

INTERNATIONAL WORKSHOP

Allergen Vaccines 2009

CURRENT PROBLEMS IN ALLERGEN VACCINE
DEVELOPMENT AND MANUFACTURING

...bringing science to industry

Varadero, Cuba, October 18-23, 2009





Program Summary

Symposiums

1. Novel Allergen Vaccines
2. Allergen Standardization
3. Regulatory Perspective
4. Pharmaceutical Development
5. Clinical Trials

Poster Sessions

1. Novel allergen vaccines
2. Standardization
3. Allergen characterization
4. Process development
5. Preclinical development
6. Regulatory aspects
7. Clinical trials of allergen IT
8. Allergen sensitization

Authors

Scientific Committee

Synopsis: Allergen Standardization and Characterization
Fernandez-Caldas E, Zakzuk J, Lockey RF

Organizing Committee

Sponsors

Sirenis La Salina, Varadero

Organized by:



**Latin American
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**Cuban Society of
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INVITATION

Dear Colleague

The Organizing Committee of the International Workshop on Current Problems in Allergen Vaccine Development and Manufacturing, **AllergenVaccines 2009**, is pleased to invite you to participate in this scientific meeting, to be held in Varadero, Cuba. AllergenVaccines 2009 is organized by the Latin American Association of Immunology (ALAI) and the Cuban Society for Immunology (SCI), with the endorsement of the Cuban Society of Allergy, Asthma and Clinical Immunology (SCAAIC), intending to promote the scientific exchange and foster collaboration worldwide in this developing field. These five days conference will gather important world-level academic researchers, company scientists, clinical investigators and practitioners, and regulatory specialists.

We are looking forward to having the opportunity of warmly welcoming you at our meeting, and will make our best efforts to arrange all travel details you may need, in order to make of your participation not only a rewarding scientific experience but a wonderful trip to our country. The venue, located in the beautiful beach of Varadero, provides a paradisiacal environment, which allows for an effective combination of science and relaxation in the friendliest atmosphere. This will be a unique opportunity to bring also your family!.

Please, make this communication available to your teammates and interested colleagues.

Best wishes,



Alexis Labrada
President Organizing
Committee



Enrique Fernández-Caldas
President
Scientific Committee



Oliver Pérez
President Cuban Society
Immunology



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 October 18-23 • Varadero • Cuba • www.sci.sld.cu/alergia2009/allergen.htm



Program Summary

Day	Morning	Afternoon	Night
18 th	Travel from Havana to Varadero (Sirenis La Salina Hotel)	Registration	Welcoming Dinner
		Opening Ceremony	
		Opening Lecture	
19 th	Symposium 1: Novel allergen vaccines	Symposium 1: Novel allergen vaccines	<u>Poster Session</u>
20 th	Symposium 2: Allergen standardization	Symposium 3: Regulatory perspective	<u>Poster Session</u>
21 th	Symposium 4: Pharmaceutical process development and validation	Free (Dolphin Show)	Social Activity
22 th	Symposium 5: Clinical trials of allergen vaccines, recombinant and sublingual vaccines	Hot Topics	Free
		Closing Ceremony	
23 th	Bilateral meetings	Check out and return to Havana	Free



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Opening Ceremony

Oct. 18

18:00 – **Alexis Labrada**
18:20 – (Organizing Committee,
Cuba)

Introductory words.

18:20 – **Enrique Fernández-
Caldas** (Germany)
19:00 – Allergy Innovations,
Germany

A1 Research and Development in
the University and in the
Pharmaceutical Industry:
Implications for the field of
Allergy Vaccination.



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Oct. 19: Symposium 1: Novel Allergen Vaccines

Session 1.I Chairs: Rudolph Valenta, Oliver Pérez

9:00-9:30 9:35-9:55 10:00-10:20 10:25-10:45	Rudolph Valenta Medical Univ. Vienna, Austria Laurence Van Overtvelt Stallergenes, France Alain Jacquet Chulalongkorn Univ., Thailand Oliver Pérez Instituto Finlay, Cuba	KNA1: Recombinant allergen-based approaches for immunotherapy C1.1 Mucoadhesive allergen formulations for sublingual immunotherapy C1.2 Immunomodulatory properties of probiotics in the context of house dust mite allergy C1.3 Proteoliposome from <i>Neisseria meningitidis</i> as allergen adjuvant
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Session 1.II Chairs: Martin Chapman, Luis Caraballo

10:55-11:25 11:30-11:50 11:55-12:15 12:20-12:40 12:40-12:55	Martin Chapman Indoor Biotech, USA Enrique Fernández-Caldas Allergy Innovations, Germany Reto Cramer SIAF, Switzerland Luis Caraballo III Univ. Cartagena, Colombia General Discussion	KNA2: Structural biology, function and rational design of allergen vaccines C1.4 Improving mite allergen extracts for diagnosis and treatment C1.5 Challenges facing novel allergen vaccines for becoming a more competitive therapeutic/prophylactic approach C1.6 The genetic basis of allergenicity and allergy vaccination
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LUNCH

Session 1.III Chairs: Momtchilo Russo, Leonardo Puerta

14:30-14:50 14:55-15:15 15:20-15:28 15:32-15:40 15:44-15:52 15:56-16:04 16:08-16:16 16:16-16:32	Momtchilo Russo Univ. Sao Paulo, Brazil Alirio Melendez Glasgow Univ., UK S. Deifl Medical University of Vienna Austria Niespodziana K University of Viena, Austria Flicker S Medical University of Vienna Austria Egea E Universidad del Norte, Barranquilla, Colombia Khosravi AR Tehran University, Iran General Discussion	C1.7 Downmodulation of Type 2 immunity by bacterial adjuvants C1.8 Anti-inflammatory/Immunomodulatory properties of SphK1 blockade during Adjuvant-allergen immunization: Potential for reducing adjuvant-inflammation and for switching Th2 towards a Th1 response P1.1 Different Toll-like receptor ligands as possible adjuvants in allergy vaccines (SOP) S1.1 A cat allergy vaccine based on Fel d 1-derived peptides fused to a viral carrier protein (SOP) S1.2 Prevention of allergy by administration of allergen-specific IgG antibodies (SOP) S1.3 Evaluation of the immunocompetence of a set of six linear synthetic oligopeptides designed from the Der p 1, Der f 1 and Blo t 1 allergens (SOP) S1.4 Evaluation of the correlation between tissue reaction and cytokines patterns induced by <i>Alternaria alternata</i> in mice (SOP)
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Oct.
20:

Symposium 2: Allergen Standardization

Session 2.I Chairs: Ronald van Ree, Domingo Barber

- 09:00-09:30 **Ronald van Ree**
 AMC Univ Amsterdam, Netherlands
- 09:35-09:55 **Domingo Barber**
 ALK-Abelló, Spain
- 10:00-10:20 **Martin Himly**
 Univ. Salzburg, Austria
- 10:25-10:45 **Murray Skinner**
 Allergy Therapeutics, UK

KNA3: Recombinant major allergens as standardization tools

- C2.1 Relevance of minor and pan-allergens for standardization
- C2.2 The physicochemical tool box for standardization of allergen products: from recombinant single allergens to polymerized high MW extracts
- C2.3 Novel approaches to standardizing grass mix product

Session 2.II Chairs: Jorgen Larsen, Sten Dreborg

- 10:55-11:25 **Jorgen Larsen**
 ALK-Abello, Denmark
- 11:30-11:50 **Sten Dreborg**
 Sweden
- 11:55-12:15 **Desirée Larenas**
 Hospital Medica Sur, AAAAI, CMICA, Mexico
- 12:20-12:40 **Alexis Labrada**
 BIOECN, Cuba
- 12:45-12:53 **Eva King**
 Indoor Biotechnologies Inc., USA

KNA4: Essential aspects of allergen vaccine standardisation

- C2.4 Biological Standardization: the Nordic concept and the ID50 intradermal method
- C2.5 Comparison between european, mexican and american allergen extracts by in-vitro and skin test methods
- C2.6 Standardization of allergen vaccines from tropical mites: *Dermatophagoides siboney* and *Blomia tropicalis*
- S2.1 A Multi-Center Ring Trial of Allergen Exposure Assessment using Fluorescent Multiplex Array Technology (SOP)

13:00-13:15 **General Discussion**



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Oct
 20:

Symposium 3: Regulatory Perspective

Session 3.I Chairs: Carlo Pini, Frank Schuler

14:30-
 15:00

Carlo Pini
 Istituto Superiore Sanita,
 Italy

KNA5: European regulatory requirements

15:05-
 15:25

Frank Schuler
 Paul Ehrlich Institut,
 Germany

C3.1 The concept of homologous allergens for registration of allergen products

15:30-
 15:50

Ingrid Mueller
 Univ. Albstadt-
 Sigamringen, Germany

C3.2 The design of a Quality Management System for pharmaceutical companies to optimize performance

15:55-
 16:15

Rolando Domínguez
 CECMED, Cuba

C3.3 Requirements for registration of allergen products in Cuba

16:25-
 16:55

Session 3.II Chairs: Sten Dreborg, Rolando Domínguez

Sten Dreborg

KNA6: Pharmacovigilance of allergen immunotherapy

17:00-
 17:20

Sweden
 Ledit Arduso
 Univ. Nacional Rosario,
 AAAIC, Argentina

C3.4 Specific Allergen Immunotherapy in Clinical Practice in Latin America

17:25-
 17:45

Ruppert Hahnstadt
 FDA Allergenic, Brazil

C3.5 Current state of registration of allergen products in Brazil (Mercosur)

17:50-
 18:10

Aznar E, Cartaya,
 Fernández L, Castro RL,
 Domínguez R.
 BIOEN, CECMED, Cuba

C3.6 Round Table: Regulatory Requirements for Clinical Trials of Allergen Vaccines in Cuba

18:10-
 18:25

General Discussion

Oct
21:

Symposium 4: Pharmaceutical Process Development and Validation

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Session 4.I Chairs: Kees van der Graaf, Jerónimo Carnés

9:00- **Kees v. der Graaf**
9:30 Citeq, Netherlands

9:35- Jerónimo Carnés
9:55 LETI, Spain

10:00- Victor Iraola
10:20 LETI, Spain

10:25- Zbynek Drab
10:45 Pharmallerga, Czech Rep.

KNA7: Mite culture process: GMP requirements

C4.1 Food allergen extracts: standardization of manufacturing process

C4.2 Allergen extracts of storage mites

C4.3 Pollens as raw allergenic materials- practical and regulatory perspectives

Session 4.II Chairs: Bev Lees, Claudio Rodríguez

10:55- **Beverley Lees**
11:25 Allergy Therapeutics, UK

11:30- Idania Caballero
11:50 CIM, Cuba

11:55- Alberto Agraz
12:15 BIOCEN, Cuba

12:20- Claudio Rodríguez,
12:40 Humberto Perez, Alexis Labrada, BIOCEN, Cuba

KNA8: Pharmaceutical Development and Validation of Grass Modified Allergen Tyrosine Absorbate (MATA) with MPL adjuvant

C4.4 Key questions on lyophilization of biopharmaceutical products

C4.5 High-scale production of biopharmaceuticals in Cuba

C4.6 **Round Table** Quality system for biopharmaceutical products, including allergen products in BIOCEN

12:40- General Discus
12:55



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Oct
 22:

Symposium 5: Clinical Trials of Allergen Vaccines, Recombinant and Sublingual Vaccines

9:00-9:30	Session 5.I Chairs: Roy Gerth van Wijk, Marek Jutel Roy Gerth v. Wijk EAACI / Erasmus Univ. Rotterdam, Netherlands	KNA9: Clinical trials for sublingual IT
9:35-9:55	Gabrielle Pauli Strasbourg Univ., France	C5.1 Clinical trials of recombinant allergens for diagnostic/therapeutic use
10:00-10:20	Karla Arruda FMRP, Univ. Sao Paulo, Brazil	C5.2 Clinical significance of recombinant allergen vaccines
10:25-10:45	Marek Jutel Wroclaw Medical Univ., Poland	C5.3 Clinical trials of SIT with recombinant allergens
10:55-11:25	Session 5.II Chairs: Miguel Casanovas, Adriano Mari Miguel Casanovas Inmunotek, Spain	KNA10: Design of Clinical trials for immunotherapy
11:30-11:50	Jorgen Larsen ALK-Abello, Denmark	C5.4 Clinical development program of the first tablet for sublingual immunotherapy
11:55-12:15	Tom Holdich Allergy Therapeutics, UK	C5.5 Challenges in performing Global Phase III studies in SIT
12:20-12:40	Barbara Bohle Medical Univ. Vienna, Austria	C5.6 Mechanisms of Allergen SIT
12:45-13:05	Adriano Mari IDI-IRCCS/Allergome, Italy	C5.7 Immunotherapy decision-making by using allergenic molecule-based diagnostics
13:05-13:20	General Discussion	



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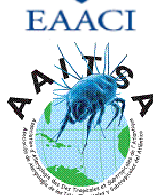
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Association d' Allergologie des Iles Tropicales et Subtropicales de l' Atlantique



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Fernandez-Caldas E, Zakzuk J, Lockey RF

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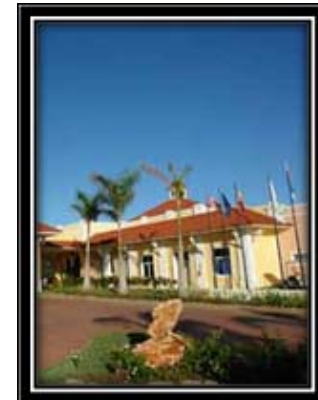
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- P1.1 Different Toll-like receptor ligands as possible adjuvants in allergy vaccines
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- P1.3 Therapeutic vaccine against food allergy
- P1.4 Hypoallergens of Art v 1, the major mugwort pollen allergen as candidate for artemisia immunotherapy
- P1.5 Probiotics' therapeutic potential in a mouse model of food allergen sensitization
- P1.6 AFPL1 adjuvant effect to House Dust Mite allergen
- P1.7 A new proteoliposomic formulation of Neisseria meningitidis outer membrane components with better structural definition, and potentiality as a Th-1 adjuvant
- P1.8 Design of a non-allergenic Der p 1 peptide-based vaccine for immunotherapy of house dust mite allergy
- P1.9 Carrier-bound peptides of the major allergen Alt a 1 for vaccination against allergy to the mould *Alternaria alternata*
- P1.10 Liposomes and Sticholysin II as immunomodulators in a murine ovalbumin model of asthma
- P1.11 Morphological response to respiratory allergen challenge in mice immunized with liposome-encapsulated allergens of *Dermatophagoides siboney*

STANDARDIZATION OF ALLERGEN PRODUCTS

- P2.1 Characterization and comparison of commercially available mite extracts for in vivo diagnosis and immunotherapy
- P2.2 Standardization of Tyrosine Adsorbate Depots for use as Subcutaneous Vaccines
- P2.3 Searching for markers of biologic/allergenic activity during house dust mite culture process for manufacturing allergen vaccines
- P2.4 Total protein concentration as an easy and cheap method for allergen extracts Standardization for routine work
- P2.5 A method for relative potency determination of new In-House Reference batches of allergen products, by parallel Skin Prick Test
- P2.6 Development of in-house references of house dust mite allergen therapeutic vaccines
- P2.7 Validation of an IgE-Inhibition ELISA as a potency assay for allergen vaccines of House Dust Mites
- P2.8 ELISA for the Group 1 Major Allergen determination in House Dust Mite vaccines, as a quality control assay: use of polyclonal vs monoclonal antibodies
- P2.9 Development of a semi-quantitative IgE Western-Blotting method for allergen composition of House Dust Mite vaccines
- P2.10 Amount of major allergen Phl p5 in different European products for Sublingual Immunotherapy



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ALLERGEN CHARACTERIZATION

- P3.1 Characterization of allergens homologous to Art v 1 in the pollen of different artemisia species
- P3.2 Protease and allergenic activity of allergen extracts of *Blomia tropicalis*, *Dermatophagoides pteronyssinus* and *Dermatophagoides siboney*
- P3.3 Cross-reactivity between the recombinant allergens Blo t13 and Der f13
- P3.4 Amino acid and cDNA sequence of the serine protease allergen (Der s 3) from *Dermatophagoides siboney*
- P3.5 Molecular cloning and characterisation of a new wheat food allergen
- P3.6 In-vivo allergenic activity of major allergens of *Dermatophagoides siboney* by Skin Prick Test
- P3.7 IgE responses to Blo t 5 and Blo t 12 allergens in mite allergic patients from Martinique, France

PROCESS DEVELOPMENT & VALIDATION

- P4.1 Effects of protease inhibitors in the preparation of allergen extracts of *Dermatophagoides pteronyssinus*
- P4.2 Concentration of *Dermatophagoides farinae* extracts by tangential flow filtration in different molecular weight fractions for diagnosis and treatment of mite allergy
- P4.3 Effect of the dialysis pore size on allergen content and allergenicity of *Dermatophagoides farinae* extracts
- P4.4 Validation of diafiltration processes of House Dust Mite allergen vaccines
- P4.5 Endotoxin content in industrially manufactured allergen extracts of House Dust Mites, including *Dermatophagoides siboney* and *Blomia tropicalis*
- P4.6 Assessment of consistency of the manufacturing process of House Dust Mite allergen vaccines
- P4.7 Evaluation of bacteriostatic and fungistatic properties of different formulations of mite allergen vaccines
- P4.8 Factors influencing the adsorption of *Dermatophagoides siboney* allergen extract into aluminum adjuvants
- P4.9 Preliminary characterization of the microbial bioburden during the culture of *Blomia tropicalis* and *Dermatophagoides siboney*
- P4.10 Process Development of Therapeutic Vaccines based on Recombinant Glycoproteins produced with Animal Cell Technology
- P4.11 Development and validation of a Western Blot assay for identifying antigens in the Outer Membrane Protein Complex of *Neisseria meningitidis*
- P4.12 Development of a lyophilization cycle and a procedure for drying rubber stoppers to increase the shelf life of hygroscopic allergen formulations



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PRECLINICAL PHARMACOLOGICAL DEVELOPMENT

- P5.1 Challenges in design of an appropriate genotoxicity package for allergen products
- P5.2 A murine model of allergic respiratory inflammation provoked by *Dermatophagoides siboney* allergens as a tool for preclinical evaluation of therapeutic antiallergic vaccines
- P5.3 Influence of a proteoliposome adjuvanted allergen vaccine on to an earlier response against *Neisseria meningitidis*
- P5.4 Preclinical evaluation in mice of the immunogenicity of a novel anti-allergic vaccine, based on *Neisseria meningitidis* proteoliposome, as adjuvant
- P5.5 Shelf-life stability study of a novel adjuvanted and adsorbed House Dust Mite allergen vaccine
- P5.6 Comparison between *Blomia tropicalis* and *Dermatophagoides pteronyssinus*-induced murine models of allergic asthma

REGULATORY ASPECTS AND QUALITY ASSURANCE

- P6.1 The Paul-Ehrlich-Institut: An Example of Quality Management in a European Regulatory Agency
- P6.2 Methodology for obtaining the Sanitary License of Pharmaceutical Operations for Allergen Vaccines in BIOGEN
- P6.3 The new regulation for therapy allergens as approach to extend the marketing authorisation to Named Patient Products (NPP) in Germany
- P6.4 Harmonization with ICH Q10 guidelines in the development, production, and commercialization of allergen products



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CLINICAL TRIALS OF ALLERGEN IMMUNOTHERAPY AND PHARMACOVIGILANCE

- P7.1 Evaluation of grass pollen specific immunotherapy: Long-time evaluation of sublingual or supralingual routes
- P7.2 Long-term Study of Hyposensitisation Therapy by means of Depot Allergen Drugs (Spring Trees and Grasses) till the Stage of Complete Remission
- P7.3 IgG4 allergen-specific antibodies are inversely correlated to IgE mediated allergic response in asthmatic patients allergic to domestic mites
- P7.4 Efficacy and safety of subcutaneous immunotherapy using standardized House Dust Mite vaccines in the treatment of allergic asthma in a Cuban population. Results from Double-Blind Placebo Controlled clinical trials
- P7.5 Associated factors to adverse reactions to immunotherapy with mite allergen extracts in allergic diseases
- P7.6 The practice of allergen specific immunotherapy by Cuban allergologists
- P7.7 Clinical Trial of Sublingual Immunotherapy in asthmatic Cuban patients using a standardized allergen vaccine of *Dermatophagoides siboney*
- P7.8 Cost-Effectiveness of Subcutaneous Immunotherapy with Mite Vaccines containing Depigmented and Polimerized Allergen Extracts plus Beclomethasone Dipropionate versus Beclomethasone Dipropionate in children with asthma in Bogota-Colombia
- P7.9 Monitoring of antibody-responses during grass pollen extract immunotherapy and after five years of discontinuation with recombinant allergens
- P7.10 Therapeutic effect in asthmatic adults treated with sublingual immunotherapy with a standardized allergen vaccine of *Dermatophagoides pteronyssinus*
- P7.11 Clinical Trial of sublingual immunotherapy with a standardized allergen vaccine of *Blomia tropicalis* in asthmatics patients
- P7.12 IgE/IgG4 ratio as a possible surrogate marker of clinical efficacy during allergen immunotherapy
- P7.13 Double-Blind Placebo-Controlled Clinical Trials of Subcutaneous Immunotherapy for evaluating the efficacy and safety of standardized House Dust Mites allergen vaccines in Cuban asthmatics
- P7.14 Cross-protection to *Tyrophagus putrescentiae* Allergy by Local Nasal Immunotherapy using Strips of *Dermatophagoides pteronyssinus*



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- P8.1 Recombinant Der p 10 as a diagnostic tool to identify patients with genuine shrimp sensitization
- P8.2 Sensitivity and Specificity of Skin Prick Test with standardized allergen extract of Dermatophagoides pteronyssinus in adults
- P8.3 IgE response to sweet orange (citrus sinensis) fruit in Cuban allergic patients
- P8.4 Skin Prick Tests Results In Children from Middle Black Sea Region, Turkey
- P8.5 Evaluation of a soy extract (Glycine max) for skin prick test in a Cuban population
- P8.6 A possible relationship between the atopic status of children with asthma and allergic rhinitis and HSV1 infection
- P8.7 Carpet Beetle (Anthrenus verbasci, Linnaeus 1767): A New Seasonal Indoor Allergen
- P8.8 Diagnostic efficacy of Nasal Provocation Test with a standardized allergen extract of Blomia tropicalis
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- P8.11 Plasmid vectors for the expression of complete allergen-specific human IgE, IgG1 and IgG4 antibodies
- P8.12 Evaluation of peanut allergenic extracts for skin prick test in Cuban population



Synopsis

Allergen Standardization and Characterization



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Introduction

There is a worldwide increase in atopic diseases, which include allergic rhinoconjunctivitis, allergic asthma, atopic dermatitis and food allergies¹. Although the reasons for this increase are unclear, allergen exposure is recognized as an important environmental risk factor in genetically predisposed individuals. The diagnosis of allergic disease requires a detailed history, physical examination, and allergy testing, i.e., skin testing or the in vitro determination of allergen-specific immunoglobulin E (IgE). Skin testing is performed by applying an allergen extract to the skin and then scratch or pricking it with an appropriate needle-like instrument. In sensitized individuals, it results in the formation of a raised wheal surrounded by an erythematous flair within 15 to 20 minutes, indicating a positive test reaction. In vitro specific IgE also supports the clinical diagnosis and helps to guide the allergist in the management of allergic diseases.

Once an individual is sensitized, symptomatic and long-term strategies, such as environmental control and immunomodulatory treatments using allergen immunotherapy, play an important role in treatment. Specific allergen immunotherapy (SIT) is effective in alleviating allergic symptoms, especially when introduced early in life and is the only known treatment that affects the natural course of allergic diseases. It also may prevent the development of asthma in patients with allergic rhinitis².



