intense research. Bacterial products such as CpG and LPS are known to activate DC and, therefore, explain an adjuvantory capacity of these compounds. The outer membrane protein complex of *Neisseria meningitidis* was coupled to form very small size proteoliposomes (VSSP) with gangliosides, a self-antigen the immune system is normally tolerant to. This new formulation proved to be safe and effective in humans. It induced both the maturation of mouse (bone marrow) and human (monocyte)-derived DC *in vitro* and the activated DC stimulated IFN-γ producing $CD4^+$ T-lymphocytes. The fact that LPS hyporesponsive C3H/HeJ also exhibits similar sensitivity suggests that the activation observed in this strain was LPS independent (Mesa, Cuba).



Number of publications on DC and immunotherapy in recent years.

Summary

We are begining to have a fuller glimpse of the vast field of DC biology and in the process we realize the immense potential of these cells to modulate the immune response. The main features of the vaccination protocols in the coming future will, necessarily, include DC as a target. Moreover, as we better understand their performance, we can also exploit their immunoregulatory potential in the treatment of autoimmune diseases. Clearly, the use of DC as natural adjuvants is not a practical alternative for many immunization protocols; however, our understanding of the ways in which adjuvants (and candidates) affect DC will be crucial in their evaluation and judicious use for vaccine development.

Oral Presentations

G protein-coupled receptors: from dendritic cells to lymphocytes

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Increasing evidence suggests the importance of neurotransmitters in the regulation of the immune response. Such neurotransmitters mostly act on G protein-coupled membrane receptors. Since chemokine receptors are also included in this family, I shall give an introduction over what we know about the structure of G protein-coupled receptors at the hand of the 3D structure of rhodopsin, about their activation mechanisms with emphasis on dimerisation, about their signal transduction mechanisms and their regulation by desensitization mechanisms. I shall further explain how the transduced signals can positively or negatively interfere with the activation of immune cells by the NF-kB pathway. I shall further summarize our knowledge about the secretion of different neurotransmitters by immune cells such as dendritic cells and lymphocytes and about the presence of functional G protein-coupled receptors in the same cells. I shall then present recent findings about the regulatory mechanisms on Th1/Th2 responses by catecholamines and histamine. These results will be discussed in the general context of immune status and in the particular context of vaccination protocols.

A human blood dendritic cell purification platform for clinical applications

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Objective: To establish a blood dendritic cells (DC) isolation strategy for cancer immunotherapy. DC generated *in vitro* by transforming monocytes with exogenous cytokines (Mo-DC), are functionally different from blood DC. We describe a platform for isolation of blood DC that are: 1) in a defined state of differentiation, 2) able to respond to physiological stimuli with the capacity to take up and process antigen, 3) more homogeneous and, 4) not exposed to exogenously added cytokines. Methods: After overnight culture of PBMC, biotinylated CMRF-44/56 were used to purify DC in a single step magnetic beads (Miltenyi Biotec, Germany) procedure. DC composition was evaluated by FACS in 10L and 15L COBE Spectra PBMC apheresis product obtained from 16 healthy individuals. Results: Using buffy coats as a source of PBMC, magnetic bead separations yielded up to 99% CMRF-44+ cells including up to 67% CMRF-44+ CD14- CD19- DC, i.e. a 100 fold enrichment from the 0.5% starting DC population. The yield of CMRF-44+ cells varied with individuals (n=53) with a mean of 52% (range 19%-99%). Blood DC (CD14- CD19- CMRF-44+) averaged 17% (range 3% - 67%). Similar results were obtained using CMRF-56 mAb (n = 10); an average of 46% CMRF-56 cells were obtained, of which 17% (range 4% – 31%) were blood DC. Overall, the procedure isolated an average of 2.2% (CMRF-44) and 1.3% (CMRF-56) of the starting PBMC, respectively. The purified CMRF-44+ cell fraction was more potent in eliciting an allogeneic MLR compared to whole cultured PBMC and no allo-stimulatory activity was observed in the CMRF-44- fraction. Moreover, MLR stimulatory capacity was identical to that found with FACS sorted CMRF-44+ CD14- CD19- cells. CMRF-56+ isolated cells efficiently generated primary immune responses restricted by both class II (KLH) and class I (HLA-A*2001) MHC molecules. We explored the yield of blood DC obtained from apheresis product in 16 healthy volunteers and established that the automated software control (6.0 Auto PBPC) yielded the better results. DC subset and activation status were not altered by the procedure, and DC numbers obtained were greater than 20 million in all cases, representing at least 20 DC immunization doses. CMRF-44+ cells were also successfully isolated from apheresis products and showed intact allo-stimulatory capacity before and after freezing. Conclusions: We describe a clinically applicable procedure for obtaining blood DC in sufficient quantity and quality for cancer immunotherapy.

Cross-priming of CTL responses by exogenous virus-like particles

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Virus-like particles (VLPs) have revealed an exceptional capacity to trigger CTL responses. However, the mechanisms of uptake, processing and presentation of these exogenous particles remain unclear. In particular, the antigen-presenting cells (APC) involved in the induction of CTL response by VLPs is still unknown. We have developed an antigen-delivery system based on non-replicative, recombinant parvovirus-virus-like particles (PPV-VLPs) formed by the self-assembly of the VP2 capsid protein of porcine parvovirus (PPV). The VP2 protein, the most abundant structural viral protein of the porcine parvovirus capsid, carrying foreign CD8+ T cell epitopes self-assembles into 25 nm pseudo viral particles after expression in insect cells. Mice immunized with these PPV-VLPs carrying a CD8+ T cell epitope from the LCMV nucleoprotein, in the absence of adjuvant, developed a CTL response against LCMV, which protected mice against a lethal intracerebral injection of LCMV, based on the induction of high-frequency of CTLs of high-avidity. In the present study, we determined the APC involved in the presentation of PPV-VLPs by MHC class I molecules, using PPV-VLPs carrying the Kb-restricted CD8+ OVA epitope SIINFEKL (PPV-VLPs-OVA). Spleen dendritic cells from mice injected with PPV-VLPs-OVA and transferred onto naïve mice elicited CTL response against SIINFEKL-coated target cells. Moreover, after i.v. injection of C57BL/6 mice with PPV-VLPs-OVA, *ex-vivo* antigen presentation assays evidenced that CD11c+ cells dendritic cells (DC) were very efficient APC and that CD8a- DCs rather than CD8a+ DCs were able to present the CTL-OVA epitope to a specific CD8+ hybridoma. Flow cytometric assays demonstrated that *in vivo* PPV-VLPs are mainly uptaken by DCs, with no differences between CD8a+ and CD8a- DCs. Therefore, PPV-VLPs are selectively presented by CD8a- DCs, and this differential antigen presenting capacity is not mediated by differ-

ences in the uptake capacity, or by on maturation level between both DCs sub-populations.

Apoptosis coupled to antigen expression in a DNA vaccine induces an antigen-dependent bystander anti-inflammatory effect

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Dendritic cells can engulf dying cells, take up antigen and present it on their own MHC class I molecules, a process known as cross-presentation. We sought to exploit this phenomenon to improve the efficacy of DNA vaccination. To this end, we coupled model antigens to an apoptosis inducing gene (E4ORF4) in a DNA vaccine in an attempt to augment cross priming of CTL. However, we found no influence on the numbers of CTL generated after intramuscular injection. When the vaccine was administered subcutaneously (sc), no CTL response could be detected. Surprisingly, when these sc-immunized animals were subsequently challenged with a vaccinia virus expressing the cognate antigen, the normal splenomegalic response to vaccinia failed to develop. Control animals (primed or challenged with irrelevant antigen, or with vaccine expressing antisense E4ORF4) developed large spleens. The difference was not due to better control of vaccinia virus, as all animals developed similar titers. We believe that we have induced antigen specific regulatory T cells that respond to antigen by secreting anti-inflammatory factors. We believe that this response was induced because dendritic cells perceive cells dying of apoptosis as "normal", and in the absence of other "danger" signals induce a protective, regulatory response which acts to suppress inflammation. These results highlight the importance of the context in which antigen is presented in the ability to prime an immune response. In addition, these results indicate that this methodology could be exploited to modulate undesirable inflammatory processes such as autoimmune disease.

VSSP: an ideal adjuvant for DC activation

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Adjuvants are an important component of many vaccine formulations. Recently a new generation of adjuvants which provide 'danger' signals that switch the immune system on has arisen. However, many of them have adverse side effects and are therefore not accepted in humans for routine vaccines. We have described a new approach in the cancer vaccines field, in which gangliosides are incorporated into the outer membrane protein complex of *Neisseria meningitidis* (Nm) to form Very Small Size Proteoliposomes (VSSP). This novel vaccine has shown the ability to overcome the tolerance normally associated with gangliosides and its use is safe in humans. This results drove our attention to the immunopotentiatory properties of VSSP. Given that DC are required for the initiation of adaptive immune responses we investigated the effects of VSSP on dendritic cell (DC) maturation. Because VSSP are essentially bacterial membranes we also designed experiments in which the effects of LPS in the VSSP could be dissected from molecules distinct from LPS. Immature DC, derived from human monocytes or mouse bone marrow were exposed to either LPS from Nm, or VSSP. Both VSSP and LPS induce similar DC maturation and stimulate the production of similar patterns of cytokines. DC matured by VSSP and LPS induce CD4+ T cell activation "in vitro" and "in vivo" and INFγ production. However, the adjuvanticity of VSSP can not be explained by the LPS component alone since VSSP induces IL12p40, IL1b, MIF and IL6 in C3H/HeJ mice which are hyporesponsive to LPS. This result also suggest that, perhaps another molecule in addition to Toll4 is required for some aspects of VSSP signaling.

Workshop on Cellular Adjuvants

Chairpersons: Agnes Le Bon[1], Oliver Pérez[2]

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ABSTRACT

The aim of the workshop "Cellular Adjuvants" was to highlight new findings concerning different aspects of the cellular immune response. In addition, results regarding the use of novel adjuvants with the capacity to stimulate dendritic cells, or to direct antigens to the cytosol and the use of components of an effective vaccine as an adjuvant were presented.

*I*ntroduction

Three talks and six posters covered the scope of this workshop. The oral presentations were presented by:

- Dr. Anselmo Otero from Havana University, Cuba, with the title: "Cytolysins: a new approach for immune deviation in vaccine development".
- Dr. Agnes Le Bon from The Edward Jenner Institute for Vaccine Research, England, with the title: "The adjuvant activity of type I interferons: a link between natural and adaptive immunity".
- Dr. Oliver Pérez from the Finlay Institute, Cuba, with the title "Th1 response induced by the B component of VA-MENGOC-BC™ overcomes the thymus independence of polysaccharide C and primes for memory in toddler".

Oral Sessions

A new antigen delivery system?

Dr. Otero addressed a very interesting question regarding how to direct the presentation of exogenous protein to the class I pathway in order to generate a good CD8 T cell response. The approach of his work was to use cytolysins. These proteins commonly expressed by bacteria are porines and allow pathogens to escape to the cytoplasm. Part of this family, Lysteriolysin from *Lysteria monocytogene* offer a good strategy to direct purified antigens or DNA in a soluble form or encapsulated into liposomes towards the class I pathway. However, the bacterial origin of this porine might raise some question about its safety for use in humans. Therefore, Dr. Otero's group looked for other sources of porines derived from non-bacterial organisms. They managed to isolate and characterize two new cytolysins derived from the sea anemone: *Stichodactyla helianthus.* The biochemical characterization of these new cytolysins revealed that their activity was pH dependent, which is compatible with their utilization to delivery antigens encapsulated in low pH sensitive liposomes. Therefore, these results are quite promising for the future utilization of these new proteins as a novel system for vaccine delivery.

A new adjuvant for humoral and cellular response?

I myself, Dr. Le Bon, presented a talk concerning the adjuvant activity of type I IFN. Based on the observation that natural infections generally induce strong immune responses and that innate cytokines such as type I interferons are normally induced to very high level shortly after infection, we have investigated the potential role of type I interferons as an adjuvant. We showed that the co-injection of type I IFN with an antigen (CGG) was greatly enhancing the antibody response specific for CGG leading to a far broader response in terms of isotype switching with the emergence of IgG2a and IgG3 isotypes. The potency of type I IFN as an adjuvant was comparable to CFA. Using mice deficient for type I IFN receptor we showed that the adjuvant activity of CFA was indeed mainly type I IFN dependent. We also showed that the presence of type I IFN during the primary response was leading to a long lasting production of antibodies as well as a good memory response. Finally, we demonstrated that the adjuvant activity of type I IFN was mediated by dendritic cells. Then, we investigated the effect of type I IFN on T cell responses. We showed that type I IFN was clearly enhancing the priming of CD4 T cells as well as their ability to produce IFN-γ. More interestingly, we showed that type I IFN had the ability to induce CD8 T cell responses to exogenous protein (OVA). Indeed, the addition of type I IFN to OVA during the priming was sufficient to induce OVA specific T cells to produce IFN-γ or to differentiate into CTL. Taken together, these data could suggest type I IFN as a very potent adjuvant.

Is VA-MENGOC-BC™ vaccine doing more than antibodies?

So far, the protective activity of vaccines against *Neisseria meningitidis* has always been related to the presence of bactericidal antibodies. Dr. Pérez presented strong evidence that the Cuban vaccine VA-MENGOC-BC™ was not simply generating antibody responses but also cellular responses. It clearly showed that young vaccinated babies were clearly able to mount a DTH response. Moreover, PBMC from young vaccinated adult were able to produce IL-2 and IFN-γ after restimulation *in vitro* but no IL-4 nor IL-5 indicating the development of a Th1 type T cell response. A second aspect of his talk concerned the response to polysaccharide C. This polysaccharide derived from serogroup C has been non covalently incorporated in the OMV based vaccine specific from *N. meningitidis B*. This polysaccharide is a T-independent antigen only able to induce the IgG_2 isotype when given on its own. When incorporated to OMV from *Neisseria* serogroup B, the response to polysaccharide C is modified towards IgG_4 and IgG_3. Moreover, the main problem with the use of polysaccharide as antigen is that the response that it generates is inadequate in terms of isotype switching and not long lasting. Dr. Pérez showed that the immunization with VA-MENGOC-BC™ was sufficient to induce a long lasting immune response to EMV and C components. Overall, Dr. Pérez presented new data highliting new understanding of the protective activity of VA-MENGOC-BC™.

Poster Session

The poster session covered several aspects of the cellular immune response and will be summarized briefly:

Possible role of neutrophils in the induction of immune response against *Neisseria meningitidis* B

M Lastre. Finlay Institute, Cuba.

The main results of this poster reveals that neutrophils might play a role in the defense against *N. meningitidis* B by producing inflammatory cytokines and chemokines (TNF-γ, IL-1b, IL-8, and MIP-1α/β). Interestingly, OMVs were also able to trigger neutrophils to produce these pro-inflammatory cytokines. The possible role of neutrophils in the immune response induction is also addressed.

Bactericidal activity against *N. meningitidis* B in relation to the Th1 response elicited by VA-MENGOC-BC™

T Rodríguez. Finlay Institute, Cuba.

This poster showed a clear correlation between the bactericidal activity of antibodies directed against *N. Meningitidis* B in vaccinated subjects and their isotype subclasses. The sera with the highest bactericidal activity were containing the highest titres of IgG1 and IgG3, which are good-complement fixing isotypes.

T-cell epitope mapping of P64K meningococcal protein in Balb/c mice

S González. Centro de Ingenieria Genética y Biotecnología, Cuba.

The aim of this work was to map dominant epitopes of p64K. Using overlapping peptides, it was shown that p64k contained a dominant T-cell epitope in a 15-amino acids sequence namely 470-485.

Enhanced IFN-γ secreting CTL activity against the HIV-1 multi-epitope TAB9 by a priming-recombinant Fowlpox virus boosting regimen after plamid-backbone manipulation (EG. Rodríguez, Centro de Ingeniería Genética y Biotecnología, Cuba.). This poster showed results concerning the improvement of the plasmid coding for HIV-1 multi-epitope TAB9 used for DNA vaccination. Indeed, insertion of multiple copies of immunostimulatory sequences into the backbone of the plamid was clearly enhancing the CD8 T cell response as measured by IFN-γ Elispot Assays.

A truncated HCV core protein formulated in different adjuvants is highly immunogenic involving a strong participation of cellular immunity components in mice

JC Álvarez-Obregón. Centro de Ingeniería Genética y Biotecnología, Cuba.

This poster presented a study of the immune response induced in mice against a recombinant HCV-core protein. When administrated in Alum or in CFA, this vaccine was leading to a high production of antibodies, an increased expression of IFN-γ gene as well as a good secondary proliferative response.

TNF-α and nitric oxide as targets in adjuvant screening

D Gil. Finlay Institute, Cuba.

This poster showed the possibility to use TNFα and nitric oxide quantification in the screening of adjuvants. The induction of TNF? and NO by different LPS was studied *in vitro.* The production of TNFa and nitric oxide by spleen cells after immunisation with different adjuvants was also examined.

As a summary the Cellular Adjuvants session was clearly covering a lot of different aspects of the cellular response from innate response (eg: neutrophils, macrophages) to the generation of good Th1 response naturally induced (using Type I IFN as an adjuvant or by VA-MENGOC-BC™), engineered (recombinant protein, DNA plasmid) or by using cytolysins to direct antigen to the cytosol and allow presentation on Class I molecules. As an overall, the results presented during this session were high quality and raised some interesting discussions.

Oral Presentations

Cytolysins: A new approach for immune deviation in vaccine development

AJ Otero. Lab of Immunology, Faculty of Biology, Havana University. aoterog@infomed.sld.cu

Current strategies for vaccine development mainly focus the immune response in terms of protective antibodies by antigen processing and presentation via phago-lysosome in the context of MHC class II molecules. The assessment of long-lasting cytotoxic T lymphocyte responses require the antigen to arrive into the cytosol for an adequate processing and presentation via MHC Class I. Bacterial porines like Lysteriolysin from Listeria monocytogenes, as a clear evasive mechanism, facilitate the escape of this pathogen to cytoplasm. These molecules have been used under novel vaccine approaches for re-directing purified antigens, attenuated bacteria, DNA sequences in a soluble form or encapsulated into liposomes. Other cytolysins from non-bacterial sources could be used for the same purposes.

Th1 response induced by the B component of VA-MENGOC-BC™ overcomes the thymus independence of polysaccharide C and primes for memory in toddler

O Pérez, M lastre, G Bracho, JM del Campo, T Rodríguez, M, Díaz, C Zayas, G Sierra. Finlay Institute, PO Box 16017, Havana, Cuba. Fax: (53-7) 2086075. oliverp@finlay.edu.cu

VA-MENGOC-BC™ is an outer membrane protein-based vaccine of serogroup B of *Neisseria meningitidis.* This also contain the serogroup C polysaccharide (PsC) which is non-covalently incorporated. We recently demonstrated the participation of other mechanisms possible involved in their protection (1). Here we show first, the characteristic Th1 response induce by this vaccine in young adults and toddler demonstrated at different levels: functional (presence of delayed-type hypersensitivity, opsonophagocytic and bactericidal activities and not immediate anaphylaxis nor passive cutaneous anaphylaxis); class and subclasses (IgG and IgG1 in human); molecular (induction of IL-2 and IFNγ RNAm and not IL-4 nor IL-5 RNAm); prost-transcriptional (production of IL-2 and IFNγ and not IL-4 nor IL-5 in supernatant of re-stimulated peripheral cells); and biological activity of γ-IFN produced. Second, a typical anti-PsC IgG secondary response was induced in toddler which demonstrated that this vaccine overcome the absence of response of the PsC, a thymus independent antigens. The main IgG subclass induced by plain PsC vaccines in human is IgG2 which is verify in our work. This response is changed by VA-MENGOC-BC™ to IgG4 and IgG3. Third, the priming induce by PsC not induced hyporesponsiveness for a natural challenge or a booster dose. Four, the long-lasting immune memory against the B and C components was demonstrated before and after a booster dose.

1. Infect and Immun. 2001;69(7):4502-8.

The adjuvant activity of type I interferons: a link between natural and adaptive immunity

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Type I interferons (IFN-I) are rapidly induced following infection and play a key role in non-specific inhibition of virus replication. Here we have investigated the effects of IFN-I on the generation of antigen-specific responses. We show that IFN-I potently enhance the primary antibody response to a soluble protein, stimulating the production of all subclasses of IgG, and induce long-lived antibody production and immunological memory. As well as enhancing antibody production, IFN-I also augmented the priming of IFN-γ-producing CD4+ T cells. In addition, endogenous production of IFN-I was shown to be essential for the adjuvant activity of CFA. Finally, IFN-I enhanced the antibody response and induced isotype switching when dendritic cells were the only cell type responding to

IFN-I. We are currently investigating the effect of IFN-I on CD8+ T cell responses and our preliminary data suggest that IFN-I strongly enhance the generation of a CTL response to co-injected protein. The data reveal the potent adjuvant activity of IFN-I and their important role in linking innate and adaptive immunity.

Le Bon A, Schiavoni G, D'Agostino G, Gresser I, Bellardelli P, David F. Tough. Immunity 2001;April: 461-70.

Can the protective effect of a *M. tuberculosis* proteins cocktail be predicted or improved by immunomodulation?

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We have cloned and purified four proteins of *Mycobacterium tuberculosis*, which are immunogenic and potential protective antigens, the 27 kDa, the L7/L12, the 85B and the ESAT-6 proteins. The cloned M. tuberculosis 27 kDa, L7/L12, 85B and the ESAT-6 genes were subcloned in pcDNA3.1, a DNA vector for DNA vaccination in addition to the cloning in the plasmids pQE for antigen preparation. We obtained with the pQE expression vectors large amount (1-6 mg/mL) of the four recombinant proteins. Groups of mice (female Balb/c 6-8 weeks, 18-20 g) were injected subcutaneously twice or three times, 3 weeks apart. Recombinant M. tuberculosis proteins, ribosomal L7/L12; ESAT-6 (6 kDa); 85B (30 kDa); and the lipoprotein 27 kDa, were used as such, adjuvanted by Ribi or entrapped in liposomes. IFN-γ was added at different doses whether free or entrapped in liposomes. Identical amount of empty liposomes was injected in the control group. The challenge was performed 4 weeks after the last immunization by injecting i.v. a dose of 10^6 CFU of the strain BCG Pasteur. The immune response resulting from the different formulations was evaluated by determining the ratios of IgG1/IgG2a and by profiles of lymphokines secreted by spleen lymphocytes during *in vitro* stimulation. Animals were sacrificed 5 weeks after the challenge. In general the immune response was a mixed Th1/Th2 response. Protection against BCG varied from nothing to a 2.5 log decrease in spleen CFU. The evaluation of the efficiency of protection according to the different ways used to present the mycobacterial antigens will be discussed.

Poster Presentations

A truncated HCV core protein formulated in different adjuvants is highly immunogenic involving a strong participation of cellular immunity components in mice

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HCV infection leads to viral persistence and chronic disease in at least 70% of cases, among which a significant proportion eventually develops cirrhosis and hepatocellular carcinoma. Current therapies are only minimally effective and no vaccines have been developed. The knowledge of anti-HCV-core responses could be of prophylactic or therapeutical concern for treatment of HCV infection. In the present study, the immunogenicity of a truncated recombinant derived HCV core protein (Co-120) in Balb/c and C57BL/6 mice was examined. Three doses of protein were adjuvanted onto either aluminum hydroxide or Freund´s adjuvant, and injected intramuscularly into Balb/c and C57BL/6 mice. The antibody response rose rapidly after the first dose, and at the end of the program both strains of mice exhibited comparable levels of anti-core IgG (titers above 1: 100 000), and a mixed IgG1/IgG2a subclass pattern of response was found. Spleen cells from Co.120 immunized mice gave a significant specific proliferative response with respect to control *($P < 0.01$)*. An appreciable induction of IFN-γ gene expression was also detected after an *ex vivo* specific stimulation of spleen cells from all immunized mice. The response showed to be independent of dose, H-2 genetic background or type of adjuvant. The results suggested that immunization with the Co.120 protein elicits a potent immune response with a strong participation of cellular immunity components, which could be taken into account to induce humoral and cellular anti-HCV responses.

Enhanced IFN-γ -secreting CTL activity against the HIV-1 multi-epitope TAB9 by a DNA priming-recombinant Fowlpox virus boosting regimen after plasmid-backbone manipulation

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Combined DNA priming-recombinant poxvirus boosting strategies have proven to be effective for generating antiviral immunity. In this work, we studied the effect of two different expression cassettes and the number of plasmid-backbone immunostimulatory sequences on the immune responses elicited by this vaccination regimen. The pAEC's transcriptional unit was replaced by another, containing the human cytomegalovirus promoter, a synthetic intron and a synthetic RBG-based termination/polyadenilation sequences. Further constructs were generated by the insertion of 5, 10, and 20 copies of the 5'-AACGTT-3' immunostimulatory motif. Balb/c mice were immunized by intramuscular injection of 200 mg of each plasmid, coding for the HIV-1 multi-epitope TAB9. After three doses of DNA, a fourth boost with plasmid DNA or a TAB9-expressing recombinant Fowlpox virus (FPTAB9LZ) was administered. The immune responses were evaluated by ELISA and ELISPOT assays. Serum IgG antibodies against TAB9 could not be detected. Only the animals receiving the new set of DNA constructs induced a detectable effector CTL response, which was further increased by the prime-boost regimen. The response was dependent on the number of immunostimulatory sequences. After *in vitro* re-stimulation with the IIIB peptide, significant CTL levels were evidenced for all the groups immunized with the new set of vectors.