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SIT is the practice of administering gradually increasing doses of allergen vaccines to reduce allergic symptoms and the need for medications. This biological response modifier is capable of influencing allergen-driven immunological responses and restoring, to a certain degree, the Th1/Th2 balance in allergic subjects. B and T cells, blocking antibodies, IL-10 and other cytokines play an important role in the response to SIT³.

Accuracy of skin tests and in vitro determinations depend on the availability of well-characterized allergen extracts. So too does effective allergen immunotherapy; allergen standardization and characterization are paramount to achieve these goals.

Testing and Standardizing Allergen Products

Allergen products to diagnose and treat allergic diseases have been used for over 100 years. Allergen extracts are biological products that are administered to humans to diagnose, prevent and treat allergic diseases⁴. Numerous double-blind placebo-controlled studies using unmodified aqueous allergens⁵ and allergoids⁶ (modified allergens) have demonstrated efficacy. "Allergoid" is a term used to describe natural allergen products that have been modified with aldehydes to decrease their allergenicity and potentially increase their safety. Some allergoids are commercially available in Europe and have demonstrated successful clinical results⁷. Sublingual immunotherapy, using drops of aqueous allergen extracts under the tongue and then swallowed⁸, or tablets⁹ with similar type of extracts, utilized the same way, also appear to be clinically beneficial, particularly for grass induced rhinoconjunctivitis.

The quality of allergen products is a key issue for both diagnosis and therapy, and the standardization of allergen extracts is thus of primary importance to improve their quality and offer physicians worldwide a reliable method to diagnose and treat allergic respiratory diseases. Effective diagnosis and treatment, using skin test reagents and SIT, requires the optimal amount of allergens for testing and the maintenance dose of vaccine for treatment. Skin test reactions should be large enough to suggest clinical sensitivity but not so large as to produce excessive discomfort or the risk for a serious systemic reaction.

The heterogeneity of allergen extracts makes it necessary to develop methodologies to assess their potency and ensure their consistency, stability and safety. Allergen products are legally considered medicines that require registration by government institutions¹⁰, such as the FDA in the United States and the Paul Ehrlich Institute in Germany, further increasing the need for standardization. Basic researchers, physicians, regulatory authorities and manufacturers have tried to define a common methodology to standardize allergen vaccines^{11, 12, 13}. The quality of mite and pollen allergen extracts is better defined today than it was in the past, and the quality of food and epithelial allergen extracts has also improved^{14, 15}. Further improvement of food allergen extracts is needed, since preliminary studies have shown efficacy using unmodified sublingual food allergen extracts¹⁶.

In Europe, because of the need to standardize allergen extracts for diagnosis and treatment, manufacturers have implemented company-specific protocols to standardize the production and quality control of the allergen extracts and to compare production batches to assure batch-to-batch consistency¹⁷. All these production and quality control issues are controlled by strict protocols and adhered to Good Manufacturing Practices¹⁸. Thus, current allergen standardization requirements concentrate on the consistency of production and the safety and potency of allergen products.



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These protocols use *in vivo* and *in vitro* standardization techniques, a representative allergic patient population, and dose-response studies to assign biological activities. Dose-response studies are mandatory and are primarily based on skin testing (intradermal or prick tests) and on inhibition of allergen-specific IgEs compared to reference extracts known as in-house reference preparations (IHRPs). Furthermore, guidelines have been issued for the clinical development of products for specific immunotherapy for the treatment of allergic diseases¹⁹. The IgE-binding potencies of the IHRPs are quantified by skin test reactivity (*in vivo* standardization) and by competitive IgE tests, such as RAST, ImmunoCAP, or ELISA inhibition assays (*in vitro* standardization). Although they may be similar, they are expressed in company-specific units.

The comparison of different products from different companies at national and international levels is complicated. Larenas-Linnemann and Cox reviewed the information obtained on unit definition and dosage of allergens from European manufacturers of allergen extracts used for sublingual immunotherapy (SLIT). They concluded that the monthly maintenance dose the manufacturers recommended for SLIT was 5-45 times higher than the recommended dose for subcutaneous immunotherapy. However, since each manufacturer in Europe uses its own IHRPs and its own units to express potencies, the comparison of different products from different companies at national and international levels is almost impossible. Even if the amount of major allergens is stated, differences in the quantification technique, the reference extracts and antibodies used can influence the outcome. Thus, for comparison of diagnostics and immunotherapeutics from different manufacturers, the same analytical methods and materials ideally should be used²⁰.

Another study quantified and compared the allergen content of different grass pollen preparations for skin prick testing (SPT) and SLIT used in Europe²¹. Protein concentrations of SPT solutions ranged from 15 to 427 µg/ml, and Phl p 5 concentrations, a major grass allergen, ranged from 0.15 to 18.3 µg/ml. Protein content of the maintenance doses of the immunotherapeutics ranged from 5 to 153 µg and Phl p 5 content ranged from 0.2 to 21.6 µg. SDS-PAGE and immunoblots confirmed the differences in protein and allergen contents.

Extracts in the United States are more homogenous with respect to total allergenic potency than the extracts produced in Europe, mainly because the FDA provides the same standardized reagent for internal use by all manufacturing companies²². However, great differences have also been shown among unstandardized mold allergen extracts in the USA²³.

The characterization of major allergen components and the development of techniques to quantify them, such as ELISA systems based on monoclonal antibodies, have led more manufacturers to provide information on the major allergen content of their extracts, even though identification of major allergen content is not currently mandatory, except for a limited number of extracts, such as cat and ragweed. The World Health Organization and some other regulatory government institutions now recommend that allergen manufacturers state the content of representative major allergens in mass units for their allergen products²⁴. As noted above, differences in assays and methodologies for measuring the major allergens may preclude direct comparisons among products of different manufacturers²⁵.



Recombinant Allergens (RAs)

Allergenic extracts consist of complex mixtures of substances with a variation in allergenic activity and allergen composition. Furthermore, patients are often not sensitized to all allergens in one extract and there may be great variability among different patients. Advances in the field of microarrays containing purified native and recombinant allergens (RAs) may be useful to identify the specific sensitivity of individual patients in order to select the proper allergen composition in the vaccine²⁶. To improve safety and efficacy of SIT, a better (alternative) approach may be to use RAs. There has been considerable progress in understanding the molecular characteristics of allergens. Many allergens have been purified from aqueous extracts or produced as recombinant molecules. Allergen sequence information is available in different databases (www.allergen.org/www.allergome.org). Molecular cloning has provided an efficient way to obtain pure polypeptides, which in their native sources, form complex mixtures and are often present in very small amounts. These polypeptides enable the mapping of B- and T-cell epitopes and the identification of their binding sites. Sequence polymorphisms that influence antibody binding and T-cell recognition for several allergens have also been established. Sequence similarity searches have identified the biological functions of many allergens. For example, Der p 1 and Der f 1 are considered major allergens based on the frequency of patients sensitized, amount of specific IgE, and content in mite extract. Der p 1 is a glycoprotein with sequence homology and thiol protease function similar to the enzymes papain, actinidin bromelain and cathepsins B and H²⁷. When an allergen does not have sequence homology with proteins of known biological function, its biochemical identity remains uncertain. Several mechanisms are involved in airway hyper-responsiveness and airway inflammation caused by these allergens. Besides acting by immunologically mediated mechanisms, Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions²⁸. Due to its high prevalence and worldwide distribution in house dust, the group 1 allergen is used as a standard to estimate environmental exposure to *Dermatophagoides* spp. in the indoor environment.

Since allergen extracts or vaccines represent several hundred allergy determinants, and individuals can be sensitized to different combinations of these proteins, sensitization profiles in atopic populations are very heterogeneous. Therefore, before RAs can be frequently utilized in routine clinical practice, epidemiological studies using RAs to identify the immune response to specific allergens in the population at large and to clarify cross-reactivity are necessary. Several immunotherapy studies have been conducted with recombinant allergens with clinical benefits^{29, 20, 31, 32}. More clinical trials are needed to compare the potential advantages and benefits of SIT with RAs versus current standard therapy with natural vaccines. This process will take years; in the meantime, optimizing SIT with naturally occurring vaccines is required to assure efficacy and safety.

The number of cloned and purified allergens has increased substantially over the past decade. RAs are useful as reagents for SIT. RAs will overcome some of the pitfalls of using natural allergen products for immunotherapy by enabling physicians to administer only the clinically relevant allergen, thus avoiding exposure to unnecessary antigens. RAs could also be rendered hypoallergenic using a variety of techniques.



The CREATE Project

A project financed by the European Union, "Development of Certified Reference Materials for Allergenic Products and Validation of Methods for their Quantification", (the CREATE Project), explores the idea of introducing standardized techniques to quantify major allergen content into standardized protocols. Mass units of major allergens would be used to quantify the active ingredients of the allergen while allowing comparison among manufacturers³³. The goals of the CREATE Project were to evaluate the potential of purified recombinant allergens as certified reference materials (CRMs) and to evaluate available ELISAs for the measurement of major allergens using the candidate CRMs as a standard. To carry forward with this project, eight major allergens originating from four of the most important inhalant allergen sources were selected: Bet v1 from birch pollen, Phl p 1 and Phl p 5 from grass pollen, Ole e 1 from olive pollen and Der p1 and 2 and Der f 1 and Der f 2 from house dust mites. Three were found to be suitable as biological reference materials; the rest, except rPhl p 1a, indicate potential for optimization, provided aspects of their protein expression processes are modified. As a result of this study, recombinant Bet v1 and Phl p 5 are being produced under "Good Manufacturing Practice" and being evaluated by the European Directorate for the Quality of Medicines as biologic reference preparations to be included in the European Pharmacopoeia as international standards. Consequently, standardization of these allergen products will become global permitting comparisons among different manufacturing sources³⁴.

Other techniques which could be used for standardization

Beyond the use of monoclonal antibodies, other physico-chemical approaches have been explored for allergen standardization and offer new possibilities. Evaluation of mass spectrometry (MS) has been performed to determine its capacity to characterize the composition of allergen extracts^{35, 36}. One advantage offered by the use of MS includes the measurement of several different allergenic components (allergens and isoforms) simultaneously rather than measuring individual allergens. This is advantageous while working with allergen preparations that contain a wide group of IgE binding proteins, such as mite extracts. Additionally, MS-based methods are available to discriminate between allergen isoforms, which is difficult to accomplish using immunologic based methods. This method could also be useful for the standardization of allergoids, since measuring major allergens is not possible in these preparations due to their chemical modification with aldehyde. The issues with MS-based protocols are that they are not quantitative and are not addressed in regulatory policies.

Conclusions and Current challenges

Allergen standardization strategies should be uniform throughout the world. The different units, which vary among manufacturers as well as global regions, are confusing and unreliable with the potential to underestimate or overestimate the potency of allergen extracts. The variations can be attributed to the variability of the raw materials used, the production methods and the lack of consistent and reliable quantification of allergy content. Major allergen measurements are essential to overcome these problems. Some recombinant allergens were found to be suitable as certified reference materials. But, results from the CREATE Project demonstrate the limitations of recombinant allergens as CRM due to incorrect folding, aggregation, poor solubility and insufficient stability³³. However, evidence gathered in studies identifies the complexity of this approach. The European Medicines Agency (EMA) recommends proving that each allergen extract contains the relevant allergens by antibody-based techniques or mass spectrometry.



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New techniques, such as nuclear magnetic resonance and small angle X ray scattering, are commonly applied to characterize allergenic molecules in the laboratory. They are slowly affecting the methods by which allergen extracts are standardized. This would be an important step forward towards a comprehensive characterization of allergen products. Future enhancements of SIT, using RAs, also require allergen standardization, which in turn, requires development of standardized methods to measure allergen content, homogeneity, folding, aggregation, solubility and stability of recombinant products. Allergen standardization will advance rapidly in the future, improving the effectiveness and safety of allergen vaccines. Allergen immunotherapy will remain an effective and safe treatment for allergic respiratory diseases.

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A1 - Research and Development in the University and in the Pharmaceutical Industry: Implications for the field of Allergy Vaccination.

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High clinical development costs along with declining success rates in drug discovery are causing productivity levels to fall in the global pharmaceuticals industry. The imminent patent expiry of several important drugs and the rise of generic alternatives is further exacerbating the situation. Despite this, the global pharmaceutical industry offers significant growth opportunity for new strategic business models. The total estimated market was estimated at USD 554.00 billion in 2004. However, the global pharmaceuticals market is forecast to register an annual growth rate of 8.2 per cent from 2004 to 2011 to reach USD 967.00 billion. Such expansion is expected to be based on the ability of pharmaceutical companies to adapt to changes in patient population, and target diseases of unmet medical need to maximize revenue potential.

Throughout the years, there has been a long standing relationship between Universities and the Pharmaceutical industry. Currently, academic scientists and the Pharmaceutical industry function in different worlds with different incentives. As a result, the gap between the two has grown rather than narrowed in the last 30 years. Some reasons may account for these differences. Understanding complex diseases requires the expertise of multiple related disciplines, which are present in the industry. The competitive reward structure of academic research prevents productive collaboration with others. The "product" resulting of medical research done in university laboratories is commonly scientific articles published in peer-reviewed scientific journals, not patient treatments. However, in many instances, these research projects also result in patents. For complex diseases, to assemble, test and validate the data reported in scientific journals from multiple unrelated investigators over the years is virtually impossible.

In the 1950s, the number of academic discoveries was more manageable and could more easily find its way to industry channels, and then be turned into treatments. Today, academic laboratories funded by the NIH and other non-profit organizations are the source of tens of thousands of incremental discoveries each year. Monitoring these discoveries for potential commercial treatments has become virtually impossible. As a result, the separation between the academic scientists and the pharmaceutical industry is growing wider as the years pass.

Promising results from academic laboratories, even those published in peer-reviewed scientific journals, are generally viewed as incomplete, lacking the level of rigor and reproducibility demanded by industry. Pharmaceutical companies are unlikely to make the multimillion-dollar investment in validating discoveries made outside their own research and development laboratories. On average it costs industry more than \$1 billion to successfully identify a single drug compound for a new target, take it through development, clinical trials and FDA approval.



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The field of Allergy vaccines is not lagging behind, although the market may be too small to experience the generalized growth seen in other disciplines. However, the increasing number of patients is sufficient to support new innovative products. Furthermore, the World Health Organisation has stated that the optimal treatment of allergy consists of four actions: avoiding the substance that causes the allergy; taking symptomatic medication; Undergoing an allergy vaccination programme and implementing patient education. The world of allergy vaccination has experienced little changes since the early 1900, when allergen vaccines were first introduced. The use of aluminum hydroxide in the 1930's and the use of allergoids in the late 1960's can be considered as major innovations. The use of sublingual immunotherapy in the late 1980's was mainly introduced to circumvent safety issues. Recent developments in the field of allergy vaccination have included the launch of sublingual tablets, promising studies with recombinant allergens, peptides, monoclonal antibodies, fusion molecules and the use of novel viral and bacterial adjuvants. These adjuvants have been administered with single molecules and whole allergen extracts. Novel routes of administration have been also suggested and shown efficacy, such as intralymphatic injections, or the administration of suppositories to treat peanut recombinant allergens. However, up to now, only the "classical" products have reached registration in Europa and the USA, although some innovative products may reach registration in the next few years.

In conclusion, the Pharmaceutical industry may significantly benefit from recent developments in the academic field of allergy research in Europe and the USA. However more efforts and funding should be available to launch some of these new innovative products. A closer, more realistic and comprehensive collaboration between Universities and the Industry is needed.



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KNA1 - Recombinant allergen-based approaches for immunotherapy

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Immunoglobulin E (IgE)-mediated allergy affects more than 25% of the population in industrialized countries. During the last years the cDNAs coding for most of the relevant disease-eliciting allergens have been isolated and expressed as recombinant allergens. Based on recombinant allergens it has become possible to reconstruct the epitope complexity of the most common allergen sources and novel diagnostic tests have been developed which allow the dissection of patients reactivity profiles down to the single molecules. Furthermore it has become possible to develop by recombinant DNA technology and peptide chemistry new types of allergy vaccines with reduced allergenic activity. The engineering and characterization of vaccines for the most common allergen sources will be discussed. Results from vaccination studies, in particular with hypoallergenic recombinant derivatives of the major birch pollen allergen, Bet v 1 and rBet v 1 wildtype allergen will be reported. Active treatment with the recombinant derivatives induced protective IgG antibodies which inhibited allergen-induced release of inflammatory mediators. Furthermore a reduction of skin and nasal sensitivity as well as an improvement of symptoms in actively treated patients was observed. Most important, rises of allergen-specific IgE induced by seasonal birch pollen exposure were significantly reduced in vaccinated patients. The new allergy vaccine based on genetically engineered allergen derivatives not only ameliorate allergic reactions, but also reduce the IgE production underlying the disease. According to the data it can be envisioned, that it will be possible to develop therapeutic and prophylactic vaccines based on recombinant DNA technology and synthetic peptide chemistry against the most common forms of IgE-mediated allergies.

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C1.1 - Mucoadhesive allergen formulations for sublingual immunotherapy

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Rationale: There is a major interest in identifying mucoadhesive allergen formulations for sublingual allergy vaccines, with the aim to enhance tolerance induction.

Methods: Candidate formulations were tested *in vivo*, in a therapeutic murine model of sublingual immunotherapy (SLIT) in BALB/c mice sensitized with ovalbumin (OVA). Airway hyper responsiveness (AHR), lung inflammation and T-cell responses were monitored by whole-body plethysmography, histology and ELISA, respectively. Their mechanisms of actions were investigated by studying their capture by antigen presenting cells (APCs) and effect on T cell priming.

Results: Only mucoadhesive and particulate formulations of the antigen such as polymerized maltodextrin and chitosan, enhance SLIT efficacy in mice by reducing AHR, lung inflammation and established Th2 responses. Antigen uptake and processing analysed *in vitro* using murine oral APCs or BMDCs, as well as *in vivo* by capture of formulated fluorescent OVA confirmed that these formulations enhance OVA uptake by APCs. T-cell priming in lymph nodes (LNs) assessed by intravenous transfer of carboxylfluorescein diacetate succinimidyl ester-labelled OVA-specific CD4⁺ T cells and flow cytometry analysis established that such antigen formulation leads to an earlier and stronger priming of suppressive T cells producing IFN- γ and IL-10.

Conclusions: Mucoadhesive and particulate allergen formulations enhance tolerance induction via the sublingual route, as a consequence of a better targeting of oral APCs and subsequent priming of Th1/Treg cells in cervical LNs. Such candidates should allow reducing the allergen dose and simplifying immunization schemes.



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C1.2 - Immunomodulatory properties of probiotics in the context of house dust mite allergy

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Probiotics are non-invasive and non-pathogenic commensal microorganisms colonizing very early the gastrointestinal tract of neonates. These bacteria drive the development of the mucosal immune system which is essential for the activation of tolerogenic mechanisms to foreign harmless antigens but also for the maintenance of intestinal homeostasis. Although immunomodulatory properties of probiotics were demonstrated in the treatment of gastrointestinal inflammatory disorders, the precise mechanism of this immunomodulation remained to be fully elucidated. Some components from these commensal bacteria might initiate various immune effects and consequently, via colonic dendritic cells (DC), may induce in the gut different immune responses through notably the production of IL-12 and/or IL-10, the key cytokines critical for the Th1 and Treg polarization respectively. Consequently, probiotics could play a beneficial role in the prevention or treatment of the Th2-biased allergic response. Moreover, epidemiological studies have reported the positive influence of lactobacilli and bifidobacteria microflora on allergy incidence. The aim of the present study was the characterization of the immunomodulatory properties of the two probiotics *L. plantarum* (Gram-positive) and *E. Nissle* (Gram-negative) at the level of the signaling pathways activated in DC. Since these bacterial strains display a strong anti-Th2 capacity, we assessed their potential to prevent the allergic response induced by the major mite allergen Der p 1. Moreover, we evaluated the capacity to use recombinant bacteria expressing allergen as live vaccine vehicles. By combining both Th1-type immunostimulatory properties and efficient allergen delivery capacity, new probiotic-based immunotherapeutics could represent promising vaccines against mite allergy.



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C1.3 - Proteoliposome from *Neisseria meningitidis* as allergen adjuvant

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Background and Aims: Allergy is a serious health problem worldwide. An uncontrolled or abnormal immune Th2 response mediated mainly by IgE or Tr1 dysfunction to otherwise non-pathogenic environmental antigens is involved. Particularly for respiratory and food allergens this response occurs in mucosa. Consequently, vaccine strategies are developing to circumvent this Th2 response inducing more physiologic Th1- or Tr1-like immune responses. Nevertheless, the induction of mucosal IgA, the main mucosal protector, has been less explored. *Dermatophagoides siboney* house dust mite is a frequent allergen source in Cuba. Adjuvants could be the best strategy to change this Th2 to a Th1 immune response. Therefore, we aimed to use AFPL1, from *Neisseria meningitidis* B and its cochleate derivate AFCo1 as adjuvants to allergens for both, parenteral or mucosal administration routes.

Results: AFPL1 induced a preferential Th1 immune response in human and mice to self antigens. As innate immune activation is essential to acquired immunity and it is mainly performed through pathogen-associated molecular patterns (PAMPs), we characterized them, showing that LPS was preferentially associated with the AFPL1 Th1 activity. AFPL1 adsorbed onto Al(OH)₃ (alum) increased its stability, redirected the alum immune response to a Th1 pattern, and also increased its overall immunogenicity. The calcium-mediated AFPL1 transformation into AFCo1 was able to increase its intrinsic stability, preserving proteins and PAMPs, and permitting its mucosal administration and function. Both, AFPL1 and AFCo1 have exceptional adjuvant characteristics: combining in the same structure the immune potentiating activity, the polarizing signals, and the delivery system capacities. The AFPL1 onto alum showed to change the Th2 pattern towards *D. siboney* allergens to a Th1 or Tr1 response and it has concluded its preclinical evaluation stage as a vaccine candidate. The capacities of AFCo1 to induce mucosal and systemic immune response to a panel of antigens were also conducted. Lastly, the development of single time vaccination strategies was conducted.

Conclusion: AFPL1 and AFCo1 adjuvants are very promising, particularly in allergen vaccine formulations.



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KNA2 - Structural biology, function and rational design of allergen vaccines

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Allergens belong to distinct protein families with a diverse array of biologic functions. They include enzymes, ligand binding proteins (e.g. lipocalins), enzyme inhibitors, structural and regulatory proteins occurring in 184 protein families in the Pfam database. While some have argued that this represents only small number (2%) of the proteins in Pfam, there is sufficient structural and functional diversity among allergenic proteins to exclude any common sequence of motif that causes IgE responses. Allergens such as mite feces contain proteolytic allergens and chitin which act as Th2 adjuvants for IgE production and, paradoxically, also contain bacterial and mite DNA which would predispose towards Th1 responses. High resolution crystal structures for the most important mite, cat, pollen and cockroach allergens are now available. Over 40 allergen structures have been resolved and these molecules constitute one of the most well defined groups of bio-medically important proteins.

Purified recombinant allergens have immuno-reactivity that is comparable to their natural counterparts and are being used to develop improved allergy diagnostics and vaccines. Static and suspension micro-arrays for allergy diagnosis have been developed using purified allergens. Initiatives such as the European Union CREATE project provide models for international allergen standardization of allergenic products using mass units of major allergens. Several pollen allergens have been produced under GMP conditions and have shown clinical efficacy in trials of recombinant allergen immunotherapy. One marker of success in these studies is a strong IgG4 antibody response among treated patients. The successful clinical outcome of these trials suggests that recombinant allergen vaccines will have a significant impact on allergy practice as these products are introduced over the next 5-10 years. Recombinant allergen vaccines offer the possibility of designing rational, safe and more effective immunologic treatments for allergic disease.