T-cell epitope mapping of the p64k meningococcal protein in BALB/c mice

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Our group has previously characterized the P64k protein of *Neisseria meningitidis.* Cloning and expression in *Escherichia coli* of the *lpdA* gene, which encodes for this protein, yielded a soluble antigen accounting for more than 20% of the total host protein [1]. Due to its relatively high molecular weight, demonstrated immunogenicity and availability, P64k was employed as a carrier protein for poorly immunogenic peptides and *N. meningitidis* serogroup C polysaccharide with good results [2]. In this study, 59 overlapping synthetic peptides that encompassed the full-length 596 amino acids of the protein were tested for proliferation in P64k-sensitized mice. The highest proliferative responses were induced against peptides P1 (amino acids 1-20) and P48 (amino acids 470-490). However, only lymph node cells obtained from either P48- or P64k-sensitized mice, produced a statistically significant proliferative response when challenged with the homologous peptide or the recombinant protein, respectively. In addition, three overlapping peptides spanning the P48 sequence were tested for proliferation in homologous peptide- and P48-sensitized mice. The highest proliferative response was obtained against a peptide that includes amino acids 470-485. We can conclude that this 15-amino-acid peptide (IPGVAYTSPEVAWVG) of P64k contains an immunodominant T-cell epitope for BALB/c mice.

1. Guillén G *et al.* Biotechnol Appl Biochem 1998; 27:189-96.
2. González S *et al.* Scand J Immunol 2000;52:113-6.
3. González S *et al.* Biotechnol Appl Biochem 2000; 32:1-8.

TNFα and nitric oxide as targets in adjuvant screening

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The tumour necrosis factor α and the nitric oxide (NO) are molecules related with the proinflammatory responses and probably with Th1 responses. For that reason we introduce the L-929 bioassay (TNFα) and Griess (NO) test for adjuvant screening. Different lipopolysaccharides (LPS, *N. meningitidis* B, *V. cholerae*, and *S. Typhimurium)* were included. *Vibrio* LPS produced less TNFα than the other two and the production of NO was similar with all LPS. The EMV induce the early production of TNFa and NO in peritoneal macrophages from immunised (with anti-*Neisseria* vaccine) and non immunised mice. TNFα was induced with lower doses of EMV than NO. TNFa levels from cells of immunised animals were higher than from cells of not immunised animals, but the production of NO was similar. In addition, these assays were used in combination with antigenicity for the evaluation of adjuvant stability.

Possible role of neutrophils in the induction of immune response against *Neisseria meningitidis* B

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The role of neutrophils (PMN) as effector cells against bacterial infection and the production of cytokines in response to polysaccharide (LPS) is well documented. However, no conclusive data of the importance of PMN as effector cell in *Neisseria* infection and no data about the possible role of PMN in the afferent arm of the immune response were reported. Therefore, we used a neutropenic rat model and infected theme with *N. meningitidis* B. The treated animals showed a 100% of mortality in less than 12 h. The bacteriolytical activity of co-cultures of human PMN and *N. meningitidis* B was demonstrated, which was increased with LPS and γIFN. In the afferent arm, the presence of high concentration of PMN around the site of immunization with anti-*N. meningitidis* B vaccine, VA-MENGOC-BC™, in hystopathologic studies was observed. In addition, the outer membrane vesicles (OMV) from *N. meningitidis* B stimulated PMN and produced pro-inflammatory cytokines and chemokines including TNFa, IL-1b, IL-8, MIP-1a/b which are increased by γIFN. IP-10 was also produced by OMV+γIFN. IL-10, a regulatory cytokine, potently inhibited TNFα, IL-1b, IL-8, and MIP-1a/b production triggered by OMV. Finally, a neutralizing anti-TNFα mAb did not influence the release of IL-8 and MIP-1b induced by OMV, therefore excluding a role for endogenous TNFa in mediating the induction of chemokine release by OMV. In contrast, the ability of LPS from *N. meningitidis* B to induce the production of IL-8 and MIP-1b was significantly inhibited by anti-TNFα mAb. In conclusion, our results establish the role of PMN as essential effector cells and that in response to OMV, PMN produce a pro-inflammatory profile of cytokines and chemokines which may not only play a role in the pathogenesis of meningitis, but may also contribute to the development of protective immunity to serogroup B meningococci.

Bactericidal activity against *N. meningitidis* B in relation to the Th1 response elicited by VA-MENGOC-BC™

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The Cuban anti-*Neisseria meningitidis* B and C vaccine, VA-MENGOC-BC™ induces opsonophagocitic antibodies, lymph proliferation, positive DTH reactions and production of INFg in humans suggesting a preferential cellular (Th1) stimulation. However, the correlate of protection for serogroup B meningococci is not currently known but for serogroup C is belived

to be the serum bactericidal assay (SBA).That is why, we evaluated the bactericidal activity against serogroup B in relation with the IgG subclasses elicited by this vaccine in healthy adults. A new colorimetric serum bactericidal assay (cSBA) was standardized and used for the determination of the bactericidal activity (1) and the IgG subclasses were measured by ELISA. 73% of evaluated sera showed high bactericidal response, corresponding to the cases with higher titters of complement fixing IgG1 subclass that was the main subclass induced fallowed by IgG3. In summary, the high levels of IgG1 and IgG3, both complement-fixing subclasses and it's functionally efficiency in the activation of the bactericidal response were in agreement with our theory that the Th1 pattern induced by VA-MENGOC-BC™ and the group of mechanisms elicited in this environment are the responsible of the efficiency showed by this vaccine [2].

1. Rodríguez T, Lastre M, Cedré B, del Campo J, Bracho G, Zayas C, Taboada C, Díaz M, Sierra G, Pérez O. Standardization of *Neisseria meningitidis* serogroup B colorimetric serum bactericidal assay. Clinical and Diagnostic Laboratory Immunology 2002. In press.
2. Pérez O, Lastre M, Lapinet J, Bracho G, Padrón J, Díaz M, Zayas C, Taboada C, Sierra G. Immune response induction and new effector mechanisms possibly involved in protection of Cuban anti-meningococcal BC vaccine. Infectious and Immunity. 2001;4502-8.

Workshop on Carriers and Immunomodulators

Chairpersons: César Pérez-Maldonado,[1] Gustavo Bracho[2]

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ABSTRACT

An important step in the development of effective vaccines is to assure the activation of cellular populations by means of specific and potent antigens. Recent advances on vaccination approaches show that the nature of adjuvants is of great importance for the immune system's ability to fight against a broad array of infectious and pathological agents. The protective effect of certain antigenic proteins is only obtained when an appropriate presentation/recognition is carried out by antigen presenting cells. Adjuvants must accomplish specific features, specially those related to a lack of toxicity as well as the non-induction of tolerance. The composition and molecular structure of the carrier have been established as "of extreme importance" for these achievements.

*I*ntroduction

Most of the oral and poster presentations in this session (3 and 11, respectively) were focused on the growing importance recently obtained by research on vaccination strategies. In our session, the industrial sector was most highly represented (9 posters and 1 oral presentation) followed by the university (3 and 1, respectively). A significant number of topics discussed were in accordance to present day worldwide research publications which stress the role of new vaccine adjuvants in the pursuit of reliable and more effective ways to improve immunological functions. The following paragraphs highlight the most important features contained in the presentations.

Impact of cytokines (IL-2, IL-4, IL-7 and γ-IFN) on the replication of primary HIV isolates from infants in the thymus

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HIV replication was inhibited by IFN-γ , IL-2, and IL-4, together, enhanced primary isolates production in thymocytes. A significantly increased replication of the X4 isolates as compared to R5 isolates was observed when stimulated with IL-4 and IL-7. These cytokines effects were related to their induction of T cell differentiation by increased chemokine receptor expression in specific subsets. IFN-γ inhibited HIV replication in thymocytes through different mechanisms.

Characterization of P64k as a carrier protein

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P64k increased the immune response against six out of seven peptides and against the serogroup C capsular polysaccharide of *Neisseria meningitidis* chemically coupled to this antigen. It was also found that P64k induced a specific humoral immune response in 15 out of 18 individuals inoculated with no induction of anti-mitochondrial antibodies.

Chemokines and their receptors in HIV-1 infected children

C Pérez-Maldonado. Cátedra de Inmunología. Escuela de Bioanálisis. Facultad de Farmacia. Universidad de Los Andes, Mérida. Venezuela.5101-A. Laboratorio de Inmunología. Hospital General. Hospitals Vall d'hebron. Barcelona, España.

RANTES is used by HIV-1 and increased plasma levels of it have been associated with HIV-1 infection resistance and immune recovery. An important RANTES augment in the infected children groups was accompanied by an improvement of the CD4+ / CD8+ index during the post HAART period which indicates that an assesment o it levels could a reliable indicator of immune recovery in the HIV-1 infection.

Cytokines production by *M. tuberculosis* H37Rv strain

AY Arce Mendoza. Facultad de Medicina. U.A.N.L. NL, México.

The way by which cytokine participates in the pathogenesis and disease, is not well understood. This study offers new information for the therapeutic management.

The biological activity of IFNα 2b in liquid and freeze-dried formulations

Ll Ruíz. Center for Genetic Engineering and Biotechnology. Havana. Cuba.

The results obtained proved the physical, chemical and biological equivalence between all of the examined formulations

IL-2 biological activity in liquid lyophilized formulations

N Reyes. Center for Genetic Engineering and Biotechnology. Havana. Cuba.

As judged by SDS/PAGE, RP-HPLC and the bioactivity assay, formulations resulting from this work are equivalent to others previously described by researchers.

Immune response induced in mice by two meningoccocal polysacharide-protein conjugates

M Cuello. Finlay Institute. Havana, Cuba.

In conclusion, at the level of IgG subclass antibodies, the conjugate PsC-OMP was able to generate a Th1 immune response, whereas the conjugate PsC-TTT generated a Th2 immune response.

Comparative immunogenecity of conjugates composed of *Neisseria meningitidis* serogroup C polysaccharide to tetanus toxoid by different spacer arms

O Cabrera. Finlay Institute. Havana. Cuba.

Higher levels of anti-TT antibodies were elicited by conjugates than TT itself.

P64k as a carrier protein of *Neisseria meningitidis* PorA peptides

G Sardiñas. Center for Genetic Engineering and Biotechnology. Havana. Cuba.

In two out of three conjugates, the anti-peptide sera reacted with native meningoccocal outer membrane vesicles in ELISA, suggesting that chemical conjugation to the carrier granted, in addition to T-cell help, a proper folding to these synthetic peptides.

Comparison of the immune response in mice against meningoccocal group C polysaccharides conjugated to three carrier proteins

A Álvarez. Center for Genetic Engineering and Biotechnology. Havana. Cuba.

The carrier protein present in the C-Ps conjugates influenced the levels of the IgG antibodies elicited to the Ps. In this regard, P64k was superior to TT and BSA.

Immunization with a peptide sequence derived from *Neisseria meningitidis* using different vaccine formulations

T Menéndez. Center for Genetic Engineering and Biotechnology. Havana. Cuba.

The best results were achieved in the group immunized with the three doses assayed of the MAP conjugated to P64k using the carbodiimide method.

Peptide synthesis containing a B-cell and a T-cell epitopes on dextran beads and evaluation of the humoral response against bead-peptide constuct

LJ Cruz. Center for Genetic Engineering and Biotechnology. Havana. Cuba.

This experiment suggest that peptide synthesis on dextran beads can be used to raise their immunogenicuty in order to use them on vaccines or therapeutics approaches.

Effect of conjugation methodology of the immunogenicity and protective efficacy of meningoccocal group C polysaccharide P64k protein conjugates

T Carmenate. Center for Genetic Engenieering and Biotechnology. Havana. Cuba.

This experiment proves that is possible to generate a protective T-cell dependent response agains meningoccocal C polysaccharide using P64k protein as carrier.

Summary

Oral and poster sessions pointed towards the P64k ability as a suitable carrier. Dr. S González (CIGB. Cuba) exposed its ability to increase the immune response whether attached to peptides or to bacterial antigens, and Dr. G Sardiñas, (CIGB, Cuba) showed its capacity to elicit a potent Th1 and Th2 response and high reactivity against native meningoccocal antigens. Dr. C Pérez-Maldonado (ULA, Venezuela) presented chemokines as immunomodulators that have a "protective effect" on certain pathological entities. Although the value of cytokine production during different stages of *M. tuberculosis* infection needs further research work, Dr. AY, Arce Mendoza (UANL, Mexico) also showed their potential impact for future therapeutical approaches. Dr. Ll, Ruiz and Dr. N, Reyes, (CIGB, Cuba) proved that new storage strategies provided important findings about the long-standing biological activity of frozen cytokine formulations. The possible peptide synthesis on dextran beads exposed by Dr. LJ Cruz (CIGB, Cuba) offers novel and very suitable method to improve a more potent and specific array of synthetic peptides.

Oral Presentations

Impact of cytokines (IL-2, IL-4, IL-7 and γ-IFN) on the replication of primary HIV isolates from infants in the thymus

L Pedroza-Martins,[1] WJ Boscardin,[1] D Schols,[2] YJ Bryson,[1] C Uittenbogaart[1]. [1]University of California Los Angeles, 10833 LeConte Avenue, Los Angeles CA, 90095, USA. [2]Rega Institute for Medical Research, Katolieke Universiteit, Leuven, Belgium. liviapm@ucla.edu

HIV infection of the thymus disrupts T cell development and may impact the outcome of disease in children. Cytokines such as IL-2, IL-4 and IL-7 play

a role in thymopoiesis and could be manipulated to increase T cell reconstitution or as adjuvants to augment vaccine efficacy. Some of these cytokines and IFN-γ are indeed induced, intentionally or not, by vaccination and in reaction to boosters and/or challenge with the infectious agents. Notwithstanding their beneficial effects in immunity, we and others have found that IL-2, IL-4 and IL-7, alone or in combinations, can enhance the replication of HIV in thymocytes, cord and peripheral blood lymphocytes. In contrast we found that IFN-γ inhibits HIV replication and CD4 depletion in thymocytes, while others found no negative effects of IFN-γ on HIV replication in peripheral blood mononuclear cells. Consequently, it is important to determine whether immunotherapy and vaccine strategies for HIV-infected children that involve cytokines could impact HIV replication in the thymus. We addressed this issue by analyzing the impact of cytokines on the replication of HIV in the post-natal thymus using well-defined *in vitro* systems (thymus organ cultures and suspension cultures). Ten HIV primary isolates obtained at /or close to birth were characterized according to their replication kinetics, response to cytokines and patterns of viral expression in thymocyte subsets at different stages of maturation. The use of CCR5 and CXCR4 as coreceptors was determined directly in the thymus by blocking studies with monoclonal antibodies and chemokine receptor antagonists.Viral tropism, thymocyte differentiation, and chemokine receptor expression were analyzed by flow cytometry. ELISPOT was used to assess cytokine production. Viral production was measured by ELISA (p24). We found that all 10 HIV primary isolates were able to replicate in thymocytes. Loss of CD4 expression was observed after infection with primary isolates using CXCR4 (X4) as well as CCR5 (R5) as coreceptors. Replication of most HIV isolates from neonates was inhibited by IFN-γ , but large differences in sensitivity were observed. IL-2 and IL-4, together, enhanced the production of all primary isolates in thymocytes. In contrast IL-4 and IL-7, significantly increased the replication of the X4 isolates as compared to R5 isolates. These effects of IL-2, IL-4 and IL-7 were related to their ability to induce T cell differentiation and increase chemokine receptor expression in specific thymocyte subsets, therefore expanding the range of thymocytes able to fully support HIV replication in the thymus. In contrast, IFN-γ inhibited HIV replication in thymocytes through mechanisms that did not involve T cell maturation or major changes in chemokine-receptor expression.

Characterization of P64k as a carrier protein

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The P64k meningococcal protein, an antigen of 64 kDa expressed in Escherichia coli, has been extensively characterized [1]. Its lipoyl-binding site was genetically modified to allow the use of P64k in prophylactic vaccines [2]. Here, we show some of the P64k properties that support its use as a carrier protein for conjugate vaccines. First, we investigated the immunogenicity in mice of several hapten-P64k conjugates. P64k increased the immune response against six out of seven peptides and against the serogroup C capsular polysaccharide of Neisseria meningitidis chemically coupled to this antigen. Moreover, it was demonstrated that P64k-induced epitope-specific suppression might occur, depending on the hapten and the pre-existing levels of immunity. Studying the cellular immune response, it was shown that lymph node cells from sensitized mice proliferated in a dose-dependent manner. Besides, a peptide containing a T cell epitope was localized, by using 59 overlapping synthetic peptides. In a Phase I clinical study, conducted in 26 healthy volunteers, the safety of two formulations of P64k, adsorbed onto aluminum hydroxide was demonstrated. Furthermore, it was found that P64k induced a specific humoral immune response in 15 out of 18 individuals inoculated with it and did not elicit anti-mitochondrial antibodies.

1. Guillén G, Álvarez A, Silva R, *et al.* Biotechnol Appl Biochem 1998;27:189-96.
2. Guillén G. Expresión en *Escherichia coli* y caracterización del antígeno P64k de *Neisseria meningitidis.* Ph.D. Thesis

Role of the macrophages and cytokines inhibiting or stimulating their activation in experimental infection by the helminth *Echinococcus granulosus*

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The infection by *E. granulosus* is a worlwide zoonosis that affects not only humans but also domestic animals and has a high degree of incidence in Uruguay [1]. The parasites biological cycle includes the transmission between dogs (definitive host) and cattle or sheep (intermediate hosts). Although humans are not natural intermediate hosts, they can infect by oncospheres intake due to the close contact with the definitive host, which occurs more frequently in rural areas [2]. The protoscoleces can differentiate to cysts in humans (secondary infection) which happens when the containing cyst breaks due to traumatisms or accidentally during surgeries. The experimental secondary infection model used for the host-parasite relationship study is the Balb/c mice infection by intraperitoneal inoculation of protoscoleces [3]. In *E. granulosus* infected mice the parasite stimulate a strong local cellular response which is not able to eliminate the parasite. However, the induction of protection is possible using proteic extracts of protoscoleces [4] or irradiated protoscoleces [5]. What is the role in the host-parasite interaction of the cytokines? It is a question that has been answered in some infection by helminths. The published results have not always agreed in such a way that there are different hypotheses on the role that these cytokines would be playing on the host

resistance or susceptibility to a determined infection by helminths. The Balb/c animals susceptible to the *E. granulosus* infection developed an early Th2 type response which is kept until the third week of infection. This response was characterised through splenocytes stimulation of infected mice with protoscoleces antigens in vitro. The results showed that the splonocytes produced high levels of IL-4, IL-5 and IL-10 and did not produced IFN-γ [6]. On the other hand, we have observed that: a) the protoscoleces are susceptible to normal macrophages *in vitro* activated with INFγ and LPS and b) molecules such as the hydrogen peroxide and nitric oxide donors such as SNAP are toxic for the *in vitro* protoscoleces. This suggests that the protoscoleces might be eliminated in vivo through a cellular defence mechanism that involves the macrophages as effect cells, with the condition, that these showed be activated during the first stages of the infection. The macrophages activation gets better in the context of a Th1 type response. Therefore, the next hypothesis is suggested: in the experimental hydatidosis a Th1 response might be associated to minor levels of infection contrary to a Th2 type response. During the first stages of the experimental infection by E. granulosus a cytokine type 2 profile is induced whose relationship with resistance or susceptibility has not been established6. The main aim of our study is to obtain results that help to clarify this point. The possible strategies to reach this aim basically consist of: 1) to *in vivo* selectively block each one of the type 2 cytokines and to evaluate the result of this treatment on the infection and on the host immune response; 2) to evaluate the susceptibility to the infection by *E. granulosus* and the cytokines response on knock out mice on type 2 cytokines; 3) to induce a response of type 1 cytokines that negatively regulates the production of type 2 cytokines and to evaluate on the immunomodulation mice the results of the infection.

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Chemokines and their receptors in HIV-1 infected children

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Chemokines are proinflammatory cytokines that attract and activate specific leukocyte subsets which receptors are used as coreceptors by some AIDS-related viral strains. CCR5 the especific ligand for β-chemokine RANTES is used by HIV-1. Increased levels of plasma RANTES have been associated with HIV-1 infection resistance and immune recovery. In a group of 28 non-infected children (age-control) and 35 vertically infected children under HAART (mean: 9 years), we evaluated CCR5 expression and RANTES synthesis. Also CD4+, CD8+, CD8/RA-, CD8/38+, CD8/28-, CD8/45HLA lymphocytes subsets, CCR5 expression and intracellular production of IL-2 and IFN-γ were determined by flow cytometry (FCM). Plasma and cell culture supernatant´s levels of RANTES were assesed by ELISA. Mean values in Group I (1 to 6 years) and Group II (> of 6 years) of infected children were: CD4+: 901 and 538 cel./mL. CD8+: 1443 and 1043 cel./mL., respectively. Basal expression of CCR5 was 18% and RANTES plasma level was 1025 pg/mL. Control groups mean values were: CD4+: 1172 and 849 cel./mL. CD8+: 839 and 703 cel./mL., respectively and their CCR5 basal expression was 38% with plasma level of RANTES was 687 pg/mL. An important RANTES augment in the infected children groups was accompained by an improvement of the CD4+ / CD8+ index during the post HAART period. RANTES levels during HAART could a reliable indicator of immune recovery in the HIV-1 infection.

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Poster Presentations

Citokines production by *Mycobacterium tuberculosis* H37Rv Strain

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The immune response against Mycobaterium tuberculosis is mediated by different cells of the immune system with several functions. The cytokines produced by the macrophage are mainly IL-1, IL-6 and TNF-α. The molecules TNF-β, IFN-γ and IL-2 release by TH1 lymphocytes are very important during the cellular immune response, on the other hand, the cytokines derived from TH2 lymphocytes are: IL-4, IL-5, IL-10, IL-13. The aim of this study was to determinate the kinetics of the production of TH1, TH2 and proinflammatory cytokines stimulated with Mycobaterium tuberculosis. This study was performed using a bacillar load of 8 X 10^6 bact/mL equivalent to a ratio of 50 bacterial per macrophage, and the cytokines was determined by ELISA immunosay. The results demonstrated that the production of different cytokines was depended of time of stimulation; first appearing proinflammatory, and thereafter TH1 and TH2 cytokines. We supouse that during the infection with *Mycobacterium tuberculosis* occurs the same phenomenon that we demonstrated in this *in vitro* experimental analysis. First are released proinflamatory citokines and later TH1 and TH2 citokines involved in the cellular and humoral immune response. It is becoming strong evidence that cytokines play an important role during the different stages of the infection but, the way by which cytokine participates in the pathogenesis and disease, is not well understand. However, this study offer new information for the therapeutic management.

The biological activity of IFNα 2b in liquid and freeze-dried formulations

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The Center for Genetic Engineering and Biotechnology (Havana, Cuba) produces interferon alpha 2b (IFN alpha 2b) since 1987 as an active ingredient and also, as a formulation. This cytokine have been formulated for parenteral use. In fact, IFN alpha 2b has been frequently used in Cuba and worldwide, as an adjuvant therapy for several carcinogenic pathologies such as respiratory papillomatosis [1].Many investigators have contributed to find storage conditions able to maintain the biological activity of this cytokine for long periods. This is an essential requirement for the achievement of the desired IFN alpha 2b *in vivo* effect. As a result, several formulations that keep intact the IFNα 2b biological activity have been developed since 1970 [2].In this work we present our IFN alpha 2b formulations, and compare them with previously developed liquid and lyophilized IFN formulations from other relevant investigations. The biological activity of IFN alpha 2b was determined by the inhibition of the cytopathic effect (ECP) produced by the Mengo virus on Hep-2 cells (ATCC No. CCL23). The physical-chemical characteristics of our products were determined by RP-HPLC and SDS/PAGE. The results obtained proved the physical, chemical and biological equivalence between all of the examined formulations.

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IL-2 biological activity in liquid and lyophilized formulations

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Antiviral therapies in a given combination of two or three antiviral agents are able to decrease the charge of the type I human immunodeficiency virus (HIV-1) to undetectable levels [1]. However, this treatment does not become definitive yet, and the restoration of the immune system has not been completed [1]. In order to assist this situation interleukin 2 (IL-2) has been investigated as adjuvant for the therapy with antiviral drugs, for attempting to induce a significant increment on the immunological activity [1]. The biological activity of this cytokine is a essential characteristic for an effective treatment; thus, this function should be carefully preserved during purification, formulation and long-term storage. For IL-2 the major degradation routes are oxidation and aggregation. Aggregation not only decreases the therapeutic potential of the product but also may increase an immune response against this cytokine. In this work we demonstrated that IL-2 produced at CGEB (Havana, Cuba), efficiently retains its biological activity even when stressing factors (e.g. freeze-drying and high temperatures) are applied to the molecule. To find conditions guaranteeing maximum stability of IL-2, a great number of solutes (aminoacids, sugars, detergents and polymers) were evaluated. The biological activity of this cytokine was estimated after freeze-drying or storage at high temperatures by the induction of the *in vitro* proliferation of CTLL-2 cells. As judged by SDS/PAGE, RP-HPLC and the bio-activity assay, formulations resulting from this work are equivalent to others previously described by other researchers.

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Immune response induced in mice by two meningococcal polysaccharide-protein conjugates

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Meningococcal infections are an important cause of morbidity and mortality worldwide. Serogroup B and C strains are responsible for most cases in the developed world. Meningococcal vaccines containing purified serogroup C capsular polysaccharide (PsC) induce protective serum bactericidal antibodies in adults but are poorly immunogenic in young children and may induce tolerance. The immunogenicity of PsC can be improved by conjugation to a carrier protein. In this study the immunogenicity and type of immune response: humoral (Th2, IgG1) or cellular (Th1, IgG2a) to two meningococcal PsC-protein conjugates was evaluated in Balb/c mice. The purified PsC was linked to a carrier protein (tetanus toxoid (TT) or Outer Membrane Protein from N. meningitidis serogroup B (OMP)), via carbodiímida-mediated reaction. The IgG and IgG subclass antibodies (IgG1 and IgG2a) anti PsC and anti OMP was evaluated in the serum of animals by an indirect ELISA. All mice that were inoculated with conjugates, induced a high titers of IgG anti PsC and anti proteins. The use of TT as carrier induced mainly IgG1 antiPsC subclass, but the OMP as carrier induced also IgG2a anti PsC. The PsC-OMP conjugate shows high titers of both IgG anti OMP subclasses, like the mice immunized with native OMP. In conclusion, at the level of IgG subclass antibodies, the conjugate PsC-OMP was able to generate a Th1 immune response, whereas the conjugate PsC-TT generated a Th2 immune response.