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C1.4 - Improving mite allergen extracts for diagnosis and treatment

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Mite allergen extracts of several species have been used to diagnose allergic respiratory diseases since the mid 1960s. Pioneer work by Voorhorst, Spieksma and Varekamp demonstrated the allergenicity of several mite species to conclude that mites from the species *Dermatophagoides pteronyssinus* were the main source of house dust allergens. Similar results were obtained by Miyamoto et al. in Japan with *Dermatophagoides farinae*. Since then, numerous mite species have also been added to the catalogue of allergenic species, including *Blomia tropicalis* and *Euroglyphus maynei*.

Mite extracts are a complex mixture of proteins (glycoproteins), carbohydrates, peptides and pigments. Many of these proteins are allergenic, and may be present in small quantities in the environment, or in mite allergen extracts. More than 20 allergenic proteins have been described in mite allergen extracts. It seems that different mite species contain very similar proteins with slight variations in amino acid sequences. Similar allergen groups have been described in numerous species, suggesting important structural similarities. However, cross-reactivity is not complete among different species. Major allergens stimulate IgE production in a majority of patients; however, any protein in a given source material has the potential to elicit an IgE response in certain individuals and are termed minor allergens. It is therefore important to ensure that all protein antigens/allergens to which humans are exposed, are contained in the raw material, especially major allergens.

In the case of mites, source materials are commonly pure mite bodies or whole mite cultures. Whole mite cultures include material from mite bodies, eggs, larvae, and fecal particles as well as some food medium. Limits for food medium contamination should be established. The medium should also be not allergenic in nature. Pure mite body extracts contain less faecal material, as well as less food medium. Clinical trials comparing vaccines based on whole mite cultures and pure mite bodies have shown similar clinical efficacy. However, we believe that more studies are needed, especially using allergoids from both types of cultures, since it seems that complete polymerization could be affected by using one or the other type of mite raw material. Other important issues include: selection of the right time culture growth for extraction, culture medium, selection of the extraction buffer, dialysis pore, length of time for extraction and freeze-drying cycle.

Before recombinant allergens will be definitively used for diagnosis and treatment of mite induced allergic diseases, we believe that there is room for better characterization and standardization of mite allergen extracts. Mite extracts should contain the relevant allergens and be similar in composition to the allergens which are inhaled by individuals. Since mite allergen extracts contain a significant enzymatic activity, the extraction time should be limited accordingly. The use of enzyme inhibitors is controversial and should be further evaluated. Other critical points such as dialysis and filtrations are also important, and may be responsible for a significant loss of allergenic materials.

In conclusion, mite allergen extracts have still room for improvement and more studies are needed to fully delineate their allergenic and non allergenic composition in order to prepare better extracts for diagnosis and treatment of mite allergic individuals.



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C1.5 - Challenges facing novel allergen vaccines for becoming a more competitive therapeutic/prophylactic approach

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Populations of allergic patients of about 65 and 87 million people in the US and Europe, respectively, indicate at a first glance a big market. In reality, however, the allergen-specific immunotherapy (SIT) market size is quite modest. Although SIT is a story of success regarding the progress in understanding the mechanisms underlying successful therapy, progress in clinical trials to improve allergic conditions is still moderate. Despite the fact that SIT is the only therapy able to cure allergic diseases, only a small minority of the patients choose this treatment mainly because it involves 30-70 doctor visits over a period of 3-5 years. SIT is performed by the administration of increasing doses of allergen extract to which the patient is allergic. A dose escalation phase consisting of weekly injections is initially employed, followed by a maintenance phase of monthly injections lasting several years. The escalation phase is required to avoid anaphylactic side-effects, the main limiting factor in SIT. In contrast to a true vaccination which induces strong humoral and cellular immune responses with a few injections in a short time, the mechanisms of action of SIT are based on immunomodulation which requires a long time to establish. To become a widely accepted therapy, SIT needs to be turned from long-term immunomodulation to a vaccination regimen which can cure the disease with a few injections. Currently the area of SIT is experiencing exciting developments which have the potential to cure allergic diseases in a very short time.



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C1.6 - The genetic basis of allergenicity and allergy vaccination

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During the last years there has been an increased interest about the origin and characteristics of allergenicity. Several approaches are currently employed to study this problem, which is essential to understand the pathogenesis of allergic diseases. They are based on different hypothesis and interpretations of a number of clinical and experimental findings. A great emphasis has been done around the intrinsic properties that could determine the allergenicity of a molecule. Here we analyze the importance of the genetic background in determining this phenomenon. There is a large number of works in animals and human showing that specific IgE response (which defines if a molecule is an allergen) is under genetic control. However, the precise genes and polymorphisms underlying those findings are mostly unknown. The explanation of this important question is nowadays a major concern in the field of molecular genetics of allergic diseases. Since specific IgE response is a complex trait that is supposed to be regulated by numerous genes and environmental factors, the task seems overwhelming but theoretically achievable, especially because great progress has been made in genomics and genotyping.

MHC genes have been traditionally studied because they are known to be directly involved in epitope recognition by T cells receptors, but now it is apparent that other genes can influence at least the general predisposition to have a Th2/IgE biased response to innocuous molecules. There have been recent important advances regarding animal models with this phenotype. In addition, several groups have explored the role of different genes controlling the IgE response in humans, providing an increasing bulk of data. Our genetic epidemiology studies in an admixed population living in the tropics show that, in addition to those in HLA genes, variants of others like *NOS1*, *LTC4S*, *BAFF* and *LIG4* are associated with the strength of the IgE response to mite and parasite allergens. We will discuss the impact of all these findings on the improvement of allergen specific immunotherapy and the prevention of allergic diseases. Also, we briefly analyze some current approaches on the search for a rational explanation of allergenicity.



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C1.7 - Downmodulation of Type 2 immunity by bacterial adjuvants

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Allergic asthma is a chronic inflammatory lung disease mediated by Type 2 T helper cells (Th2) and is characterized by airway eosinophilia, airway hyper-reactivity (AHR), mucus hyper-secretion and elevated levels of IgE. Epidemiological studies suggest a direct relationship between high living standards/hygiene conditions and an increased risk to develop allergic diseases. The Hygiene Hypothesis proposes that a reduced contact with infectious diseases such as tuberculosis, measles and others, may favor atopy. The Toll-like receptors are an important family of innate immune response that appears to be involved in the protection or susceptibility to asthma. Indeed, epidemiological evidence indicates that endotoxin lipopolysaccharide (LPS); a prototypic cell wall component of gram-negative bacteria that activates immune cells via the transmembrane toll-like receptor 4 (TLR4) can influence the development of asthma. Because endotoxin LPS acts as Th1-prone adjuvant, the role of LPS in modulating Th2 immunity is anticipated, but the results obtained by different groups of investigators are quite conflicting. Experimental data obtained with the murine ovalbumin (OVA) model of asthma indicated that exposure to LPS could either protect against asthma or exacerbate it. Controversy also exists as to whether TLR4 and MyD88 molecules are required for optimal development of Th2 immunity. Another Th1-favoring adjuvant is *Mycobacterium bovis* Bacille Calmette-Guérin (BCG). Epidemiological data also indicated an association between the exposures to *Mycobacterium tuberculosis* and a reduced risk of developing asthma. Experimentally, it has been shown that BCG and other mycobacterial strains can attenuate allergen-induced Th2-type cytokine responses a finding that reinforced the hygiene hypothesis. We are currently investigating the utility of microbes and microbial products for modulation of inflammation in experimental asthma. First, I will show specifically the effect of LPS during the sensitization phase. Second, I will show the effect of recombinant BCG (rBCG) strain that express the genetically detoxified S1 subunit of pertussis toxin (rBCG-S1PT) on asthma development. In both cases, the end result is down modulation of type 2 immunity. The cellular and molecular mechanisms involved in the down regulation of allergic responses will be highlighted.



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C1.8 - Anti-inflammatory/Immunomodulatory properties of SphK1 blockade during Adjuvant-allergen immunization: Potential for reducing adjuvant-inflammation and for switching Th2 towards a Th1 response

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Introduction: Sphingolipids are being recognised as bioactive molecules critical in mediating inflammatory responses. We have recently reported that blockade of Sphingosine kinase 1 (SphK1) prevents experimental anaphylaxis and asthma but the mechanisms of SphK1 effects are not fully elucidated. Here we present data, suggesting that SphK1-blockade has a potential therapeutic application in allergen vaccination by skewing the allergen-induced Th2 response towards a protective Th1 one.

Methods: Mouse bone marrow-derived dendritic cells were stimulated with Alum-OVA in the presence or absence of SphK1 blockers to evaluate the secretion of cytokines. The *in vitro* immunomodulatory properties of SphK1-blockade were characterized and its prophylactic potential was evaluated in an Alum-OVA-sensitization murine model of experimental asthma.

Results: Mouse dendritic cells stimulated by Alum-OVA triggered the release of interleukin-1b, (IL-1 β), IL-6, IL-10, IL-12 p40/p70 and tumour necrosis factor-alpha (TNF- α). Blockade of SphK1 considerably reduced the levels of IL-1 β , IL-6 and TNF- α but not that of IL-10 and IL-12. Prophylactic treatment of mice with SphK1-siRNA, or a specific SphK1-inhibitor prevented the development of the typical Th2 allergic response by a drastic reduction of specific IgE and the induction of protective allergen-specific IgG2a antibodies. Moreover, the mice pre-treated with the siRNA-SphK1, or a SphK1-inhibitor, had reduced airway eosinophilia, IL-4, IL-5 and Eotaxin secretion following OVA-challenge.

Conclusion: SphK1 blockade inhibits the inflammatory parameters seen in experimental asthma while inducing high level of anti-OVA IgG2a protective immunization, suggesting the usage of SphK1-inhibitors as a promising novel approach to develop allergen-vaccines, while reducing the adverse effects caused by strong adjuvants.



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S1.1 - A cat allergy vaccine based on Fel d 1-derived peptides fused to a viral carrier protein

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Introduction: Allergen-specific immunotherapy (SIT) is based on the administration of subcutaneous injections of gradually increasing quantities of the disease-causing allergens to allergic patients but can induce severe side effects. The aim of this study was to engineer recombinant fusion proteins based on viral components and surface-exposed peptides derived from the major cat allergen, Fel d 1 which lack allergenic activity.

Methods: Two peptides, one from chain 1 and a second from chain 2 of the Fel d 1 protein, each comprising approximately 30 amino acids were expressed in *E. coli* as recombinant fusion proteins with a viral carrier protein and purified by a nickel affinity chromatography. Allergic patients' IgE reactivity was measured by ELISA and the allergenic activity was compared with that of the rFel d 1 allergen by *in vitro* basophil activation tests.

Results: The fusion proteins showed neither reactivity to IgE antibodies from cat allergic patients nor allergenic activity when exposed to their basophils. Upon immunization of mice and rabbits, the hybrids did not sensitize against rFel d 1 but induced protective IgG antibodies which inhibited the binding of allergic patients' IgE to the complete rFel d 1 as well as the allergen-induced basophil degranulation.

Conclusion: Fusion proteins consisting of allergen-derived peptides without IgE and T cell reactivity may represent safe allergy vaccines which induce protective IgG responses through carrier help.

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S1.2 - Prevention of allergy by administration of allergen-specific IgG antibodies

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Introduction: Allergic sensitization occurs early in childhood. Our aim was to investigate the effect of prophylactic administration of allergen-specific IgG antibodies on allergic sensitization in a murine model of grass pollen allergy.

Methods: Naïve BALB/c mice received intra-peritoneally Phl p 5-specific rabbit polyclonal IgG antibodies and for control purposes, rabbit polyclonal IgG antibodies specific for an unrelated grass pollen allergen, Phl p 2. Mice were then subjected to a robust sensitization protocol based on Aluminium-hydroxide adsorbed major grass pollen allergen, Phl p 5 and Bet v 1, the major birch pollen allergen. Blood samples were taken before prophylactic antibody treatment and after sensitization and analyzed regarding the presence of the administered IgG antibodies, IgE and IgG1 reactivity to Phl p 5 and Bet v 1 in ELISA and IgE-mediated allergic reactions using rat basophil leukemia (RBL) assays and intra-dermal skin tests.

Results: Prophylactic administration of Phl p 5-specific IgG antibodies specifically prevented the development of mouse Phl p 5-specific IgE and IgG1 antibodies but had no effect on the development of mouse IgE and IgG1 antibodies directed to Bet v 1. Moreover, Bet v 1- but no Phl p 5-induced basophil degranulation and skin reactivity could be elicited in mice that had received Phl p 5-specific IgG antibodies. Mice treated with Phl p 2-specific IgG antibodies developed Phl p 5- and Bet v 1-specific IgE and IgG1 antibodies and showed basophil degranulation and skin reactivity to Phl p 5 and Bet v 1.

Conclusion: Prophylactic administration of allergen-specific IgG antibodies prevents allergic sensitization.

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S1.3 - Evaluation of the immunocompetence of a set of six linear synthetic oligopeptides designed from the Der p 1, Der f 1 and Blo t 1 allergens

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Introduction: The use of short peptides of native allergens has been evaluated in a variety of studies performed in animal models. The majority of them have demonstrated to produce allergen specific immuno-modulation. We report here a set of six oligopeptides of the group 1 of mite allergens that produced allergen specific IgY in a aviar model.

Methods: Peptides were designed using the following software: Blast, Clustalw, protscale, and Epitopeviewer. Peptides were 16 to 19 mer and were synthesized using an automated peptide synthesizer with a Fmoc strategy purified with a HPLC and confirmed with mass spectrometry. Its sequences were obtained by homology alignments with the native proteins of the Der p 1, Der f 1 and Blo t1 from the Swiss-Prot database. Hens 16 old-weeks were immunized intramuscularly once using 100 micrograms of each six peptides in complete Freund adjuvant. Subsequent doses were injected at 3, 5 and 7 weeks in incomplete Freund adjuvant. Controls hens were treated with BSA and PBS. Igy were detected by a indirect ELISA assay using microtitre plates coated with purified Derf1 and Derp1 allergens. Elisa titers were calculated based on the analysis of the absorbance of each eight thiophilic column purified IgY suspensions.

Results: IgY were able to recognize and bind to allergens. Taken together the data indicate that this experimental model was successful for priming cell of immune system and produce a specific allergen IgY response.

Conclusions: These findings suggest that those peptides could be used for future immunomodulation protocols and experimental immunotherapy.



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S1.4 - Evaluation of the correlation between tissue reaction and cytokines patterns induced by *Alternaria alternata* in mice

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Introduction: *Alternaria alternata* is well-known as source of allergenic components in the cell wall and cytoplasm of conidia and hyphae that cause respiratory allergic disorders. The purpose of this study was to evaluate tissue reaction and Th2 cytokines in mice exposed to *A. alternata*.

Methods: *Alternaria alternata* was cultured and fungal extract was prepared by freeze-defreeze and sonication methods. BALB/c mice in one group were sensitized by two intraperitoneal injections of *A. alternata* extract and then intranasally challenged with spores suspended in sterile normal saline solution, and in another group, mice only received spores intranasally. Blood sampling and necropsy were performed in 1 and 72 hours after spore inhalation. The sera were analyzed by ELISA method to determine serum levels of IL-4 and IL-13 in immediate response and late-phase reaction, respectively.

Results: Histopathologic study demonstrated an inflammation response with inflammatory cells including lymphocytes, macrophages, neutrophils and eosinophils, and mucus hypersecretion in the lungs, and airways epithelial cells hyperplasia and necrosis in sensitized and nonsensitized mice. Increase in Th2 cytokine (IL-4 and IL-13) levels in the sera was also observed in the sensitized and challenged mice. The results showed exposure to extract and then spores of *A. alternata* induced rapid and highly elevated production of IL-4 and IL-3. These cytokines were associated with respiratory histopathologic changes.



KNA3 - Recombinant major allergens as standardization tools

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Major allergen measurements have relevance for the standardization of allergen extracts for immunotherapy and for epidemiologic studies into the cause of allergic diseases. Standardization is still largely centered on overall IgE-binding potencies (biological standardization). Major allergen levels show significant correlation with IgE-binding potencies, but ratios of the two can differ 5- to 10-fold between individual extracts. Major allergen quantities needed for effective and safe subcutaneous immunotherapy are proposed to be between 5 and 20 µg per maintenance shot. Although this figure is not really based on dose-finding studies, it has reached the status of a guiding principle. It is necessary to add major allergen measurements to standardization requirements to design adequate dosage schemes and elucidate the dose-response relation between major allergen dose and therapeutic effect. This will also help clarify to what extent sublingual immunotherapy requires higher doses of major allergen. Fine specificity of different assays toward isoforms and other variants of single allergens often results in diverging allergen measurements. Standardization should be based on certified major allergen references based on recombinant molecules and accompanying assays that are cross-reactive enough to recognize all variants to facilitate comparability. This will also ensure that primary and secondary prevention strategies aiming at regulating allergen exposure will stay on solid ground.



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C2.1 - Relevance of minor and pan-allergens for standardization

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Introduction: The aim of allergen standardization has been to overcome the allergen extract heterogeneity and variability. Allergenic extracts for therapeutic use are crude mixtures of proteins and non-protein components isolated from natural sources. Control of relevant components (so-called major allergens is demanded). Methods: By mean of a Component Resolved Diagnosis (CRD) approach we wanted to investigate the role of minor allergens in allergy disease Results: Minor allergens sensitized a significant percentage of patients in areas with high pollen counts. Sensitization to minor allergens was a marker of a more severe diseases and in particular case a risk factor during immunotherapy. Conclusions: Control of minor allergens is a need for correct standardization. Identification of population sensitized to minor allergens can be easily done by mean of CRD approaches.



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C2.2 - The physicochemical tool box for standardization of allergen products: from recombinant single allergens to polymerized high MW extracts

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Allergic diseases ranging from rhinitis to asthma represent increasing problems in many countries in the world. However, allergy diagnosis and specific immunotherapy (SIT), the only curative approach towards the treatment of IgE-mediated disorders, are still performed like in their very beginnings more than a century ago. The use of allergen extracts of undefined contents bears the risk of anaphylactic side effects and sensitization to new allergens in the course of immunotherapy. During SIT a modulation of the immune system takes place including a Th2 to Th1 shift and induction of regulatory T cells. Recombinant allergens are being used in new concepts of allergy diagnosis and immunotherapy. These developments aim to replace currently used crude allergenic extracts by well-characterized recombinant molecules. Furthermore, the concept of using hypoallergens for SIT may be facilitated by genetic engineering of molecules bearing less IgE-reactive epitopes than their wildtype counterparts. Another concept for SIT with reduced IgE-mediated side-effects involves chemically modified extracts. There, allergens are cross-linked by formaldehyde or glutaraldehyde and the resulting high molecular weight allergoids are claimed to induce even higher titers of non-IgE antibodies than native extracts. Although allergoid-based products showing improved safety and efficacy have been used routinely for SIT by clinicians the demand for better standardization has been rising in the past year. We have established in our laboratory a comprehensive set of methods to evaluate recombinant (hypo-)allergen candidates for identity, purity, structure, homogeneity, and biological activity (allergenic activity). Furthermore, we have shown the availability of mass spectrometry-based methods for determination of allergenic content and have applied light scattering techniques for molecular size measurements of allergoids.



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C2.3 - Novel approaches to standardizing grass mix product

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An important pre-cursor to product standardization is that the biological reference is characterised. This is challenging as many allergenic products are composed of multiple soluble proteins, and available international standards may be composed of different proteins. In addition, a product based upon a pool of allergens from multiple species certainly makes sense in targeting a more diverse patient population but further complicates characterisation of the drug. Allergy Therapeutics produce a vaccine against grass allergy consisting of 13 different grass pollens. There are several soluble proteins in each grass pollen that are extracted to produce the vaccine. These consist of glycosylated and non glycosylated proteins of different sizes, structures and physiochemical properties. Group 1 and group 5 allergens are believed to provoke a strong allergic response amongst the majority of sensitised patients. Allergy Therapeutics have used a novel combination of methods including SDS-PAGE, RP-HPLC, SEC, IgG & IgE ELISA and I.E.F to determine whether soluble allergen extracts are immunologically consistent when the pollens are extracted at the same time in the same vessel and when they are extracted separately and mixed. Allergens from several species have been isolated and characterised providing an insight to the roles and means of control of multi-allergen products.



KNA4 - Essential aspects of allergen vaccine standardisation

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Practicing allergologists are dependent on reliable products for allergen specific diagnosis and safe, efficacious allergen specific immunotherapy. Vaccines for allergen specific immunotherapy are produced by aqueous extraction of natural source materials, which are inherently variable by disposition. Effective measures to control variability, i.e. standardization, need to be implemented in order to obtain a reliable product and to achieve reproducible results when treating allergic patients.

The aim of standardization is to ensure a constant composition of the active protein ingredient. Since every protein is a potential allergen all proteins should be subject to standardization. Standardization is dependent on choices with respect to which components to measure, how to measure them as well as release criteria applied in quality control. As a result the quality of standardization varies considerably among different manufacturers, and therefore also the quality of products.

Standardization should ideally comprise all aspects of the production processes. Essential aspects include control of raw materials, use of optimised and validated production processes, choice of batch release criteria, as well as stability and storage of the vaccines. It is essential to perform batch release as a three-step procedure including 1) a quantitative or semi-quantitative biochemical separation technique, 2) a quantitative assessment of major allergen(s), and 3) determination of overall IgE binding potency. All assessments should be performed relative to a thoroughly characterized standard ideally proven useful in practical immunotherapy.

With optimally standardized products the physician achieves safety and efficacy as well as reproducibility in the results obtained using allergen specific immunotherapy.



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C2.4 - Biological Standardization: the Nordic concept and the ID50 intradermal method

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Introduction: Biological standardization (BS) means trying to define a clinically relevant potency of allergens. There have been two major methods described, the FDA intradermal method (FDAM) and the Nordic method (NM), based on skin prick testing. The FDAM aims at finding a concentration of each allergen not causing general reactions in sensitive patients at IDT. The aim of the NM is to equilibrate the potency between extracts of common inhalant allergens. The FDAM includes highly sensitive patients, whereas the NM includes consecutive patients, clinically sensitive to the allergen tested, attending a specialized allergists office. The FDAM is applied to new batches, whereas the NM should be applied once to the In House Reference preparation (IHR), testing BS in a region where the allergen is a major problem.

Results: The results using the NM have been repeatable using the same IHR in different regions of Europe, whereas the FDAM showed a higher potency of a Bermuda grass extract in a region with high than in one with low pollen counts.

The Nordic Biological Unit/ml correlates with the major allergen content of common inhalant allergens, *i.e.* equilibrates the potency between extracts of different allergens. The FDAM shows similarity between new batches and the FDA reference preparation (RP) per species. The potency of cat and short ragweed extracts, as measured by the FDAM, correlated to the major allergen content, *i.e.* to the Fel d 1 and Amb e 1 conc., respectively.

The FDAM can be applied to all types of allergens, since the aim is safety, whereas the NM can only be applied to common inhalant allergens with a defined clinical history (pollens, animal danders, etc.), but not to *Hymenoptera* venoms or food allergens.

Conclusions: The aim of the NM is fulfilled by proper determination of major allergen(s). As time goes, whole extracts will be replaced by recombinant allergens or peptides. Thus, the most relevant standardization of future allergen preparations for diagnosis would be by mmol's of molecules with at least two B-cell epitopes/ml using CREATE standards or similar. The method for standardization of preparations for treatment may be expressed in terms of induced clinical effect or correlating *in vitro* measures.



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C2.5 - Comparison between european, mexican and american allergen extracts by in-vitro and skin test methods

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Background: US and European standardization techniques vary. In a previous study ELISA inhibition (CBER/FDA competition ELISA) testing of Bermuda grass (BG) and cat diagnostic extracts of 3 European manufacturers showed relative potencies between 5-25% (BG) and 2.1-29 Fel d 1 Units/mL (cat) in comparison to the FDA standard of 10.000 BAU/mL and 18 Fel d 1 Units/mL. Here we report a comparative study of the biologic potency using quantitative SPT

Methods: Diagnostic extracts of BG and cat from six different manufacturers (2 Mexican, 3 European, 1 US = 10.000 BAU/mL reference) were tested in SPT in 21 and 14 patients as a concentrate and two serial 2-fold dilutions. All extracts and dilutions were applied in quadruplicate during two SPT sessions on the forearms of subjects with allergic rhinitis, who had tested positive to BG or cat in a routine SPT

Results: Most extracts showed a good dose-response in wheal size for the concentrate in comparison with the two dilutions (steep part of the curve). The Wilcoxon test for linked random samples was used in each group to investigate whether the distribution of the Reference Extract differed from each of the test extracts to a statistically significant degree (test level $\alpha = 0.05$). BG: European (2-sided asymptotic significance $p = 0.001$ to 0.005) and Mexican ($p = 0.005$ to 0.022) extracts were less potent than the reference extract. Cat: only Eur2 ($p=0.013$) and Mex2 ($p=0.005$) were less potent. Expressing the potency relative to the 10.000 BAU/mL FDA standard, results can be seen in fig 1 (BG) and 2 (cat).

Conclusions: Diagnostic extracts of BG used in Europe and Mexico are less potent than those used in US. Testing sensitivity and specificity of US and EU extracts would be very helpful in defining the ideal test-extract concentration. There might be inter-continent differences as natural exposure and genetics are different

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C2.6 - Standardization of allergen vaccines from tropical mites: *Dermatophagoides siboney* and *Blomia tropicalis*

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Background: House Dust Mites are the most relevant allergen sources in Cuba, associated to respiratory allergy, particularly asthma. *Dermatophagoides siboney* (Ds) is an endemic species closely related to *D. farinae*, whereas sensitization to *Blomia tropicalis* (Bt) is also important. This work describes the development of standardized allergen extracts of these two species in addition to the well-known *Dermatophagoides pteronyssinus* (Dp).

Methods: Product standardization was based on development of analytical immunoassays for total allergenic activity and allergen composition (using IgE antibodies from allergic patients) and for quantification of Major Allergens with mono and polyclonal antibodies. The Nordic definition of Biological Units (BU), based on Skin Prick Test and Histamine HCl 10 mg/mL as standard, was used. Nordic and European Guidelines, as well as local regulations on allergen products were followed.

Results: The Relevance of Der s 1 major allergen, contributing to 50-70% of total allergenic activity was demonstrated, both by in-vitro and in-vivo methods. Group 1 content was significantly correlated to in-vivo allergenic activity in several batches ($r=0.88$ $p<0.01$), therefore supporting its use as an activity marker. For Bt, the Low Molecular Weight Fraction (LMWF) containing Blo t 5, Blo t 12 and Blo t 13, showed to respond for 70% of the in-vitro IgE-binding activity of the whole extract. The following equivalence was found 1BU = 2ng Der p 1; 4ng Der s 1 and 10ng LMWF_{Bt}. The precision of IgE-based assays (Inhibition ELISA and IgE-Western Blotting) was enough for releasing the product batches using only one assay, according to international acceptance criteria (50-200%). Peak height at 15 and 25kDa for Dp/Ds, and 17kDa for Bt, was chosen as a quantitative parameter of the allergenic profile, over peak weight, due to its lower variability. Among individual allergen assays, polyclonal-based ELISA against Der s 1 or Der p 1 showed the highest precision ($CI_{95\%}=13-17\%$) and similar sensitivity as compared to MAbs-ELISA. The manufacturing process that included application of in-process controls on intermediate products was able to achieve consistent results at the end product level with a likelihood of success over 94%. The stability of freeze-dried final products was evidenced during 60 months at 4°C. Clinical trials for diagnostic use by Skin Prick Test showed that the optimal concentration, regarding Sensitivity and Specificity was 20 000 BU/mL, using a cut-off diameter of 3mm. Six clinical trials of subcutaneous immunotherapy in allergic asthma, using the same dosing scheme showed similar and homogeneous clinical outcomes (homogeneity test, $p<0.05$).

Conclusions: Three standardized allergen vaccines of relevant in Cuba domestic mites were licensed for both indications: diagnostic and immunotherapy, becoming the first registered allergenic products in Cuba. The value of standardization methods was evidenced, achieving manufacturing and clinical consistency. Availability of industrially manufactured standardized allergen vaccines becomes a valuable tool for expanding the etiological approach for asthma management in our country.



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S2.1 - A Multi-Center Ring Trial of Allergen Exposure Assessment using Fluorescent Multiplex Array Technology

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Background: Monitoring the performance of allergen assays is essential to ensure reproducibility of allergen exposure assessments. We evaluated inter-laboratory and quality control variability of a Multiplex ARay for Indoor Allergens (MARIA) which measures the eight most common indoor allergens in a single test. The 8-plex array analyzes Der p 1, Der f 1, Mite Group 2, Fel d 1, Can f 1, Rat n 1, Mus m 1 and Bla g 2.

Methods: We performed a multi-center ring trial in nine laboratories across the US and Europe to determine within and between laboratory variability. All MARIA reagents required for the trial, as well as aliquots of 151 dust samples were sent to nine participating centers and analyzed by each laboratory on three separate occasions. A hierarchical model was applied to the nested data (repeat nested within laboratories, laboratories nested within samples).

Results: Results shown are based on more than 32,000 individual allergen measurements. Allergen levels covered a wide range for all allergens from below detection limit to greater than 100µg/g (Mus m 1 <35µg/g). Results were reproducible within as well as between laboratories. Within laboratories, 84% of correlation coefficients were >0.95, more than 50% of intra-laboratory results were within the 5% CV and 75% within the 10% CV margin. Results between laboratories also showed highly significant positive correlations for all allergens (~0.95, p<0.001). Overall means of results were comparable and inter-laboratory CV% ranged between 19% and 27%.

Conclusion: The ring trial demonstrates that MARIA produced results that are reproducible within as well as between laboratories. Application of multiplex technology will provide standardized and consistent allergen measurements that will streamline allergen exposure assessments.



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KNA5 - European regulatory requirements

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Allergen preparations have been used in the diagnosis and in the immunotherapy of allergen-induced diseases for many decades. Their manufacturing process as well as their standardization have been regarded in an very confused way by regulators and clinicians, and until '90ies they have been essentially regarded as outside any regulatory framework, with the exception of a defined approach taken by the Nordic Countries (Nordic Council on Medicines. Registration of allergenic preparations. Nordic guidelines, 1989) covering the quality safety and efficacy of these preparations. More recently, European directive 89/342/EEC, amending directive 65/65/EEC and 75/319/EEC, was issued and for the first time allergen preparation for in vivo diagnosis as well as immunotherapy became medicinal products, with all the implications of this new harmonized status at the European level. Despite the big achievement of having a common directive in Europe, establishing the legal status of these products, and despite the large diffusion of these preparation in various Member States, only a few of them really and practically implemented at the national level the European text. Briefly, besides the medicinal product status, the directive was underlining two basic concepts; the first one was that the directive itself applied to industrially manufactured products only (so called named-patient products are excluded): the second one was that no detailed indications about the type of documentation to be required by Regulatory Authorities for this products was given, in relation to the huge number of different preparations available and the basic requirement of having each of them individually authorized. As a consequence of this directive, it was subsequently felt by the Competent Authority that the directive itself might deserve some help in interpreting a number of concept; a Guideline was then issued at European level in 1992 and revised mainly editorially in 1996. Moreover, in order to try to set also specific qualitative and quantitative parameters, the European Pharmacopoeia similarly decided to issue a general monograph on allergenic products, trying to establish the analytical characteristics of raw materials, bulks and final products covered by the European Directive and industrially manufactured. Subsequently, directive 2001/83/EEC, as amended, was issued confirming the medicinal product status of industrially manufactured allergenic products. These three pieces of documents have been in force for more than 10 years, and have been used in a variety of approaches by various European Member States (such as Germany, France, Sweden, Denmark and other countries). After a certain period, it has been recently recognized the need for an update of both the Guideline and the European Pharmacopoeia Monograph, in the light of the enormous increase in scientific knowledge in the field, of the regulatory experience of some member states and also taken into account the emerging approach in the production not only of allergen extracts but also of recombinant allergens taken by a number of Manufacturers in the field. Therefore, both the Guideline at the EMEA level and the Monograph at the Pharmacopoeia have been recently covered by a revision process, whose outcome can be summarized as follows. For the Guideline, one of the characteristics of the original text was to group the huge number of preparations according to taxonomic families.



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This means that some information had to be provided at the National Authority not for each individual allergen preparation but for a group of allergens belonging to the same taxonomic family. This aspect could not be supported anymore and in the revised guideline a full quality section is foreseen for each individual product. However, the concept of taxonomic families has been somehow maintained but converted in the more scientifically based concept of homologous groups (see relevant presentation) which can be followed for the planning of efficacy studies for extracts belonging to the same group. Moreover, in the new Guideline recombinant allergens are now clearly included and covered, giving further specific advice to this category of products. Finally, in the new Guidelines on allergens, the non clinical and clinical parts are now extensively covered, being these aspects only marginally addressed by the old guideline, which was essentially focused on quality aspects. As already anticipated, the revision of the European Guidelines, carried out under the remits of the BWP/CHMP at the EMEA level, was accompanied by the revision of the European Monograph text, which was similarly quite old, carried out by EDQM. A number of rather minor changes have been introduced, but as a major point it was decided to extend the field of application of the text not only to industrially manufactured products but also to named patients products, thus opening the possibility to have a general improvement on the quality of these preparations. The new guideline will be put into force as per 20th May 2009 and the new European Pharmacopoeia Monograph will be effective as per 1st January 2010. The various implications at the national as well as at the European level of these two new set of documents will be discussed.



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C3.1 - The concept of homologous for registration of allergen products

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According to European regulations complete dossiers have to be provided for marketing authorisation of each individual biological medicines including allergen products. Therefore complete data on quality, safety and efficacy have to be provided by the applicant regardless of the similarity of the source material or the allergen composition. This means that e.g. for a finished product containing active substances from various grass pollen complete data on quality, safety and efficacy would have to be provided for each grass species. To reduce the amount of data to be generated by industry for the approval of allergen products and to ensure availability of the existing great variability of allergens the concept of taxonomic families was introduced by the "Note for Guidance on Allergen Products" (CPMP/BWP/243/96) allowing the extrapolation of data on stability, safety and efficacy from one to another member of the same taxonomic family. In the light of new scientific knowledge the concept of taxonomic families was replaced by the concept of homologous groups along with the new "Guideline on Allergen Products: Production and Quality Issues" (EMA/CHMP/BWP/304831/2007). The concept of homologous groups allows to a limited extent the extrapolation data on quality, as well as a more complete extrapolation in the field of safety and efficacy. In general extrapolation is possible from one member ("representative allergen") to other members ("non-representative allergens") within the same homologous group. The grouping is based on following criteria: (i) comparable physiochemical and biological properties of the source material (ii) cross-reactivity/structural homology of the allergens, (iii) identical formulation of the finished product and (iv) identical production process of the allergen extract and of the finished product. Several groups are defined by the "Guideline on Allergen Products: Production and Quality Issues" (EMA/CHMP/BWP/304831/2007), but additional groups may be defined or new members may be included in existing groups by applicants on the basis of a justification. The concept of homologous groups therefore provides a scientific rationale for the grouping of allergens and at the same time the flexibility needed for dealing with the number of allergens used for the manufacturing of allergen products.



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C3.6 - Round Table: Regulatory Requirements for Clinical Trials of Allergen Vaccines in Cuba

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The field of allergenic extracts and vaccines is developing all over the world. In Cuba these preparations are considered as pharmaceuticals products with the requirement of evidencing their safety and efficacy in clinical trials. General methodological aspects in the design of clinical protocols and Good Clinical Practice (GCP) should be considered, intending to assure adequate protection of the rights and safety of subjects involved in those trials, as well as the quality and integrity of the resulting data. A system for GCP certification of clinical institutions involved in Clinical Trials in Cuba is under development. Specific requirements for authorization of clinical trials for allergenic products by the National Regulatory Authority (CECMED) have been included in the Regulation on Registration on Allergen Products in Cuba (Reg. 30-2002). The taxonomical family concept can be applied also to clinical trials. Separate clinical trials should be designed for evaluation as in-vivo diagnostic products (skin tests) and for allergen specific immunotherapy. Allergen products for immunotherapy are considered also therapeutic vaccines therefore, the methodological guidelines intended for these products should also be taken into consideration. Specific pharmaceutical and non-clinical studies can be required prior to authorization of clinical trials including toxicity studies, particularly for innovative and adjuvanted allergen vaccines. Indications for clinical use should be approved by the CECMED according to evidence from clinical trials. A system of severe adverse event reporting to CECMED during clinical trials is also enforced. In the last 10 years, 14 clinical trials with allergen products have been authorized by CECMED, all of them, using standardized freeze-dried allergen extracts of three species of House Dust Mites (VALERGEN, BIOCEN, Cuba), 13 have finished already, 4 were designed for evaluation as diagnostic products (skin tests), 7 for injection immunotherapy and 3 for sublingual immunotherapy. These allergen vaccines have been approved and introduced into Allergy Services all over the country.



C3.2 - The design of a Quality Management System for pharmaceutical companies to optimize performance

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Why is it important to talk about "The design of a quality Management System for pharmaceutical companies to optimize performance"?

During the last 15 years the European Medicines Agency, called EMEA, documented and published the results of their inspections made in Member states of the European Countries as well as in Third Countries. Out of 425 inspections they reported nearly 10.000 findings. What is obvious upon the very first view on these findings is that concerns over documentation which means the quality system head the list by a significant margin. Similar results were documented in other countries. The consequence of these results are that everybody are looking for a better understanding of the flaws in the quality system what is emphasized in a large number of regulations and norms concerning the quality system.

A Quality Management System has to be a set of interrelating and interacting elements.

The most important parts of a quality system are the Quality Manual, the Site Master file and the Document Management System.

In the Quality Manual basic ideas of the philosophy regarding quality are written down.

The Site Master File gives information about the company itself. For example: Size, products, employees.

Documentation has a basic role within pharmaceutical development and manufacturing. Within a company a lot of documents exist, so you have to structure them within a Document Management System so that they can be identified and found very quickly without mix ups.

Already Aristotle gave thought to quality. He remarked: "Quality is not an act, it's is a habit". So being concerned with quality has a long tradition.



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KNA6 - Pharmacovigilance of allergen immunotherapy

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Introduction: Subcutaneously administered allergen is rapidly distributed throughout the body with the aim to reach immuno-competent cells. However, injected allergens also reach other organs, *e.g.* the skin or lung. In case these organs are reactive the provocation by circulating allergens may provoke severe reactions. To reduce the risk of severe allergic reactions, allergen extracts have been modified to slow down the release and allergen molecules or have been made "hypoallergenic".

So far the literature on allergic side effects of immunotherapy is limited but points on the high risk of severe reactions in asthmatic patients. There are no reports from authorities or manufacturers who would have the best overview. ADR forms used by authorities aim at registering events related to pharmaceuticals, but are not suitable for identification of causes of severe ADR related to immunotherapy (or other biologicals).

Methods: I have been pharmacovigilance officer within several companies but not been able to publish due to secrecy agreements. Thus, this report is based on non-identifiable data.

After a reported death or life threatening event the case was thoroughly investigated by personal visits to interview and secure vials and other data. I organized a "crash commission", visiting the site within days. A questionnaire concentrating on batch control, diagnosed concomitant allergies, allergen exposure and asthma control has been successively developed.

Aluminum-hydroxide adsorbed allergens can induce local nodes, local reactions related to allergen exposure and general aluminum contact allergy. Even these reactions need specific penetration.

Results: In the majority of severe general reactions and deaths, there have been two dominating interactions: Severe, symptomatic asthma despite pharmacotherapy or non-treated asthma and/or exposure to a known allergen, *e.g.* dog, cat or pollen, prior to the injection. There are few reactions in connection with infections.

Subcutaneous nodules with concomitant aluminum contact allergy are not uncommon.

Conclusions: Severe reactions to immunotherapy can be avoided by proper diagnosis of concomitant allergies and asthma and proper control of lung function and allergen exposure. Investigations of severe events should be by external experts.



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C3.4 - Specific Allergen Immunotherapy in Clinical Practice in Latin America

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In Latin America, allergic diseases have a very high prevalence, comparable to that of many other countries of the world, and that prevalence is constantly increasing. Within the region, the number of allergy specialists is quite high, although allergy is not recognized as a full specialty in all countries.

During the last 20 years, the practice of immunotherapy in Latin America has much improved, especially because of the intensive educational effort, the worldwide spreading of the international guidelines and the adoption of the paradigm of the EBM.

In this regard, indications, contraindications, and limits of immunotherapy, are currently well acknowledged. However, they are not always appropriately applied. There are many published practice parameters and guidelines for immunotherapy, mainly coming from the American Academy of Allergy, Asthma and Immunology, the American College of Allergy, Asthma and Immunology and the European Academy of Allergy and Clinical Immunology.

The latest is the "Allergen Immunotherapy: a practice parameter second update" (*JACI 2007; 120,3*) which is nowadays, one of the most complete and comprehensive documents about this subject and which addresses patient selection and extract preparation.

Allergen immunotherapy is effective when appropriate quality and clinically relevant allergens are used. For the latest, an Accurate Etiologic Diagnostic is needed and it depends on the quality of the extracts used for skin and/or laboratory test and on the allergologist's skill. He have to perform a detailed history and an appropriate physical exam, select laboratory tests, perform adequate skin test technique and have knowledge of local aerobiology. In Argentina not all extracts have good quality and not all allergologists perform an adequate skin test technique as we can see in the results of the survey: "Skin Tests for the diagnosis of respiratory allergic diseases in Argentina" carried out by the AAAeIC Scientific Committee of Allergens, Diagnostic Tests and Immunotherapy in 2006.

Clinical research in immunotherapy in Latin America is minimal, in some cases non-existent. There is few data published in indexed papers coming from Latin America research teams.

Two surveys on immunotherapy, one of them named "Immunotherapy in allergic respiratory diseases. Situation in Argentina" (Arduzzo LRF et al, 2008) carried out in Argentina and the other named "Experience with sublingual immunotherapy in Latin America" (Huerta Lopez JG et al, 2008), a survey which consisted of 19 questions regarding the SLIT practices through a questionnaire hosted in both SLAAI (www.slaai.org) and SBAI (www.sbai.org.br) websites and answered by physicians belonging to 19 Latin American countries, give us an idea of how immunotherapy is carried out in Latin America.

The results gained from these two surveys show among other findings that SLIT modality is increasingly used as an alternative route to SIT; and that although there are many position statements on practice parameters for skin test and immunotherapy, physicians not always follow these guidelines in their practice in Latin America.



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C3.5 - Current state of registration of allergen products in Brazil (Mercosur)

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The field of allergenic extracts is hardly developing worldwide, the standardization of the extracts, the quality control and the production processes have been improving, resulting in products of best quality and efficacy. The production of allergenic extracts started in the early times of the XX Century as artisanal products, and only almost 70 years later these products were considered as pharmaceutical products and must fulfil several requirements enforced by the regulatory agencies of different countries.

In South America, the regulatory control of allergenic extracts started only in the 1990's, with the first resolution 102/98 of ANMAT (Administración Nacional de Medicamentos, Alimentos y Tecnología Médica), from Argentina, that regulates the importation of in vivo diagnostics products and later the resolution 6826/02 that regulates the production and the special requirements for this peculiar industry. The same happened in Brazil only in the beginning of 2000, with the RDC 324/03, from ANVISA (Agência Nacional de Vigilância Sanitária), in 2003, based on the ANVISA point of view, the European Pharmacopea monography and FDA regulation. This resolution was extremely necessary to regulate the allergenic extracts production in Brazil, since all local producers were considered not legal, because it was not possible to register the extracts in accordance to the current regulations, which included only general requirements to biological products as immunizing vaccines and sera. The new regulation determined that all extracts should be registered in 2 years and included all requirements of raw materials, quality control, GMP (the same as for any pharmaceutical industry) and registration of the products.

The RDC 324/03 was modified in 2005, by a new resolution the RDC 233/05. The modification was about the characteristics of the companies that could produce allergenic extracts and allergenic products. The allergenic extracts (IMP); vaccination and diagnostic industrialized products (finished products) have to be produced by pharmaceutical industries with product registration. However, it is also authorized the preparation of allergen vaccines and diagnostic products in Patient Named or Medical Professional Named basis, by specialized manipulation pharmacies without registration, but always from registered allergenic extracts.

As the result of new regulations, such as the specific to allergens and GMP, the impact over allergenic extracts industry was dramatic and currently only one, among 10 companies, has allergenic extracts registered in Brazil. The GMP requirements, scientific and quality control data for registration of the extracts are the main difficulties that the Brazilian producers are facing. This new situation requires hard work, flexibility and frequent technical discussion with the Brazilian Regulatory Agency (ANVISA), however the last one can be considered a real challenge.



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C3.3 - Requirements for Registration for allergen products in Cuba

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Worldwide scientific and regulatory developments of allergenic products of biological origin, including allergen extracts derived from natural source material, as medicinal products, has urged the National Regulatory Authority of Cuba (CECMED) to develop national requirements for the registration of these products.

Joint efforts between CECMED, the Expert Group on Allergy of the Ministry of Health (MOH) and a local manufacturer of allergenic extracts were made to draw national requirements for the licensing of standardized extracts. Requirements were approved in 2002 for the presentation of chemical/biological, manufacturing and control (CMC) part of the dossier as well as for the non clinical and clinical information. In the preparation of this guideline, the concept of taxonomical allergen families was also considered.

The guideline also provides guidance to the industry on the development of allergen products and contributes to improve the availability in the market of novel allergen products with enhanced clinical efficacy and safety and thereby benefits public health.

To date three standardized allergenic extracts has been licensed for their use as *in-vivo* diagnosis of immunoglobulin E (IgE)-mediated allergic diseases as well as for specific immunotherapy of allergic diseases. The licensing process of these three allergens was based on the national guideline.

In this presentation we are describing the main steps and the evolution of allergenic extracts regulation in Cuba, as well as the main requirements for the registration of these medicinal products.



KNA7 - Mite culture process: GMP requirements

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Differences exist between the European and the United States registration procedures needed for the production of mites as source material. We present an overview of the formal procedures for Europe and the United States concerning source materials.

The overview will be focused mainly on the situation occurring in Europe, however, the registration procedure by the Food and Drug Administration (FDA) in the US will be discussed briefly. The FDA requirements are source materials with less than 1% foreign material, while the European situation is less defined.

The process of mite production and the registration of end products are based historically. The history of the mites, its predecessor house dust and their allergenic properties in perspective to the new regulations are presented. An important example is using human and/or animal food as growth medium.

Good Manufacturing Practice or GMP is accepted worldwide for the control and management of manufacturing and quality of pharmaceutical products. All present background information is needed to place mite source materials in the chain of active ingredients used in allergy vaccines and therefore GMP guidelines are necessary to define the quality of mite end products. However, many pharmaceutical companies must follow GMP procedures; they usually create their own GMP guidelines.