Comparative immunogenicity of conjugates composed of *Neisseria meningitidis* serogroup C polysaccharide bound to tetanus toxoid by different spacer arms

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The influence in immune response of structure spacer arms used in *Neisseria meningitidis* serogroup C polysaccharide (MGCP) – Tetanus toxoid (TT)

conjugate was evaluated in Balb/c mouse. 1,6- diaminohexane (AH), 1,8-diaminooctane (AO), 6-aminohexanoic acid (AA) and adipic acid dihydrazide (ADH) were used like spacer arms with different structure linked to MGCP and TT using carbodiimide –mediated coupling. The IgM antibody anti MGCP, IgG anti MGCP and IgG anti TT was evaluated in serums of animals by an indirect ELISA. We also evaluated the IgG subclasses (IgG1 and IgG2a) anti MGCP and Bactericidal activity of antibodies present in sera. All mice that were inoculated with conjugates, made high titers of IgG anti MGCP, but the higher titers were found for ADH. In this group was found too the higher titers of IgG2a and the higher Bactericidal titers. Higher levels of anti TT antibodies were elicited by conjugates than TT in itself. The possible basis for differences in the immunogenicities of these conjugates is discussed.

P64k as a carrier protein of *Neisseria meningitidis* por A peptides

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Class 1 protein from the outer membrane of *Neisseria meningitidis* is an attractive target for vaccine development. Previously, cyclic peptides corresponding to VR1 and VR2 regions of the meningococcal class1 protein have been designed and employed as candidate antigens with good results [1]. To increase the humoral immune response against these cyclic synthetic peptides, we conjugated the peptides to P64k, a novel carrier protein from the same bacterium expressed in *Escherichia coli* [2]. In addition, one of these peptides was restricted to a linear conformation before it was chemically coupled to the carrier. The conjugates were administered to mice in a three-dose immunization schedule, resulting in a potent anti-peptide immune response, which suggested that chemical conjugation to this carrier provided T-cell help. Antisera directed to the conjugates reacted with *N. meningitidis* outer membrane PorA upon immunoblot analysis. Moreover, in two out of three conjugates, the anti-peptide sera reacted with native meningococcal outer membrane vesicles in ELISA, suggesting that chemical conjugation to the carrier granted, in addition to T-cell help, a proper folding to these synthetic peptides.

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Comparison of the immune response in mice against meningococcal group C polysaccharides conjugate to three carrier proteins

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Objective: To study the nature and kinetics of the serum antibody response in mice to meningococcal group C polysaccharide (C-Ps) conjugates to three carriers: Bovine serum albumin (BSA), Tetanus toxoids (TT) and recombinant P64k. Design: Meningococcal group C polysaccharide was conjugated using carbodiimide as coupling reagent and adipic acid (ADH) as spacer between the C-Ps and the carrier. Carrier proteins, conjugates and free C-Ps were employed to immunize Balb/C mice. Aluminum hydroxide was used as adjuvant. The murine humoral immune responses were evaluated by ELISA after the third dose. Result: The polysaccharide-protein ratios of the three conjugates were: TT-1.26, BSA-1.13 and P64k-0.4 mg/mL. The immune response against the three C-Ps conjugates was higher than against free C-Ps. The levels of antibodies detected in the sera of mice immunized with C-Ps-P64k conjugate were higher than those detected against C-Ps-TT and C-Ps-BSA conjugates. Conclusions: The carrier protein present in the C-Ps conjugates influenced the levels of the IgG antibodies elicited against to the Ps. In this regard P64k was superior to TT and BSA.

Immunization with a peptide sequence derived from *Neisseria meningitidis* using different vaccine formulations

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In a previous work we have selected a peptide sequence after the screening of a phage library with a monoclonal antibody directed against the capsular polysaccharide from *Neisseria meningitidis.* In the present work, a multi antigen peptide (MAP), containing four copies of the selected peptide, was synthesized. The MAP was coupled to the carrier protein P64K using different conjugation methods. The MAP, conjugated and unconjugated, was employed to immunize BALB/c mice, using doses of 5, 25 and 50 ug of each immunogen. The preinmune and 4th dose sera antisera were evaluated by ELISA, using as coating antigen the unconjugated MAP. Specific anti-peptide antibodies were elicited after immunization. The best results were achieved in the group of mice immunized, with the three doses assayed, with the MAP conjugated to P64K using the carbodiimide method (1-ethyl-3-(dimethylaminopropyl)carbodiimide)) combined with previous conversion of amine residues from P64K in acid residues using succinic acid, and in the group of mice immunized with the MAP coupled to P64K using maleimide propionic acid N-hydroxysuccinimide ester as coupling agent.

Peptide synthesis containing a B-cell and a T-cell epitopes on dextran beads and evaluation of humoral response against bead-peptide construct

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Cross-linked dextran beads were chemically modified with Fmoc-bAla to give an amino functionalized support suitable for solid-phase peptide synthesis.

On this support, a peptide comprising B-cell and T-cell epitopes in tandem was synthesized manually by Fmoc procedure monitoring the coupling reaction by bromophenol blue procedure and standard Kaiser test. The quality of peptide synthesis was verified by RP-HPLC and mass spectrometry. The size and morphologic characteristics of dextran beads were conserved after peptide synthesis. Furthermore, we analyzed immunogenicity in mice of the T-B peptide on dextran beads compared with T-B peptide immunogenicity when administered in CFA/IFA. In both cases, titers were high and there was not a significant difference in antibody titers between groups ($p<0.05$). But in contrast CFA they are biocompatible and did not induce any adverse reaction at the site of injection. This experiment suggests that the peptides synthesis on dextran beads can be used to raise the immunogenicity of synthetic peptide in vaccines or therapeutics.

Effect of conjugation methodology on the immunogenicity and protective efficacy of meningococcal group C polysaccharide-P64k protein conjugates

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Neisseria meningitidis serogroup C polysaccharide was conjugated to the carrier protein P64k using two different conjugation procedures carbodiimide with adipic acid dihydrazide as spacer and reductive amination method and the condensation mediated by carbodiimide. BALB/c mice were immunized with the resultant polysaccharide–protein conjugates and the immune response was evaluated. All conjugates assayed generated a higher immune response than the free polysaccharide. The reductive amination method rendered the best conjugate named PsC-P64k$_R$. PsC-P64k$_R$ was able to elicit an antibody titer statistically different from the plain polysaccharide ($p< 0.001$). The bactericidal response obtained against this conjugate was three folds higher than against the plain polysaccharide and it was able to protect against the challenge with the meningococci in the infant rat protection model. The influence of polysaccharide chain length was also studied. Three different conjugates were obtained with polysaccharide molecular relative sizes of 20 000 - 40 000 Da, 40 000 - 100 000 Da or 100 000 - 500 000 Da but no differences were detected in the immune response obtained against the three conjugates. Our experiment demonstrate that it is possible to generate a protective, T-cell dependent response against meningococcal C polysaccharide using the P64k protein as carrier.

Workshop on Adjuvants for Therapeutic Vaccines

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Oral Presentations

Hyperthermia and heat shock proteins as adjuvants for cancer immunotherapy

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Increased temperature is a cardinal feature of inflammatory responses and yet, it is usually overlooked as a potential immunoregulator. We have analyzed the effects of a mild whole body hyperthermia (WBH) which is similar to that obtained during a normal febrile episode (ie: 39.5-40 ºC for 6-8 h) on various immunological effector cells and systems testing the hypothesis that this treatment can serve as an adjuvant for cancer therapy. Fever-range WBH results in several changes in lymphocytes including alterations in the organization of the cytoskeleton and in homing and adhesion properties, reorganization of several PKC isoforms, and induction of heat shock proteins. More recent studies measuring leukocyte population distributions in Balb/c mice after a fever-range WBH treatment revealed the loss of lymphocytes from blood and spleen, suggesting their movement into the periphery. WBH treatment of mice has also been shown to enhance LPS induced TNF-a and IL-6 levels in the serum. Furthermore, WBH treatment after application of the elicitation dose of antigen enhanced the ear swelling response of a contact hypersensitivity (CHS) model in BALB/c mice. The effects of this treatment in the CHS response appear to be due to regulation of both the dendritic cells in the skin (i.e., Langerhans cells) as well as lymphocytes. *In vitro* studies using an ear skin culture system have further revealed that fever-range hyperthermia (40 ºC for 6 h) enhances the kinetics by which the Langerhans cells are actively induced to migrate out of the skin and into the culture supernatant. Because of this evidence for the capacity of WBH to stimulate immune cells and alter their motility and homing potential, we have also examined the impact of fever-range WBH on tumor growth *in vivo.* Interestingly, treatment with mild WBH alone resulted in tumor growth delay in SCID mice bearing human tumors and BALB/c mice bearing syngeneic tumors. This effect appeared to be a result of lymphocyte and/or NK cell activity. WBH also appeared to induce changes in tumor vasculature that might be partly responsible for the anti-tumor effects. Overall, these data suggest that this physiological type of "heat stress" can contribute to beneficial anti-tumor responses at the level of the organism's immunity. A

phase I clinical trial using this fever-range WBH treatment has recently been completed at our institution and a second is now underway. Supported by the CRI (J.R.O.), NIH grants CA71599 and CA16056 (E.A.R.) and NIH grant CA79765 (S.S.E.)

Antisense targeting of thymidylate synthase in human tumour cells: effects on cell cycle, proliferation, and drug sensitivity

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Killing cancer cells by interfering with their increased proliferative and metabolic activity has long been a goal of cancer chemotherapy. Unfortunately, drug-resistant tumor variants often arise, resulting in treatment failure. To address this problem, we have used antisense oligodeoxynucleotides (ODNs) that target thymidylate synthase (TS), an essential enzyme for *de novo* thymidylate production and DNA synthesis, and a target for a number of chemotherapeutics including 5-fluorouracil and raltitrexed. Antisense treatment effectively reduces TS mRNA and protein, inhibits cell proliferation, and sensitizes human HeLa cervical carcinoma and HT29 colon carcinoma cells to TS-directed chemotherapeutic drugs *in vitro*. Tumor growth in immunocompromised mice is inhibited by intraperitoneal administration of TS antisense ODNs, and antisense ODN treatment enhances the antitumor activity of raltitrexed. Cell cycle analysis of cultured tumor cell lines using flow cytometry indicates that antisense ODN treatment results in accumulation of cells in the G2/M phase, in contrast to G1/S arrest by 5-fluorouracil or raltitrexed treatment, suggesting a previously unknown connection between TS protein (or mRNA) and the G2/M cell cycle checkpoint. The current focus is to uncover the molecular mechanism mediating G2/M arrest following antisense ODN treatment, by investigating known cell cycle regulators such as p53 and cyclin/cdk complexes, and by analysing global changes in gene expression using cDNA microarray technology. Our results indicate that TS antisense ODN treatment improves the efficacy of anti-TS chemotherapeutic drugs *in vitro* and *in vivo*, is effective in overcoming tumor cell resistance to chemotherapeutic drugs, and has the potential to become an important anti-tumor therapy. Supported by a grant from Imperial Oil Canada, Ltd.

VSSP: A novel solution for ganglioside based cancer vaccines

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Gangliosides are tumor-associated antigens that constitute potentials targets for cancer immunotherapy. A major drawback of ganglioside vaccines, however, is the poor immunogenicity of these glycolipids. Particularly the presence of substantial amounts of GM3 ganglioside on human melanomas and other tumors, together with its peculiar biological properties, makes this glycolipid a unique target for cancer immunotherapy. Recently, we hydrophobically incorporated purified GM3 into the outer membrane protein complex from *Neisseria meningitidis* to form Very Small Size Proteoliposomes (GM3/VSSP). The antitumor properties of GM3/VSSP were studied in B16 murine melanoma and are discussed. The results of a Phase I clinical trial in stage III, IV breast cancer patients, testing the GM3/VSSP vaccine, will be also discussed. This clinical trial showed that GM3/VSSP/Montanide ISA 51 vaccine is safe and remarkably immunogenic, since most of the patients were able to generate an specific immune response against the most tolerated ganglioside.

Sinusitis autovaccine

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Surgery and the indiscriminate administration of antibiotics has been the traditional treatment of sinusitis; sometimes with poor results. Besides the relapsing of the desease has been frequently observed; all these unfavorable features go hand in hand with the relatively high cost of the treatment. In this study we prepared a polyvalent autovaccine with clinical material collected from rinitis and sinusitis patients. Previous to a clinical and radiological diagnosis of the infection a sample was taken from each patient taken at the medium meatus and the laryngopharyngeal zone. This sample was put on agar-blood medium and incubated at 37ºC in aeroplylic conditions. A vaccine was prepared with the isolated microorganism according to Kolmers method. 581 patients were treated with their respective vaccine. The dose injected subcutaneously was administered during 33 to 44 days, and increased every 3 or 4 days; 15 days after treatment began, a reinforcing dose was applied, and 6 months after another and last dose was injected. The medical evaluation revealed an effectivity of 77%. A bacteriological study was conducted on 108 patients affected with sinusitis which resulted in the isolations and identification of *Streptococcus viridans* in 23% of these cases. *S. viridans* has been sporadically reported in the literature as an etiological agent of the sinusitis infection.The autovaccine could become a potentially more effective and specific alternative for treating sinusitis.

Workshop on Adjuvants for DNA Vaccines

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ABSTRACT

DNA vaccine technology has provided important hopes and intense investigation for the past decade. DNA vaccines are typically comprised of plasmid DNA molecules that encode an antigen(s) derived from a pathogen or tumor cell. Following introduction into a vaccine, cells take up the DNA, where expression and immune presentation of the encoded antigen(s) take place. DNA-based immunization of animals with plasmid DNA has been shown to elicit both cellular and humoral immune responses against a wide variety of antigens. However, immune responses generated by using this methodology, remain frequently insufficient. A great deal of effort is now being applied to the development of delivery vehicles and adjuvants for DNA-based vaccines. The workshop session "Adjuvants for DNA vaccines" is focused at enhancing DNA vaccine by different approaches including DNA vaccine formulation, vector modification and delivery systems. Different additives and protein combinations were evaluated to increase the immune response generated after the administration of DNA based-vaccines. Particularly, the use of Opc, an outer membrane protein from *Neisseria meningitidis* complexed to pCMVβ-galactosidase plasmid, expressing β-galactosidase was described. The use of plasmid DNA-protein complexes was associated with the induction of a humoral response in serum and also with a proliferative response in the spleen. Additionally, a novel polysaccharide adjuvant acemannan consistently modulated the anti-hepatitis C virus core antibody response elicited by DNA immunization. Combinations with other additives like sonicated calf thymus DNA and polyetylenglicol or co-immunization with interleukin15-encoding plasmid also transiently modulated the anti-hepatitis C virus core immune response elicited by DNA vaccination. On the other hand, constructs expressing polyprotein variants were successfully evaluated to enhance the immune responses induced after DNA immunization.

*I*ntroduction

It is now well established that the injection of plasmid DNA through a wide range of routes induces both humoral and cellular immune responses against the encoded immunogenic proteins in several hosts. Moreover, immune responses induced by DNA immunization have lead to protection against various viral, bacterial and parasitic pathogens. However, while initial human clinical studies demonstrated the priming of immune responses with naked DNA vaccines, potency remains a significant limitation. While most of the experiments conducted up to date have used phosphate buffer saline as a diluent for the injected DNA, novel additives are currently investigated. Such reagents may increase the uptake of DNA, reduce the number of doses for immunization, and enhance subsequent immune responses. Some systems currently under investigation are dendritic cells, cationic liposomes, immunostimulatory oligonucleotide sequences, cytokines, poly(lactide-coglycolide) (PLG) microparticles and monophosphoryl lipid (MPL) A.

The aim of the workshop session "Adjuvants for DNA vaccines" was to discuss different approaches including DNA vaccine formulation, vector modification and delivery systems to enhance the immune response generated against the encoded antigens. The workshop session included two oral presentations and two posters.

As a part of the oral presentations Dr. Alexis Musacchio from the Center for Genetic Engineering and Biotechnology (Havana, Cuba) described the use of plasmid DNA-recombinant Opc protein complexes, for nasal immunization. On the other hand, Santiago Dueñas-Carrera, also from the Center for Genetic Engineering and Biotechnology (Havana, Cuba), showed his work on the enhancement of the immune response generated against the hepatitis C virus envelope proteins after DNA vaccination with plasmids managed to express polyprotein variants of the envelope proteins.

At the poster session, Jeny Marante and Ariel Viña, both from the Center for Genetic Engineering and Biotechnology (Havana, Cuba) presented interesting papers on the adjuvant effect of IL-15 gene and acemannan on the anti-core antibody response elicited in mice by HCV DNA-based vaccine.

*O*ral Presentations

Immunization with plasmid DNA-protein complexes. Optimal induction of mucosal immunity in general requires targeting antigens to the specialized antigen presenting cells of mucosal immunity associated to lymphoid tissues. The nasal mucosa may provide a simple, non-invasive route to deliver DNA encoding the introduced gene to stimulate immunity. In this way, nucleic acid vaccines represent a new approach to the control of infectious agents. To evaluate preliminarily the feasibility of this approach, Dr. Alexis Musacchio investigated a model system, where protein-DNA complexes were used. The outer membrane Opc protein from *Neisseria meningitidis*, was selected due to its role in attachment of meningococci to the endothelial and epithelial cells. Opc-DNA complexes may facilitate a better interaction between this inmunogen and mucosal cells. An expression vector, containing the β-galactosidase reporter gene, under the control of the human cytomegalovirus promoter (pCMVβ-galactosidase) was used by Dr. Alexis Musacchio *et al*. To characterize the complexes, they performed gel filtration analysis, transmission electronmicroscopy and transfection experiments in cos-7 cells. Balb/c mice were intranasally (i.n.) and intramuscularly (i.m.)-immunized to study the im-

mune response. The humoral immune response against Opc and β-galactosidase was measured by ELISA and immunoblot. The proliferative response in the spleen was also measured. Antibody response was evaluated fifteen days after the fourth inoculation. Antibodies specific to β-galactosidase were detected in groups i.m. immunized with pCMV-β galactosidase DNA, and also in mice where Opc-pCMV-β galactosidase DNA complex was i.n.administered. Antibody levels were significantly higher in animals inoculated i.m. with pCMV-β galactosidase plasmid. However, after two and a half months this difference in antibody levels between these groups was not observed. A proliferative response specific to β-galactosidase protein was also detected, having no appreciable differences between groups. From the resulting data, Dr. Alexis Musacchio concluded that, the use of plasmid DNA-protein complex was associated with the induction of a humoral response in serum and also with a proliferative response in the spleen.

Immunization with polyprotein-encoding plasmids. The hepatitis C virus (HCV) is the major causative agent of non-A, non-B viral hepatitis. No prophylactic vaccine against HCV is available and therapeutic treatments are effective in less than 40% of the cases. Many studies have been published on the development of DNA-based vaccines against HCV. However, weak or transient anti-HCV immune responses are frequently elicited by using this immunization strategy. Plasmids expressing variants of the hepatitis C virus (HCV) core, E1 and E2 proteins individually or as polyproteins were administered to BALB/c mice by Santiago Dueñas-Carrera *et al.* All plasmids induced a detectable and specific antibody response. Antibody titers against C, E1 and E2, 19 weeks after the primary immunization, ranged from 1:50 to 1:4500 depending on the inoculated plasmid and the HCV antigen evaluated. Constructs expressing HCV envelope proteins as polyprotein variants induced statistically stronger antibody response than plasmids encoding individual E1 and E2. Particularly, the pIDKE2 plasmid, expressing the first 650 aa in the viral polyprotein, induced a potent and multispecific antibody and lymphoproliferative response against the HCV core, E1 and E2 proteins. Anti-E2 antibodies generated by pIDKE2 immunization were cross-reactive to hypervariable region-1 peptides from different genotypes. Immunization with the pIDKE2 also generated a positive cellular response against the core antigen, determined by IFN-γ ELISPOT assay, and induced detectable levels of IFN-γ but not IL-4 in vaccinated mice. Taking all these data together, Santiago Dueñas-Carrera concludes that plasmids expressing the HCV structural antigens as polyproteins could induce a potent, multispecific and cross-reactive anti-HCV immune response. A transient enhancement of the anti-HCV immune response generated by the DNA vaccine was also described by Santiago Duenas-Carrera by the use of additives like sonicated calf thymus DNA and polyetylenglicol.

Poster Presentations

IL-15 gene adjuvant. In many animal models for infectious diseases, DNA vaccines induced a broad range of immune responses, including antibody, CD8+ cytotoxic T lymphocyte (CTL) and CD4+ helper T (Th) lymphocyte responses. The magnitude and nature of these immune responses to DNA vaccines can be further manipulated by the use of cytokine genes. The study presented by Jeny Marante (Center for Genetic Engineering and Biotechnology, Cuba), was conducted to investigate whether the expression of human IL-15 could enhance the immune response elicited by a DNA vaccine expressing the structural proteins of HCV (Core, E1 and E2) in mice. Two eukaryotic expression vectors encoding for human IL-15 were constructed. One of them comprises the coding sequence for human IL-15 including the own signal peptide; in the other one, the IL-15 coding sequence is fused to the erithropoyetin signal peptide. A faster seroconvertion rate and a stronger antibody response against the core protein were observed in mice immunized with the IL-15 encoding plasmids compared with animals vaccinated with the HCV antigens-encoding plasmid alone. These results suggest that the co-expression of IL-15 influences the specific immune response elicited after immunization with HCV antigen-encoding plasmids.

Acemannan as an adjuvant for DNA vaccines. Acemannan has been capable of rising and/or modulating the immune response of mice to vaccinal antigens at serum and mucus levels, while plasmid expressing the HCV nucleocapsid alone often elicited strong cellular but weak and/or transient humoral immunity. Ariel Viña (Center for Genetic Engineering and Biotechnology, Cuba) investigated the influence of the acemannan on the Ab response elicited by a DNA vaccine. Mice were intramuscularly immunized with the pIDKCo plasmid alone or in combination with acemannan. The pIDKCo encodes the first 176 aa of the hepatitis C virus nucleocapsid protein. Six weeks after immunization, animals immunized with pIDKCo combined with acemanane 0.1 and 0.3 mg/mL had statistically higher level of anti-core Ab titers than the others ($p<0.05$). At week 19, 0.3 mg/mL acemannan still increased the anti-core Ab response ($p=0.038$). However, acemannan did not modify the IgG1/IgG2a ratio of anti-core Ab. Altogether, these data indicate that acemannan modulates the anti-core antibody response elicited by DNA immunization.

Summary

An important issue for effective vaccines is the development of potent adjuvants that can facilitate induction or increase of immunity. Adjuvants are known to strongly enhance immune responses generated by traditional vaccines, but less is known about the effects of adjuvants on vaccination with DNA. The workshop summarized here focuses on recent advances in several strategies that have been used to improve and modulate the immune response induced by DNA vaccines. The combination of DNA vaccines with different additives transiently influences the immune response elicited in animals. In fact, the use of the acemannan adjuvant or the Opc-DNA complexes evidenced sustained enhancement of the immune response against the encoded antigens. While all these adjuvants are promising, further works are needed to better define the mechanisms of adjuvant action. The access of antigens to antigen presenting cells (APCs)

appears to be a rate-limiting step in the generation of immune responses to DNA vaccines. Thus, ultimately, the development of more potent adjuvants, DNA vectors or delivery systems targeted to APCs may allow DNA vaccines to be used as both, therapeutic and prophylactic agents.

Oral Presentations

Plasmid DNA–recombinant Opc protein complexes, for nasal immunization

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Optimal induction of mucosal immunity in general requires targeting antigens to the specialized antigen presenting cells of mucosal immunity associated lymphoid tissues. The nasal mucosa may provide a simple, non-invasive route to deliver DNA encoding the introduced gene to stimulate immunity [1]. In this way, nucleic acid vaccines represent a new approach to the control of infectious agents [2]. To evaluate, preliminarily, the feasibility of this approach, we have investigated a model system, where protein - DNA complexes were used. We selected the outer membrane Opc protein from *Neisseria meningitidis*, due to its role in attachment of meningococci to the endothelial and epithelial cells [3]. Opc-DNA complexes may facilitate a better interaction between this inmunogen and mucosal cells. An expression vector, containing the β-galactosidase reporter gene, under the control of human citomegalovirus promoter (pCMVβ-galactosidase) was used [4]. Optimal conditions of interaction between recombinant Opc protein and pCMVβ-galactosidase DNA were established. To characterize the complexes, gel filtration analysis, transmission electronmicroscopy and transfection experiments in cos-7 cells were performed. Balb/c mice were intranasally (i.n.) and intramuscularly (i.m.)-immunized to study the immune response. The humoral immune response against Opc and β-galactosidase was measured by ELISA and immunoblot. The proliferative response in the spleen lymph nodes was also measured. Antibody response was evaluated fifteen days after the fourth inoculation. Antibodies specific to β-galactosidase were detected in groups i.m. immunised with pCMV-β galactosidase DNA, and also in mice where Opc- pCMV-β galactosidase DNA complex was i.n. administered. Antibody levels were significantly higher in animals inoculated i.m with pCMV-β galactosidase plasmid. However, after two and half months this difference in antibody levels between these groups was not observed. A proliferative response specific to β-galactosidase protein was also detected, having no appreciable differences between groups. From the obtained data, we can conclude that, the use of plasmid DNA-protein complexes was associated with the induction of a humoral response in serum and also with a proliferative response in the spleen lymph nodes.