We present the impact of the GMP guidelines for source materials during production and show results for growth medium, time for culturing mites and growth environment.

Although mites are highly undefined organisms, by good control and management, it is possible to have consistent and reliable products. Due to the fact that mites are living creatures a watching eye has to be kept on possible toxicity agents to be monitored by all available techniques.



C4.1 - Food allergen extracts: standardization of manufacturing process

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Allergen standardization is considered as fundamental in control of lot-to-lot variability of allergen extracts, to guarantee the consistency of the extracts used in the allergenic vaccines and hence to maximize patient safety. Standardization of pollen, mite, epithelium or mould allergenic extracts has been established for many years using different techniques and units based on the potency or biological activity of the extracts. However, food allergenic extracts have not been included in this group of allergenic standardized extracts, principally because food immunotherapy remains undeveloped, but also due to the variability and intrinsic complexity of food allergenic extracts. Different approaches to standardize food allergenic extracts have been based on the total *in vivo* potency, or by quantifying the major allergen content using monoclonal or polyclonal antibodies. The lack of development of food immunotherapy is related to clinical recommendation for control through strict avoidance of the offending allergen sources, the unknown etiopathology of food allergy in some cases, together with poor extract characterization, unknown allergenic profiles, or differences between food varieties or maturation steps. During the last few years our group has selected tomato allergy as a model for the study of food allergy using a population of 1750 individuals. Tomato extracts have been characterized, differences in the antigenic composition of varieties have been elucidated depending on the ripening stage, cross-reactivity studies have been performed with pollens and other foods, and the importance of individual allergens has been analyzed. The objective of these studies has been to establish the characteristics of tomato extracts for human treatment.



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C4.2 - Allergen extracts of storage mites

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In recent years, the allergological importance of different mite species not belonging to the family Pyroglyphidae has been demonstrated. These mites, commonly named storage mites, include *Blomia tropicalis*, *Lepidoglyphus destructor*, *Glycyphagus domesticus*, *Tyrophagus putrescentiae*, *Acarus siro*, *Aleuroglyphus ovatus* or *Suidasia medanensis*. Many allergens, especially from *B. tropicalis*, have been purified, sequenced and cloned, showing sequence homology and biological function similar to those previously described in *Dermatophagoides* spp. Main allergens described in storage mites include digestive enzymes, fatty acid or lipids binding proteins, tropomyosin and paramyosin homologues, apoliphorine like proteins, alfa-tubulines and other, such as group 5 and 7 allergens, which definitive biological function has not been described yet. Besides, allergenicity of other species such as *Acarus farris*, *Austroglycyphagus malaysiensis*, *B. kulagini* and *B. tjibodas*, *Cheyletus eruditus*, *Chortoglyphus arcuatus*, *Gohieria fusca*, *Thyreophagus entomophagus* and *Tyrophagus longior* has been investigated.

However, the lack of studies using internationally standardized extracts of storage mites hampers, to a certain extent, the complete understanding and full analysis of the clinical significance of sensitization to these mite species. An exact taxonomic identification, a correct selection of the raw material used (mite bodies, faeces, whole culture), the knowledge of enzymatic activity, among other characteristics should improve the quality of these extracts.



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C4.3 - Pollens as raw allergenic materials- practical and regulatory perspectives.

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Pollens, as raw allergen materials, possess a number of unique qualities that powerfully influence the requirements for their handling and processing. Unlike other allergens, pollens have a great variety in starting parameters, based on realistically unpredictable and uncontrollable external factors typically related to climate, like the particular temperature curve, sunlight availability and rainfall in the year of collection. These facts require the adoption of a very specific practical and regulatory approach.

Due to the great variety at input, it is imperative the particular processing method be fine-tuned to the particular material at hand to guarantee a predictable high-grade final product, which means that a standardisation of processing methods is only possible in the form of general guidelines. The properties of pollen further change with factors such as humidity, air pressure and temperature even during processing. For example, different mesh sizes are required in different kinds of weather for the sieving of the same species, even in controlled conditions. For another example, the volume of defatting agent to be applied is proportional to the content of fat in the material.

In either case, the process cannot be formally specified beyond general terms.

It is possible to draw a parallel between pollen and, for instance, wine. In the making of wine, the method must be determined on a case-by-case basis to best suit the biological material harvested, in order to guarantee a fixed and reasonably predictable outcome. Fixation of the process, however, would make the outcome unpredictable.

The determination of optimum processing method is therefore a matter of some experience and practice, and the training of new professionals is a process that takes a number of years.

While it is possible and desirable to apply restrictive standardisation to pollen (the setting of qualitative limits, prohibition of non-essential toxic substances in processing), positive standardisation (precise and uniform specification of processing method) is on the contrary impossible. While the former encourages standardised high quality in products, the latter would effectively prevent it. When working with pollens, the standardisation of process and the standardisation of outcome are frequently in a relationship of mutual exclusivity.



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KNA8 - Pharmaceutical Development and Validation of Grass Modified Allergen Tyrosine Absorbate (MATA) with MPL adjuvant

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The design of Grass MATA MPL and its manufacturing process, to consistently deliver the intended performance and meet the need of customers. Including all stages of manufacture : drug substance, drug product, novel excipient , process and analytical development. AT created a project team including, Validation, Production operators, QA, Development, and Engineering. A URS was issued detailing teams requirements and a formal validation Programme (DQ, IQ, OQ, PQ, PV) followed. Logical, detailed batch records and procedures were developed. Production operators played a key role in `walking through' procedures and batch records; time spent here is key in minimising risks of errors in the future. Technology transfer activities were performed to transfer product and process knowledge between development and manufacturing within Allergy Therapeutics to form the basis for the manufacturing process, control points, process validation and ongoing continual improvement. Process Validation involves the evaluation, sampling and testing of the critical parameters and quality attributes. Appendix 15 of the "Rules and Guidance for Pharmaceutical Manufacturers and Distributors 2007" (Orange Guide) describes the various approaches to Process Validation. Once in manufacturing in order to achieve `right first time' product, maintain a state of control and encourage continual improvement. The Quality system ensures that Grass MATA MPL is routinely made, suitable process performance is achieved, controls are appropriate, improvement opportunities are identified and knowledge is developed. This includes sourcing raw materials, maintaining facilities, production, QC, release, storage and distribution.



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C4.4 - Key questions on lyophilization of biopharmaceutical products

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It is well-known that many biopharmaceutical active substances including allergen extracts and vaccines are sensitive to heat and light. In many cases lyophilization or freeze drying is the only way to withstand storage for a long period of time without losing their biological activity. In spite of this, there are only a few freeze-dried allergen products in the market. This work describes key aspects to be considered for the development of a reliable lyophilization process. An understanding of the thermal properties of any solution to be freeze dried is critical to successful drying because the formulation composition impacts directly on the freezing properties of any material for lyophilization. Other studies should be addressed on some other practical aspects, like innovative non-invasive software methods to monitor the lyophilization cycle or the design of cleaning process, process validation problems and a proper method to determine the residual moisture within an aseptic production. Often, the selection of packaging materials is considered late in the development cycle. The processing and selection of packaging materials, in particular, the elastomeric closures, plays a critical role in preserving the lyophilized product over the intended shelf life.



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C4.6 - Round Table Quality system for biopharmaceutical products, including allergen products in BIOCEN

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ABSTRACT NOT AVAILABLE



C4.5 - High-scale production of biopharmaceuticals in Cuba

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Cuban biotech sector is now twenty-five years old, and during this time grew up from almost zero to the international recognition, not only because of scientific results but mainly for its ability to close the full product cycle of development, manufacturing and commercialization.

In all these years, the technology for producing protein pharmaceuticals from natural sources or by recombinant technology has been developed by our scientists and technicians. We could say that almost all expression, fermentation, and downstream systems existing around have been used or developed by our biotech industry. Some of them have been granted with process or product patents.

More than fifteen different molecules have been produced, all of them with the proper validation. Different host expression systems have been used for recombinant proteins: yeast, bacteria and mammalian cells; expressing both soluble and insoluble proteins. Here we present key aspects to be considered for high scale development of recombinant proteins for pharmaceutical use as injections. Results based on our experience on the development of Hepatitis B surface antigen expressed in *P. pastoris* and Streptokinase expressed in *E. coli*, are discussed.

On the other hand, these productions have been audited and certified by national and international regulatory authorities. Particularly, the manufacturing process of the recombinant HepB Vaccine by CIGB and BIOCEN has been continuously certified by WHO during the last eighth years. Of this vaccine, it has been produced more than 200 million doses, which have been used safely in more than thirty countries all-over the world.

The primary target of the Cuban biotech is the National Healthcare System and therefore the Cuban population, who have been benefited in these years by solving relevant health problems, such as the almost complete eradication of Hepatitis B and Meningitis infections in children. Cuban biotech system is now a serious and mature consolidated system, with implemented state-of-the-art technologies and moving forward. The experience gathered so far can be now put in function to the development of new medicines and products for the world wellbeing.



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KNA9 - Clinical trials for sublingual immunotherapy.

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Introduced: in 1911 allergen specific immunotherapy. Although this subcutaneous injection therapy is effective in patients with allergic rhinitis and asthma it has also disadvantages in terms of safety and patient acceptance. To overcome these drawbacks alternative routes of administration have been developed of which sublingual immunotherapy (SLIT) have attracted the most attention.

Until now 60 randomized DBPC trials of SLIT, among of which 41 conducted with grass or HDM extract. 48 trials provided positive results, whereas 12 trials were negative. Several meta-analyses also provided positive results, however the interpretation of the outcome of these meta-analyses is hampered by heterogeneity in underlying studies. Also reviews of meta-analyses yielded contradictory conclusions. In recent years large studies with high dose sublingual extracts appeared to be effective in both adults and children. The question has to be addressed as to whether such well-performed large studies are more important and convincing than the current meta-analyses, although the latter approach is considered to provide the highest level of evidence. Apart from the level of evidence, other factors such as the balance between desirable and undesirable effects, the values and preferences of the patient, costs and resource allocation are important. Such factors are taken into account in the new GRADE system.

One of the main advantages of sublingual immunotherapy is its safety profile. According to the recent ARIA update on subcutaneous and sublingual immunotherapy in all reported DBPC trials, SLIT was well tolerated. Today, a few severe systemic reactions have been reported.

Less is known of the long term aspects of SLIT. A few studies suggest that SLIT may prevent the development of new sensitizations and asthma.

Looking at the future of sublingual immunotherapy, there are still unmet needs. Research to the efficacy with other allergens should be intensified. Optimal dosing and the ideal duration of treatment has to be established. There is also a need for solid research in asthma.

Conclusion: SLIT appears to represent a valuable extension of the therapeutic arsenal for the benefit of allergic patients. However, there is substantial room for research to optimize this therapeutic approach.



C5.1 - Clinical trials of recombinant allergens for diagnostic/therapeutic use

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Natural allergen extracts are complex mixtures of allergens; not all are well characterized and there hardly exists any standardization between the different manufacturers. The development of recombinant allergens allows the use of well-defined allergenic extracts with consistent pharmaceutical quality in defined mass units of major allergens (van Ree et al. Allergy 2008). Skin testing with recombinant allergens from birch were first performed in 1996 (Pauli et al JACI 1996) and since then a great number of recombinant allergens have been tested (more than 20 on 1600 patients in 2001 (Schmid-Grendelmeir and Cramer Int Arch Allergy Immunol 2001)). Performances of recombinant allergens for diagnosis depend on the choice of major recombinant allergens included in the diagnostic tests, which must be relevant for a given population.

For therapeutic use the value of new allergenic products in terms of efficacy and safety must be demonstrated in double-blind, placebo controlled, randomized studies (optimally in comparative studies including natural extracts) and by the use of appropriate statistical methods). Animal experiments had indicated that it was possible to convert important allergens into hypoallergenic derivatives with decreased IgE-binding capacity, which explains why molecular modified derivatives of recombinant Bet v 1 were also used in clinical trials. The most important results, all published in full length in peer-reviewed journals, are reported here. Two studies were performed with wild-type recombinant allergens, one with a cocktail of 5 recombinant allergens from grasses (Jutel et al. JACI 2005), and one with rBet v 1 (Pauli et al. JACI 2008), and a single study was performed with hypoallergenic derivatives of Bet v 1, including two groups treated with either a trimer or a mix of two fragments of Bet v 1 (Purohit et al. Clin Exp Allergy Immunol 2008). One study applied a mixture of five *Phleum pratense* major allergens in a maximum dose of 40 µg protein (10 µg Phl p 1, 5 µg Phl p 2, 10 µg Phl p 5a, 10 µg Phl p 5b, 5 µg Phl p 6) in 29 actively treated and 28 placebo patients. The clinical efficacy showed a significant efficacy with about a 40% reduction in the combined scores (symptoms and medication) ($p = 0.44$) for actively treated patients compared to placebo. Systemic side effects were observed in 7 out of 29 actively treated patients (generalized urticaria, rhinoconjunctivitis). The second study, using birch wild-type recombinant allergen, compared treatments performed with molecular compounds (natural (n=24) and recombinant Bet v 1 (n=27)) with treatments performed with a commercial birch pollen extract (n=27) and placebo (33); after a build-up phase, monthly injections of 15 µg Bet v 1 allergen were given for 2 years. Combining symptom and medication scores (equivalent weight) indicate a 59% reduction in disease severity in patients treated with birch pollen extract, 57% for rBet v 1 and 57% for nBet v 1 compared to placebo. The side effects were mild reactions, except for one patient in the nBet v 1 group who developed anaphylaxis. In both studies (grass and birch) active treatment induced a statistical significant increase in IgG1, IgG2 and IgG4 specific antibodies in the actively treated groups. The third study compared treatments performed with rBet v 1a trimer (created by expressing three copies of the gene) (n=28), Bet v 1 fragments (two recombinant peptides that together represent the whole sequence of Bet v 1) (n=19) and placebo (n=37). The immunotherapy schedule included monthly injections of 80 µg protein after a build-up phase.



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There was no clinical difference between the trimer-group and placebo and the mean difference between the fragments-treated group and placebo was 28% (NS), during the birch pollen season. Active treatment induced a statistical significant increase in IgG1 and IgG4 Bet v 1 specific antibodies. Systemic side effects (generalized urticaria) were observed in 21% of fragments-treated and 16% of trimer-treated patients.

The proof of the concept is given by the clinical efficacy of the immunotherapy in the 2 studies performed with wild type recombinant allergens, but for the birch system, it was also shown that recombinant allergens are as clinically effective as purified allergens and the crude allergen extracts. The advantages of using recombinant allergens for immunotherapy are obvious, though clinical studies on a larger scale are needed before the overall value in terms of efficacy and safety can be determined.



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C5.2 - Clinical significance of recombinant allergen vaccines

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ABSTRACT NOT AVAILABLE



C5.3 - Clinical trials of SIT with recombinant allergens

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Although, a large number of allergens have been cloned and characterized, only a few have been used in clinical studies. Two types of recombinant allergen-based vaccines have been developed and tested in clinical trials. The first type is based on recombinant allergens that equal the natural allergens (ie, recombinant wildtype-based vaccines). However the recombinant allergen technology also provides the possibility to create allergen derivatives with reduced IgE-reactivity. Thus, the second type of vaccines is based on genetically engineered hypoallergenic molecules.

The selection of the relevant recombinant allergen molecules is an important step during the vaccine development. The availability of pure recombinant allergens will make it possible to perform component-resolved diagnosis and to produce individual's sensitization profile. The information will also help to elucidate the role played by cross-reacting allergens in clinically relevant sensitizations in certain patients populations. A perfect vaccine would exactly match this pattern. This concept called patient tailored immunotherapy is very attractive. However, the issue of costs and time consumption have put this approach to some delay in the practical clinical application.

Currently, standard combinations of allergens from one source, or in some cases single allergens, which are most frequently implicated in allergic disease are used and evaluated in clinical studies.

1.1.1.1 Studies in birch pollen allergic patients

Effective vaccines containing a single allergen can be developed for the treatment of birch (Bet v 1) or cat (Fel d 1) sensitized subjects. In fact the first clinical trials with recombinant allergen-based vaccine were performed with genetically modified hypoallergenic derivatives of the major birch pollen allergen, Bet v 1. In parallel, several studies, which analyzed the effects of such vaccination on the immune parameters, have been performed. The recombinant Bet v 1 (rBet v 1) fragments and rBet v 1 trimer showed a more than 100-fold reduced allergenic activity compared with the natural Bet v 1. When skin prick testing was performed at concentrations of 100 µg/mL in birch pollen-allergic subjects who had not received specific immunotherapy, 18 of 23 and 15 of 23 failed to react to the fragment mixture and the trimer, respectively.

In a multicenter, randomized, double blind, placebo-controlled study, 124 patients with birch pollen allergy received a single preseasonal course of injection immunotherapy with a mixture of 2 recombinant Bet v 1 fragments or a recombinant trimer of Bet v 1. The recombinant preparations were adsorbed to aluminium hydroxide suspensions at concentrations of 100 µg/mL, and immunotherapy was performed with a course of eight preseasonal injections of increasing concentrations from 1 to 80 µg of total protein. Subjects were assessed at baseline, after treatment, during and after the pollen season, and after 12 months. The patients were able to tolerate maintenance doses of 80 µg active ingredient per injection. The cumulative dose reached by the majority of the patients was more than 150 µg active preparation.



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Symptom medication scores and interval scoring in the per protocol-treated population (n = 84) was assessed to prove the clinical efficacy. There were trends towards improvement in the subjects' well-being and clinical symptoms (nasal scores), although comparisons with a placebo group did not show statistical significance in the main end-point, the combined symptom-medication score.

Local injection-site reactions were most frequent in the trimer group affecting 59.5% of patients as opposed to 37.8% and 30.6% in the fragment and placebo groups, respectively. Systemic reactions were elicited more frequently by fragments.

In addition, very significant effects on the immunologic parameters of Bet v1 specific response were demonstrated. Results from 71 patients showed that both active preparations induced Bet v 1-specific IgG1, IgG2, and IgG4 antibody responses against Bet v 1 and Bet v 1-related pollen allergens from alder (Aln g 1) and hazel (Cor a 1), and food allergens (ie, apple, Mal d 1; carrot, Dau c 1; and celery, Api g 1). Interestingly, no relevant rises of allergen-specific IgA were detectable in serum and nasal secretions. Responses to the trimer were stronger, and this preparation also induced IgM antibodies, indicating its good immunogenic properties. There was significant association between IgG1 antibody titers and the reduction of immediate-type cutaneous and nasal sensitivity to Bet v 1 and inhibited Bet v 1-induced basophil degranulation in vitro as well as an improvement in clinical symptoms. The Bet v 1-specific IgE responses during the pollen season were blunted in the treatment groups but not in the placebo group. Bet v 1-specific lavage fluid IgG1 levels were significantly raised at the end of the pollen season in subjects who had received the active preparation when compared with placebo subjects. At the end of the birch pollen season, there was a correlation between nasal IgG4 and reduced specific nasal sensitivity. The nasal antibody levels mirrored those in serum. The reduced nasal sensitivity may be accounted for by the inhibitory effect of the antibodies on basophil and mast cell mediator release as demonstrated for the serum antibodies in vitro.

Analysis of epitope-specific immune responses induced by vaccination with structurally folded and unfolded recombinant Bet v 1 allergen derivatives showed that both types of vaccines induced a comparable IgG1 and IgG4 response against new sequential epitopes which overlap with the conformational IgE epitopes of Bet v 1. This response was 4- to 5-fold higher than that induced by immunotherapy with birch pollen extract. Trimer more than fragments induced also IgE responses against new epitopes and a transient increase in skin sensitivity to the fragments at the beginning of therapy.

In the trimer-treated patients a suppression of Bet v 1-specific TH2 responses - significant reductions in interleukin-5 (IL-5) and IL-13 producing cells was observed. This may be due to the high treatment dose or the intrinsic ability of the Bet v 1 trimer to induce higher TH1 responses than the recombinant wild-type allergen.

Thus, in spite of its very significant effect on the immunological parameters of allergen specific immune response a single course of injection immunotherapy with Bet v 1 allergen derivatives showed trends towards improved well-being and reduced reactivity to specific allergen provocation, but did not yield significant improvement in the combined symptom medication score.

An alternative strategy for developing a hypoallergenic vaccine is the application of a folding variant of rBet v 1 (rBet v 1-FV). This folding variant exhibits a stable random coil structure that can be clearly distinguished from the secondary structure of the native molecule.



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Currently, data from 2 studies are available. The first study was conducted in a group of 24 patients with birch pollen allergy treated with rBet v 1-FV versus a reference group composed of 27 individuals receiving a standard birch pollen extract-based vaccine according to a preseasonal treatment regimen. Patients received very high maintenance doses of 80 µg rBet v 1-FV, fourfold higher than the Bet v 1 content of the natural allergen preparation, reaching a cumulative dose of 157.7 µg. They developed very strong Bet v 1-specific IgG1 and IgG4 responses. Results obtained during the pollen season after the initiation of SIT showed that the combined symptom-medication score for subjects receiving the recombinant preparation was lower than that for subjects receiving the natural pollen extract (a 53% reduction in symptom medication score compared with a parallel group). However, during the following pollen season after the second course of treatment both groups showed similar range of symptom medication score reduction indicating that further cumulative dose increase might not result in more vaccine effectiveness. Safety data indicated that the preparations were comparable with respect to the occurrence of adverse events. The immunogenic activity of the recombinant preparation was confirmed by its ability to induce strong IgG1 and IgG4 antibody responses.

The same compound was tested in a large phase III multicentre, double-blind placebo controlled study, which included 228 subjects (116 in the active group). Clinical efficacy was confirmed by improvement in symptom medication score as compared to placebo group ($p=0.0137$). The median reduction of symptom medication score during the second pollen season was 5.1 per day. Very robust IgG1 and IgG4 antibody responses were also shown. No serious adverse effects were reported. Recently, results from a large trial investigating the effectiveness of the recombinant wild-type allergen-Bet v 1a isoform in patients with birch pollen allergy were published. In this multicenter, randomized, double-blind, placebo-controlled study 134 adult patients were included. 3 different vaccines i.e. recombinant birch pollen allergen vaccine (rBet v 1a), licensed birch pollen extract, natural purified birch pollen allergen (nBet v 1) were compared. Patients received 12 weekly injections followed by monthly injections of the maintenance dose containing 15 µg Bet v 1 for 2 years. Significant reductions (about 50%) in rhinoconjunctivitis symptoms, rescue medication, and skin sensitivities were observed in the 3 actively treated groups compared with placebo during 2 consecutive pollen seasons. Clinical improvement was accompanied by marked increases in Bet v 1-specific IgG levels, which were higher in the rBet v 1-treated group than in the birch extract or nBet v 1-treated groups. New IgE specificities were induced in 3 of 29 patients treated with birch pollen extract, but in none of the 32 rBet v 1-treated or 29 nBet v 1-treated patients. No severe systemic adverse events were observed in the rBet v 1-treated group.

This study was the first to demonstrate in a direct comparison that a single recombinant allergen is as effective as treatment with a natural extract for the treatment of respiratory allergy.

1.1.2 SIT with recombinant grass pollen allergens

Another study with a preparation equaling the natural allergens was performed with an equimolar mixture of 5 aluminum hydroxide-adsorbed recombinant timothy grass pollen allergens—Phl p 1, Phl p 2, Phl p 5a, Phl p 5b, and Phl p 6—in patients with grass pollen allergy. In pollen or mite sensitizations the sensitization patterns are more complex than in birch pollen or cat allergic subjects. Grass pollen has 11 different allergens that have been identified and characterized in detail and some of these are known to occur as several different isoforms. Only some of these allergens are associated with a high prevalence of sensitization in the grass pollen allergic population and thus accounting for a large part of the specific IgE directed against the whole pollen extract.



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A double-blind placebo-controlled clinical trial was performed with 62 grass pollen allergic patients suffering from rhinoconjunctivitis with or without asthma. The recombinant allergen adsorbate was administered in subcutaneous injections of increasing concentrations at 7-day intervals prior to the pollen season in 2002, starting with 0.02 mg total protein, followed by 0.16 mg and then doubling to 40 mg total protein (0.8ml). The maximum dose contained 10 mg Phlp1, 5mg Phlp2, 10 mg Phlp5a, 10mg Phlp5b and 5mg Phlp6. Maintenance injections were continued until after the subsequent pollen season with a 50% reduction during each pollen season. The median cumulative dose was 490 mg total protein, corresponding to 122.5 mg each of Phl p 1, 5a and 5b, and 61.25 mg of Phl p 2 and 6. A symptom–medication score was the primary outcome measure to assess efficacy. A per protocol analysis included 24 active treatment and 25 placebo patients. A combined symptom–medication score adopted as primary end-point showed a 39% improvement in the active treatment group relative to placebo ($p < 0.041$). Symptoms alone improved by 37% ($p < 0.015$) and the use of symptomatic medication decreased by 36.5% relative to placebo. A validated rhinitis quality of life questionnaire (RQLQ) was a secondary end-point. Questionnaires were completed every two weeks during the pollen seasons and the questionnaire following the maximum pollen count was used for analysis. Benefits were registered for those subjects on active treatment during the first pollen season. However, the per protocol evaluation during the second pollen season showed even greater differences between active and placebo treatment with an overall significant benefit ($p < 0.024$), providing further evidence of clinical efficacy. Significant effects were seen in five of seven domains tested. Conjunctival provocation tests were performed prior to therapy (inclusion criterion) and at the end of the study using a standardized 6-grass allergen extract. The concentration was increased in half-log steps starting with 5 BU/ml until a positive reaction was obtained or a maximum concentration of 5000 BU/ml (0.18 mg group 5 allergen/drop) was reached. A favorable trend was observed ($p < 0.081$) with an increase in the threshold dose in favor of treatment with the active preparation. The failure to achieve a significant result was most likely attributable to the small numbers of patients. Active treatment induced highly significant increases in both IgG1 and IgG4 grass pollen specific antibody concentrations together with a significant decrease in IgE. IgG1 increased approximately 60-fold, peaking during the first 12 months of the study. IgG4 showed a continuing upward trend, achieving an approximately 4000-fold increase by the end of treatment. Specific IgE levels were not significantly different between groups at the beginning of the study, but thereafter the active treatment group showed a downward trend with values significantly less than baseline. Four subjects in each group had no Phl p 5a/b specific IgE prior to the study, but reacted to Phl p 1 and other grass pollen allergens. None of these subjects developed Phl p 5a/b IgE antibodies during the study, although the four subjects receiving active treatment developed strong IgG4 and IgG1 Phl p 5a/b responses consistent with induction of a protective immune response. This observation obviously needs to be substantiated and it will also be of interest to look at the prophylactic effects of the treatment in guarding against the development of new sensitizations. Treatment related adverse events were seen in association with 78 injections (10.7%) in the active treatment group and 44 injections (5.9%) in the placebo group. Local reactions involving erythema and swelling in the vicinity of the injection site, with or without pruritus, accounted for nearly all these reactions. Single systemic reactions were seen in seven active treatment and two placebo subjects, the former including general urticaria (two cases), local urticaria (two cases), dyspnea (one case), rhinoconjunctivitis (one case) and one case of asthma two days following the injection. All these subjects continued treatment without further problems and it was concluded that the preparation showed a favorable safety when compared with findings from other immunotherapy studies.



Conclusion

The clinical trials performed with recombinant wild-type allergens and genetically modified hypoallergenic allergen derivatives indicate that these vaccines are safe and at least equally effective as the standard extract-based preparations both for immunotherapy in sensitization against allergen sources containing 1 predominant allergen as well as for complex allergen sources. Identification and inclusion of the relevant allergens in the vaccine is crucial for its efficacy.

TABLE Immunotherapy trials with recombinant allergen-based vaccines

Molecules	Allergen source	No. of patients	Treatment groups	Mode of administration	Adjuvant	Study design
Recombinant wild-type allergen rPhl p 11, rPhl p 21, rPhl p 5a1, rPhl p 5b1, rPhl p 6	Grass pollen	n = 62	Allergens, placebo	Subcutaneous	Alum	DBPC, randomized single-center
Recombinant wild-type allergen rBet v 1	Birch pollen	n = 134	rBet v 1, nBet v1, birch pollen extract, placebo	Subcutaneous	Alum	DBPC randomized multicenter
hypoallergenic derivatives rBet v 1 fragments rBet v 1 trimer	Birch pollen	n = 124	rBet v 1 fragments rBet v1 trimer/placebo	Subcutaneous	Alum	DBPC randomized, multicenter
rBet v 1-FV	Birch pollen	n = 51	rBet v 1-FV/birch pollen extract	Subcutaneous	Alum	Open/reference group, multicentre-center

Notes: Alum: Aluminum hydroxide; DBPC: double-blind, placebo-controlled.



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KNA10 - Design of Clinical trials for immunotherapy

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The clinical efficacy of specific immunotherapy has a solid documentation based on controlled clinical trials and meta-analysis. However, according the current requirements, many of the past studies have flaws, leading the need for studies according the current "state of the art".

The clinical trials with allergen specific immunotherapy should follow the principles of Good Clinical Practice and the Guidelines adopted by Health Authorities. The studies should have a clear objective coupled with a summary of the methods for analysis of the results; the design must allow quantitative assessment of the efficacy by a valid comparison with a control group and the protocol should accurately define the design and the duration of the study, sample size issues, clear description of the method of the patient selection and treatment assignment, methods for bias minimization (eg. blinding), and the description of appropriate measures for assessing patient response.

In spite of the Guidelines related to the design, conduction, analysis and reporting Clinical Trials, there are issues related to the specific field of allergen immunotherapy that should be taken into account. These issues are described in the "Guideline on the Clinical Development of Products for Specific Immunotherapy for the Treatment of Allergic Diseases" (CHMP/EWP/18504/2006), and are related to patient characteristics, therapeutic agents other than allergens, the strategy and designs of clinical trials, efficacy in paediatric populations and safety.



C5.4 - Clinical development program of the first tablet for sublingual immunotherapy

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A large proportion of vaccines for allergen specific immunotherapy are marketed without authorisation as named patient products. This practice is under pressure from the authorities aiming for proper authorisation of medicines including biological products. Some products have obtained market authorisation in individual countries based on a single or a limited number of clinical trials. The clinical development program for Grazax[®] sets a new standard for development of products for allergen specific immunotherapy. Through a centralised procedure with the European Medicines agency, EMEA, market authorisation in 27 European countries was achieved simultaneously.

A comprehensive clinical development program was conducted to address thoroughly safety and efficacy issues associated with the new drug, a tablet for sublingual immunotherapy of grass pollen induced rhino-conjunctivitis and asthma. Three phase I tolerability studies (n=174) were conducted demonstrating tolerability of a maximal dose corresponding to 13 times the marketed dose. A large phase II dose-finding study (n=855) was conducted to identify the optimal dose in rhinitis patients, followed by a safety and efficacy study (n=114) in asthma patients. A large phase III study (n=634) currently ongoing is addressing efficacy of three years treatment including follow-up one and two years after end of treatment. Two safety studies (n=60) and an efficacy study (n=250) in children supported the indication in children. A total of 1516 patients have been exposed during the clinical development program leading to approval in adults as well as in children.

This comprehensive development program positions Grazax[®] as the best documented product for allergen specific immunotherapy.



C5.5 - Challenges in performing Global Phase 3 studies in SIT

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Introduction: Allergen Immunotherapy has been in clinical use for nearly 100 years. A recent Cochrane review revealed over 1000 publications in the literature indicating its efficacy. However, only in the last few years have formal international multi-centre Phase 3 studies been conducted to GCP standards for the purpose of product registration.

Methods & Results: In the conduct of international Phase 3 studies there are several areas that require consideration and agreement with Regulatory Authorities, IRBs and Investigators with differing practices of SIT. These include: Standardisation of products dose regimens Study design & duration Methods for the selection of appropriate patients Initiatives to recruit large cohorts of patients Establishing acceptable assessment scales Requirements for data handling & analysis Furthermore, the evaluation of the outcome of recent international Phase 3 studies may require re-assessment of the expectations of SIT. Key areas include: Transitioning from Phase 2 to Phase 3 Variability vs validity of the data European vs North American outcomes Relevance of immunology and Quality of Life

Conclusions: The conduct of Global Phase 3 studies has led to new advances in the understanding of SIT.



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C5.6 - Mechanisms of Allergen SIT

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Although allergen-specific immunotherapy (SIT) has been performed for almost a century, the immune mechanisms underlying this treatment are still not entirely solved. During SIT increasing doses of allergen extracts are administered subcutaneously (SCIT) or sublingually (SLIT) to the patient to generate clinical tolerance to natural allergen exposure. We monitored the changes of allergen-specific antibody and T cell responses during SLIT with birch pollen in patients with birch pollinosis and associated food allergy. The major birch pollen allergen Bet v 1 and its highly cross-reactive homologue in apple, Mal d 1, served as model allergens. After 4 weeks of SLIT, Bet v 1-induced proliferative responses were significantly reduced reflecting allergen-specific peripheral tolerance. During this early phase we found regulatory CD4⁺ T cells suppressive *via* IL-10 in the peripheral blood of the individuals. After 52 weeks, Bet v 1-induced proliferative responses remained significantly reduced as compared to before SLIT. However, at this time point no evidence for regulatory T cells was found but we observed the switch from the allergen-specific Th2-like response typical for allergic patients to a more Th0/1-like response characteristic for non-allergic individuals. In summary, our data indicate that different immune mechanisms are operative during early and later treatment phases of SIT. Early on, tolerance is mediated by regulatory T cells. Later on, immune deviation is induced whereas the mechanisms suppressing allergen-induced proliferation still remain to be determined. Of note, SLIT with birch pollen only altered the T cell response to Bet v 1 but not to Mal d 1. In the same individuals only Bet v 1-specific IgG4 antibodies were induced by SLIT. To follow this up, we investigated the induction of allergen-specific IgG4 antibodies during SCIT with birch pollen. All patients developed Bet v 1-specific IgG4, however, only around one third of them also developed Mal d 1-specific IgG4. We conclude that the immune mechanisms induced by SIT are highly allergen-specific. This immunological observation may explain why SIT with birch pollen is not effectively curing associated food allergy.



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C5.7 - Immunotherapy decision-making by using allergenic molecule-based diagnostics

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Allergenic extracts have been in use by allergists for both diagnosis and immunotherapy for more than a century to date. During the last twenty years an increasing amount of research work has been done focusing identification of the real sensitizing and triggering structures, namely allergenic molecules. The current knowledge embraces data on more than 1588 structures (www.allergome.org/script/statistic.php on June 2009). At the beginning it was thought that the allergenic molecules were going to replace the extracts by mimicking their historical use. It was just a thinking about how to make a better extract. Since the single allergens entered in the clinical use, it became evident that it was the beginning of a new era for allergy diagnosis. The availability of allergenic molecules, mainly on well established in vitro systems, allowed us to explore the intrinsic feature of many allergenic molecules, starting to classify them as "markers" of a clinical conditions, as "signature" of a sensitization profiles, as "genuine" when identifying source-related sensitizations, as "panallergens" when describing unrelated multiple source sensitizations.

The advent of micro-technology applied to medicine is further speeding up the process of using allergenic molecules for routine allergy diagnosis. Biochips are bringing the allergy diagnosis to a broader and general level allowing for instance immunotherapy decision-making easier. Waiting for the commercial availability of allergenic molecules for immunotherapy, the basic for this decision-making process is anyway the full description of extracts' allergenic content. Having the full matching of the patient's profile and the perfect knowledge on allergenic components in each therapeutic extract any allergist may decide which product to use.



P1.1 - Different Toll-like receptor ligands as possible adjuvants in allergy vaccines

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Introduction: The use of Toll like receptor (TLR) ligands as adjuvants in vaccines for specific immunotherapy (SIT) of Type 1 allergy is considered a promising approach to promote the SIT-induced shift from an allergenic Th2-type immune response towards a more physiologic Th0/Th1-like immune response. To date, 11 TLR are known in humans which are differentially expressed in different cell types. For two of these TLR, ligands have been found that promote allergen-induced immune deviation in favour of Th1 responses. For TLR4, which is present on monocytes and myeloid dendritic cells (DC) this is monophosphoryl lipid (MPL) A. For TLR9, a receptor on B cells and plasmacytoid DC this is oligodeoxynucleotides containing CpG motifs (CpG-ODN). TLR2 and TLR5 are present on myeloid DC, monocytes and T cells. In this study we wanted to compare flagellin from *Salmonella typhimurium*, a TLR5 ligand, and aA-crystallin, a TLR2 ligand, with MPL A and CpG-ODN with regard to their possible adjuvant capacity.

Methods: Peripheral blood mononuclear cells (PBMC) were isolated from birch pollen-allergic individuals and stimulated with different TLR ligands. Production of different pro- or anti-inflammatory cytokines was determined by ELISA.

Results: Both, flagellin and aA-crystallin induced the production of IFN-g and IL-10 but not TNF-a. Experiments to determine the cellular source of these cytokines are currently performed.

Conclusion: Our results indicate that flagellin and aA-crystallin might be possible adjuvants in vaccines for SIT.



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P1.2 - Generation of vaccine candidates for birch pollinosis-associated food allergy

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Introduction: Birch pollen allergic patients often display adverse reactions following ingestion of plant-derived foods caused by cross-reactive IgE antibodies. To date, specific immunotherapy represents the only curative approach towards allergy treatment. Although successfully applied for birch pollinosis, permanent amelioration of associated food allergies can hardly be achieved. The aim of this study was the generation of vaccine candidates targeting both symptoms of birch pollen and associated food allergies.

Methods: Based on a structural variant of the birch pollen major allergen Bet v 1, corresponding hypoallergens were designed in silico for the Bet v 1 homologues from apple (Mal d 1) and hazelnut (Cor a 1). The respective proteins were recombinantly produced in *Escherichia coli*, purified by standard chromatography techniques, and tested for their structure and IgE reactivity.

Results: Both mutant molecules could be efficiently produced as recombinant proteins. They displayed altered structures and significantly reduced IgE-binding capacity when compared to wild type Mal d 1 and Cor a 1.

Conclusions: We were able to identify a distinct position in the hydrophobic cavity of Bet v 1 family members, where the introduction of a single lysine residue leads to a distortion of the overall Bet v 1-fold resulting in significant reduction of IgE binding. Such hypoallergenic fold variants represent promising candidates for the treatment of both birch pollinosis and associated food allergy.



P1.3 - Therapeutic vaccine against food allergy

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Introduction: For the treatment of food allergy, specific immunotherapy (SIT) is at present not available. In the current study a novel SIT against food allergy is explored, which is an antibody-based fusion protein containing a recombinant allergen linked to a unit targeting an inhibitory pathway in the immune system.

Methods: As a model allergen, the major allergen in shellfish, tropomyosin, was chosen. Shrimps of the species *Pandalus borealis* were collected from the Oslofjord (Norway), and a cDNA clone containing the tropomyosin gene was generated.

Results: The tropomyosin gene was sequenced and its corresponding amino acid sequence appeared to have 96 % identity with tropomyosin from the shrimp species *Farfantepenaeus aztecus* within the 5 identified IgE binding regions. The cDNA was identical to tropomyosin from *Pandalus eous* within these regions.

Conclusion: a cDNA clone with tropomyosin from *P. borealis* was obtained that can possibly be used for inclusion in a candidate vaccine against shellfish allergy.



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P1.4 - Hypoallergens of Art v 1, the major mugwort pollen allergen as candidate for artemisia immunotherapy

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Introduction: Patients sensitized to Art v 1, the major allergen from mugwort pollen commonly display IgE antibodies against the disulfide bond-stabilized defensin domain. In addition, some individuals develop IgE antibodies against mono- β -arabinose sugar residues attached to hydroxylated proline residues in the C-terminal domain. Our aim was to generate hypoallergenic variants by targeting disulfide bridges and O-glycans, the two prominent post-translational modifications of Art v 1.

Methods: Site-directed mutagenesis was used to disrupt disulfide bridges responsible for the stabilization of IgE-binding epitopes located in the N-terminal defensin domain Art v 1. Engineered constructs were expressed in *E. coli* to produce non-glycosylated variants and recombinant proteins were tested for IgE and T cell reactivity as well as for their physicochemical characteristics.

Results: Three non-glycosylated cysteine variants (C22S, C47S, and C49S) exhibited extremely low IgE-binding activity in immunoblot and ELISA using sera from mugwort pollen-allergic patients. Mediator release assays using rat basophil leukemia cells showed that these variants displayed a 1×10^5 -fold reduced allergenic potency as compared to wild-type Art v 1. All variants were able to activate allergen-specific T cells in PBMC, as well as Art v 1-specific T cell lines and clones.

Conclusions: The low allergenicity and high immunogenic activity of the non-glycosylated Art v 1-C49S variant was accomplished through manipulations of posttranslational modifications. Disruption of the disulfide bond formed by the pair Cys26-Cys49 altered IgE-binding epitopes and exposed hydrophobic patches on the surface of the protein, which correlated with an increased capacity to activate allergen-specific T cells.



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P1.5 - Probiotics' therapeutic potential in a mouse model of food allergen sensitization

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Introduction: The immunological mechanisms responsible for the immune-modulating and anti-allergic effects of probiotic bacteria are still poorly defined. They have been only in part explored in suitable animal models to elucidate the in vivo processes that inhibit allergy responses.

Methods: The therapeutic activity of VSL#3 mixture, which contains eight different bacterial strains (four lactobacilli, three bifidobacteria, and one *Streptococcus thermophilus*), was assessed in a mouse model of oral sensitization and anaphylaxis to the food allergen Shrimp Tropomyosin (ST) from *Metapenaeus ensis*. C3H/HeJ mice were orally sensitized with purified ST plus cholera toxin as adjuvant. ST-specific serum IgE, IgG1, IgG2a responses were evaluated by ELISA. To induce in vivo anaphylaxis, mice received an oral challenge with ST. Local and systemic anaphylactic reactions were scored according to symptoms observed. Local IgA production and histamine levels were evaluated in faecal samples. Mice were then orally treated for three weeks with VSL#3 preparation. The effects of the probiotic treatment were evaluated as above.

Results: Oral therapeutic treatment with live VSL#3 was able to significantly reduce symptoms of anaphylaxis, as well as histamine levels in faecal extracts. Serum antibody levels were not affected by probiotic treatment.

Conclusions: These results support the therapeutic potential of the oral administration of a probiotic mixture, associated to the sensitizing allergen, on an established food allergen sensitization. The capacity of probiotics, or their products, to induce protective immune responses linked to counterregulatory local and systemic immune responses might become an effective strategy in the immunotherapy of type I allergy



P1.6 - AFPL1 adjuvant effect to House Dust Mite allergen

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Introduction: Allergic diseases are caused by altered Th2 immune response, where IgE is the main antibody involved. Consequently, vaccine strategies are developing to circumvent this Th2 response inducing a non-pathological Th1 or Tr1 responses. The AFPL1 adjuvant (Finlay Institute) containing the Proteoliposome from *Neisseria meningitidis* serogroup B exerts a Th1 effect. *Dermatophagoides siboney* (Ds) house dust mite is a common cause of respiratory allergy in Cuba. Therefore, we sought to determine the influence of AFPL1 over Ds allergen-specific response in mice.

Methods: Mice immunization experiments using AFPL1 and Ds adsorbed onto Aluminum Hydroxide (Alum) or Ds onto Alum were performed. Total and specific IgE, specific IgG and IgG subclasses, and cytokines after immunization and before and after allergen challenge were determined.

Results: Significant reduction in total and anti-Ds IgE, induction of IgG2a prominent response, IFN γ and not IL-4/5 after AFPL1 + Ds in comparison to Ds+Alum, were detected. The Ds challenge induced neither pulmonary inflammatory signs in lung tissues nor eosinophilia in AFPL1 + Ds immunized mice, without affecting the IL-10 local production in bronchoalveolar lavage. The vaccine was well tolerated without severe toxicity symptoms.

Conclusion: AFPL1 is a promising adjuvant for developing therapeutic or prophylactic allergen-based vaccines.



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P1.7 - A new proteoliposomic formulation of *Neisseria meningitidis* outer membrane components with better structural definition, and potentiality as a Th-1 adjuvant

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Introduction: Vaccines and adjuvants based on proteoliposomes have been successful; however it would be helpful to improve its structural definition preserving its effectiveness and stability, without the addition of the aluminum hydroxide to the final vaccine formulations, thus diminishing also its reactogenicity. This work presents the results obtained in Balb/c mice immunized with a new formulation of Neo-proteoliposomes (NPL), prepared starting from components of more defined molecular structure.

Methods: Final assembling in proteoliposomic form was evaluated by transmission and scanning ME. These formulations were compared with the classic formulation of the Cuban vaccine against *Neisseria meningitidis* (VAMENGOC-BC), based on aluminum hydroxide as depot adjuvant. Antigen proteins purified from the outer membrane of *Neisseria meningitidis* B were used. The specific immune response mediated by antibodies, delayed hypersensitivity reaction, spleen Index, and markers of local and systemic toxicity were evaluated.

Results: The new anti-meningococcal formulation was similar in terms of immunogenicity as compared to the classic formulation with aluminum hydroxide, with a more potent T-cellular immune response, similar immunological memory, lower reactogenicity and better structural definition.

Conclusion: This new formulation could be applicable to new anti-infective, and therapeutic anti-allergic and anti-anticancer vaccines.



P1.8 - Design of a non-allergenic Der p 1 peptide-based vaccine for immunotherapy of house dust mite allergy

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Introduction: More than 80% of mite-allergic patients show IgE-reactivity to the major mite allergen Der p 1. Our aim was to design a vaccine based on non-allergenic Der p 1 peptides with reduced T cell-reactivity which after binding to a carrier induce IgG antibodies blocking allergic patients IgE reactivity to Der p 1.

Methods: Eight Der p 1 peptides of approximately 30 amino acids were synthesized and tested for IgE reactivity. Mice and rabbits were immunized with KLH-coupled Der p 1 peptides and IgG antibodies were tested for reactivity to Der p 1 and their ability to inhibit patients' IgE binding to Der p 1. Lymphoproliferative and cytokine responses to Der p 1 and Der p 1 peptides were determined in PBMC cultures from mite allergic patients.

Results: None of the Der p 1 peptides showed any detectable IgE-reactivity and they induced reduced lymphoproliferative responses in PBMCs from mite allergic patients compared to Der p 1. Two N-terminal and one C-terminal peptide were identified which induced IgE-blocking IgG responses against Der p 1.

Conclusion: The three identified peptides should be useful for safe vaccination against house dust mite allergy because they allow by-passing of IgE and T cell-mediated side effects. Supported by grants F1803, F1805, F1815 of the Austrian Science Fund, by Biomay, Vienna, Austria and the Christian Doppler Research Association, Austria.



P1.9 - Carrier-bound peptides of the major allergen Alt a 1 for vaccination against allergy to the mould *Alternaria alternata*

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Introduction: Specific immunotherapy (SIT) is the only allergen-specific and disease-modifying approach for the treatment of allergy. The high incidence of asthma among mould sensitized patients and the poor quality of fungal extracts limit the application of SIT for mould allergy. The aim of our study was to develop a safe vaccine for the treatment of allergy to *Alternaria*.