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Enhancement of the immune response generated against the hepatitis C virus envelope proteins after DNA vaccination with fusion proteins-encoding plasmids

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Hepatitis C virus (HCV) is the major causative agent of non-A, non-B viral hepatitis. No prophylactic vaccine against HCV is available and therapeutic treatments are effective in less than 40% of cases. Many studies have been published on the development of DNA-based vaccines against HCV [1]. However, weak or transient anti-HCV immune responses are frequently elicited by using this immunization strategy [2]. In this work, plasmids expressing variants of the hepatitis C virus (HCV) core, E1 and E2 proteins individually or as fusion proteins were administered to BALB/c mice. All plasmids induced a detectable and specific antibody response. Antibody titers against C, E1 and E2, 19 weeks after primary immunization, ranged from 1:50 to 1:4500 depending on the inoculated plasmid and the HCV antigen evaluated. Constructs expressing HCV envelope proteins as fusion variants induced statistically stronger antibody response than plasmids encoding individual E1 and E2 proteins. Particularly, the pIDKE2 plasmid, expressing the first 650 aa in the viral polyprotein, induced a potent and multispecific antibody and lymphoproliferative response against HCV core, E1 and E2 proteins. Anti-E2 antibodies generated by pIDKE2 immunization were cross-reactive to hypervariable region-1 peptides from different genotypes. Immunization with the pIDKE2 also generated a positive cellular response against the core antigen, determined by IFN-γ ELISPOT assay, and induced detectable levels of IFN-γ but not IL-4 in vaccinated mice. These data indicate that plasmids expressing the HCV structural antigens as fusion proteins could induce a potent, multispecific and cross-reactive anti-HCV immune response.

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Second Generation DNA Vaccine strategies

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DNA vaccine technology has provided great enthusiasm and intense investigation for the past decade. Gene-based vaccination of animals with plasmid DNA has been shown to elicit both cellular and humoral immune responses against a wide variety of antigens. However, while initial human clinical studies demonstrated priming of immune responses with naked DNA vaccines, potency remains a significant

limitation. We have sought to address the issue of DNA vaccine potency using several different approaches, including vector modification, formulation and delivery systems. In one such approach, alphavirus RNA replicon technology was incorporated into plasmid DNA vectors, whereby the RNA polymerase II promoter launches a self-amplifying RNA transcript encoding the desired antigen. This "layered" expression strategy not only induces an antigen-specific immune response, but also provides a means for stimulating the innate immune response through the presence of cytoplasmic dsRNA intermediates. Animal studies with a variety of antigens demonstrated significantly increased potency, requiring as little as 10- to 1000-fold lower dosages of replicon plasmid DNA for immune induction, as compared to conventional plasmid DNA. In addition, alphavirus replicon and other plasmid DNAs have been formulated by adsorption onto the surface of cationic poly(lactide-coglycolide) (PLG) microparticles to increase the efficiency of delivery. Again, for a variety of antigens, PLG-formulated DNA was shown to be substantially more potent than corresponding naked DNA vaccines, increasing CD8(+) T-cell and antibody responses by approximately 100- to 1,000-fold, respectively. These two strategies represent a powerful means for increasing the overall potency of DNA vaccines.

Poster Presentations

Enhancement of the anti-core antibody response elicited in mice by HCV DNA-based vaccine though the use of IL-15 gene adjuvant

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DNA vaccines containing genes for antigenic portions of viruses have been developed as a novel vaccination technology. An important advantage of this vaccination method is that *in vivo*-synthesized viral proteins can enter both major histocompatibility complex (MHC) class I and class II antigen-processing pathways to activate specific immunity. In many animal models for infectious diseases, DNA vaccines induced a broad range of immune responses, including antibody, CD8+ cytotoxic T lymphocytes (CTL) and CD4+ helper T (Th) lymphocyte responses. The magnitude and nature of these immune responses to DNA vaccines can be further manipulated by the use of cytokine genes. We have investigated the utility of DNA-based immunization as a strategy for the development of an effective vaccine against hepatitis C virus (HCV). The present study was conducted to investigate whether expression of human IL-15 could enhance the immune response elicited by a DNA vaccine expressing the structural proteins of HCV (Core, E1 and E2) in mice. Two eukaryotic expression vectors encoding for human IL-15 were constructed. One of them comprises the coding sequence for human IL-15 including the own signal peptide; in the other one, the IL-15 coding sequence is fused to the erithropoyetin signal peptide. A faster seroconvertion rate and a stronger antibody response against the core protein were observed in mice immunized with the IL-15 encoding plasmids compared with animals vaccinated with the HCV antigens-encoding plasmid alone. These results suggest that co-expression of IL-15 influence the specific immune response elicited after immunization with HCV antigens-encoding plasmids.

Acemannan enhances anti-HCV core Ab response elicited by DNA immunization in mice

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Injection of plasmid DNA induces both humoral and cellular immune responses against the encoded proteins in several hosts (1). However, immune responses generated against several antigens remain insufficient (2). A great deal of effort is now being applied to the development of delivery vehicles and adjuvants for DNA-based vaccines. Acemanane has been capable of rising and/or modulating the immune response of mice to vaccinal antigens at serum and mucus levels, while plasmid expressing the HCV nucleocapsid alone often elicited strong cellular but weak and/or transient humoral immunity (3). We investigated the influence of the acemanane on the Ab response elicited by a DNA vaccine. Mice were intramuscularly immunized with pIDKCo plasmid alone or in combination with acemanane. The pIDKCo encodes the first 176 aa of the hepatitis C virus nucleocapsid protein. Six weeks after immunization, animals immunized with pIDKCo in combination with acemanane 0.1 and 0.3 mg/mL had statistically higher level of anti-core Ab titers than the others ($p<0.05$). At week 19, 0.3 mg/mL acemanane still increased the anti-core Ab response ($p=0.038$). Additionally, acemanane modified the epitope specificity but not IgG1/IgG2a ratio of anti-core Ab. Altogether; these data indicate acemanane modulates the anti-core antibody response elicited by DNA immunization.

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Workshop on Antigen-Delivery Systems

Chairpersons: Claude Leclerc,[1] Dania Vázquez Blomquist[2]

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ABSTRACT

Conventional vaccines based on inactivated pathogens, live pathogens, subcellular components from bacteria and viruses or recombinant proteins have been successfully used to elicit neutralizing antibodies against surface proteins, in order to prevent or detain the infection. In recent years, the vaccine scenario has changed and most of the experimental vaccines currently tested in humans are based on recombinant viruses, recombinant proteins, DNA vectors, purified subunits, peptides and some others targeting well-defined antigens. In some of the cases, an efficient delivery system should be used to target the antigens to the antigen presenting cells (APC), stabilize the antigen *in vivo* and/or release the antigen over an extended period of time. The workshop "antigen-delivery system" included well-known bacterial, viral or particulated systems for antigen delivery by different routes of immunization, including *Salmonella typhi,* BCG, a toxin from *Bordetella pertussis,* Fowlpox viruses, Adenoviruses, microparticles and biopolymers. The novel aspects were related to the model antigens and new strategies to control very extensively studied diseases such as tetanus, HIV-1, HBV, Cholera and tuberculosis. The study of new natural polymers was also shown. The use of bacteria-derived toxins co-administered with antigens is well documented as a potent mucosal adjuvant. The inclusion of CTL epitopes onto a detoxified adenylate cyclase (CyaA) toxin from *B. pertussis* was shown to be a TAP dependent $CD8^+$ T cell epitope delivery system. The perspective of vaccines based on antigens expressed in plants was discussed, taking as an example the oral delivery of de HBsAg expressed in potatoes. One of the most widely discussed topics was Dendritic Cells as professional APC and the ways to improve antigen targeting and delivery to their cytosol, but also the induction of maturation and the expression of MHC class I molecules to increase CTL epitopes presentation.

*I*ntroduction

Current vaccinology is moving toward the search of more defined antigens to be included in vaccines that sometimes show a lower immunogenicity than the conventional inactivated or attenuated vaccines. In some infectious diseases the importance of eliciting a cellular response against different antigens or a humoral/cellular mucosal immunity has become more relevant. In this sense, the search of new formulations, adjuvants and more efficient delivery systems for antigens, has been enhanced. The only approved FDA adjuvant for human trials has been aluminium salts, and lately MF59 for the Influenza vaccine. These two adjuvants preferentially promote a Th2-type immune response by a systemic route. Oral administration is being actively investigated using live bacterial vectors and proteins inserted into mucosal delivery systems. The use of strong mucosal adjuvants as detoxified toxins such as Cholera Toxin (CT) or *E. coli* LT is hampered by their very high toxicity, but point mutation strategies have been carried out to dissociate the toxicity and adjuvant properties. Other adjuvants have been tested in some Phase I clinical trials against mortal diseases such as HIV-1, including, MF59 + Muramil Tripeptide, Incomplete Freund Adjuvant, Montanide ISA51 or ISA720, Ribi and others. Besides the adjuvants which stimulate and increase humoral and/or cellular immune responses against antigens through different mechanisms, the delivery systems are generally, physical structures assuring the presentation of the vaccine antigens to the APC or stabilizing and releasing the antigen over an extended period of time. Vaccine delivery systems are mostly particulated e.g. emulsions, microparticles, ISCOMS, liposomes. Alternatively, we could also consider a delivery system with viral or bacterial vectors; *Salmonella typhi*, for example, is widely used for mucosal delivery.

The aim of the "Antigen-Delivery System" workshop was to discuss different and novel vehicles to target the antigens to the APC and/or release the antigen over an extended period of time. This session of Adjuvant 2001 included five oral presentations and eight posters.

As a part of the oral presentations Dr. OG Gómez-Duarte from the Department of Pedriatrics in Sinai Hospital, Baltimore showed the results after a Phase I clinical trial with an attenuated strain of *S. typhi* expressing fragment C from tetanus toxin. Dr. Claude Leclerc from Institute Pasteur, France gave an interesting presentation on detoxified adenylate cyclase (CyaA) toxin from *B. pertussis* carrying CTL epitopes and Dania Vázquez from the Center for Genetic Engineering, Cuba showed her work using Fowlpox viruses as a carrier vector for HIV-1 multi-CTL epitope polypeptides. Dr. HM Vordermeier from VLA Weybridge, Addlestone, UK proposed a *Mycobacterium bovis* synthetic peptide vaccine adsorbed onto nano- and microparticles to control tuberculosis in cattle. Lastly, Dr. Yasmin Thanavala from Roswell Park Center Institute, Buffalo, USA presented a novel and surprising edible Hepatitis B Virus vaccine in plants, using *Agrobacterium* plasmid where the HBsAg coding DNA was cloned.

Dr. W.G Metzger from Max-Planck-Institut für Infektionsbiology, Berlin, Germany represented the poster session showing a pilot study with the *S. typhi* vaccine expressing *Helicobacter pylori* urease, followed by the tetanus vaccination using Adenovirus vectored nasal and epicutaneous vaccines by Dr. B. Bjarnason from University of Alabama, USA. A. Acosta and M.E Sarmiento, both from Finlay Insti-

tute, Cuba presented the expression of a B subunit of cholera toxin in BCG and *Salmonella typhi*, respectively. L. González and E. Muñoz from Finlay Institute, Cuba and LL. Ruiz and A. Pérez from the Center for Genetic Engineering and Biotechnology, Cuba presented four posters on particulated systems as vehicles to deliver antigens. Those results included natural biopolymer A, biodegradable microparticles based on Poly (D, L lactide-co-glycolide), bio-adhesive polymers and acemannan plus calcium salts.

Oral Presentations

Dr. Oscar Gómez-Duarte *et al.*, used *Salmonella* as a vaccine vector for heterologous antigens and they constructed an attenuated CVD 908htrA vaccine strain, able to induce strong cellular and humoral immune responses in mice and humans following mucosal immunization. They cloned and expressed a variety of foreign antigens in CVD 908htrA including, tetanus and diphtheria toxoids, and *Plasmodium falciparum* antigens and mice immunized with these bacterial constructs by the intranasal route that induced specific immune responses to these antigens indicating that *S. typhi* can express and deliver heterologous antigens *in vivo* and successfully present such antigens to the mammalian immune system. Phase I clinical studies showed that CVD 908htrA expressing fragment C of tetanus toxin also elicited specific antibody responses not only to the bacteria itself but also to the Fragment C of the tetanus toxin. The titer of anti-tetanus toxin antibody response elicited by the *S. typhi* vaccine construct was equivalent to the titer attained by the conventional tetanus vaccine.

Dr. Claude Leclerc showed the possibility of the rational design of new vectors based on the Adenylate cyclase (CyaA), one of the major toxins produced by *Bordetella pertuss*i that is able to enter eukaryotic target cells. The catalytic domain (AC) is located within the first 400 amino acids and the carboxy-terminal 1306 residues are responsible for binding the toxin to target cell membranes and the subsequent delivery of the catalytic moiety into the cell cytosol. Exogenous peptides can be inserted into various permissive sites within the catalytic domain of CyaA without hampering its ability to enter eukaryotic cells. One particular permissive site located in the center of the catalytic domain between amino acid 224 and 225 of CyaA, has been used for the construction of recombinant toxins harbouring $CD8^+$ T cell epitopes. Using various APC, they showed that $CD8^+$ T cell epitopes genetically inserted into the AC domain of detoxified CyaA molecules are presented to $CD8^+$ T cells by a mechanism requiring 1) proteasome processing; 2) TAP and 3) neosynthesis of MHC class I molecules. *In vivo*, detoxified CyaA toxoids carrying viral or tumoral $CD8^+$ T cell epitopes induce protective anti-viral and anti-tumoral CTL responses. Moreover, CyaA hybrid molecules were shown to induce both CTL and $CD4^+$ T cell responses, characterized by IL-2 and IFN-γ production, indicative of a Th1-like cytokine profile. They also explored the capacity of the CyaA vector carrying several different $CD8^+$ T-cell epitopes inserted into sites previously identified to stimulate CTL responses. Each of these epitopes was processed upon delivery by CyaA and *in vivo*, in the case of the CyaA toxoid carrying the polyepitope triggered specific CTL responses for each of the three epitopes. These results highlighted the potency of the adenylate cyclase vector to induce protective CTL responses with multiple specificity and/or broad MHC restriction. It was also demonstrated that the CyaA binds to target cells via the a_Mb_2 integrin (CD11b/CD18). Thus, the cellular specificity of CyaA allows its specific targeting to dendritic cells *in vivo*.

Another way to deliver antigens into the cytosol of APCs is the use of viral vectors such fowlpox virus, a member of Poxvirus family that replicates in the cytoplasm. Dania Vázquez *et al.*, obtained recombinant fowlpox viruses expressing multi-epitope polypeptides (MEP) from HIV-1: TAB9 (FPTAB9LZ) and CR3 (FPCR3). Gene tab9 encodes for a protein with six copies of the V3 loop from HIV-1 isolates: LR150, JY-1, RF, MN, BRVA, IIIB, joined by AGGGA sequence and fused to the N-terminal of P64K protein from *Neisseria meningitidis*. Gene cr3 encodes for multiple epitopes from HIV-1 proteins, targeted for helper or cytotoxic T responses (gp120, gp41, vpr, RT, Nef, p24). Balb/c mice immunized with FPTAB9LZ developed a potent Th1 and cellular responses, demonstrated by Cr^{51} release assay, IFN-γ ELISPOT, and ELISA for IFN-γ and IL4 cytokines, mediated by CD8+ lymphocytes and mainly directed against a 9 mer immunodominant epitope from IIIB strain. This response was responsible for the 2.7 to 4.7 log decreases in ovary titres, when mice were challenged with a vaccinia virus expressing a similar MEP. Balb/c mice immunization with FPCR3 also induced an IFN-γ secreting-cells response against CR3, Gag and Nef proteins in ELISPOT. A pilot therapeutic trial in HIV-1^+ subjects receiving HAART, with FPCR3 will be started soon.

One approach for the efficient delivery of a peptide vaccine is the use of structures that ensure that antigens are slowly released and are protected from a rapid clearance. Dr. HMVordermeier talked about the need for a novel vaccine that protects cattle against bovine tuberculosis. They chose a peptide vaccine composed by promiscuous epitopes adsorbed in nano- and microparticles as a vaccine. They previously identified two immunodominant *M. bovis* antigens, ESAT-6 and CFP-10 that are frequently recognized by bovine T cells in a MHC promiscuous manner and also demonstrated to be 'xeno-promiscous'. Expanding their observation of cross-species promiscuity further, they demonstrated that human MHC binding prediction programs were able to predict 8/10 promiscuous epitopes that were empirically identified in *M. bovis* infected cattle indicating that such programs can also be used to predict bovine promiscuous epitopes.

The new approach of an oral (Edible) vaccine against Hepatitis B was presented by Dr. Yasmin Thanavala who exposed the disadvantages of current needle stick-based vaccines, mainly in very poor and distant populations, where the cold chain to preserve the vaccine doses is a limitation. Moreover, the impossibility to vaccinate at the same time a high number of people, the increased cost of vaccines because of the devices or the infections associated with the vaccination procedure in rural and poor areas are additional disadvantages. The possibility to use plants

expressing antigens as a "vaccine" opens new feasible possibilities. She spoke of experience expressing virus-like particles of HBsAg in potatoes. Experiments in mice showed the appearance of an antibody response and the establishment of an immunological memory, which supports this strategy as one possibility for certain vaccines in the future.

Poster Session

Bacteria and viral vectors. Dr. WG Metzger *et al.*, showed the results of a pilot study of a *Salmonella typhi* (Ty21a) vaccine expressing *Helicobacter pylori* urease (Ty21a (pDB1)). Ten out of 12 volunteers developed humoral immune responses to the *Salmonella* carrier but only two volunteers seroconverted. A total of 5 volunteers showed responses in one or two out of three assays for cellular responses to the carrier, and of the volunteers that had received Ty21a (pDB1) three showed a weak but significant T cell response to *Helicobacter* urease, while no volunteer had detectable humoral responses to urease. Interestingly, two of the responders to urease had previous contact to *S. enterica* serovar Typhi suggesting that pre-existing immune responses to the *Salmonella* carrier might enhance responses to heterologous antigens. Dr. B Bjarnason *et al.*, showed protection against tetanus by needle-free inoculations of adenovirus-vectored nasal and epicutaneous vaccines. They reported that the administration of a single intranasal dose of this vaccine was 100% protective against the intramuscular injection of 6 x 10^3 *C. tetani* cells, and that the administration of a single dose topically was protective in 80% of the mice with the protection rising to 100% when three doses were administered topically. The titres of anti-tetC antibodies of animals elicited by epicutaneous inoculation were relatively low when compared to those elicited by intranasal inoculations. Intranasal inoculation could completely overcome the pre-existing immunity to adenovirus, while the epicutaneous route could partially overcome it. The efficiency of vector absorption into the skin was very low, with approximately 1 in 4,000 particles being taken within one hour of the topical application. No adverse effects associated with the inoculation were observed in any of the immunized mice during the course of these studies. A Acosta *et al.*, expressed the B subunit of cholera toxin in BCG and Balb/c mice immunised with the recombinant BCG showed a vigorous lymphoproliferative response in splenic lymphocytes in the presence of CTB. A priming effect of the specific humoral response was demonstrated in mice immunised with the recombinant BCG followed by a booster with CTB. M.E. Sarmiento *et al.*, also expressed the B subunit of cholera toxin but in *Salmonella typhi* Ty21a. The immunisation of mice with the recombinant vaccine by the oral route showed an increased protection capacity after the challenge with *Salmonella typhi* Ty2 compared with the immunisation with *Salmonella typhi* Ty21a, inactivated cells or polysaccharide Vi.

Biopolymers. The biopolymer A is a polysaccharide obtained from natural sources to which immunostimulating properties have been adduced. L. González *et al.* evaluated its adjuvanticity using the *Leptospire* particulate antigen, exploring different concentrations and routes of immunization. The best results were with the vaccine formulation containing 2 mg/mL intramuscularly and the challenge assay demonstrated a protecting effect by inducing antibodies in a similar way to that of the preparation adjuvated in aluminium hydroxide. E. Munoz *et al.* characterized poly (D, L lactide-co-glycolide)-based microspheres by size, morphology, surface absorbed substances, encapsulation efficiency and release kinetics and LL. Ruiz *et al.* evaluated the influence of some bio-adhesives polymers and their viscosity on releasing the IFN alpha 2b across synthetic membranes. They showed that viscosity did not significantly affect the IFN alpha 2b biological activity, but the releasing profile notably varied when the viscosity of the polymers was increased. The effect of acemannan and calcium salts on the immune response against different antigens exposed by A. Pérez, closed the poster session. Different formulations of acemannan (0.3 mg/mL), calcium oxalate (0.5 mg/mL) and calcium phosphate (0.5 mg/mL) were administered alone or in combination with the HBsAg and TAB9 antigens and through different immunization routes in mice. They concluded the administration route and the type of antigen are important issues in the behaviour of the resulting formulation.

Summary

Besides targeting an antigen or DNA to APCs, delivery systems may enhance vaccine stability *in vivo* and provide a depot effect, whereby the antigen is kept in the site for continual immune stimulation. For mucosally delivered vaccines, this may enable efficient presentation and uptake by M cells, followed by transcytosis into Peyer´s patches and presentation to lymphocytes for the induction of mucosal immunity. For certain formulations, the vaccine may be maintained inside a physical structure for a long period of time, releasing the antigen slowly in order to function as a one-dose vaccine. The discussion of these issues was fulfilled by this session where different strategies were presented. The use of recombinant bacterial or viral vectors for antigen delivery was accompanied by the use of biopolymers and microparticles as physical vehicles and the oral vaccine for HBV in row potatoes, which open a new path in vaccinology. The discovery of and experimentation with new adjuvants and delivery systems may allow the development of vaccines against infectious agents that do not naturally elicit protective immunity or the improvement of current vaccines.

Oral Presentations

Attenuated *Salmonella* vaccine as a vector for Heterologous antigens

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Typhoid fever caused by Salmonella enterica serovar Typhi *(S. typhi)* is associated with significant

morbidity and mortality in poor areas around the world. Typhoid fever vaccines are recognized as an important public health tool to prevent infections from highly antibiotic resistant *S. typhi* strains. We constructed the attenuated S. Typhi CVD 908htrA vaccine strain, an aroA-aroD-htrA triple mutant capable to induce strong cellular as well as humoral immune responses in mice and humans following mucosal immunization. We cloned and expressed a variety of foreign antigens in CVD 908htrA including, tetanus and diphtheria toxoids [1], and Plasmodium falciparum antigens [2]. Mice immunized with these bacterial constructs by the intranasal route induced specific immune responses to these antigens indicating that S. Typhi can express and deliver heterologous antigens in vivo and successfully present such antigens to the mammalian immune system. Phase I clinical studies showed that CVD 908htrA expressing fragment C of tetanus toxin also elicited specific antibody responses not only to the bacteria itself but also to the fragment C of tetanus toxin. The titer of anti-tetanus toxin antibody response elicited by *S. typhi* vaccine construct was equivalent to the titer reached by the conventional tetanus vaccine [3]. More phase II and phase III clinical studies will be necessary to demonstrate that *Salmonella* vectors may deliver heterologous antigens and elicit protective immune responses to microbial pathogens from bacterial and parasitic origin.

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Rational design of new vectors

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Adenylate cyclase (CyaA), one of the major toxins produced by *Bordetella pertussis*, is able to enter eukaryotic target cells where, upon activation by endogenous calmodulin (CaM), it synthetizes high levels of intracellular cAMP, that cause impairment of cellular functions. The CaM-dependent adenylate cyclase catalytic (AC) domain is located within the first 400 amino acids. The carboxy-terminal 1306 residues are responsible for the binding of the toxin to target cell membranes and the subsequent delivery of the catalytic moiety into the cell cytosol. Exogenous peptides can be inserted into various permissive sites within the catalytic domain of CyaA without hampering its ability to enter eukaryotic cells. One particular permissive site located in the middle of the catalytic domain between amino acid 224 and 225 of CyaA, has been used for the construction of recombinant toxins harboring $CD8^+$ T cell epitopes. Using various APC, we showed that $CD8^+$ T cell epitopes genetically inserted into the AC domain of detoxified CyaA molecules are presented to $CD8^+$ T cells by a mechanism requiring 1) proteasome processing; 2) TAP and 3) neosynthesis of MHC class I molecules. *In vivo*, detoxified CyaA toxoids carrying viral or tumoral $CD8^+$ T cell epitopes induce protective antiviral and anti-tumoral CTL responses. Moreover, CyaA hybrid molecules were shown to induce both CTL and $CD4^+$ T cell responses, characterized by IL-2 and IFN-γ production, indicative of a Th1-like cytokine profile. We have also explored the capacity of the CyaA vector carrying several different $CD8^+$ T-cell epitopes inserted into sites previously identified to stimulate CTL responses. Each of these epitopes was processed upon delivery by CyaA and *in vivo*, the CyaA toxoid carrying the polyepitope triggered specific CTL responses for each of the three epitope. These results highlighted the potency of the adenylate cyclase vector to induce protective CTL responses with multiple specificity and/or broad MHC restriction. Very recently, we also demonstrated that the CyaA binds to target cells via the a_Mb_2 integrin (CD11b/CD18). Thus, the cellular specificity of CyaA allows its specific targeting to dendritic cells *in vivo*. Thus, altogether, these results demonstrate that CyaA is targeted to professional APC, leading to an exceptional capacity to induce immune responses.

Induction of specific cellular response after the immunization of BALB/c mice with recombinant fowlpox viruses expressing HIV-1 multi-epitope polypeptides

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The vaccination with avipoxvirus vectors has been an effective approach to generate a cellular response against HIV-1, with the additional advantage to be safe in humans where they do not replicate. We obtained recombinant fowlpox viruses expressing multiepitope polypeptides (MEP) from HIV-1: TAB9 (FPTAB9LZ) and CR3 (FPCR3). Gene *tab9* encodes for a protein with six copies of the V3 loop from HIV-1 isolates: LR150, JY-1, RF, MN, BRVA, IIIB, joined by AGGGA sequence and fused to the N-terminal of P64K protein from *Neisseria meningitidis*. Gene *cr3* encodes for multiple epitopes from HIV-1 proteins, targeted for helper or cytotoxic T responses (gp120, gp41, vpr, RT, Nef, p24). Balb/c mice immunized with FPTAB9LZ developed a potent cellular response, demonstrated by Cr^{51} release assay and IFN-γ ELISPOT, mediated by $CD8^+$ lymphocytes and mainly directed against a 9mer immunodominant epitope from IIIB strain. This response was responsible of the 2.7 to 4.7 log decreases in ovaries titres, when mice were challenged with a vaccinia virus expressing a similar MEP. Balb/c mice immunization with FPCR3 also induced an IFN-γ secreting-cells response against CR3, Gag and Nef proteins in ELISPOT. A pilot therapeutic trial in HIV-1+ subjects receiving HAART, with FPCR3 will start very soon.

Oral (Edible) vaccine for hepatitis B: from laboratory studies to clinical reality

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The incredible success of vaccination in contributing to public health is attributable, in part, to the simplicity of vaccines. For polio, the simple incorporation of attenuated polio virus on sugar cubes resulted in an oral vaccine that was universally acceptable. The impressive logistical advantage of orally administered vaccines were exemplified by two national vaccination days in 1996, when 121 million Indian children were vaccinated against polio at 650,000 immunization centers. Orally delivered vaccines made this enormous endeavor achievable at reasonable costs. Unfortunately, a simple oral formulation is not easily achieved for the new generation of subunit vaccines which hold the greatest promise for disease prevention in the twenty-first century. There is an urgent need to make the technically sophisticated field of subunit vaccines (which are almost exclusively biotechnology products) available in the poorer countries of the world where infectious diseases are still the primary cause of death.

The Children's Vaccine Initiative (CVI) called for universal immunization against major infectious diseases. The CVI focused attention upon the need for new technologies which would make vaccines cost effective and reliable in both production and delivery, especially for the developing world. However, for such a program to be effective, it must reduce dependence on a cold chain, as well as promote the development of vaccines that can be administered without needles and syringes.

In contrast to the large variety of currently available injectable vaccines that provide systemic immunity, vaccines administered non systemically are rare. Recently, however, there has been a surge of interest in developing novel strategies for vaccine development with oral and nasal delivery as the preferred routes.

In my presentation, I will describe experiments where oral immunization with transgenic plants expressing hepatitis B surface antigen has moved successfully from preclinical efforts through to a phase I clinical trial.

Micobacterium bovis-derived determinants are recognized promiscuously by bovine T cells and can be presented to the immune system in the form of synthetic peptides adsorbed to nano or micro-particles

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Novel vaccines that protect cattle against bovine tuberculosis (BTB) are needed because the only available TB vaccine, *Mycobacterium bovis* BCG, has variable protective efficacy in cattle (as in humans). In addition, BCG vaccination compromises the specificity of tuberculin-based diagnostic tests, which are central to current test and slaughter control strategies for BTB in many countries including Great Britain. Subunit vaccines, particularly those based on fully synthesized peptides, may have considerable advantages over other types of vaccines, especially in terms of quality control and safety to consumers. In order for peptide vaccines against *M. bovis* to be a viable option two pre-requisites have to be fulfilled. First, peptides that are recognized in the context of multiple cattle MHC alleles (promiscuous epitopes) have to be identified, and secondly, suitable adjuvants or delivery systems have to be employed to induce strong and lasting immunity. We have previously identified two immunodominant *M. bovis* antigens, ESAT-6 and CFP-10, that are frequently recognized by bovine T cells in a MHC promiscuous manner (1,2). The identification of these promiscuous peptides satisfies the first pre-condition for the development of peptide-based vaccines. Interestingly, T cells from *M. bovis* infected guinea pigs, mice and human TB patients also recognized the ESAT-6 epitopes that were recognized promiscuously in cattle. This suggests that some T cell determinants are recognized not only across the MHC spectrum within one species but also across host species ('xeno-promiscously'). Expanding our observation of cross-species promiscuity further, we have demonstrated that human MHC binding prediction programs were able to predict 8/10 promiscuous epitopes that were empirically identified in *M. bovis* infected cattle indicating that such programs can also be used to predict bovine promiscuous epitopes. Finally, in the search for adjuvants with which to develop peptide vaccines, we have preliminary data to indicate that adsorption of synthetic peptides onto nano- and microparticles improves antigen recognition by bovine T cells and therefore may be useful for the development of peptide-based vaccines.

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Alphavirus replicon vectors for gene-based antigen delivery to dendritic cells

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Dendritic cells (DCs) are the most potent antigen presenting cell population and play a pivotal role in eliciting the humoral and cellular immune responses central to successful vaccination. We have used two approaches to develop alphavirus-based replicon particles for efficient antigen delivery to human DCs. Alphavirus replicon particles provide an exciting platform for gene-based vaccines, with high-level transient expression of antigens and availability of stable packaging cell lines that make these vectors amenable to production in sufficient quantities for clinical testing. In one approach, variants of the prototype alphavirus, *Sindbis virus* (SIN) were derived, which efficiently transduced immature human DCs derived from peripheral blood monocytes. A specific mutation responsible for DC tropism was mapped to the E2 envelope glycoprotein gene and then engineered into SIN replicon particles, resulting in highly efficient vector transduction of immature DCs. Costimulatory (CD80, CD86) and MHC mol-

ecules were upregulated on SIN replicon transduced DCs *in vitro* and *in vivo*, highlighting the natural adjuvant activity of these vectors. In a second approach, the natural DC tropism of a pathogenic alphavirus, Venezuelan equine encephalitis virus (VEE), was exploited. Alphavirus replicon particle chimeras were engineered such that SIN replicon RNA molecules were packaged within VEE-derived envelope glycoproteins. Although interactions between SIN replicon RNA and VEE structural proteins are less efficient than their homologous counterparts, a series of defined modifications significantly increased production of such chimeric particles. These novel alphavirus systems represent a highly efficient modality for gene delivery to DCs and should facilitate the development of potent vaccines for a variety of human diseases.

Polyphosphazenes: A unique polymer platform for the rapid synthesis of adjuvants and delivery vehicles for vaccines and immunotherapies

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The development of a number of important vaccines and immunotherapies has been stymied by the inability to induce appropriate immune responses in the recipient. Adjuvants are essential components of new subunit and killed vaccines that boost the immune responses to an antigen creating protective immunity in the vaccinated individual. The only existing FDA approved adjuvant, Alum has poor immunostimulatory properties and some safety concerns. Attempts to develop new adjuvants over the last thirty years in both the academic and commercial arenas have not been successful. One of the principal problems with adjuvants to date is that they induce a specific immune response that cannot be modulated or enhanced. The increasing use of vaccines and immunotherapeutics to control a range of medical problems including infectious disease, cancer, neurological and addictive disorders, will require improved adjuvants with innovative properties. This presentation will outline the development of a flexible polymer platform for adjuvants and vaccine delivery using polyphosphazene polyelectrolytes. Polyphosphazenes are hybrid organic/inorganic polymers comprised of a backbone of alternating nitrogen and phosphorous atoms with two side groups attached to each phosphorous moiety. Polyphosphazenes can be synthesized with properties such as controlled architecture, molecular weight and critical biological characteristics like biocompatibility, safety, controlled biodegradability and immunostimulation. Moreover polymer polyelectrolytes are potent adjuvants in the aqueous phase and when conformed into hydrogel microspheres. The first generation product, Poly [di(carboxylatophenoxy)phosphazene] referred to as PCPP was licensed to Aventis-Pasteur for use in the development of injectable vaccines. PCPP in the soluble form was formulated with injectable vaccines and shown to be safe and effective in over 1000 human subjects. Aventis-Pasteur is currently in Phase II clinical development for RSV utilizing PCPP and an existing preclinical package and DMF for PCPP is on file with the FDA. Preliminary data suggests that PCPP is an effective adjuvant with a variety of viral and bacterial antigens. Parallel chemical synthesis was used to prepare twenty five structural variants of PCPP. Twenty percent of these polymers exhibited an enhancement in both the extent and nature of immune responses induced in animals. In fact a polymer was shown to generate immune responses four hundred times greater than those of PCPP. PCPP was also used to develop a proprietary microencapsulation system using aqueous coacervation and ionic crosslinking which rapidly produces uniform and monodisperse microspheres in the nanometer to micrometer range. The process is commercially scalable requiring no complex manufacturing equipment, elevated temperatures, generation of aerosols or the use of organic solvents. These microspheres were shown to generate mucosal vaccines combining efficient antigen encapsulation, mucosal delivery and adjuvant activity that induced mucosal and systemic immune responses. These combined data imply that a limited group of novel polymers will be defined that modulate the TH1/TH2 balance and provide an adjuvant and vaccine delivery platform for injectable and mucosal vaccines and immunotherapies.

Poster Presentations

Two pilot studies of a *Salmonella typhi* (Ty21a) vaccine expressing *Helicobacter pylori* urease

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Helicobacter pylori urease was expressed in the common live typhoid vaccine Ty21a yielding Ty21a(pDB1). In a first pilot study, nine volunteers received Ty21a(pDB1) and three control volunteers received Ty21a. No serious adverse effects were observed in any of the volunteers. Ten out of 12 volunteers developed humoral immune responses to the *Salmonella* carrier as detected by antigen-specific antibody-secreting cells but only two volunteers seroconverted. A total of 5 volunteers showed responses in one or two out of three assays for cellular responses to the carrier (proliferation, IFNγ-secretion, IFNγ-ELISPOT). Three of the volunteers that had received Ty21a(pDB1) showed a weak but significant T cell response to *Helicobacter* urease, while no volunteer had detectable humoral responses to urease. Ty21a(pDB1) is a suitable prototype to optimize *Salmonella*-based vaccination for efficient cellular responses that could mediate protective immunity against *Helicobacter*. Interestingly, two of the responders to urease had previous contact to *S. enterica* serovar Typhi suggesting that pre-existing immune responses to the *Salmonella* carrier might enhance responses to heterologous antigens. In a second pilot study, this hypothesis is

being tested, and results and conclusions are discussed here.

Protection against tetanus by needle-free inoculation of adenovirus-vectored nasal and epicutaneous vaccines

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Tetanus continues to be a threat to public health with more than half a million fatalities each year being associated with infection with *Clostridium tetani* and vaccination is the most effective medical intervention for protection of the public against this deadly disease. An alternative new modality is noninvasive vaccination onto the skin (NIVS) by topical application of epicutaneous vaccines. This study was undertaken to determine if administration of a vaccine consisting of an adenovirus (Ad) recombinant (AdCMV-tetC) encoding the immunogenic but atoxic tetanus toxin C-fragment (tetC) can protect against a lethal challenge with live *C. tetani* when administered either intranasally or by an epicutaneous patch. We report that administration of a single dose of this vaccine intranasally was 100% protective against intramuscular injection of 6 X 10^3 *C. tetani* cells, and that administration of a single dose topically was protective in 80% of the mice with the protection rising to 100% when three doses were administered topically. The titers of anti-tetC antibodies of animal elicited by epicutaneous inoculation were relatively low when compared to those elicited by intranasal inoculations. Intranasal inoculation can overcome the pre-existing immunity to adenovirus, while epicutaneous route can partially overcome it. The efficiency of vector absorption into the skin was very low, with approximately 1 in 4,000 particles being taken within one hour of the topical application. No adverse effects associated with inoculation were observed in any of the immunized mice during the course of these studies. This is the first time to demonstrate that topical application of epicutaneous vaccines can protect recipients against live pathogenic bacteria in a disease setting.

Expression of the B subunit of cholera toxin in BCG. Evaluation of the immunogenicity in mice

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BCG is one of the most promissory systems of antigen delivery (1-3). In order to evaluate the feasibility to express the B subunit of cholera toxin (CTB) in BCG a shuttle vector *E. coli*-mycobacteria was constructed containing the CTB gene with the signal peptide of the labile toxin B of *E. coli* (LTB) under the control of tac promoter. After the transformation by electroporation of *M. smegmatis* and Moreau BCG strain the expression of CTB was detected in the supernatant of culture. Balb/c mice immunised with the recombinant BCG shown a vigorous lymphoproliferative response of the splenic lymphocytes in presence of CTB. A priming effect of the specific humoral response was demonstrated in mice immunised with the recombinant followed by a booster with CTB. These results demonstrate the feasibility to express CTB in BCG as a potential system of delivery of antigens by different routes.

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Increase of the protective capability of *Salmonella typhi* Ty21a expresing the B subunit of cholera toxin

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The expression of heterologous antigens in *Salmonella typhi* Ty 21a is one of the options for the development of antigen delivery systems (1-3). With the objective to evaluate the impact of the expression of B cholera toxin (CTB) in the protective capability of the attenuated strain, *S. typhi* Ty 21a was transformed with a plasmid containing the gene of CTB with the signal peptide of the labile toxin B of *E. coli* (LTB) under the control of the inducible tac promoter. The transformed strain expressed CTB in the periplasmic space both constitutively and in an inducible way. After the administration by different routes (O, EV, IP) the persistence in different organs was detected. The administration of the recombinant to Balb/c mice had a priming effect on the specific humoral response. The immunisation of mice with the recombinant by the oral route shown an increase of the protection upon challenge with *Salmonella typhi* Ty2 compared with the immunisation with *Salmonella typhi* Ty21a, inactivated cells or polysaccharide Vi. These results demonstrate the adjuvant effect of the expression of CTB *S. typhi* Ty21a.

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Evaluation of immunoadjuvant capacity of biopolymer using *Leptospira* particulate antigens

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The biopolymer A is a polysaccharide obtained from natural sources to which have been adduced

immunostimulating properties. In this paper we evaluated the polymeric adjuvant´s capability by means of a *Leptospire* particulate antigen. We formulated vaccine preparations using different polysaccharide's concentrations and they were compared with the placebo formulations. We used both, the antigen adjuvanted in gel of aluminium hydroxide and without it as controls. The formulations were administrated subcutaniusly (sc) and intramuscularily (im). When evaluating the responses produced by induced antibodies using microaglutination (MAT), ELISA, and Dot Blotting, we obtained the best result with the vaccine formulation containing 2mg/mL via im. The challenge assay demonstrated that the protecting effect of induced antibodies was similar to that obtained with preparation adjuvanted with gel of aluminium hydroxide. The toxicity preliminary test suggested that polymer A was harmless and safe in the used dosage.

Influence of formulation variables on the *in vitro* release from biodegradable microparticulate systems in vaccine candidates

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Poly (D,L lactide-co-glycolide) containing BSA (bovine serum albumin) as test, tetanic toxoide, polysaccharide C of *Neisseria meningitides* and DNA were prepared by a method of solvent evaporation using double emulsion. These microspheres were characterized for size, morphology, surface absorbed substances, encapsulation efficiency and release kinetics. The influence of two formulation variables like: (the procedure to obtain the first emulsion and procedure having dried off once encapsulated the protein, polysaccharides and DNA) on the physical characteristics and the behavior of release they were also investigated. The microspheres made with lower energy of cavitation were more general spheric although there have some changes according to the nature of the substances employee too. The effects of drying end also had its influences in the physical characteristics from the one encapsulated to other being notably different the process for drying with Speed Vacuum to Liophilizationn method being significantly better this last .

Effect of viscosity of bio-adhesive polymers on IFN alpha 2b release across synthetic membranes

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Since 1976 scientists began to use interferon (IFN) as an adjuvant therapy after the surgical treatment of respiratory papillomatosis, showing effectiveness for the relapse control of the disease [1]. Other pathologies such as multiple myeloma and colon cancer, have also been treated with IFN as adjuvant, often using the parenteral via for the delivery of the cytokine. Bioadhesive polymers have increasingly been thought as a good tool for the development of controlled release systems. These polymers can enhance drug release through an optimal contact with adsorption surfaces. In addition, they can extend the drug residence period, reducing the therapeutic doses that are needed in the treatment [2]. Other administration routes (nasal, sublingual, ocular, rectal) are potentially attractive to be evaluated using formulations based on these polymers. In this work, we evaluated the influence of some bioadhesives polymers as well as their viscosity on releasing IFN alpha 2b across synthetic membranes. Release of the cytokine was evaluated on a homemade cell designed from the Formulation Development Department (CIGB, Havana, Cuba). The concentration of IFN alpha 2b was determined by a sandwich-type enzyme-linked immunosorbent assay (ELISA). We also ascertained the effect of these polymers on the physical/chemical (by RP-HPLC and SDS/PAGE) and biological stability of IFN alpha 2b. The later parameter was estimated by determining the inhibition of the cytopathic effect (ECP) produced by the Mengo virus on Hep-2 cells (ATCC No. CCL23).Viscosity did not significantly affect the IFN alpha 2b biological activity, but the releasing profile notably varied when the viscosity of the assayed polymers increased.

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Effect of acemannan and calcium salts on the immune response against different antigens

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The development of more effective vaccines against viral diseases is a priority of international health organizations (WHO, PHO). Efforts are being made to reduce the number of administrations and to modulate the immune response to vaccine antigens to increase their efficcacy. In this work, we studied the effect of acemannan and calcium salts on the immune response induced against several proteins. Different formulations of acemannan (0.3 mg/mL), calcium oxalate (0.5 mg/mL) and calcium phosphate (0.5 mg/mL) were administered alone or in combination with the antigens and through different routes of immunization, in mice. Total IgG antibody titers were measured by ELISA, after each dose. A mixed pattern of antibody subclass was evidence for the HBsAg. A prevalence of IgG2a subclass was observed for TAB 9, a recombinant polypeptide coding for different V3 peptides. In general we conclude that the route of administration and the type of antigens are important issues in the behaviour of the resulting formulation.

Workshop on Mucosal Adjuvants

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ABSTRACT

Several adjuvants and adjuvant strategies were presented in the session Mucosal Adjuvants. Two oral presentations were based mainly on the work with the attenuated strain of *Vibrio cholerae* 638 (El Tor Ogawa) as a vaccine candidate for cholera. A new CTX?-negative hemagglutinin/protease defective candidate cholera vaccine strain was examined for safety and immunogenicity in healthy adult Cuban and Ecuadorian volunteers. The Cuban first generation attenuated strain 81 and JBK70 was inoculated in a group of Cuban volunteers as the reactogenic control. In a double-blind placebo controlled study, no significant adverse reactions were observed in volunteers ingesting strain 638. Four volunteers out of 42 which ingested the 638 strain and 1 out of 14 placebo experimented loose stools. *V. cholerae* 638 at doses ranging 4 x 10^7 to 2 x 10^9, elicited significant serum antibodies shown by vibriocidal and ELISA titers and anti-Ogawa IgA antibody secreting cells in Cuban and Ecuadorian adult volunteers.

The mucosal response was evaluated in a second paper on the 638 cholera strain. The IgA secreted in saliva was analyzed and detected with maximum values at day 9 after inoculation. These results suggested that the 638 strain induces a good mucosal response.

Different vaccine formulations based on HBV antigens were presented. The purpose of one study was the evaluation of the use in humoral immunity with a combined formulation of hepatitis B virus antigens in mice administered nasally with different formulations. The systemic and mucosal responses were characterized for the acemannan/HBsAg and HBcAg/HBsAg formulations. A high immunogenicity of recombinant HBcAg after nasal inoculation was also evaluated and demonstrated as well as the general high immunogenicity of the formulations under study. A strong adjuvant effect of HBcAg on HBsAg immunogenicity in serum and vaginal secretions, similar in intensity to CT and acemannan was evidenced. A higher and significant increase was found in the IgG2a/IgG1rate against HBsAg for the group immunized with the HBcAg/HBsAg combination, following the Th1-like response also observed for the HBcAg subclass analysis. These results have implications on the design of mucosal therapeutic and preventive vaccines against HBV infection.

In a last paper, a mucosal administration of the Hepatitis C core was also studied and evidenced the same effects as the previously described interaction of HbcAg and HbsAg on the resulting immune response.

Oral Presentations

638 attenuated strain cholera candidate vaccine

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Vibrio cholerae 638 (El Tor Ogawa) a new CTX?-negative hemagglutinin/protease defective candidate cholera vaccine strain, was examined for safety and immunogenicity in adult healthy Cuban and Ecuadorian volunteers. Cuban first generation attenuated strain 81 and JBK70 was inoculated in a group of Cuban volunteers as reactogenic control. In a double-blind placebo controlled study, no significant adverse reactions were observed in volunteers ingesting strain 638. Four volunteers out of 42 which ingested 638 strain and 1 out of 14 placebo experimented loose stools. The strain colonized well the human small bowel as evidenced by it isolation from the stools of 37 out of 42 volunteers inoculated. JBK70 and 81 strains orally administered showed a significant adverse reaction meanly in the number and severity of diarrheic events. No difference was observed between the isolation from stools of volunteers inoculated with 638 or two others strains. The immunogenicity was evaluated in inoculated human sera by vibriocidal and ELISA test and in blood by ELISPOT for detecting anti-Ogawa IgA antibody secreting cells. Vibriocidal test were done against VC12 strain (El Tor Ogawa) and plates of ELISA and ELISPOT tests were coated with purified LPS. *V. cholerae* 638 at doses ranging 4 x 10^7 to 2 x 10^9, elicited significant serum antibody showed by vibriocidal and ELISA titers and anti-Ogawa IgA antibody secreting cells in Cuban and Ecuadorian adult volunteers.

Mucosal response to 638 attenuated cholera strain

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Cholera is a serious health problem in developing countries. Vaccines are not yet available, but they under development will be administered by mucosal route in order to induce a protective IgA response. In Cuba, a 638 attenuated cholera strain has been developed. This vaccine candidate induces a good response in animal and in preliminary human study has proven to be safe and immunogenic. However, the specific IgA at mucosal sites and comparison of 638 strain with the known reactogenic JBK70 strain are not demonstrated. Therefore, we evaluated the production of anti-LPS (El Tor Ogawa) anti-

body secreting cells (ASC) by ELISPOT in the blood and the anti-LPS antibodies in the parotid saliva by ELISA to explore mucosal responses in human volunteers inoculated with one or other strains. Three groups of 638 (10^9, 10^8, and 10^7 CFU), one group of JBK70 (10^9 CFU) and one placebo group were enrolled. The IgA+ASC response predominated and IgG+ASC was only found with the higher doses. The salival secretory IgA was consistently detected with maximum values at day 9 after inoculation. These results suggest that salival IgA is locally produced. In conclusion, our results show that 638 induces a good mucosal response similar to those induced by JBK70 strain.

Combined formulation of Hepatitis B Virus antigens as a nasal vaccine candidate

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The purpose of this study is the evaluation of the humoral immunity raised with a combined formulation of Hepatitis B Virus antigens.We have immunized groups of 8 to 10 female Balb/c mice with different formulations containing HBsAg and HBcAg through the nasal route in a total volume of 50?L per mice. The IgA, IgG, IgG1 and 2a titers in sera and vaginal washes were determined by ELISA. The interaction between HBsAg and HBcAg on HBsAg immunogenicity was carried out using as control adjuvants cholera toxin (CT) and acemannan. Initially we demonstrated a high immunogenicity of recombinant HBcAg after nasal inoculation. Then we evaluated the effect of antigen combination on HBsAg immunogenicity co-administering both antigens nasally. We observed a strong adjuvant effect of HBcAg on HBsAg immunogenicity in serum and vaginal secretions, similar in intensity to CT and acemannan. We also achieved a higher and significant increase in the rate IgG2a/IgG1 against HBsAg for the group immunized with HBcAg and HBsAg, following the Th1-like response also observed for HBcAg subclass pattern. We conclude that the inoculation of the soluble formulations of HBsAg and HBcAg enhanced the immunogenicity of HBsAg, in serum and mucosal secretions, and modulated the IgG subclass pattern to the Th1-like pattern.

Poster Presentations

Intranasal immunisation of Balb/c mice with recombinant *Micobacterium tuberculosis* antigens using cholera toxin (CT) as adjuvant

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Many attempts are being done for the development of new generation vaccines against tuberculosis [1, 2]. In order to evaluate the immunogenicity and the protective effect of the administration of a mixture of recombinant M. tuberculosis antigens (85B, L7/L12, 27 kD, esat-6) plus CT, Balb/c mice were immunised by the nasal route. In the immunised animals there were a significative increase of the specific IgG antibodies against L7/L12, 85B, 27 kD and esat-6 antigens. A protective effect upon challenge with BCG was demonstrated in the immunised animals although this effect was lower than the results obtained in the group immunised with BCG.

1. Horwitz MA, *et al*. Proc Natl Acad Sci USA 97:13853-8.
2. Hess J, *et al*. FEMS Immunol Med Microbiol 27:283-9

Inhibition of anticolonizing effect of positive serum from humans inoculated with the attenuated strain 638 *Vibrio cholerae* O1

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As a part of the study to obtain an oral vaccine against cholerae, the anti-colonizing capacity of the human serum, extracted after an oral dose with the attenuated strain 638, was evaluated. Inhibition studies with somatic antigens of *Vibrio cholerae* O1 like, Ogawa and Inaba lipopolysaccharide (LPS), outer membrane proteins (OMP) and the 20 kDa antigenic complex as well as the LPS of the O139 serogroup, were done.The neonatal mice model was utilized. The positive serum showed a high anti-colonizing activity. In the antigen inhibition studies, was observed that the Ogawa LPS reduced the anti-colonizing capacity of the positive serum. The 20 kDa antigenic complex, the Inaba LPS and the OMP showed a low and differential inhibitory effect. These results confirm that the most protective antigen is the homologous LPS, although there are others cellular surface antigens involved in the protective immunity induction. The results here described contribute to understand the protective immunity mechanism against *vibrio cholerae* and stimulate to use of conjugated and subunits vaccine.

Nasal monovalent vaccine formulation of HBsAg against Hepatitis B Virus infection

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The aim of this work was the evaluation of the humoral immunity raised with a monovalent formulation of Hepatitis B surface antigen (HBsAg) and the natural polysaccharide acemannan to obtain a nasal vaccine candidate improving the immune response in systemic compartments and mucosal tissues against the HBV. Groups of 8 Balb/c mice 8 to 12 weeks old, were immunized three times, 2 weeks apart by nasal and systemic routes, with equal doses of HBsAg. The concentration of acemannan was determined by the Antrona colorimetric method. IgG and IgA titers in sera and vaginal washes were measured by ELISA. Titers were compared using the Student's t test. Serum IgG and secreted vaginal IgA response for the acemannan group was higher than that obtained with HBsAg in saline-

phosphate buffer. No difference was observed for the seric IgG titers after i.m. and s.c. inoculations using the same dose of antigen in alum after three inoculations. Nasal inoculations of HBsAg-acemannan formulations gave a high length and stronger responses compared to cholera toxin and alum formulations. In conclusion, we have demonstrated in mice that using acemannan-HBsAg formulation, the nasal route can be as efficient as systemic routes in the induction of strong and high length anti-HBsAg antibody responses in sera, with the advantage of inducing strong mucosal responses.