Methods: Four non-IgE-reactive peptides derived from the major *Alternaria* allergen, Alt a 1, of approximately 30 amino acids length were coupled to KLH and used to immunize rabbits. Peptide-induced rabbit IgG antibodies were tested for their ability to recognize Alt a 1 and to inhibit the binding of allergic patients' IgE to the allergen.

Results: A complete lack of IgE reactivity was demonstrated for each of the four peptides by ELISA and dot blot. The three C-terminal Alt a 1 peptides induced IgG antibodies which inhibited allergic patients' IgE binding (n = 18) to Alt a 1 (mean > 30%). A combination of antibodies specific for two peptides yielded a mean inhibition of more than 50%.

Conclusion: We developed a safe peptide vaccine that could be used for immunotherapy of *Alternaria alternata* allergic patients.

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P1.10 - Liposomes and Sticholysin II as immunomodulators in a murine ovalbumin model of asthma

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Allergic asthma is a chronic inflammatory lung disease mediated by Th2 cells, characterized by airway eosinophilia, airway hyper-reactivity, mucus hyper-secretion and elevated levels of IgE. Liposomes have been used as immunoadjuvants of different antigens including allergens. Different strategies have been employed such as the co-encapsulation of cytolytic toxins or peptides with antigen into liposomes in order to activate Th1 CD4⁺ and/or cytotoxic T CD8⁺ lymphocytes. Sticholysin II (St II) is a toxin isolated from the Caribbean Sea anemone *Stichodactyla helianthus* forming pores into membranes. Previous results of our group evidenced that the co-encapsulation of St II with the main allergens from *Dermatophagoides siboney* into liposomes improved antigen-specific IgG2a/IgG1 ratio suggesting the presence of a Th1 response pattern. In this work we used a protocol to induce asthma-like response in mice employing ovalbumin (OVA) as antigen, which consists basically of two subcutaneous immunizations of Balb/c mice with OVA followed by two intranasal challenges with the antigen in PBS. The aim of our study was to evaluate the ability of liposomes, comprised of DPPC:Cholesterol and co-encapsulating OVA and St II, to reverse the OVA-specific asthma-like response in mice. For that, 24 hours after second OVA challenge, mice were sacrificed to determine: i) total and differential cells of bronchoalveolar lavage fluid, ii) peribronchial and perivascular lung inflammation and the mucus index by histological analyses, iii) OVA-specific IgE, IgG1 and IgG2a antibody levels on the sera, and iv) IL-5 and IFN- γ secreting cells into lymph nodes and lung by FACS. Financial support: CNPq-CITMA project No. 004/2007-07-20



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P1.11 - Morphological response to respiratory allergen challenge in mice immunized with liposome-encapsulated allergens of *Dermatophagoides siboney*

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Introduction: Mites from the genus *Dermatophagoides* are a major source of allergen in house dust and play an important role in the pathogenesis of allergic asthma. Liposomes are non toxic and biodegradable lipid vesicles, which can be used as adjuvants to induce Th1-skewed immune response. In this work we characterized the morphological response in lung tissue after challenge with allergens of *Dermatophagoides siboney* (Ds) in mice previously immunized with Ds encapsulated in to dipalmitoyl phosphatidylcholine (DPPC) liposomes.

Methods: Four groups of Balb/C mice were intraperitoneally immunized with Ds dissolved in PBS, or encapsulated into DPPC liposomes (CEP, Biology Faculty, University of Havana), or adsorbed onto Alum. Injections containing 5 µg Der s1 each were administered on weeks 0 and 2. Control group was injected with PBS. Ten days after the last immunization, mice were exposed to the allergen challenge using aerosolized Ds extract during 1 hour. The output of the nebulizer was 0.3 mL/min and Der s 1 concentration was 100 µg/mL. 24 hours after the challenge mice were sacrificed; their lungs fixed in 10% formalin and submitted to histological examination. Lungs sections were embedded in paraffin, sectioned and stained with haematoxylin-eosin.

Results: Mice immunized with Ds encapsulated into DPPC liposomes or dissolved in PBS showed normal lung histology similar to control group, in contrast with those receiving Ds adsorbed onto Alum. In this group, extensive peribronchovascular inflammation was found, featuring eosinophilia and mucus secretion.

Conclusion: Encapsulation into liposomes could be considered as a promising alternative for developing novel allergen vaccine formulations counteracting the allergic inflammatory response in the airways.



P2.1 - Characterization and comparison of commercially available mite extracts for in vivo diagnosis and immunotherapy

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Introduction: In the field of allergic diseases, both in vivo diagnosis and specific immunotherapy are primary tools in clinical practice. This study aims at evaluating commercially available mite extracts for in vivo diagnosis and sublingual immunotherapy from eight manufacturers.

Methods: Dermatophagoides pteronyssinus and D. farinae extracts were analyzed for total protein content by Bradford. SDS-PAGE, IgE-immunoblotting and skin prick tests were also carried out.

Results: As regards extracts for diagnosis, the protein amount ranged from 27,7 ug /ml extract to 361,1 ug/ml (D. pteronyssinus) and from 20,3 ug/ml to 353,0 ug/ml (D. farinae). As regards extracts for immunotherapy, total protein amount ranged from not-detectable level to 103,0 ug/ml (D. pteronyssinus) and to 78,4 ug/ml (D. farinae). SDS-PAGE experiments showed that some components are poorly represented or absent in extracts from most manufacturers. Similar results were obtained by IgE-immunoblotting. Ten mite allergic patients were subjected to skin prick test with the extracts for diagnosis and a broad spectrum of reactivity of the extracts in the same subject was confirmed.

Conclusions: Immunochemical analysis showed a heterogeneous amount of component/s among mite extracts from different manufacturers. These data were confirmed by in vivo testing, suggesting that, for some of the tested patients, the absence of relevant allergens could strongly affect the diagnosis. Furthermore, this heterogeneity could also strongly affect immunotherapy, due to lack of correct identification of patient sensitisation profile and to poor quality of the therapeutic preparations.



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P2.2 - Standardization of Tyrosine Adsorbate Depots for use as Subcutaneous Vaccines

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There is a need for accurate particle size and dispersion data to ensure tyrosine adsorbate products can be manufactured reproducibly. Traditional methods of particle characterisation are unsuitable for use with such products due to the partial solubility of each particle. As the solution is diluted to a working concentration to enable accurate solubility or size analysis the particles reduce in size and the solubility data becomes irrelevant. In order to overcome these issues two methods were developed. The first method used Flow Particle Image Analysis to orientate particles with an image sensor to enable repeatable and accurate particle size analysis. As such, particle size is measured as a distribution rather than an average particle size value as this would be inappropriate due to the proportion of varying sizes present. Using known particle size data of different vaccine batches, a spectroscopic method was also developed in which dilutions of the tyrosine adsorbate were made in both a saturated solution and in water. This provides information relating to particle dispersion as well as dissolution. The findings demonstrate that the rate of dissolution was constant irrespective of particle size.



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P2.3 - Searching for markers of biologic/allergenic activity during house dust mite culture process for manufacturing allergen vaccines

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Introduction: Current standardization of allergen vaccines is based mostly on allergenic activity as measured by IgE competition immunoassays, using human sera from allergic patients. Alternative approaches comprising individual allergen content and enzymatic activity have been proposed. The aim of this work was to assess the relationship between the allergenic activity of the House Dust Mite *Dermatophagoides siboney*, enzymatic activity and content of its major allergen Der s1 (a cysteine-protease), during the mite culture process.

Methods: For this purpose, samples of cultures, maintained in a hypoallergenic growth medium, were collected weekly during 10 weeks. Total allergenic activity was measured by IgE inhibition ELISA, protein composition by SDS-PAGE, Der s1 content by mab-ELISA and protease activity by a kinetic test using casein as substrate and by a gelatinolytic zymogram method.

Results: Both, the allergenic potency and Der s1 content achieved peak values after 6 weeks of culture. Allergenic activity was significantly correlated to Der s1 content ($r=0.84$, $p=0.005$) and even to greater extent to enzymatic activity ($r=0.95$, $p<0.0001$). The zymogram showed that the enzymatic activity was focused in the 25kda band corresponding to Der s1, which showed a marked time-dependent increase by SDS-PAGE. The consistency of the Der s1 content was confirmed in 28 consecutive culture batches during a period of 4 years, with a mean geometric value of 3.91 mg/g (Geometric CV: 2.42)

Conclusions: These results support the introduction of Der s1 content and enzymatic activity as surrogate markers of biological potency during mite culture process.



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P2.4 Total protein concentration as an easy and cheap method for allergen extracts Standardization for routine work.

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Introduction: Since its foundation, the Allergy and Clinical Immunology Clinic of Hospital General de México has prepared its own allergen extracts for diagnosis and treatment purposes. Traditionally, extracts were prepared in a weight/volume basis (1:100). Total protein content of pollen extracts determined by Bradford's method varies considerably between pollen species and different lots. Since 2004 in an effort to "standardize" extracts potency, all pollen extracts have the same total protein content.

Methods: Pollen was extracted with Evan's solution in a 1:10 weight/volume ratio. Total protein were determined by Bradford's method and concentration was adjusted. Skin prick tests (SPT) were performed in patients attending our clinic for specialty attention. To assess the impact of this approach, we determined the frequency of positivity for all pollen species monthly for two years, and compared the results with those obtained prior to 2004.

Results: Positivity frequencies showed a better correlation with pollen season, consistent year to year, than those obtained before total protein content standardization, and SPT results were consistent with taxonomical relationship between pollen species.

Conclusions: Total protein concentration is a cheap and easy method for pollen allergen extracts standardization when biologically standardized extracts are not available.



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P2.5 - A method for relative potency determination of new In-House Reference batches of allergen products, by parallel Skin Prick Test

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Introduction: In-House Reference Preparations (IHRP) of allergen products should be biologically standardized, preferably, on the basis of skin reactivity. Biological Units (BU) are defined regarding reactivity to Histamine; nevertheless, for establishing a new IHRP batch, both the old and the new IHRP should be tested in parallel. The aim of this work was to establish a parallel skin test for this purpose.

Methods: Freeze-dried allergen extracts of *Dermatophagoides pteronyssinus* (Dp), *D. siboney* (Ds) and *Blomia tropicalis* (Bt) (BIOCEN, Cuba) were used. SPT was performed using three nominal concentrations for each batch: 200K; 20K and 2K BU/ml; and the wheal diameter (d) and area were measured. A dose-response regression line was built for each batch. The test was regarded valid for $d > 3\text{mm}$ at 20K BU/ml, and if it passed the linearity ($r > 0.90$) and parallelism tests ($p < 0.05$) for both regression lines. Relative Potency (RP) was calculated using the parallel lines statistical method. The first consecutive 10 valid patients were selected for RP calculations for each IHRP, and the final value was calculated as a weighted mean according to the inverse variance method.

Results: The RP results were: for Dp: 0.82 (CI: 0.39-1.72); Ds: 1.29 (0.83-2.02); Bt: 1.29 (0.98-1.68). These values were in good agreement with the absolute potency results as determined using Histamine hcl 10mg/ml, and with the in-vitro RP values as measured by ige inhibition ELISA. The overall precision of the method was a $[\log_{pr}] = 0.116$, better than for the absolute potency test.

Conclusion: this method is suitable for assigning potency values to new IHRP batches.



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P2.6 - Development of in-house references of house dust mite allergen therapeutic vaccines

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Introduction: Standardization of therapeutic allergen vaccines is mainly based on In-House manufacturer's References (IHRs), which are required for routine quality control. Freeze dried IHRs were developed for *Dermatophagoides pteronyssinus* (VALERGEN-DP), *Dermatophagoides siboney* (VALERGEN-DS) and *Blomia tropicalis* (VALERGEN-BT) House Dust Mite allergen vaccines (BIOCEN, Cuba).

Methods: The IHRs were characterized regarding in-vivo (Skin Prick Test) and in vitro allergenic activity (IgE Inhibition ELISA), protein and allergen composition (SDS-PAGE and IgE Western Blotting, respectively). The activity was expressed in Biological Units (BU), which is related to the skin reaction size produced by Histamine 10 mg/ml.

Results: The allergenic potency for *Blomia tropicalis* was 115600 BU (CI95%: 69900-191200); for *Dermatophagoides pteronyssinus*: 81800 BU (CI95%: 64740-103450), and for *Dermatophagoides siboney*: 128700 BU (CI95%: 111920-148080). Replacement of old IHR batches by new ones has been accomplished by both, in-vivo and in-vitro comparative tests, using the parallel line statistical approach. Stability was assessed by means of an accelerated study at 4 temperatures (-70, 4, 37 and 60°C) during one year. The results predicted the stability at -70°C for more than 10 years. At 60°C, despite the collapse of the freeze-dried cake and marked change in appearance, the allergenic activity was kept within the specified limits during the first 6 months. The on-going stability of the first IHR batches was confirmed by tests during 10 years: from 1997 to 2007.

Conclusion: New IHRs were developed and established according to national and international requirements assuring the quality control of these vaccines.



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P2.7 - Validation of an IgE-Inhibition ELISA as a potency assay for allergen vaccines of House Dust Mites

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Introduction: Determination of total allergenic activity as a concept of biological potency is required for standardized allergen vaccines. The IgE-inhibition ELISA, using a sera pool from allergic patients is the method of choice. The aim of this work was to validate an in-house IgE-inhibition ELISA as a quality control assay for allergen vaccines of *Dermatophagoides pteronyssinus*, *D. siboney* (Ds) and *Blomia tropicalis* (Bt) manufactured in BIOCEN.

Methods: This assay determines the ability of an allergen sample to inhibit the IgE binding to a solid-phase bound allergen In-House Reference Preparation (IHRP), and thus, it measures the Relative Potency (RP) using the parallel lines statistical method. Two different serum pools obtained from Cuban allergic patients allergic to Ds/Dp and to Bt were used.

Results: The validation study demonstrated an analytical sensitivity (quantification limit) of 18 BU/ml to Ds and Dp and 133 BU/ml to Bt. Linearity of the log-transformed inhibition curve was satisfactory in the 20-80% range ($r > 0.95$). Intermediate precision, expressed as the 95% confidence interval for one assay, was 0.601-1.663 RP, which is less than the regulatory acceptance interval for allergenic potency: 0.5-2.0. This value was similar to the report of the FDA homologous method. Assay accuracy was validated in the rank of 20-20 000 BU/ml. A revalidation was accomplished after replacing both serum pools, which showed no substantial changes regarding sensitivity and assay range.

Conclusion: This assay showed to be appropriate as a potency analytical method for application to both finished non-modified allergen products and active substances.



P2.8 - ELISA for the Group 1 Major Allergen determination in House Dust Mite vaccines, as a quality control assay: use of polyclonal vs monoclonal antibodies.

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Introduction: The Group 1 allergen from Dermatophagoides House Dust Mite species can be used as a reliable marker of total allergen activity in allergenic extracts. Commercially available assays for Der p 1 and Der f 1 are based on monoclonal antibodies (MAb). The aim of this work was to compare performance parameters of MAb-based sandwich ELISA, with an in-house method based on polyclonal antibodies.

Methods: For Der p1 a commercial MAb-ELISA was used (INDOOR, UK); for Der s1 (Dermatophagoides siboney), a MAb-based ELISA developed by BIOCEN, Cuba, was used. Polyclonal antibodies (PAb) were raised in rabbits against MAb-affinity purified Der s1, which were able to recognize both Der s1 and Der p1 in the respective allergen extracts. An indirect ELISA assay was developed using these PAb, for quantification of Der s1 or Der p1 using specific standard curves for each allergen.

Results: The working range of the PAb ELISA was 2-60ng/mL for Der s1, and 5-80ng/mL for Der p1, not very different from MAb ELISAs. In contrast, validation studies showed that PAb-ELISA has better performance parameters regarding inter-assay variability (CV=8.8%; assay precision: $CI_{95}\% = \pm 17\%$) as compared to MAb-ELISAs for Der s1 (CV=16.3%, $CI_{95}\% = \pm 31\%$) or Der p1 (CV=15.6% $CI_{95}\% = \pm 31\%$). Both assay types showed the same accuracy and specificity as regards possible interference from process contaminants.

Conclusion: Therefore, the PAb based ELISA, in spite of losing the ability to distinguish between species, showed its overall advantage concerning robustness, precision and simplicity; features that are very relevant as a quality control assay.



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P2.9 - Development of a semi-quantitative IgE Western-Blotting method for allergen composition of House Dust Mite vaccines

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Introduction: An assay of allergenic composition or profile is required by the European Pharmacopeia and European guidelines for standardized allergen vaccines. IgE Western Blotting (WB) is currently the preferred method. The goal of this work was to establish a WB method, evaluating the use of semi-quantitative variables for quality control of allergen extracts of *Dermatophagoides pteronyssinus* (Dp), *D. siboney* (Ds) and *Blomia tropicalis* (Bt).

Methods: Immunoblotting was performed after SDS-PAGE, followed by transfer to a nitrocellulose membrane, incubation with sera pool from allergic patients, Peroxidase-labelled anti-IgE MAb, addition of a chemiluminiscent (ECL, AP Biotech) substrate and exposure to a Polaroid film. Finally, this film was subjected to scanner-densitometric analysis. Peak intensity (height) at 15 and 25 kDa (for Dp/Ds) and at 17 kDa (for Bt) were selected as quality parameters since showed less variability than peak weight (area under the curve). Peak intensity was measured relative to an IHRP.

Results: The method showed linearity in the range of 400-10 000 BU (for Dp/Ds) or 2000-50 000 BU for Bt. The limit of detection was below 1000 BU for Dp/Ds and close to 5000 BU for Bt. These parameters showed a satisfactory precision (CV=12.3%), which, when expressed in terms of 95% confidence interval for one assay, was estimated to be ± 0.240 . This error value is less than the tolerance range for assays for individual allergens (0.5-2.0) and even less than the IgE-inhibition ELISA error.

Conclusion: Thus, this analytical method was validated to be used as a quality control assay for allergen vaccines in BIOCEN.



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P2.10 - Amount of major allergen Phl p 5 in different European products for Sublingual Immunotherapy

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Rationale: Allergic rhinitis induced by grass pollen can be treated by means of allergen-specific immunotherapy. The sublingual administration route has garnered considerable attention this past decade, and a number of products are currently commercially available for routine sublingual immunotherapy (SLIT). It has been acknowledged that the dose for SLIT should be higher than for subcutaneous immunotherapy and that the dose is decisive for clinical efficacy. We investigated the content of major allergen Phl p5 in different SLIT products.

Methods: SLIT products from main European allergy companies were purchased and the concentration of Phl p 5 was determined using an ELISA method. The analyses were performed by Dr I. Sander, BGFA - Forschungsinstitut für Arbeitsmedizin der Deutschen Gesetzlichen Unfallversicherung Institut der Ruhr Universität Bochum, Bochum, Germany. Products based on drops were diluted directly and tablet products were first solubilised, and then diluted. Phl p 5 was calculated in ug (microgram) per maintenance dose, using the instructions for administration as provided by the manufacturers.

Results: The analysis revealed the following results: SLIT-One-plus (ALK-Abello, 2500 STU/ml): 1.7 ug Phl p 5; Grazax (ALK-Abello, Tablet 75.000 SQ-T): 5.0 ug Phl p 5; SUBLIVAC (HAL Allergy, 10.000 AUN/ml): 6.2 ug Phl p 5; TOL (Leti, vial C, 100 HEPL/ml): 0.4 ug Phl p 5; Oralair (Stallergenes Tablet 300 IR) 5.2. The Phl p 5 amounts of Grazax, SUBLIVAC and Oralair are in the same range and represent a high dose of major allergen. Clinical data show that products dosed in such high concentrations give good clinical efficacy. SLIT-One-plus and TOL are considerably lower than Grazax, SUBLIVAC and Oralair. Preliminary data on potency measurement, also taking into account the contribution of grass allergens other than Phl p5 indicate a similar deviation between the different products.

Conclusion: We conclude that Grazax, SUBLIVAC and Oralair are in the same range with regard to strength, while SLIT-One-plus and TOL are considerably lower.



P3.1 - Characterization of allergens homologous to Art v 1 in the pollen of different artemisia species

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Introduction: Among the large genus of *Artemisia*, the herb mugwort (*Artemisia vulgaris*) has been extensively characterized as one of the main causes of late summer pollinosis. Its major allergen Art v 1, a modular glycoprotein homologous to plant defensins, is recognized by more than 95% of mugwort-sensitized patients. Apart from mugwort, information on allergens from plants belonging to the genus *Artemisia* is still missing. Therefore, we screened for Art v 1 homologous proteins in leaves and pollen from different *Artemisia* species.

Methods: Aqueous pollen and/or leave extracts of *A. absinthium*, *A. annua*, *A. californica*, *A. dracunculoides*, *A. frigida*, *A. ludoviciana*, *A. tridentata*, and *A. vulgaris* were analyzed by gel electrophoresis, carbohydrate staining, immunoblotting, as well as ELISA and microarray inhibition experiments. Art v 1 homologues were purified from the respective pollen extracts and subjected to sequence analysis by Edman degradation and mass spectrometry. Finally, cDNAs of the Art v 1 homologues were cloned.

Results: IgE-reactive proteins homologous to Art v 1 could be identified in pollen but not in the leaves of all *Artemisia* species investigated. Primary structure evaluation revealed more than 90% of sequence similarity to Art v 1.

Conclusion: The Art v 1 family of pollen allergens is highly conserved in the genus *Artemisia*. Worldwide geographical distribution and commercial cultivation of *Artemisia* species contribute to the importance of this allergen family. Due to the high degree of homology, a single molecule might be sufficient for both diagnosis and therapy of *Artemisia* pollen allergy.



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P3.2 - Protease and allergenic activity of allergen extracts of *Blomia tropicalis*, *Dermatophagoides pteronyssinus* and *Dermatophagoides siboney*

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Introduction: Molecular characterization of several mite allergens has elucidated their enzymatic proteolytic properties, which can be linked to allergenicity. On the other hand, the presence of active proteases in biopharmaceutical products could lead to protein degradation affecting product stability. The objective of this work was to study the gelatinolytic protease profile of allergen extracts of *Dermatophagoides siboney* (Ds), *D. Pteronyssinus* (Dp) and *Blomia tropicalis* (Bt) and its relation to IgE binding-activity.

Methods: Zymogram using gelatin as substrate and SDS-PAGE followed by IgE Western Blotting (WB).

Results: The gelatinolytic profile of Bt consisted of 8 separate bands. Only the 30 kd band was resistant to incubation in denaturing conditions at 100°C, during 5 min. In the Dp extract, 4 gelatinolytic bands were detected, 2 of them thermo-resistant, including the most intense, which corresponds to the major allergen Der p 1. In spite of the taxonomic and allergenic similarity between Dp and Ds, in the last specie, it was detected up to 6 enzymatically active bands, whereas only Der s 1 was thermoresistant. Oxidation of Ds extracts using metal ions (Zn⁺, Cu²⁺) showed inhibition of some bands. The overall comparison of zymograms with the allergenic profiles, as measured by WB, suggests that most allergenic components have enzymatic activity in the case of Bt, in contrast to Dp or Ds. The incubation at high temperatures removed most of the enzymatic bands, not affecting IgE-binding bands, i.e. allergenic activity.

Conclusions: These results suggest that inactivation at high temperature could be used for the development of allergen vaccines with reduced enzymatic activity.



P3.3 - Cross-reactivity between the recombinant allergens Blo t 13 and Der f 13

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Introduction: Group 13 of mite allergens belong to the fatty acid binding protein family, which has conserved molecular structure and biological function. In the last years several allergens of this group have been cloned and several isoforms, which may display different IgE reactivity, have been identified. The allergenic characteristics of these molecules are not totally known.