Mucosal immunogenicity of HBcAg, a carrier protein

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In this study we explored the immunogenicity of the recombinant hepatitis B virus core antigen (rHBcAg) in Balb/c mice by several mucosal immunization routes. Four doses of 5 µg each were administered at days 0, 15, 30, and 210. We evaluated the IgG antibody response kinetic and studied the IgG subclasses response in serum and the IgA antibody response in mucosal lavages after four doses by ELISA. The immunogenicity of the rHBcAg by the several mucosal immunization routes was high, resulting the intranasal route the one that generated the highest response. The intranasal route also generated the higher specific IgA response at mucosal level. In general, the obtained results strongly suggest that the immunogenicity of the rHBcAg by mucosal routes follows the decreasing order: [intranasal]>> [intrarrectal]> [sublingual]> [oral]> [intravaginal], being the intranasal route able of generating responses of strong intensity as the one generated by systemic route.

HCV core, a mucosal adjuvant

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The capacity of both the entire Hepatitis C virus (HCcAg) produced in *P. pastoris* and a truncated variant comprising the first 120 aa (HCc.120) produced in *E. coli* to form particles was studied. Size exclusion chromatography suggested that HCcAg and HCc.120 assembled into high molecular weight structures [1, 2]. A serum from a chronic HCV carrier patient also specifically recognized them. Both antigens migrated with buoyant density values similar to those obtained for native nucleocapsid particles from infected patients when analyzed using sucrose density gradient centrifugation. The analysis by electron microscopy of purified HCcAg and HCc.120 showed aggregates resembling virus-like particles (VLPs) with an average diameter of 30 nm. These results indicated that these antigens assembled into VLPs resembling HCV nucleocapsid particles in a mature stage. Afterwards we study the *in vitro* self-assembly properties of HCcAg. HCcAg was purified as a low molecular weight species by electroelusion under denaturalizing conditions for confirmation of its self-assembly properties. After renaturalization, the electron microscopy showed that HCcAg assembled into spherical particles of 30 nm. HCcAg also showed homogeneity and was specifically recognized by a serum from a chronic HCV carrier patient. The data indicated that *in vitro* assembly of HCcAg into virus-like particles resembling HCV nucleocapsid particles in a mature stage is an intrinsic quality of this protein. Finally, HCc.120 obtained as a VLP in *E. coli* was coinoculated nasally with HBsAg [3]. This formulation evidenced a synergistic effect on the immunogenicity of both antigens suggesting its usefulness as a mucosal adjuvant.

1. Acosta-Rivero N, *et al*. Biochem Biophys Res Commun. Sep 14;287(1):122-5.
2. Lorenzo L, *et al.* Biochem Biophys Res Commun 281,962-965.
3. Aguilar JC, *et al*. CU 98/ Dec, PCT/CU/99/00006.

Workshop on Adjuvants for Viral Vaccines

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ABSTRACT

The oral session was a scheduled debate over several strategies to deal with different viral infections in man and animals. There were presentations on studies in mice to increase cross-reacting antibodies to HIV-1; afterwards, delegates were alerted to a potential drawback of Morbillivirus vaccines; also, DNA vaccination for BHV-1 using a lipopolysaccharide from Gram-negative bacteria (RN-205) was subjected to discussion; and finally, also the immune regulation of hepatitis B vaccine by MAbs. The poster session was devoted to study the mouse as a model for screening the inactivated BHV-1 vaccine using different adjuvants (SL-CD/S/W, Acridine, RN-205 and oleous adjuvant); the use of Algammulin as an adjuvant for HBsAg in mice; the use of several additives ($CaCl_2$, PEG-6000, Freund's Adjuvant, sonicated Calf Thymus DNA and Co.120) for a naked-DNA coding HCcAg; also, the protection induced by a recombinant envelope protein from Dengue virus was presented; additionally, the adjuvant effect of a truncated HCV core protein regarding the E2 protein was illustrated; and finally, preliminary results were shown on the possible mechanisms of virus clearance from the Central Nervous System of mice infected with the Measles virus. It was an heterogeneous workshop considering that discussions were on different viruses; but that was in fact the strongest point. This combination provided an exceptional opportunity to exchange experiences.

Introduction

The aim of the workshop was to discuss practical approaches to viral vaccines stressing the use of classical and novel adjuvants. Chairpersons in this session were Dr. SL Cosby (The Queens University of Belfast, United Kingdom) and E Iglesias (Center for Genetic Engineering and Biotechnology, Cuba). There were four oral presentations and six posters.

Main body

The oral debate started with the lecture "Increased immunogenicity and cross-reactivity induced by the conjugation of V3-based multiple antigen peptides (MAP) to carrier proteins" by E Iglesias (Center for Genetic Engineering and Biotechnology, Cuba). He showed promising results using synthetic peptides for inducing antibodies with a capacity to positively recognize highly variable V3 peptides from HIV-1. From his work it was concluded that a possible approach to increase the generation of cross-reacting antibodies, at least in mice, may be through the conjugation of MAPs to carrier proteins. However, the carrier protein exerts its influence on the quality of antibodies. Because of the flexibility of the synthetic approach different presentation forms to the immune system were explored. It was shown that the mixture of V3-MAP and CD4bp-MAP (CD4bp: CD4 binding peptide from gp120 of HIV-1) conjugated to different carrier molecules enhances the anti-V3 antibody response and the level of cross-reacting antibodies. However, the best response is obtained using a single "heterologous" CD4bp-V3-MAP conjugate.

At the second hour Dr. SL Cosby (The Queens University of Belfast, United Kingdom) gave the talk "Morbillivirus vaccines and virus-induced immunosuppression". Morbillivirus infections induce severe disease in their natural host, causing high morbidity and mortality. Virus-induced immune suppression may influence the high mortality. She showed data on the co-expression on the surface of presenter cells of the fusion protein (F) and the hemagglutinin (H) for all members of Morvillivirus as the cause of immune suppression. Her results showed that vaccine strains of RPV in cattle and peste des petite ruminants virus (PPRV) in goats, cause immunosuppression by this mechanism. Therefore, H and F protein interactions must be considered when designing recombinant morbillivirus vaccines and the use of adjuvants.

The session continued with Dr. P Zamorano's (Centro de Investigación en Ciencias Veterinarias, Argentina) talk "DNA Vaccination of mice against BHV-1: effects of the RN-205 adjuvant in the modulation of the immune response". In her work the speaker studied the influence of the RN-205 adjuvant (lipopolysaccharide from Gram-negative bacteria) on the cellular and humoral immune response of three DNA vaccines for BHV-1. RN-205-treated mice showed higher levels of proliferation, and IL-4 and γ-IFN secreting cells in comparison to untreated mice.

At the end Dr. A Basalp (Research Institute for Genetic Engineering and Biotechnology, Turkey) talked on "Regulation of the immune response to hepatitis B virus vaccine by monoclonal antibodies". In spite of the success of commercially available HBV vaccines prepared with alum several immunizations are required to achieve a protective response. Also, they are less effective in the elderly and immune compromised people. So, there is still a growing need for vaccine systems that effectively enhance the immune response. Dr. Basalp analyzed the antibody response elicited by both the IgM and IgG-HBV vaccine (GenHevac B Pasteur, France) immunocomplex in mice. An enhanced immune response was observed when the HBV vaccine was complexed to IgM in a range from 1 to 5 mg. In the case of IgG an antibody excess was necessary; however, at concentrations equivalents to the vaccine an enhance immune response was observed after the second dose. Then, it was concluded that monoclonal antibodies coupled to HBV vaccines might be a useful strategy to improve their effect.

In the poster session Dr. Z Cinza (Center for Genetic Engineering and Biotechnology, Cuba) showed data regarding the use of two Algammulin formulations as adjuvants for the HBsAg in mice. It was concluded that the AG-43F (fine formulation; 1 mm) was superior to the AG-38ff (ultra fine formulation; < 1 mm) and to the vaccine in alum. On the other hand, Dr. L Álvarez from the same institute studied the effect of different additives on the antibody response to a naked DNA immunization using the plasmid pIDKCo coding for a truncated HCV core protein. For the tested additives PEG-6000, Freund´s adjuvant and sonicated calf thymus DNA elicited higher antibody responses regarding the DNA in PBS and no difference was evident among them. A positive IgG2a/IgG1 ratio was found in all cases. In another poster Dr. L Hermida again from the same Institute in Havana City assessed the protective effect of a recombinant envelope protein form Dengue virus 4 produced in yeast *(Pichia pastoris)* and adjuvated in alum and Freund´s adjuvant. Mice immunized with both formulations were protected after an intra-cranial challenge in more than 60%. Interesting data were posted by Dr. SL Cosby (The Queens University of Belfast, United Kingdom) in her work "Mechanisms of virus clearance from the central nervous system of mice persistently infected with measles virus". She unveiled possible mechanisms to clear persistent Central Nervous System viral infections in a mouse model. Initial results showed that virus clearance occurs following treatment with immune serum. The data highlights the importance of an increased neutralizing antibody and/or cytokine response. A last poster by Dr. G Martínez (Center for Genetic Engineering and Biotechnology, Cuba) showed the adjuvant effect of a truncated HCV core protein (Co120) produced in *E. coli*. Mice immunized with a mixture or conjugate of Co120 and the E2 from the same virus enhance the humoral response to the latter.

Oral Presentations

Immunogenicity and efficacy of subunit vaccines for respiratory syncytial virus infection in a nonhuman primate model

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Respiratory syncytial virus (RSV) is the major cause of upper respiratory system infection in infants, elderly, and transplant recipients. Infection in infants may result in morbidity to mortality depending upon the healthcare support and system. Several vaccine strategies are being evaluated for prophylaxis against RSV infection, and include cold adopted, attenuated, and purified subunit F and G protein vaccines. In the present study we report the immunogenicity and efficacy of subunit vaccines in the baboon model for RSV. Infant baboons (4 week old) exposed to RSV by the intranasal route develop characteristic clinical signs and symptoms of infection. Due to delayed development of immunocompetence they respond poorly to RSV. Therefore, the role of RSV specific maternal antibodies in prevention or reduction in severity of infection in infants was evaluated by immunizing pregnant baboons with either F protein or F + G protein vaccines. Thirty adult female baboons seronegative for RSV were intranasally inoculated with RSV to simulate natural exposure and after resolution of infection they were placed in a breeding program. Pregnancy was confirmed by sonogram and the animals were immunized with either the F vaccine (n=10), or F + G vaccine (n=10) and adjuvant alone (n=10). Infants derived from dams in the 3 experimental groups were challenged with RSV by the intranasal route at 4 weeks of age to determine the effect of RSV specific maternal antibodies. Maternal vaccination markedly reduced the number of infected infants when compared to the rate of infection in infants born to mother given adjuvant alone. The results suggest that maternal vaccination strategy can markedly reduce the incidence of RSV infection in newborn infants.

Increased Immunogenicity and cross-reactivity induced by conjugation of V3 based multiple antigen peptides to carrier proteins.

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Multiple antigenic peptides (MAP) comprising eight and four synthetic peptides corresponding to the V3 loop of HIV-1JY1 (subtype D) were compared regarding Ab titers and level of cross-reaction to several subtype B V3. The MAP8 induced higher titers and cross-reaction level than MAP4 after four doses. The increased recognition of heterologous peptides is correlated to a broad interaction with aa residues in the primary sequence. MAP8 did not differ to a quimeric protein comprising several V3 peptides in Ab titers to JY1 peptide. But the MAP induced higher level of recognition against a panel of several V3 peptides in ELISA. To increased the immunogenicity and cross-reaction JY1-MAP8 was coupled to KLH, HBsAg and P64k proteins. The conjugated MAP were more inmunogenic than the respective peptide-conjugate and were more or as immunogenic as four times more MAP8 immunized alone. Also, conjugates to HBsAg and KLH enhanced the cross-reaction to heterologous V3 peptides in comparison to JY1-MAP8. Pooled sera from different immunization schedules were set at a similar titer and compared regarding cross-reaction to a panel of several V3 peptides. The analysis showed that MAP-based immunogens are more prompt to elicite cross-reactive Abs than lineal polymers or peptides couple to dextran.

1. Cruz L, *et al.* Biotecnol Apl 17:35.
2. Cruz L, *et al.* Letters in Peptide Science *7:229*.
3. Cruz L, *et al.* J Pept Sci *Accepted for publication*.

Non-replicating vaccines for prevention of damaging congenital HCMV infections: Current status and new problems

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Human cytomegalovirus (HCMV) remains an important cause of disease in immunocompromised allograft recipients and individuals with AIDS. In addition, HCMV is the most common cause of congenital viral infection in humans and a major cause of central nervous system (CNS) damage in infants and children. In the US, congenital HCMV infection occurs in 1% of live birth and up to 3000 infants have CNS damage each year as a result of this infection. Studies have suggested that this infection is potentially vaccine modifiable because pre-existing maternal immunity has been thought to limit disease in the infected fetus. Current vaccine approaches include a replicating vaccine and subunit vaccines. A subunit vaccine currently under testing which consists of the major envelope glycoprotein of HCMV, gB, combined with an oil/water emulsion has been shown to induce virus neutralizing antibodies; however, these antibodies wane with time. Virus neutralizing antibodies and T lymphocyte reactivity, including HCMV specific CTL, were induced in mice by immunization with gB combined with the saponin derivative, QS21. Although these conventional approaches are well founded, recent data has indicated that immunity following natural infection does not prevent fetal infection and disease following congenital infection. Several possible reasons could account for these observations including reinfection by antigenically distinct viruses. As an example, gN, which is an abundant viral envelope glycoprotein and a target of virus neutralizing antibodies, has been shown to exhibit considerable amino acid divergence in primary isolates. The role of antigenic variation in this virus and characteristics of the natural history of this virus infection in the design of non-replicating vaccines must be reconsidered in the design of protective vaccines for this congenital virus infection.

Morbillivirus vaccines and virus-induced immunosuppression

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Morbillivirus infections induce severe disease in their natural hosts, causing high morbidity and mortality. Virus-induced immune suppression may influence the high mortality, allowing secondary bacterial infections to flourish that are lethal to the host. This

can also give rise to problems with live attenuated vaccines in susceptible individuals. A hallmark of such virus induced immunosuppression is the reduced capability of freshly isolated peripheral leucocytes (PBL) to proliferate in response to a variety of stimuli. We have shown that all members of the morbillivirus exhibit this property and that, as for measles virus, co-expression of both the fusion (F) and the haemagglutinin (H) proteins of rinderpest virus (RPV), on the surface of the presenter cells is necessary and sufficient to induce immune suppression *in vitro* [1]. Our results show that vaccine strains of RPV in cattle and peste des petits ruminants virus (PPRV) in goats, cause immunosuppression by this mechanism, although this is less severe than with wild type strains. Furthermore, challenge with wild type virus, following vaccination, still gives rise to a degree of immunosuppression, due to replication of the challenge virus. Therefore, H and F protein-cell surface interactions must be considered when designing recombinant morbillivirus vaccines and use of adjuvants.

1. Heaney J, Barrett T, Cosby SL. J Virol (2001, in press).

DNA Vaccination of mice against BHV-1: effects of the adjuvant RN-205 in the modulation of the immune response

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It is well documented that adjuvants improve the immune response generated by traditional vaccines, but less is known about the effects of adjuvants on the immune response elicited by DNA vaccines. In this study, we have investigated the use of RN-205 (lipopolysaccharide from Gram-negative bacteria) as an adjuvant and analyzed the humoral and cellular specific immune response elicited by DNA vaccines based on the glycoprotein D from BHV-1. We have compared the antibodies induced by a cocktail of three versions of gD (membrane-anchored, secreted and cytosolic) with and without RN-205. The cellular immune response of RN-205-treated mice was higher, not only in the indexes of proliferation tests but in the number of IL-4 and IFN-γ secreting cells. When total spleen cells were marked with specific monoclonal antibodies, a significant augment in the macrophage population from all the groups receiving RN-205 have been observed, although CD8 and CD4 positive cells were increased, the differences were not so important. The antibody titers of RN-205-treated mice decreased after booster vaccinations compared with those induced by DNA vaccination without adjuvant (although the differences were not significant). Together, our results indicate that the incorporation of RN-205 into DNA vaccines induces an increase of the cellular specific immune response.

Regulation of immune response to hepatitis B virus vaccine by monoclonal antibodies

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Hepatitis B Virus (HBV) infection is a major cause of acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma. Approximately 2 billion people have evidence of current or past HBV infection, thus represents a serious worldwide problem. Commercially available HBV vaccines prepared with aluminum hydroxide are safe and effective but multiple immunization schedule is required to elicit a protective immune response. Besides, it has less effect in the elderly and the immuno compromised and also the titers of antibodies to HBV may decline in the course of 6 months following third immunization. Thus the risk of acquiring HBV infection increases as anti-HBV levels become undetectable. While alum has a good safety record, it mainly augments the humoral immunity and not effective in raising the cellular immunity and there is still a growing need for an appropriate vaccine systems which effectively potentiate the immune response to HBV with fewer immunizations. In the present study, both IgM and IgG type anti- HBV monoclonal antibodies were complexed to commercially available HBV vaccine (GenHevac B Pasteur, France). BALB/c mice were immunized with HBV vaccine complexed to IgM and IgG antibodies at varying concentrations of antibodies. An enhanced immune response was observed when HBV vaccine was complexed to IgM in the dose range of 1 to 5 µg. On the contrary, increased antibody levels were detected when mice were immunized with HBV vaccine complexed to IgG in excess of antibody (5, 10, 20 µg). When IgG antibody is used at a dose near or equivalance with HBV vaccine (0.5, 1, 1.5 µg) an enhanced antibody response was obtained especially after secondary immunization compared to those immunized by vaccine alone. The results indicate that complexed monoclonal antibodies to HBV vaccine may induce an immunopotentiating effect against HBsAg.

Poster Presentations

A comparison of methods for measuring antibody responses in mice and cattle following vaccination against BHV-1 (use of adjuvants in vaccines)

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Infectious Bovine Rhinotracheitis is a disease which causes severe economical losses in Argentina due to respiratory and reproductive disorders. Vaccination is a common preventive measure. Vaccines usually prevent the development of clinical signs and reduce the shedding of virus after infection. Inactivated vaccines against BHV-1 are usually poor immunogens and require adjuvants to induce a significant immune response. To assess the possibility of using the murine system as an indicator of the vaccine´s immunogenicity, antibody levels induced by different vaccines in mice were compared with those in cattle. We used a murine system because mice are genetically identical and less expensive than cattle. The study was conducted by the development of an ELISA test using a

recombinant gD protein from BHV-1. Mice and bovine were immunized with inactivated vaccines against BHV-1 containing different immunomodulators (SL-CD/S/W-non mineral oil and biodregradable, Avridine, RN-205, oleous adjuvant) and viral concentrations. In this report we demonstrate a close similarity between mice and cattle in terms of humoral immune response (similar antibodies profile and similar levels). In addition, we were able to detect differences between vaccines with different viral amounts. In summary, we propose the murine model for vaccine screening before the vaccination in the natural host.

The effect of high hydrostatic pressure on herpes simpex virus type 1 (HSV-1) evidences for changes on immune

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Herpes Simplex Virus Type I belongs to Herpesviridae family and was the first virus associated with latent or innaparent infections primarily in sensory ganglia. Their lesions are limited to oropharynx and are transmitted by direct contact of a susceptible individual with infected secretions, i.e.vesicular fluids. In animal models, Balb/c, Natural Killer cells and lymphokine responses (IFN-γ , IL-6) have been implicated as important host defenses against HSV infections. Although humoral immune response exist, the development of vaccine is difficult due to recurrences in HSV-1 infections. Inactivated (or killed) virus appear to show weak protection to the animal host. In our lab we demonstrated in other works that high hydrostatic pressure is a tool for inactivation of virus particle [1, 2]. The use of this method alters the conformational state of the proteins in the capside. When returned to atmospheric pressure this new conformational state enhances its immunogenicity. It was also successful with *Leptospira bovis* [3]. In this work we demonstrated that HSV-1 submitted to pressure (41Kpsi) for one, 4 and 8 hours reduces the infectivity titer by at least five order of magnitude. Furthermore analises with Natural Killer cells, IFN and NO demonstrate that this method alters the antigenic presentation.

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Algammulin as adjuvant of the HBsAg in BAlB/c mice

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Algammulin is a vaccine adjuvant that is formed when inulin is crystallized in a suspension of alum and the two are enmeshed to form similar ovoids particles that are electron-dense. These both particles carry antigen and activate the Alternative Complement Pathway. Inulin strongly enhances murine Th1 and to some extent Th2 immune response pathways. Alum is a potent immune modulator emphasizing Th2 response. Thus the type of response to Algammulin (Th1 or Th2) may be predicted to depend on the proportion of the Algammulin surface occupied by inuline or alum [1, 2]. Here it is shown the immunogenicity when are applied several formulations of Algammulin, as adjuvant, and the Cuban HBsAg produced in yeast *P. pastori* [3], as antigen, in BALB/c mice. In conclusion, some formulations of Algammulin can produce a superior response of anti-HBs when they are applied with HBsAg in BALB/c mice using this antigen adjuvated with alum as control.

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Additives modulate the antibody response elicited by a hepatitis C virus core-encoding plasmid in mice

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Humoral and cellular immune responses are currently induced against hepatitis C virus core following vaccination with core-encoding plasmids [1]. However, the anti-core antibody response is frequently weak or transient. In this work, we evaluated the effect of different additives and DNA-protein combinations on the anti-core antibody response. BALB/c mice were intramuscularly injected with an expression plasmid (pIDKCo), encoding a C-terminal truncated variant of the HCV core protein, alone or combined with $CaCl_2$, PEG-6000, Freund´s adjuvant, sonicated calf thymus DNA and Co.120. Mixture of pIDKCo with PEG 6000 and Freund´s adjuvant accelerated the development of the anti-core Ab response. Combination with PEG 6000 also induced a bias to IgG2a subclass predominance among anti-core antibodies. The kinetics, IgG2a/IgG1 ratio and epitope specificity of the anti-core antibody response elicited by Co.120 alone or combined with pIDKCo was different regarding that induced by the pIDKCo alone. Our data indicate that the antibody response induced following DNA immunization can be modified by formulation strategies.

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Evaluation of the recombinant envelope protein from dengue-4 virus formulated in two different adjuvants

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Envelope protein (E) of Dengue virus (DEN) is an important target of immunological response in humans

[1]. For this reason, is considered a suitable component of a recombinant subunit Dengue vaccine. Several laboratories have reported the obtention of the E protein in different hosts like: insect cells [2], *E. coli* and and yeast [3]; all of them were capable to induce functional antibodies and protection in mice. In our work, we obtained the E protein of DEN-4 virus (E4 rec) in *Pichia pastoris* yeast associated to the host cell membrane. The immunological evaluation was carried out using two different adjuvants: Freund's and aluminium hydroxide. The E4rec formulated in both adjuvants was capable to induce a functional immune response as well as a partial protection against lethal virus challenge.

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Enhanced anti-HCVE2 antibody response is elicited in mice by mixing or conjugation with a truncated variant of the HCV core protein

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Hepatitis C virus (HCV) remains persistently in about 85% of infected individuals, and chronic HCV infection is associated with cirrhosis and hepatocellular carcinoma. The structural E2 glycoprotein has been considered a potential antigen for use in a vaccine against Hepatitis C. Both humoral and cellular immune response against E2 protein are likely to be important for controlling HCV infection and are indispensable requirements to take into account for develop an effective HCV vaccine. The HCV genome encodes structural proteins (core, E1, E2) and nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B). A truncated variant of HCV core protein (Co.120), has previously elicited a strong immune response in mice. In the present work, two C-terminal truncated forms of hepatitis C virus E2 glycoprotein (384-605) and (384-680) produced in *E. coli* and yeast *(Pichia pastoris)*, respectively, were mixed or conjugated to Co.120 protein with the purpose to enhance the immune response against E2 protein. The humoral immune response was substantially increased in mice immunized with the mixture E2(680):Co and the best titers against E2 protein were obtained with conjugated E2(680)-Co. While using E2(384-605) protein, the highest humoral response were obtained in mice immunized with E2(605) only in PBS. The predominant antibody subclass observed for both proteins was IgG1. Additionally, we studied the specific cellular immune response induced by E2(680) protein by *in vitro* lymphoproliferative assays, no significant difference was observed between immunized groups. These results indicate that immune response to E2(680) B-cell epitopes could be significantly enhanced by mixture or conjugation of E2(680) to Co120 protein.

Use of the immunomodulators and various schemes of vaccination for increasing of efficiency of vaccination against influenza in the persons with immunodeficiency

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Peculiarities of anti-influenza immunity formation after alive (AIV) and inactivated influenza vaccination (IIV) in professional collectives of poultry farms were studied. In such collectives infection of *T. gondii* is an occupational harm and seropositivity reached 73,8% (30,6% in control). Different irregularities of immune state (such as decreasing of rates of T-lymphocytes, T-helpers, antihemagglutinin (AGA) levels to actual influenza virus strains and different classes of immunoglobulines) were determined in infected people. The highest sickness rates of influenza and acute respiratory diseases (ARD) with broncho-pulmonary complication and long time disability (up to 44%) were registered. Inoculation of the AIV and IIV was not effective and did not lead to the valid humoral response in seropositive persons with the immunodeficiency. Earlier we presented the results of the use of different immunomodulators (antiparasitic drugs Piremetamin and Delagil, which gives an effect of immunostimulation in low dosage, Levamisol, Tactivin, Timogen and Natrii nucleinat) in animal models (mice and rabbits) with experimental chronic T.gondii infection in complex with AIV and IIV. On the base of these experiments we selected some immunomodulators with the best stimulating effect and used them in infected persons. Materials and methods: intranasal inoculation of AIV-A(H1N1), parenteral inoculation of IIV – A(H3N2), A(H1N1), ELISA, RIF with Toxoplasma antigens, RHI with the commercial Influenza antigens: A(H1N1), A(H3N2), B. The primary AIV vaccination gave a low levels of AGA and protection against Influenza, but treatment of pharynx and glands with levamisol fresh solution in 0,05-0,1-0,2% concentration for 6 - 8 days before AIV resulted in 2 times decreasing of morbidity of toxoplasma infected people. After repeated AIV inoculation high prophylactic effect and decreased morbidity of toxoplasma seropositive people in 3,3 times in comparison with not vaccinated were observed. The usage of Piremetamin by proposed schedule (half of day's dose (12,5 mg) during 6-7 days after inoculation of IIV) in seropositive persons led to the significant rise of the AHA titers for the both vaccine strains and reduction of Influenza and ARD morbidity. Use of the above mentioned immunoimprovers is recommended for increasing of immune response against Influenza in immunocompromised persons and decreasing of ARD morbidity.

Mechanisms of virus clearance from the central nervous system of mice persistently infected with measles virus

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Measles virus (MV) is the causative agent of a variety of central nervous system (CNS) disorders i.e.

acute measles encephalitis (AME), measles body inclusion encephalitis (MIBE) and subacute sclerosing panencephalitis (SSPE). In SSPE virus can persist for long periods in the CNS and is not cleared by the immune response. To investigate whether it is possible to clear virus from the CNS, by enhancing the immune response, a persistent MV infection of the CNS in mice was established where virus can be detected up to 90 days after infection [1]. Clearance of virus from the CNS was observed over a 5 day period following re-exposure to virus in the periphery (intraperitoneal boost). Expression of Interferon gamma (IFNγ), Interleukin 10 (IL10) and Interleukin 6 (IL6) mRNA were detected in the CNS of boosted and non-boosted animals (concentrations to be determined). An highly, significant increase in the titre of neutralizing antibody was recorded up to 10 days after the animals received the boost. Initial results also show that virus clearance occurs following treatment with immune serum. The results suggest that induction of an increased antibody and/or cytokine response may be sufficient to clear virus from the brain and raises the possibility of vaccination therapy in persistent CNS infections.

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Workshop on Proteoliposomes, Liposomes, and Virosomes

Chairpersons: Mark Chambers,[1] Gustavo Sierra[2]

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*I*ntroduction

This session was on liposomes, which are artificial vesicles typically formed from phospholipids. Liposomes have been used successfully for a number of years to deliver encapsulated enzymes and drugs to the body (Koff & Fidler, 1985), sometimes in a manner that directs them to specific cellular targets (Norley *et al.*, 1986). The use of liposomes as adjuvants and delivery systems for vaccines is a relatively new, exciting and expanding field of research (O'Hagan *et al.*, 2001). Proteoliposomes and virosomes are liposomes that additionally contain proteins, usually proteins from the viral envelope in the case of virosomes (Kaneda, 2000).

Dr. Lockhoff from Bayer AG, Germany presented his work using a new class of synthetic glycolipid adjuvants (GLA) of which BAY R1005 is an example. These GLA are not liposomes, in that they resemble the structure of ceramide, rather than forming vesicles. The GLA BAY R1005 is of particular interest since it acts as an adjuvant even when administered to the antigen by a different route. In this sense it acts like a drug. It is relatively easy to chemically synthesise and to produce to high purity since it forms crystals: an unusual property for a lipid-containing molecule. In relation to safety, BAY R1005 produces little to no local irritation, has low acute and sub-chronic toxicity and is free of T-cell mitogenicity. BAY R1005 has been evaluated in mice, sheep and cattle with a variety of particulate and soluble antigens of viruses and bacteria, as well as purified virus vaccines. The use of BAY R1005 as the adjuvant in an attenuated trivalent bovine respiratory viral marker vaccine (IBR-Marker+BRSV+ PI3) and an attenuated bivalent bovine respiratory viral vaccine (BRSV + PI3) looks particularly encouraging.

As mentioned previously, liposomes are usually made from phospholipids. However, a series of paucilamellar liposomes composed of non-phospholipids, sterols, oils and buffers (Novasomes™) (Wallach & Philippot, 1993) have been made by Novavax, Inc., USA and show considerably promising as adjuvants. Dr. Chambers from the Veterinary Laboratories Agency, England presented data using formalin-inactivated whole mycobacterial cell preparations mixed with a variety of Novasomes™ as part of their programme to develop improved and safer tuberculosis vaccines. The vaccines produced no adverse reaction in guinea pigs and a number of the preparations protected guinea pigs from challenge with a low dose aerosol of viable *Mycobacterium bovis.* In some cases, the levels of protection were equivalent to that achieved with the current live TB vaccine, BCG. The formalin-inactivated whole mycobacterial cell preparations were ineffective in the absence of the adjuvant. Novasomes™ can be produced rapidly in litre quantities with little batch to batch variation, they can be manufactured to possess different surface charge and membrane fusogenicity, and incorporate other molecules such as proteins. These properties make Novasomes™ an attractive alternative to conventional phospholipid-based liposomes.

Dr. Sierra from the Finlay Institute presented studies on the adjuvant activity of a proteoliposome derived from N. meningitidis. The highly efficacious vaccine VA-MENGOC-BC was the first effective vaccine against *N. meningitidis* B and the first proteoliposome vaccine licensed for humans and clinically applied. Over 45 million doses of the vaccine have been administered in Latin America and the first European trial is scheduled to begin in Belgium. The vaccine consists of purified Outer Membrane Proteins (OMP) from *N. meningitidis* B and purified C polysaccharide from *N. meningitidis* C. The OMP self-assemble into vesicular proteoliposomes that are stabilised with alum to form one of the major components of the VA-MENGOC-BC vaccine. The OMP proteoliposome adjuvant has been demonstrated to be a broad-spectrum Th1 adjuvant and is currently being evaluated for other vaccine targets as diverse as cholera and salmonella, parasites, cancer, and allergy. The technology is patented in 25 countries.

This report was expanded by Dr. Bracho, also of the Finlay Institute, Cuba with a thorough description of the immune response elicited to injections with VA-MENGOC-BC. The vaccine induced delayed type hypersensitivity, the opsonophagocytic killing of bacteria and nitric oxide. There was rapid (in 2 hours) production of tumour necrosis factor-alpha (TNF-α) by polymorphonuclear cells, as well as monocytes after the injection of the vaccine. The production of interferon-gamma (IFN-γ) followed 8 hours post-injection. Bactericidal antibodies were stimulated after the injection of the vaccine, but also when given intranasally and gastrically. Salivary IgA was additionally produced following intransal vaccination. The cytokine pattern elicited by *in vitro* re-stimulation of peripheral blood mononuclear cells from vaccinated volunteers was also examined. Messenger RNA for IFN-γ and IL-2 was demonstrated, reaching a peak at 14 hours and 24 hours, respectively and remaining high until 72 hours. Bioactive IFN-γ was detected in the culture media. In contrast, IL-10 and IL-4 were not detected. Since OMV proteoliposomes have a good safety profile and generate a Th1-biased response in humans, they may be applicable as adjuvants to a number of vaccines.

In the final presentation, Dr. Alonso from the University of Havana, Cuba presented his finding on the use of DPPC:Cholesterol (Cho) liposomes to deliver recombinant human epidermal growth factor (rhEGF). Whilst principally used by these workers as a model antigen with which to study the modulating ability of these vesicles upon the immune response in mice, hEGF and its receptor are over-expressed in certain tumours, making hEGF and its receptor targets for cancer immunotherapy. A vaccine comprised of hEGF conjugated to carrier protein (P64K protein from *Neisseria meningitidis)* plus the conventional adjuvant aluminium hydroxide (AlOH) is currently undergoing pre-clinical trial. Using liposomes as the adjuvant, these workers have demonstrated in mice that it was possible to simplify the immunisation protocol from four to two doses without changes in the total anti-EGF antibody titres. Liposomes of saturated phospholipids (DPPC:Cho and DSPC:Cho) were more immunostimulatory than unsaturated phosphatidylcholine from soybean (sPC:Cho) or AlOH, in terms of the titre of IgG generated and their ability to block the interaction between rhEGF and its receptor. These workers concluded that DPPC:Cho and DSPC:Cho liposomes are better adjuvants for rhEGF than AlOH because they enhance the levels of antibody, and induce DTH responses and lymphocyte proliferation, even in the absence of carrier proteins, at least in an experimental system.

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Oral Presentations

BAY R1005, a new specific adjuvant for veterinary vaccines

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The identification of new potent adjuvants plays a major role in modern vaccine development. An adjuvant must not only enhance the immune response but should also drive this response to achieve protective immunity. Additionally, adjuvants must fulfil increased safety standards. We have identified a new class of synthetic glycolipid adjuvants (GLA) which structurally resemble the lipid second messenger ceramide. BAY R1005 is the most prominent GLA representative which has been developed as adjuvant for veterinary vaccines (Figure).

In mice BAY R1005 elicits humoral immune responses *in vitro* and *in vivo* against a variety of particulate and soluble antigens in a dose dependent manner. The increase in antibody synthesis is specifically dependent on the antigen and is not the result of a polyclonal stimulation. BAY R1005 in combination with purified virus vaccines or subunit vaccines led to increased protection of virus-challenged mice. Parenteral immunization with recombinant urease mixed with BAY R1005 induced strong Th1 and Th2 response and thereby elicited better protection against *H. pylori* infection than adjuvants which induced a Th2 type response only. In cattle an attenuated trivalent bovine respiratory viral marker vaccine (IBR-Marker+ +BRSV+PI3) and an attenuated bivalent bovine respiratory viral vaccine (BRSV + PI3) both adjuvanted with BAY R1005 have been developed that are exceptionally efficacious even when applied parenterally to very young calves in the presence of maternally derived antibodies. The vaccines are safe in all categories of cattle, including the most susceptible seronegative calves and pregnant animals. Due to the gE-deletion of the BHV1 vaccine strain, the trivalent vaccine can be used in modern BHV1 control programs.

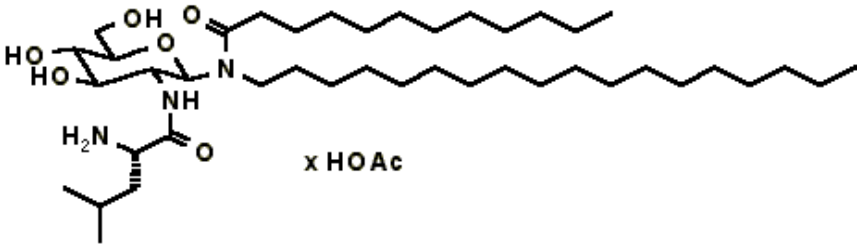

Figure. Chemical structure of BAY R1005.

Adjuvant activity of proteoliposome from *N. meningitidis B*

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Vesicles of lipid bilayers have been investigated as drug delivery vehicles for more than two decades. More recently vesicles of protein + lipid bilayers with self-assembly capacity have been incorporated to this investigation tendency, more specifically proteo-lipidic bilayers vesicles (Proteoliposomes) self-assembled after the purification of Outer Membrane Proteins (OMP) of different bacteria, with the aim to obtain effective vaccines against this bacteria. A purified vaccine VA-MENGOC-BC consisting of purified OMP(s) from *N. meningitidis B* and purified C polysaccharide from *N. meningitidis C* presented as a protein-polysaccharide complex based on a Proteoliposome model has been developed and carefully tested. In a well controlled, randomized, stratified, prospective, double blinded placebo-vaccine trial this vaccine has shown an efficacy of 83% ; 95% Cl (42%-95%). After ten years (1987-97) of massive application in Cuba and many other countries of more than 40 millions of VA-MENGOC-BC doses under epidemic and endemic situations an effectivity ranging from 75 to 98% have been proved under different trial conditions (cohort studies, case-control studies, etc..). Very important results have been obtained in laboratory as well as field clinical-epidemiological studies when measuring the effectivity of the vaccine against the C meningococcus. A clear difference has been shown when VA-MENGOC-BC was compared with the classical C polysaccharide vaccine. VA-MENGOC-BC was more efficacious against C Meningococcus than the C polysaccharide vaccine. Monosialoganglioside GM3 and its derivaties seen to be potential molecular targets for experimental therapeutic cancer vaccines for example in human breast tumors in which this molecule is relatively highly expressed. The main problem is the low immunogenicity of this molecule in human, as well as many experimental animal models. This problem has been avoided presenting to the immune system an OMP-GM3 complex. At least in the murine animal model, the OMP-GM3 complex was able to induce a potent IgG response. Neoglicoconjugates of oligosaccharides from cholera LPS conjugated with OMP from *N. meningitidis* has also shown its capacity to destroy target cholera strains in vibriocidal assays. Synthetic as well as natural oligosaccharides (PRP)n from Haemophilus b conjugated with *N. meningitidis* OMP (s) presented in a proteoliposome complex have shown equivalent or higher specific immune response when tested against Hib target strains in comparison with commercially available anti Hib vaccines.

Examples of the use of proteoliposome-based vaccine formulations.

PLS ———— OMP (NMB) + Poly C
PLS ———— OMP (NMB) + PRP(n) natural
PLS ———— OMP (NMB) + PRP(n) Synther
PLS ———— OMP (Hib) + PRP(n) natural
PLS ———— OMP (NMB + GM3)
PLS ———— OMP (NMB + Oligo cholera)

The proteoliposome basis for the presentation of oligosaccharidic antigens as well as other antigen types to the immune system has shown very adequate properties for new generation vaccines in children and in young animal models. A well defined Th-1 pattern have been demonstrated to be induced by the proteoliposome and the proteoliposome containing formulations.

Novasome™ adjuvanted killed mycobacterial whole cell vaccines that protect guinea pigs against aerogenic *Mycobacterium bovis* challenge

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Tuberculosis has been the scourge of man and animals for centuries and continues to inflict a huge cost, both in terms of human and animal health and financially. This is particularly disappointing since for over 70 years a vaccine has been available for tuberculosis: bacille Calmette-Guerin (BCG). BCG possesses many of the qualities of an ideal vaccine: it is cheap to produce and administer, it is safe and has been shown to be efficacious in many circumstances [1]. Unfortunately, administration of live BCG to HIV infected patients has resulted in disseminated BCG infection. This and other problems surrounding the lack of universal efficacy and safety of BCG have resulted in increased efforts to develop a new generation of tuberculosis vaccines. Vaccines based on killed whole cell preparations of mycobacteria should be safe and would present a wide repertoire of antigens to the recipient. However, such killed vaccines have classically conferred little to no specific protection to subsequent challenge with virulent mycobacteria [2]. In order to re-evaluate the vaccine potential of killed whole mycobacterial cell preparations, we inactivated cultures of *M. bovis* and BCG strains Pasteur and Tokyo using formalin. The cultures inactivated in this way were found to be completely non-viable as determined by culture on growth medium and inoculation into severe combined immunodeficient (SCID) mice. The formalin-inactivated preparations were mixed with a range of Novasome™ adjuvants (paucilamellar liposomes composed of non-phospholipids, sterols, oils and buffer) [3] and were used to vaccinate guinea pigs. The vaccines produced no adverse reaction in the animals. Significantly, a number of the killed vaccines protected guinea pigs from challenge with a low dose aerosol of viable *M. bovis.* In some cases, the levels of protection were equivalent to that achieved with the gold-standard vaccine, live BCG Pasteur. These data give hope for the development of a safe, efficacious vaccine for tuberculosis based on Novasome™ adjuvanted, inactivated whole cell cultures of mycobacteria.

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Th1 cytokines profile induced by VA-MENGOC-BC™

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An effective vaccine should stimulate the T cells subset (Th1 or Th2) that efficiently activate the appropriated defense mechanisms. Cytokine pattern induced after antigen challenge direct T cells to differentiate into Th1 or Th2 response. Meningoccocal Meningitis is a widely spread health problem either in developing and developed countries. Meningoccoco serogroup B and C constitute the main causes of meningitis. VA-MENGOC-BC® is an anti B and C menigoccocal vaccine that had demonstrated high efficacy and safety in masive vaccination trials and is composed by outer membrane vesicles (OMV) from serogroup B and polysaccharide from serogroup C. The defense mechanisms induced by this vaccine include delayed type hipersensitivity, bactericidal antibodies, opsonophagocitic killing of bacteria and nitric oxide. Additionally we determine the cytokine pattern elicit by *in vitro* re-stimulation of peripheral blood mononuclear cells from vaccinated volunteers. The induction of mRNA of IFNγ and IL-2 was demonstrated, raising a peak at 14 h and 24 h remaining at high levels until 72 h. IFNγ mRNA was translated and secreted to the culture media eliciting IFNγ elicit biological. In contrast, IL-10 and IL-4 was not detected indicating a prevalence of a Th1 activation. Ours results demonstrate the preferent induction of a Th1 immune response by vaccination with VA-MENGOC-BCÒ. Finally, our result support the possibility to use the main component of this vaccine as adjuvants or immune modulators. The availability and easy modification for new antigens inclusion on OMV make is suitable for this purposes

Adjuvant effect of liposomes encapsulating human recombinant epidermal growth factor

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For the last few years, we have been using hrEGF, as a model antigen, encapsulated into DPPC:Colesterol (Cho) Liposomes (LP) in order to study the modulating ability of these vesicles upon the immune response in mice. Previously, it was demonstrated that it is possible to induce anti-hrEGF antibodies by immunization with this antigen conjugated with P64K protein from *Neisseria meningitidis*, included in conventional adjuvants. In this work we demonstrate that it is possible to simplify the immunization protocol from four to two doses without changes in the total anti-EGF antibody titers. The sensibilization of animals with LP/hrEGF, in the absence of P64K protein, and challenged with this antigen in Aluminium hydroxide (Al), showed antibody levels similar to those observed in the groups immunized with the conjugate (hrEGF-P64K) encapsulated into LP or included in Al, after two immunization. The isotype levels (IgG2a and IgG2b) in mice treated with LP containing only hrEGF was similar to those observed in the groups immunized with hrEGF-P64K in Al or in PBS, and higher than that treated with Al/hrEGF. Liposomes of saturated phospholipids (DPPC:Cho and DSPC:Cho) potentiate more efficiently the immune response against hrEGF than unsaturated phosphatidylcholine from soybean (sPC:Cho) or Al in terms of: IgG titers, the induction of IgG2a and IgG2b isotypes and the antibody ability to block the hrEGF and its receptor (R-EGF) interaction. Liposomes are better adjuvant for the hrEGF than Aluminium hydroxide because they enhance IgG2a and IgG2b levels, induce DTH response and lymphocyte proliferation, even in the absence of P64K. This might be indicating a switch in the anti-EGF immune response toward a Th1 pattern associated with a cellular response. Taking into account these results, it is possible to suggest that it is not necessary the use of P64K as a carrier protein when hrEGF is encapsulated into Liposomes, at least in experimental models.

Workshop on Adjuvants for Parasitic Vaccines

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ABSTRACT

A number of vaccine candidates have been tested in animal models and they have been able to successfully prevent the disease and provide efficient long-lasting immunity. However, it has not been possible to translate this knowledge into clinical use, due to various technical hindrances. The most important one is the limitation of using adjuvants strong enough to elicit the level of the immune response required to prevent the disease. Recently, some potent adjuvants have been tested and the results are very promising. In this workshop a successful case of immunization in humans, a novel methodology for delivering antigens and an innovative diagnostic assay were discussed. The use of Montanide ISA-720 was shown to generate a comprehensive and efficient immune response in humans immunized with a synthetic peptide comprising the C-terminal region of the circumsporozoite protein of

P. falciparum. In this Phase I/II human trial, naïve individuals reached levels of antibodies, $CD4^+$ and $CD8^+$ lymphocyte responses comparable to those observed in individuals from endemic areas. Most strikingly, this is the first report of a protein vaccine generating high levels of IFN-? producing $CD8^+$ lymphocyte responses, known to be essential in the eradication of the disease. A model for the introduction of *P. falciparum* sequences into BCG in which two sequences present in proteins of the parasite were successfully introduced into BCG to be used in human vaccination was discussed. Finally, the use of an immunodiagnostic method utilizing an antigen-based agglutination test for schistosomiasis and malaria that may detect very low levels of circulating antigen was described.

Introduction

Three talks covered the scope of this workshop and they were presented by:

José Alejandro López from the Mater Medical Research Institute, South Brisbane, 4101, (Australia) with the title: "Phase I clinical trial of long synthetic malaria peptides using different adjuvants".

Mohd Norazmi from the School of Health Sciences, University Sains Malaysia, Kelantan (Malaysia) with the title: "Recombinant BCG containing selected epitopes of *Plasmodium falciparum"*.

Oscar Noya from the Instituto de Medicina Tropical, Facultad de Medicina, Universidad Central de Venezuela, Caracas (Venezuela) with the title: "Immunogenicity of schistosoma synthetic peptides".

Main Body

The use of natural sources for immunization has been applied to malaria research quite successfully. In fact, the complete prevention of the disease has been achieved with the use of irradiated sporozoites. However, due to technical limitations in the production of large quantities of sporozoites (manually dissected from mosquito glands) an alternative option needs to be found. For the first time in humans, a vaccine has been developed which is capable of inducing strong responses from the three arms of the immune system (López *et al*, 2001. *Eur. J. Immunol.* 31:1989). The vaccine preparation included a 100 amino acid long synthetic peptide representing the C-terminal region of the circumsporozoite protein delivered in two immunizing doses (100 and 300 mg) at 0, 1, and 6 months in the presence of two adjuvants (Alum and Montanide ISO 720). It elicited antibody titers higher than those observed in individuals from endemic areas, similar to those reached with the classical tetanus toxoid vaccine, and most importantly, they recognized (by IFAT) the infective form of the parasite, the sporozoite with titers comparable to those of "immune" adults from endemic areas. Additionally, the vaccine also elicited strong proliferative responses that produced high levels of IFN-γ secretion. Perhaps the most striking feature of this preparation was the successful generation of $CD8^+$ lymphocyte responses upon immunization; as measured by antigen specific IFN-γ ELISPOT. The levels of response obtained were comparable to those observed in individuals from endemic areas. Two adjuvants were evaluated, Montanide IS0-720 and Alum and both appeared to generate CD8 responses. However, Montanide appeared to be superior in the generation of antibodies. Proliferative responses were observed similarly in both groups. An interesting finding of dose-scaling study was that the injection of 300 ?g peptide appeared to have a deleterious effect on the magnitude of the immune response. The one hundred microgram injection appeared to be the level necessary for an optimal response (López, Australia).

New vehicles for the delivery of antigenic sequences have been explored in many systems. In the case of malaria antigens, a model for the insertion of various sequences into BCG was presented. A fragment of region II of EBA-175 and the repeated (NANP) region of the circumsporozoite protein of *P. falciparum* were inserted by assembling PCR into BCG. The data showed that the inserts were present and that specific antibodies could be generated against the delivered proteins. Data on the immunogenicity of the constructs in animal models are underway (Norazmi, Malaysia).

A sensitive antigen detection system has been developed utilizing peptides from various parasite proteins. In the case of malaria parasites, a combination of synthetic peptides present in the histidine rich protein II (HRPII), glutamate rich protein (GLURP), falciparum interspersed repetitive antigen (FIRA) was used to generate specific antibodies in rabbits capable of detecting very low levels of circulating antigen. A similar strategy was employed for schistosoma antigens cathepsin B (Sm31) and asparaginil endopeptidase (Sm32) to generate an agglutination-based antigen detection system (Noya, Venezuela).

Summary

Major advances in the field of adjuvant development have now reached the development of parasitic vaccines. For the first time, a malaria vaccine successfully elicited strong responses from the three arms of the immune response and promise to convey clinical protection. The use of lower peptide concentrations in the presence of potent adjuvants appeals as an optimal combination. Other interesting advances in the field include the use of cellular forms for delivery systems such as BCG and the utilization of more efficient diagnostic kits for the early and accurate identification of the disease; in the case of the later technology, an additional product from research led to the identification of promising proteins, potential vaccine candidates against schistosomiasis.

Oral Presentations

Phase I clinical trials of long synthetic malaria peptides using different adjuvants

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Objective: Evaluate the safety and immunogenicity of synthetic peptide *Pf CS 282-383* with different

adjuvants.Methods: This peptide, produced according to good laboratory practice and spanning the C-terminal region of the circumsporozoite protein of *P. falciparum* strain *NF-54* was evaluated as a vaccine candidate in an open, non-randomized, phase I trial. Sixteen volunteers in 4 groups received 3 injections (at months 0, 1 and 6) of peptide (100 or 300µg) combined with Montanide ISA-720 or Alum as adjuvants. Immunogenicity was evaluated by 1) antibody titers (ELISA) and lymphocyte proliferation responses to peptide *Pf CS 282–383*, 2) antibody response to the *Pf NF-54* (IFAT) 3) inhibition of sporozoite invasion (ISI) and 4) CTL response to the two epitopes *Pf CS 327-335* and *299-308*) (ELISPOT). Results: In only 11 of 48 injections mild local pain were reported. In Montanide groups, particularly with the 100µg dose of peptide, antibody responses to the peptide and to the *Pf NF-54* sporozoite were similar or higher than those observed in adults from endemic area in Burkina Faso. In contrast, immunization with Alum resulted in weaker responses. A vigorous cellular response was observed in all volunteers which did not correlate with the humoral response. In 6 out of 8 HLA-A*0201 volunteers, a specific CTL response against one or both mentioned epitopes was detected with a frequency of $9x10^2$ and $7.5x10^3$ specific CTL per million $CD8^+$ cells. The results concerning the inhibition of sporozoite invasion are currently analyzed. Conclusions: The malaria vaccine formulation using *Pf CS 282-383* with Montanide ISA-720 or Alum as adjuvants was safe, well tolerated and immunogenic.

Recombinant BCG containing selected epitopes of *Plasmodium falciparum*

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Mycobacterium bovis bacille Calmette Guerin (BCG) has been suggested to be an attractive vehicle for the delivery of various vaccine candidates, including those for malaria [1, 2]. We have previously shown that cloning of a synthetic malarial epitope constructed with mycobacterium codon bias into *M. smegmatis* increased the transformation efficiency and growth of the transformants, as well as the expression levels of the fusion protein [3]. The objective of this study is to employ the same strategy, known as assembly PCR, to clone two malarial epitopes; a fragment from the so-called region II of EBA-175 (F2RII-EBA) and three NANP repeat sequence of CSP, generated from a series of oligonucleotides, into BCG. In addition, we incorporated the 65 kDa hsp promoter of *M. tuberculosis*, a signal peptide, a 6 His tag and 2 T-cell epitopes from *M. tuberculosis* ESAT-6. The technique involved the generation of the full 1,413bp fragment of the composite sequence from 36 overlapping, 25 to 45bp oligonucleotides, designed in favour of mycobacterium codon usage. The fragment was cloned into the cloning vector pCR2.1 TOPO (Invitrogen) and the resultant clone converted into a shuttle vector by insertion of the mycobacterial replicon. Cloning success was verified by sequencing. Transformation of the shuttle vector into BCG was achieved by electroporation. Western blot analyses using a polyclonal anti-F2RII-EBA antibody raised against the native EBA epitope, confirmed the expression of the fusion protein in the culture supernatant with minimal reactivity in the cell pellet - suggesting that the epitope of interest could be expressed in its native form and that the signal peptide is functional. Immunogenicity studies are currently being performed in mice. Assembly PCR is a flexible technique which would facilitate the cloning of composite candidate molecules coded for by genes located in different chromosomes or from different organisms.

1. Haeseleer *et al.* Mol Biochem Parasitol 1993;57:117.
2. Matsumoto *et al.* J Exp Med 1998;188:845-54.
1. Norazmi *et al.* Biotech Tech 1999;13:458-89.

Antigenicity and immunogenicity of synthetic peptides derived from parasite proteins

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Synthetic peptides have been recently used as candidate antigens for immunodiagnosis and vaccines. Two mayor parasitic diseases, schistosomiasis and malaria lack effective vaccines and ideal diagnostic tests. In the case of malaria, 29 peptides derived from ten different excretory-secretory molecules from *Plasmodium falciparum* have been synthesized in order to use them in antibody and antigen-based diagnostic agglutination tests. Peptides derived from the Glutamate Rich Protein (GLURP) have shown the highest sensitivity and specificity when evaluated with human sera, by ELISA. Antibodies raised in rabbits against HRPII (histidine rich protein II), GLURP and FIRA (falciparum interpersed repetitive antigen), were able to detect circulating antigens down to 1 ng of protein in the plasma of infected patients. For the diagnosis of *Schistosoma mansoni*, we have basically worked with the Sm31-cathepsin B and the Sm32 – asparaginil endopeptidase; two prominent proteases, from the gut of this trematode. Three peptides from the Sm31 were antigenic when evaluated with patients sera (range 49 – 86% sensitivity and 100% specificity). Antibodies raised against two peptides from the Sm31 (IMT 172 and 180) and three from the Sm32 molecules (IMT12, 14 and 64) where highly immunogenic, eliciting antibodies against these two enzymes. These antibodies are currently evaluated in an antigen-capture assay. Based on previous works and also on the preferential recognition of antigenic enzymes from non permissive schistosome hosts, we have identified potential protective epitopes from the GST-28 (glutathione-S-trasferase), Sm32 and TPI (triose phosphate isomerase). Preliminary results have shown the protective effect of GST-28 and Sm32 derived peptides. All this work has been carried out immunizing the animals subcutaneously and using complete and incomplete Freund´s Adjuvant. However, based on the interest to primarily elicit a potent Th2 response in both experimental models, other adjuvants, like QS-21 and other routes of inoculation, need to be evaluated.

Th1 immune response induced by KM+ lectin: a consequence of IL-12 production

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The outcome and severity of some diseases is directly correlated with the dominance of either the T helper 1 (Th1) or Th2 immune response, which is stimulated by IL-12 or IL-4, respectively. In the present study we demonstrate that spleen cells from BALB/c mice stimulated with the lectin KM+, a mannose-binding lectin from Artocarpus integrifolia, produced interferon gamma (IFN-γ) in dose-response manner. In order to determine if the IFN-γ production by spleen cells was dependent on a soluble mediator induced in macrophages, the supernatant of J774 cells stimulated with KM+ was depleted of lectin by adsorption to immobilized mannose and added to a non-stimulated culture of mouse spleen cells. This supernatant induced expressive production of IFN-γ, an effect blocked by anti-IL-12 monoclonal antibody. In fact, when macrophages from several sources, including cell lines, spleen and peritoneal cavity, were stimulated with KM+ they produced IL-12. Furthermore, this KM+-induced IL-12 production by macrophages was remarkably inhibited by D-mannose, but not by D-galactose. Additionally, IFN-γ production by spleen cells stimulated with KM+ in the presence of either D-mannose or mannotriose were inhibited in a dose-dependent manner, whereas no inhibition was determined by carbohydrate when spleen cells were directly stimulated with IL-12. These observations indicate that KM+ induces IL-12 production by macrophages via its carbohydrate recognition domain.

The FML-vaccine: a second generation candidate for vaccination against murine and canine leishmaniasis

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The FML antigen is a complex glycoprotein fraction isolated from *L. donovani* promastigotes. This antigen was used to immunize mice using the adjuvants: IFA, alum, BCG, IL-12, saponin, QS21 like fraction and QuilA. Antibody responses were primarily IgG1, IgG2a and IgG2b. Highest titers were found in mice receiving FML+QS-21-like, followed by FML+QuilA, FML+saponin, FML+IL-12, and FML+BCG and FML+alum; no Ig2a response were observed for mice receiving FML with either alum or BCG. All vaccinees showed a significantly enhanced DTH response. Significant protection was observed in FML+QuilA (93%) and FML+saponin (73%) mice. Sapogenins were obtained by chemical removal of the oligossacharides from saponin and QuilA. Despite of the reduction of toxicity in both sapogenins, only QuilA fraction maintained protective potential (85%) and significantly enhanced DTH and antibody response. Recent studies employing saponin and the GP36 component of FML antigen complex indicate that this molecule can provide significant protection in the murine model (BALB/c) against *L. donovani* infection (reduction of parasite burdens of 68%). The protection provided by vaccination with FML-vaccine was examined in dogs naturally exposed in an endemic area in Natal, Brazil. After 2 years of monitoring the dogs, data indicated that the FML vaccine induced a 92% significant protection (76% of vaccine eficacy). With the FML-QuilA formulation a 95% protection (80% of vaccine efficacy) against canine kala-azar was achieved. No significant difference between the two formulations was found. 3.5 years after vaccination, saline controls showed positive PCR for Leishmania DNA and FML-serology with no DTH reaction. Higher seropositivities and DTH with no Leishmanial DNA were detected in vaccinees. Both FML-vaccines induced a significant, long-lasting and strong protective effect against canine kala-azar in the field. Support: PCDEN and PNUD- FNS; FINEP; CNPQ; CAPES; MCT/ PRONEX; CNPQ; RHAE-CNPQ; FUJB-UFRJ; FAPERJ; CEPG-UFRJ, Brazil.

QS-21 saponin adjuvant in the FML-vaccine against experimental visceral leishmaniasis

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The development of a successful vaccine formulation for subunit antigens requires the use of a potent adjuvant that induces both humoral and cellular response. In the present work, we analyzed the protective potential of the FML antigen formulated with QS-21 fraction obtained from Quil-A saponin (Superfos), according to Kensil *et al.,* 1991 (J. Immunol 146: 431-7). Briefly, the QuilA was applied on a semipreparative HPLC column (Alltima-Altech C18 5U, 5mm particle size, 3000 nm pore size, 10 mm I.D. x 25 cm length). The gradient was 30 to 40% 0.1% TFA/acetonitrile/ 30min, 40% /15 min at a flow rate of 4 mL/min and saponins peaks were monitored by absorbance at 214nm. As described by Kensil *et al.,* four different peaks were detected. The major peak showed retention time and sapogenin polarimetric values ([a]20D= + 55,6) compatible with QS-21 ([a]20D= + 56,1). Swiss Albino females were immunized with three weekly doses of the FML150 µg and QS-21100 µg by s.c. route. Saline and adjuvant treated animals were included as controls. The parameters analyzed were: anti-FML antibody levels (types and subtypes) and delayed type of hypersensitivity (DTH) against LD1S f/t promastigote lysate antigen. The anti-FML antibody response (titre log2: IgM/ total IgG/ IgG1/ IgG2a/ IgG2b/ IgG3) before and after infeccion was: 5/8/6/9/9/7 and 10/5/8/12/9/4 for saline, 6/5/7/6/7/7 and 8/4/5/7/6/4 for adjuvant and 11/ 18/19/19/19/17 and 10/16/18/22/16/16 for FML+QS-21, respectively. The DTH response was specific and significantly higher than saline controls ($p<0.005$) in FML-vaccinees both before and after infection (0.580/ 0.042mm and 0.384/0117, respectively). Compared

to previous work with IL12, QuilA and Riedel De haen saponin, these results show the success in purification of the active component of the QuilA mixture and the remarkable potency of QS-21 adjuvant against visceral leishmaniasis. Support: PCDEN and PNUD-FNS; FINEP; CNPQ; CAPES; MCT/PRONEX; CNPQ; RHAE-CNPQ; FUJB-UFRJ; FAPERJ; CEPG-UFRJ, Brazil.

Poster Presentations

The adjuvant effect of KM+, a mannose-binding lectin, in experimental toxoplasmosis

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In order to determine if KM+, a mannose binding lectin extracted from *Artocarpus integrifolia,* could induce a protective immune response in an experimental model of murine toxoplasmosis, a total of fifty Balb/c mice was divided into four groups and immunized with: Group 1- PBS (n=12); Group 2- Soluble antigen of *T. gondii*, STAg (n=12); Group 3- KM+ (n=13); Group 4- STAg plus KM+ (n=13). The experimental groups of animals received a total of three injections, with doses of 25 micrograms of STAg and/or 0.5 mg of KM+ per inoculation, in weekly intervals. Animals from control group received three injections of PBS only. Ten days after the last injection, part of the animals from each group was challenged with 1,000 tachyzoites from RH strain of *T. gondii*, via intraperitoneal. The remaining animals were perorally challenged with 50 cysts from ME-49 strain or sacrificed in order to count the number of cells from spleen, mesenteric and popliteal lymph nodes, as well as, to analyze the secretion of nitric oxide under stimulation with Con-A, STAg, KM+ or medium only. It was observed that, when challenged with RH strain, mice from Groups 3 and 4 presented higher percentage of survival than animals from Groups 1 and 2. However, when challenged with ME-49 strain, animals from Group 3 presented the highest number of brain cysts and only those from Group 1 presented significant rate of mortality. In addition, animals from Group 3 showed the highest number of spleen and lymph node cells when compared with other groups. The nitric oxide production was detected in all groups, mainly under KM+ stimulation. It can be concluded that KM+ modified the profile of immune response for both strains of *T. gondii* and therefore further studies should be done to determine its role as immunologic adjuvant in this system. Support: FAPESP, FAPEMIG, CNPq and CAPES

Th1 immune response induced by KM+ lectin: protection against leishmania infection

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When experimentally infected with *Leishmania major,* an obligate intracellular parasite of macrophages in mammalian hosts, most of inbred mice strains are resistant to leishmaniasis. In contrast, BALB/c mice are susceptible, develop severe lesions and do not become immune to reinfection. Murine resistance and susceptibility are clearly related to the development of the polarized CD4+ T helper 1 (Th1) and Th2 response, respectively. The differentiation of T cells to Th1 and Th2 cells has been associated with production of interleukin-12 (IL-12) and IL-4, respectively. Since KM+, a mannose-binding lectin from *Artocarpus integrifolia,* induces IL-12 production *in vivo*, we aim here to verify if KM+ could switch BALB/c mice susceptibility to *L. major* infection. When BALB/c mice were immunized with soluble leishmanial antigen (SLA) in combination with KM+, they became resistant to *L. major* infection. Draining lymph node cells from these immunized BALB/c mice, in contrast to cells from animals immunized only with SLA, secreted high levels of IFN-γ and low levels of IL-4, which characterized a Th1 rather than a Th2 response pattern. The footpad thickness of BALB/c mice immunized with SLA plus KM+ and challenged with *L. major* was similar to that of uninfected mice. In contrast, control mice immunized only with SLA and challenged with *L. major* showed footpad thickness as large as of infected mice treated only with PBS. The beneficial effect induced by KM+ against leishmanial infection was blocked by pretreatment of these mice with anti-IL-12 antibody. These observations indicate that KM+ has an adjuvant effect on the induction of a protective immune response to *L. major* infection.

Role of IL-4 in experimental infection by the helminth *Echinococcus granulosus*

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Type-2 cytokine responses have been proposed as protective in infections by helminth. The experimental infection by *E. granulosus* induces an early type-2 response which is unable to impair parasite establishment. The aim of this work was to evaluate in vivo the role of IL-4 in the susceptibility to *E. granulosus* infection. Cytokine production by spleen cells, nitrite production by peritoneal adherent cells and major spleen cellular populations were analysed in: infected and treated with anti-IL-4 MoAb mice, infected and treated with homologous antibodies non-specific for IL-4 mice and normal non-treated mice, on days 3, 7 and 14 post-infection. Mice treated with anti-IL-4 MoAb showed reduced levels of IL-4, IL-10 and IL-5 compared with control infected mice. Blocking of IL-4 did not induce IFN-γ production. Treatment with anti-IL-4 MoAb led to an increase in nitrite production on day 7 post-infection in both peritoneal adherent and spleen cells respect to control infected mice. No significative differences were observed at chronic infection in the levels of cytokines and in the percentages of the major cellular populations in spleens between mice infected and treated with anti-IL-4 MoAb and control infected mice. Comparison of the percentage of infection and size of the cysts developed in mice treated with anti-IL-4 MoAb and infected controls mice, showed no significative differences. In conclusion, in vivo blocking of IL-4

produced early in experimental infection by the helminth E. granulosus seemed not to affect the establishment of infection and parasite growth. Supported by: CONICYT Fondo Clemente Estable (grant 4002); IFS (grant F/2930-1); CSIC; Regional Norte-Sede Salto Universidad de la República

Posters on Vaccines and Others

Comparative study of the immunogenicity and protective capacity of adsorbed and non adsorbed vaccines against Leptospirosis in hamsters

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The increasing incidence of Leptospirosis in Cuba at the beginning of the 80's, conditioned the production of the first Cuban leptospiral vaccine for use in man. Two experimental vaccines based on inactivated whole cells were formulated: non adsorbed (NAV) and adsorbed in aluminium hydroxide (AV), containing 50-80 x 10^6 cells of serovars *canicola*, *copenhageni* and *mozdok* per 0.5-mL dose. The immunogenicity and protection were evaluated in hamsters. Two assays were developed; in the first one 120 hamsters were inoculated by intramuscular (IM) route with two dose of 0.5 mL each, six weeks apart. Blood samples were taken at 0 (T0), 6 (T6) and 9 (T9) weeks. The IgG immune response was evaluated by ELISA and the protection induced, by challenge against virulent strains of *canicola*, *copenhageni* and *mozdok*. In the second assay 180 hamsters were inoculated with a single dose and challenge 1.5, 3 and 6 months later. The results showed that IgG response of hamsters vaccinated with AV was significantly higher than the response of the ones vaccinated with NAV. Both vaccines protected against challenge with 1000-10 000 LD_{50} of the strains, but AV induced a longer immunity and protected also against renal infection.

Comparison of three types of oily emulsions used in the formulation of a vaccine for pigs

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Three oily formulations (oil in water, water in oil and double emulsion) of several compositions and phase distribution are evaluated as vehicles for vaccination in pigs using K99 and K88ab enterotoxigenic *E. coli* fimbriae as antigens. The physical properties and immunological properties in rabbits were appropriate for each emulsion. The three variants were inoculated to sows at eight and fourteen weeks of gestation, and to piglets at the ages of one and three weeks old. The double emulsion was the most attractive for vaccine formulation, because the immune response is greater or equal to the other two emulsions used. Moreover, the low viscosity makes the double emulsion easier to inoculate and more fluid into the tissues. The biological stability of both double and water in oil emulsions is 2 years.

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Immunomodulating effects of polysaccharide fractions from mycelium of *Pleurotus ostreatus*. I. *In vitro* macrophage-stimulation activity

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Water soluble, complex carbohydrate derived from natural sources have been shown to augment various facets of immune responsiveness in human and animals. These natural product carbohydrate polymers belong to the class of drugs known as biological response modifiers (BRMs) [1]. The ability of BRMs, including polysaccharides isolated from the mycelium, fruiting bodies, and culture media of fungi, to exert beneficial immunomodulating and antitumor effects has stimulated research into their potential biomedical applications [2]. Since macrophages have been suggested to play important roles in immunological surveillance, the purpose of this study was to assess the *in vitro* effects on macrophage activation of five water soluble polysaccharide fractions (F-I to F-V) from mycelium of *Pleurotus ostreatus*. Murine peritoneal macrophages were obtained as adherent cells from peritoneal exudate collected with Hanks´ solution. After incubation of macrophages with polysaccharides (500 mg/mL) or LPS used as a positive control (100 mg/mL) for 48 h, the glucose concentration was measured in the supernatant using the Rapi GlucoTest kit (EPB Carlos J. Finlay). The glucose consumption of the macrophages significantly increased to 1.8-2.9 times that of the control without polysaccharides. A large quantity of glucose is known to be consumed by macrophages to synthesise the ATP and NADPH required for phagocytosis and to produce active oxygen species [3]. The lysosomal enzyme acid phosphatase activity was determined in cell lysate by the amount of nitrophenol released (mmoles) per 1x10^5 macrophages per 60 min. The enzyme activity was also enhanced positively compared with physiological saline control group. This evidence could be of interest, since it has been reported that the lysosomal enzyme in macrophages participates in the antitumor activity. In conclusion, these results suggested that resident macrophages are stimulated by *Pleurotus* polysaccharides *in vitro*.

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Characterization of the antibody response elicited after immunization of human healthy volunteers with recombinant p64k meningococcal protein

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Recombinant P64k, expressed in *Escherichia coli*, acts as an efficient carrier protein [1] and is safe and immunogenic in human healthy volunteers, as ascertained in a Phase I clinical trial [2]. The volunteers received three doses of recombinant P64k and were boosted 9 months after the third dose. In the present study, we further characterized the human antibody response against this protein, by using volunteer sera collected during the trial. As expected, IgG1 was the main subclass of anti-P64k antibodies all over the study. However, after the booster dose, a statistically significant amount of anti-P64k IgG4 was detected in the sera. A maturation of the antibody affinity was found in most of the sera. The presence of antinuclear and antimitochondrial antibodies in paired volunteer sera was examined by immunofluorescence. None of the sera contained such autoantobodies. Additionally, 84 overlapping synthetic peptides spanning the entire sequence of P64k were probed by SPOTscan [3], where the assayed sera recognized more frequently 12 peptides. We can conclude that IgG1 and IgG4 are the main subclasses of anti-P64k antibodies developed after immunization, the antibodies do not react with mammalian tissue and the continuous B-cell epitopes they recognized are mainly exposed in the protein.

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Histopathological study of the inoculation site of rats inoculated with adjuved vaccines

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Lesions at the inoculation site caused by different kinds of adjuvants included in vaccines have been thoroughly studied. However, regulatory agencies demand further in-depth research on this topic for new candidate vaccines. In the present work, the microscopic lesions caused at the inoculation site by three different adjuved vaccines were studied. Tissue samples were taken from the inoculation site of young adult male/female rats enrolled in the studies of local irritation of DPT, VA-MENGOC-BC™ + DPT and combined MEMB vaccines. Samples were fixed in 10% neutral formaldehyde, processed by the technique of inclusion and cut in paraffin and stained with haematoxiline-eosin. Those rats inoculated with either Aluminum hydroxide or pertusiss antigen had granulomatous macrophagic processes in the inoculation site. Both substances are recognized as responsible for eliciting that kind of local lesions, which are part of the immune response developed.

Effect of *Aloe barbadensis* on opsonophagocitic rate in burnt patient

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Behavioral results of opsonophagocytic rate in 40 severe, highly severe and critical burned patients distributed in two groups is presented: group I (treated with *Aloe barbadensis* Miller extract and usual treatment) and group II (usual treatment). Blood samples were taken in different times (24 h and 10 and 21 days) were analysed and phagocyte parameters were determined at 15-60 min. First, during a few hours, behaviour between groups was similar, but at the 10^{th} days a significant difference in opsonophagocytic rate of *Aloe* group at 60 min was noticed and neutrophiles increased their possibility of carrying microorganism.

Some toxicity mechanisms of immunological adjuvants

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The search of a potent and safe immunological adjuvant for routine human use is a scientific challenge. In this way the most important issue is the safety, specially for preventive vaccines, for this reason, only alum has been licensed by regulatory organisms like FDA since 1926 as adjuvant for human vaccine. The adverse effects of adjuvants can be a direct consequence of the inclusion of toxic or non metabolizable components in the formulation, or use of elements overstimulating of the immune or inflammatory response. These effects are differentiated as local (since local and erythema until delay hypersensibility, abscess, ulcers, granulomes and cysts) or general effects (flu like symptoms, autoimmune disorders and changes of the hepatic metabolism). Also extrinsic factors can determinate apparitions of side effects, like: doses, frequency and routes of application, age, genetic factors and health history. In this work are considered some mechanisms that can explain many of the reported side effects in preclinical and clinical assays of adjuvants.

A repeated doses toxicity study of magnetized CM 95

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Was performed a repeated doses toxicity assay of magnetized CM 95, immunomodulator developed in Biotechnology Studies Center *(CEBI)*, using Sprague Dawley rat. According to Guidelines 407, 1995 Organization for Economic Cooperation and Development (OECD), using three levels of magnetization of the product, via intraperitoneal routes and two control group: non magnetized CM 95 and physiologic saline solution. The endpoints evaluated were: clinical signs of toxicity (weight loss, piloerection, conductual disturbs), macro and microscopic anatomopathology, relative weight of thymus and spleen, bone marrow

cellularity, global and differential leucocytes, glycemia, total proteins, alaninaminotransferasa enzyme, creatinine and serum immunocomplex. Not was detected significant modifications on the endpoints analized in treated and control rats, except a lightly diminutions of glycemia on the highest level of magnetization group, reported before in previous preclinical studies. Looking together all these results, there is not evidence of general toxicity under the experimental conditions employed.

Immune response to house dust mite allergens in a mouse model

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Allergic sensitisation to house dust mite allergens have been described as a major cause of asthma and other allergic diseases worldwide. Immune response to these inhaled allergens in allergic patients is characterised by IgE production and a Th2 bias. Allergen Immunotherapy is an effective treatment against respiratory allergy, which consists in injecting increasing amounts of the allergen, achieving a decrease of Th2 cytokines. The aim of this work was to study the antibody (Ab) response to allergens of *Dermatophagoides pteronyssinus* (DP), *D. siboney* (DS) and *Blomia tropicalis* (BT) mites, either in aqueous (PBS) or alum-adsorbed (Al) form, in a mouse animal model. Freeze-dried VALERGEN Allergen extracts were obtained from BIOCEN, Cuba. The strength of the products is expressed in terms of the major allergen content (Der p1/Der s1) or biological activity (Biological Units, BU). Reconstituted allergen extracts were adsorbed on aluminium hydroxide gel at a final concentration of 10 mg/mL Der p1/Der s1 for DP and DS, and 2000 BU/mL for BT. Immunisation was carried out at the weeks 0 and 3. Each mouse (Balb/c) received a 0.5 mL dose of either individual or mixed preparations. Bleeding was performed at the weeks 5 and 7. Serum IgG, IgG1, IgG2a and IgG2b specific to each antigen and total IgE were measured using ELISA (Pharmingen, USA). It was observed a predominant IgG1 response to DS, while the response to DP was more balanced between IgG1 and IgG2a. On the contrary, the Abs to BT were predominantly of the IgG2a subclass (Figure). The adsorption on Alum did not affect importantly the IgG2a/IgG1 ratio, but certainly increased IgG and total IgE Abs. It produced also a time shift in the peak values. However, the effect of mixing the three allergens (Tri), did produce a significant decrease of the IgG2a/IgG1 ratio to BT, suggesting a non-specific influence (Figure). It is concluded that the IgG2a/IgG1 ratio (and presumably the Th1/Th2 balance) to common mite allergens can be affected by the allergen nature and concomitant administration of other antigens.

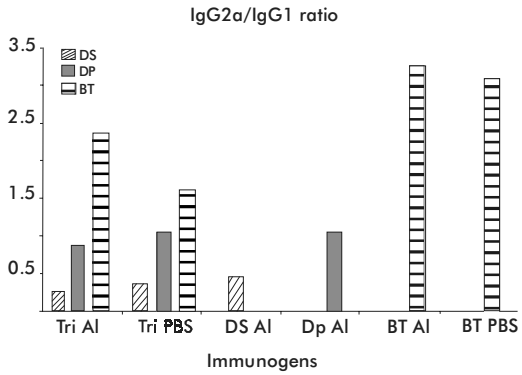

Figure. Antibody response to allergens.

Adjuvanticity of specific immunocomplex of surface system (HbsAg–anti HBsAg) of hepatitis virus

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With the objective to increase the immunogenicity of the HbsAg through the use of biological adjuvants, we have developed a procedure for obtaining immunocomplex in an excess of antibodies by means of immunological methods in solid phase, from alum adjuvanted vaccines and in liquid phase. They were given to experimental animals (sheep); susceptible humans with high risk of exposition and persons chronically infected (reservoirs of hepatitis B virus). The sheep antibody titers obtained post vaccination were higher than 10^6 IU/L, and the short period of latency was an outstanding subject by using this procedure. This fact, allows us to early select the hyper-responders animals. In non reservoir human, it was observed in only seven days after the booster dose; and in the reservoirs humans it has not been obtained any type of side effects or undesired response. For these reasons, the possibility to use this procedure as a form of therapy can not be discarded, then, as it is known, it does not exist an effective therapy for this disease at the present time.