Methods: Sera from mite allergic patients of Cartagena, Colombia were selected to perform ELISA and cross inhibition assays with the recombinant allergens from *B. tropicalis* (Blo t 13) and *D. farinae* (Der f 13), isoforms DF414 and DF1096. Molecular modeling and Bioinformatics approach was used to predict shared epitopes.

Results: The prevalence of IgE reactivity was 15.7 % (14/89) for Blo t 13 and 13.4 % (12/89) for DF414; the serum IgE levels to both allergens correlated very well. The maximum inhibition of IgE binding to Blo t 13 in solid phase by DF414 was 44.6%; when DF414 was in solid phase the maximum inhibition produced by Blo t 13 was 79.4%. Using ELISA the monoclonal antibodies anti-Blo t 13 (5G3) showed reactivity to DF414 and DF1096. Molecular modeling predict six antigenic patches common to Blo t 13 and DF414 which might be involved in the moderate cross-reactivity between them.

Conclusion: Sensitization to Der f 13 in the Colombian allergic patients seems to be induced mainly by exposure to *B. tropicalis*.



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P3.4 - Aminoacid and cDNA sequence of the serine protease allergen (Der s3) from Dermatophagoides siboney

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Introduction: Dermatophagoides siboney has been described as a major allergenic source in Cuba. Major allergens Der s1-3 have been isolated from allergen extracts. The goal of this work was to clone and sequence the cDNA corresponding to the 30kDa trypsin-like protease allergen Der s3.

Methods: Primers were designed according to conserved nucleotidic sequences from the homologous Group 3 Dermatophagoides allergens, to amplify the cDNA sequence corresponding to the mature protein. Gene-specific primers were further designed to be used in 5'RACE reactions in order to obtain the full cDNA, including the signal sequence and the propeptide region. Der s3 native protein was purified from D. siboney mite extract using affinity chromatography with immobilized benzamidine, a serine-protease inhibitor.

Results: The full length cDNA sequence comprised 854 nucleotides, showing 98.1% identity to previously published Der f3 cDNA. Four single nucleotide substitutions led to amino acid changes as compared to Der f3: two of them were located in the mature protein and the rest in the proenzyme region. Single nucleotide sequence variation was found in 18 positions, indicating possible polymorphisms, not previously reported for Der f3 or Der p3. The homology analysis between the inferred aminoacid sequence and the previously published N-terminal protein sequence showed significant differences, indicating that the native protein previously known as Der s3, should be indeed renamed as Der s6. In order to confirm this finding, the affinity purified native Der s3 protein was subjected to Mass-Spectrometry analysis, confirming the results from cDNA sequencing.

Conclusion: Overall, this work confirmed the high sequence homology between serine protease allergens of different Dermatophagoides species explaining their extensive cross-reactivity.



P3.5 - Molecular cloning and characterisation of a new wheat food allergen

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Introduction: Wheat (*Triticum aestivum*) and wheat products are a major element in nutrition but it can be responsible for IgE-mediated food allergy. Avoidance of wheat products is currently the only therapy for wheat allergic patients whereas allergen-specific approaches such as immunotherapy would require a detailed knowledge and availability of the culprit allergens. Aim of this study was the identification, isolation and characterisation of new allergens recognized by patients suffering from wheat-induced food allergy.

Methods: A *Triticum aestivum* expression cDNA library was constructed in lambda gt11 and screened with serum IgE antibodies from wheat food allergic patients. cDNA clones coding for allergens were analyzed regarding DNA sequence and IgE reactivity. The cDNA coding for a novel wheat food allergen, a-purothionin, was identified by sequence analysis, cloned into pET17b vector and expressed in *Escherichia coli* as C-terminal His₆-tagged protein, purified and characterized regarding molecular, structural and immunological properties.

Results: We identified a-purothionin as a novel wheat food allergen and purified the recombinant allergen. It is a small, cysteine-rich protein in the endosperm of wheat seeds and because of their toxicity for bacteria, yeast, fungi and animals may represent a defense protein. The structural gene includes regions encoding a typical signal peptide, a thionin domain (5 kDa) and a C-terminal acidic extension. The C-terminal acidic extension domain was identified as IgE epitope-containing portion.

Conclusion: Recombinant a-purothionin may be useful for the diagnosis and possibly immunotherapy of IgE-mediated wheat food allergy.

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P3.6 - In-vivo allergenic activity of major allergens of *Dermatophagoides siboney* by Skin Prick Test

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Introduction: Major allergens of the endemic House Dust Mite *Dermatophagoides siboney*, Der s1 and Der s2 have been identified before based on their in-vitro IgE-binding activity. The objective of this work was to determine allergenic activity in-vivo of the isolated Der s1 and a fraction of the allergen extract containing both Der s1 and Der s2, as compared to the whole extract.

Methods: Der s1 was purified using Mab-affinity chromatography, rendering purity higher than 95%. The fraction containing Der s1 and Der s2 (s1/s2), was obtained by salting out and gel-filtration, using Superdex-200. The 25 and 15kD components represented 90% of the total protein content. The allergenic activity was tested in-vitro by IgE Inhibition ELISA using a serum pool of allergic patients, and in-vivo, in 34 allergic patients by Skin Prick Test The reaction size was recorded, and used to calculate the activity in Biological Units (BU) using Histamine HCl as standard.

Results: Up to 70 and 95% of the total IgE binding activity of the extract was inhibited in-vitro by Der s1 and s1/s2, respectively; 96% of patients showed positive tests to s1/s2 and only 58% to Der s1, whereas 94% were positive to the Der s1-depleted extract. The in-vivo activity of s1/s2 was close to the whole extract (90%, N.S.), whereas the activity of Der s 1 was approximately the half ($p < 0.05$).

Conclusion: The fraction containing both allergens Der s1 and Der s2 seems to be a good candidate for substituting the whole extract for developing a vaccine with more defined composition. The classification of Der s1 as a major allergen was confirmed in-vivo.



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P3.7 - IgE responses to Blo t 5 and Blo t 12 allergens in mite allergic patients from Martinique, France

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Background: In the Island of Martinique, 95% of mite allergic patients are sensitized to this domestic mite species. However, there is no data about the specificity of the IgE response. The evaluation of the allergenic potential of purified allergens is essential for clinical purposes. We sought to identify if Blot 12 and Blot 5, which are relevant allergens in other populations, are also important in this area.

Methods: Blot5 cDNA was cloned from a *Blomia tropicalis* cDNA library and expressed as a His-tag-fusion protein in *E. Coli*. Blot12.0101 was produced in *Pichia pastoris*. Since Blot 12 is hyperglycosylated and this condition negatively influences its IgE-binding capacity, we deglycosylated it with TMSF. IgE reactivity against both allergens was determined by ELISA in 69 *B. tropicalis* allergic patients from Martinique who had allergic rhinitis and/or asthma.

Results: The obtained Blot 5 cDNA had a 100% of identity with the published sequence. IgE reactivity against Blot 5 and Blot 12 were 79% and 25%, respectively. Mean sIgE levels to Blot 5 (1.24+0.61) were higher than to Blot 12 (0,64+0,49). sIgE levels against Blo t 5, but not Blo t 12 were significantly correlated with IgE levels to *B. tropicalis* ($r=0,66$ $p=0,00$ and $r=0,2$ $p=0.1$, respectively).

Conclusion: IgE sensitization frequencies against these allergens in Martinique are similar to others reported in tropical regions, supporting that Blo t 5 is an epidemiologically important allergen and that response to Blo t 12 is more moderate. These data indicate the importance of including them as reagents for diagnosis/immunotherapy in this tropical island.



P4.1 - Effects of protease inhibitors in the preparation of allergen extracts of *Dermatophagoides pteronyssinus*.

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Introduction: Protease inhibitors are frequently used for their potential inhibition capacity of the protease activity in mite extracts. However, there is little information on the effect of protease inhibitors on the allergenicity and enzymatic activity of mite allergen extracts. The objective of this study was to compare the influence of a commonly used protease inhibitors cocktail (PIC) in the production of *Dermatophagoides pteronyssinus* extracts.

Methods: Extracts were manufactured from 4 different batches of raw materials of *D. pteronyssinus*. The preparation of the extracts was performed with or without the addition of the protease inhibitor cocktail (Sigma, P2714). Briefly, mites were incubated in PBS at 1:20 w/v ratio including, or not, 100 ml of 1x PIC per 20 g mites. Specific IgE-levels were tested by direct ELISA for batch control and quantification of the IHRP. The allergenic potency was evaluated by ELISA inhibition experiments using extracts prepared in the presence, or not, of PIC and western blot analysis. The major allergen content (Der f 1 and Der f 2) was measured with monoclonal antibodies. The enzymatic activity was detected by a zymogram.

Results: Only one of four extracts prepared in presence of protease inhibitors exhibit a better IgE recognition than the extract prepared without it ($p = 0.042$ (Wilcoxon)); in the rest of the extracts, the difference was not significant. In the extracts prepared with PIC, Der p 1 (7.0 ± 5.6 ug/ml) and Der p 2 (3.0 ± 3.4 ug/ml) levels were not significantly different than in those prepared without PIC; Der p 1 (6.8 ± 5.9 ug/ml) and Der p 2 (2.6 ± 2.9 ug/ml). There were no significant differences in the evaluation of the allergenic potency between the extracts prepared with (0.05 ± 0.02 ug in test) and without PIC (0.13 ± 0.12 ug in test). Extracts containing protease inhibitors show in general a lower protease activity according to the intensity of the digested bands in the zymogram. Immunoblots analysis with scanning densitometers showed no significant difference between with PIC (74.29 ± 13.65 kpixel/lane) and without PIC (64.43 ± 10.02 kpixel/lane).

Conclusions: Our experiments show that the use of protease inhibitors in the preparation of mite extracts does not increase the potency of these extracts. In general, mites prepared in the presence of PIC presented better defined bands in immunoblots.



P4.2 - Concentration of Dermatophagoides farinae extracts by tangential flow filtration in different molecular weight fractions for diagnosis and treatment of mite allergie

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Introduction: There is little information on the effect of major allergen after tangential flow filtration and concentration in different molecular weight size ranges. The objective of the study was to compare the allergenicity and major allergen content of concentrated versus nonconcentrated allergen extracts of *D. farinae*.

Materials and Methods: We prepared 4 different batches of raw material of *D. farinae* from semipurified whole cultures. Five fractions were prepared from each batch of raw material. The extracts were processed to obtain the following 5 fractions: a) extracts dialyzed in membranes with 3 kDa; b) extracts dialyzed in 5 kDa membranes; c) extracts which contained allergens between 3 and 50 kDa; d) extracts between 5 and 50 kDa and e) extracts with molecular weights > 50 kDa. We used the flat bed dialysis system (PALL system, CENTRAMATTM) to dialyze and concentrate all these extracts. The process was stopped when the conductivity reached 375 ppm, or less. The time needed and the pressures used in the dialyses were monitored. The allergenic potency was evaluated by ELISA inhibition experiment against an In House Reference Preparation, and western blot analysis. Der f 1 and Der f 2 allergen levels were measured with monoclonal antibodies.

Results: Der f 1 levels (mean \pm SD) in the c) and d) extracts (17.6 ± 4.2 ug/mg) (20.8 ± 1.2 ug/mg), respectively, were significantly higher than in extracts a) (9.3 ± 2.2 ug/mg) and e) (5.4 ± 1.2 ug/mg) but levels of extract b) was significantly higher to d) but not to c) (13.6 ± 3.8 ug/mg) ($p = 0.006$) ($p > 0.05$). There were no significant differences in Der f 2 levels (mean \pm SD) in extract a) (2.0 ± 0.5 ug/mg); in b) (1.8 ± 0.8 ug/mg); in c) (2.3 ± 0.5 ug/mg); and in d) (2.2 ± 0.7 ug/mg); except in e, which is significantly lower (0.3 ± 0.06 ug/mg) ($p < 0.05$). In 50% inhibition values were not significantly different in extracts a, b, c and d (a: 0.43 ± 0.04 ug/ml); (b: 0.58 ± 0.17 |jg/ml); (c: 0.47 ± 0.09 |jg/ml); and (d: 0.41 ± 0.09 ug/ml); except in e, which was significantly higher (0.92 ± 0.19 ug/ml) ($p < 0.05$). SDS-Page and Western blot confirmed these results.

Conclusion: In this study we present a novel way to concentrate allergenic compounds with tangential flow filtration. The data confirms that Der p 1 concentrations are greater between 5 and 50 kDa, and that allergen extracts greater than 50 kDa contain significantly less allergenic activity.



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P4.3 - Effect of the dialysis pore size on allergen content and allergenicity of *Dermatophagoides farinae* extracts

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Introduction: There is little information on the effect of the dialysis pore size on the concentration of major allergens of mite extracts. The objective of the study was to evaluate the influence of 3 and 5 kDa tangential flow filtration membranes (TFFM) on allergen content and allergenicity of *Dermatophagoides farinae* extracts.

Methods: Semipurified *D. farinae* cultures were extracted in PBS overnight. Five different charges were processed and further analyzed. Once extracted and filtrated, the extracts were placed in a TFFM system (PALL, CENTRAMATETM). The extracts were divided into two groups: 5 extracts were dialyzed against purified water through 3 kDa, and 5 through 5 kDa membranes. Dialysis was stopped when the conductivity was below 400 ppm. The dialysis waters of these 10 extracts were also lyophilized and saved for further analyses. The time and pressure needed for dialyses were also monitored. The allergenic potency was evaluated by ELISA inhibition and western blot analysis using a serum pool of highly allergic individuals. Der f 1 and Der f 2 content was measured with monoclonal antibodies.

Results: The mean \pm SD time to dialyze the extracts with the 3 kDa membrane was 145 ± 6 minutes and with the 5 kDa membrane, 84 ± 10 minutes ($p = 0.0005$). In the extracts dialyzed through 5 kDa extracts there was a higher content of Der f 1 (mean 10.0 ± 4.3 ug/mg) than in those dialyzed through 3 kDa membranes (mean 7.8 ± 3.2 ug/mg); ($p = 0.04$). There were no significant differences in Group 2 allergen levels between the extracts dialyzed through 5 kDa membranes (mean 1.5 ± 0.5 ug/mg) and through 3 kDa membranes (mean 1.8 ± 0.8 ug/mg); ($p = 0.06$). Der f 2 was detected in the fraction below 5 kDa (0.08 ± 0.05 ug/mg) and in the fraction below 3 kDa (0.02 ± 0.02 ug/ml) ($p = 0.008$). In general, SDS-PAGE and immunoblots revealed a similar protein/allergen profile in both types of extracts. No significant differences were obtained for the 50% inhibition values; 0.50 ± 0.36 ug/ml in extracts dialysed with 3 kDa membranes and 0.23 ± 0.06 ug/ml in 5 kDa membranes ($p = 0.08$). The < 3 and < 5 kDa allergen extracts produced significantly higher 50% inhibition values (178.0 ± 52.8 ug/ml) (16.9 ± 7.3 ug/ml).



P4.4 Validation of diafiltration processes of House Dust Mite allergen vaccines

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Introduction: Process validation is a requirement of pharmaceutical industry. Validation studies are designed to demonstrate the expected results of each process. Ultrafiltration is used in the manufacturing process of allergen extracts as a purification step after a prior clarification, intending to remove low molecular weight irrelevant components. The aim of this work was to validate the diafiltration process, using Hollow Fibber cartridges with a cut-off of 10 kDa, for allergen extracts obtained from the whole culture of *Dermatophagoides pteronyssinus*, *D. Siboney* and *Blomia tropicalis*.

Methods: The following studies were performed: Operational Qualification of the DC-10 Amicon equipment; Validation of the Cleaning In Place (CIP) cycle, regarding the removal of cleaning agents, and Performance Qualification.

Results: Washing with Sodium Hydroxide 0.2 mol/L and Formaldehyde 0.5% followed by 3 rinsing cycles with purified water, was able to remove completely the remains of cleaning agents and allergen products, as measured by Total Organic Carbon test (<500 ppb), pH and conductivity, maintaining the microbiological counts within the specification limits. Cartridge performance and integrity assessment demonstrated to retain more than 99% of Human Albumin 1% solution, after a full diafiltration-concentration cycle. The performance qualification using four batches of allergen products showed the expected values regarding recovery of Group 1 Major allergens and other allergenic proteins, and the corresponding shift in conductivity and absorbance.

Conclusion: This validation work demonstrated the proper performance of this important step in the context of the manufacturing process, contributing to guarantee the quality of the final product.



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P4.5 - Endotoxin content in industrially manufactured allergen extracts of House Dust Mites, including *Dermatophagoides siboney* and *Blomia tropicalis*

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Introduction: Endotoxin is an ubiquitous inflammatory agent derived from Gram-negative bacteria, which poses a safety concern for injection drug products and, at the same time, is a potent immunomodulator. Endotoxin can be present in variable amounts in allergen vaccines from House Dust mites, although its content is not subjected yet to regulatory limits. The aim of this work was to assess the endotoxin content in industrially manufactured standardized allergen extracts of House Dust Mites, including *Dermatophagoides pteronyssinus* (*Dp*) and the tropical species *D. siboney* (*Ds*) and *Blomia tropicalis* (*Bt*).

Methods: Three batches of each product in lyophilized form were tested (100 000 BU, VALERGEN, BIOGEN, Cuba) at a strength of 20 000 BU/ml, as indicated for Prick Test, using the Limulus Amebocyte Lysate clot assay (LAL).

Results: The LAL results were much higher for *Ds* (up to 1093 EU/ml) as compared to *Dp* or *Bt* (<100 EU/ml). Pyrogen test in rabbits confirmed a mild pyrogen effect *in-vivo* for *Ds* with a mean increase of $1.4^{\circ}\text{C} \pm 0.4$ body temperature. These results agree with previous reports indicating more intrinsic endotoxin content in *D. farinae* (close to *Ds*) than in *Dp*. The values were well below the endotoxin content of allergenic extracts from collected house dust (mean value: 3722 EU/ml ± 1134). In spite of the protease activity of these extracts, no significant inhibition of the LAL activity was detected at the adjusted dilution.

Conclusion: These results can be relevant to the problem of establishing limits to endotoxin content in allergen vaccines.



P4.6 - Assessment of consistency of the manufacturing process of House Dust Mite allergen vaccines

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Introduction: Consistency of quality parameters of the final and intermediate products is a requirement of the pharmaceutical processes, and it is of special concern for biological complex products, such as allergen extracts. The objective of this work was to evaluate the consistency of consecutive batches of industrially manufactured allergen extracts of *Dermatophagoides pteronyssinus*, *D. siboney* and *Blomia tropicalis*.

Methods: The following specific tests were used: allergenic potency, as measured by IgE-inhibition ELISA, allergen profile by Western Blotting, SDS-PAGE. Major allergen (Der p1 and Der s1, as measured by ELISA) and total protein content were monitored during the whole manufacturing process. A total of 32 batches were monitored over a period of 6 years and process capability index was calculated.

Results: No batches were found out of the specification or $\pm 3\sigma$ limits. The maximum failure probability was estimated to be 6% for the allergenic potency. A similar value was observed for allergen composition parameters (intensity of the bands corresponding to major allergens). Lower failure probabilities were found for protein composition (0.2%) and Der p1/Der s1 allergen content (3%), both at the finished product or the active substance stage. These values are regarded as satisfactory considering the high variability of these analytical methods (which is estimated to be much greater than the intrinsic process variability) and are found to be similar to the rejection rate of standardized allergen extracts in USA.

Conclusion: It was demonstrated the consistency of the main quality parameters of standardized allergen vaccines, which is very relevant to clinical safety and efficacy.



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P4.7 - Evaluation of bacteriostatic and fungistatic properties of different formulations of mite allergen vaccines

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Introduction: The evaluation of inhibitory activity to microbial growth is a prerequisite for developing a validated sterility testing for biopharmaceuticals intended for injection, including allergen vaccines. The aim of this work was to assess the intrinsic bacteriostatic and fungistatic properties of standardized allergen extracts of *Blomia tropicalis* and *Dermatophagoides siboney* house dust mites, formulated with different preservative agents (phenol or thiomersal) in aqueous form or adsorbed into aluminium hydroxide.

Methods: Specifically, it was evaluated the inhibitory effect to the growth of *Pseudomonas aeruginosa* ATCC 9027, *Bacillus subtilis* ATCC 6633, *Candida albicans* ATCC 10231 y *Aspergillus niger* ATCC 16104, representing different microbial groups, using the USP membrane filtration method.

Results: The results indicated that the vaccine depot formulation containing aluminum hydroxide and thiomersal exerts the greatest inhibitory effect to *C. Albicans* y *A. Niger*. No inhibitory intrinsic effect was detected for the allergen extracts alone. Proper neutralization methods were established for each vaccine formulation, consisting of repeated rinsing of membranes with 100 ml aliquots of 0.1% meat peptone saline solution or adding sodium thioglycolate 0.5 g/L, which proved to counteract this inhibitory effect.

Conclusion: These results assure the validity of the sterility test by membrane filtration, for House Dust Mite allergen vaccines.



P4.8 - Factors influencing the adsorption of Dermatophagoides siboney allergen extract into aluminum adjuvants

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Introduction: Allergen-specific immunotherapy consists of periodic administration of allergen vaccines, particularly from House Dust Mites (HDM), for desensitization and amelioration of allergic symptoms. The mite Dermatophagoides siboney has been commonly found in house dust in the Caribbean and it is associated to allergic asthma. In order to obtain a depot HDM vaccine containing aluminum adjuvant, a lyophilized allergen extract of D. siboney was adsorbed into aluminum hydroxide (AH) and aluminum phosphate (AP) gels, aiming to establish the parameters that determine the highest adsorption capacity.

Methods: Allergen adsorption was measured by Lowry total protein assays and by Der s 1 allergen-specific MAb-ELISA. Immunogenicity was assessed in Balb/C mice using two doses of 5 ug Der s 1, by subcutaneous route.

Results: AH showed better adsorption capacity when compared to AP. The best adsorption conditions using AH were: 0.9 % NaCl at pH 8 in 30 min. Sodium phosphate buffered solution showed a negative effect on the allergen adsorption into AH, both, when used during the mixing process or added later. The within-batch consistency of the adsorption process in absence of phosphate buffer was demonstrated, as well as the immunogenicity of this formulation, regarding induction of allergen-specific IgG antibodies in mice.

Conclusion: The immunogenicity and quality consistency of this optimized formulation can be relevant for the development of improved adsorbed allergen vaccines.



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P4.9 - Preliminary characterization of the microbial bioburden during the culture of *Blomia tropicalis* and *Dermatophagoides siboney*

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Introduction: House Dust Mites coexist in their natural habitat with different microorganisms, possibly with symbiotic interactions. From the pharmaceutical point of view, GMP require the establishment of limits for microbial contamination in Raw Materials and intermediate drug products, including the Allergenic Raw Material to be used for manufacturing allergen vaccines. The objective of this work was to perform a preliminary evaluation of the microbial burden during the culture process of the tropical mites *Blomia tropicalis* and *Dermatophagoides siboney*.

Methods: Samples of different culture flasks were collected and subjected to microbiological analysis. Total count of Colony Forming Units (CFU) was accomplished by spread plate method. For detection of enterococcus and Gram-negative bacteria, selective chromogenic media were used.

Results: The CFU count peaked in the first two-three weeks, achieving 10^2 - 10^3 CFU/g, with a later decrease, correlated with mite density growth and media consumption. The most frequent bacteria were *Bacillus* sp and *Staphylococcus* sp. The most frequent fungi were *Penicillium*, *Aspergillus*, *Cladosporium*, *Trichoderma* and *Mucor*. Overall, *Blomia* cultures showed higher counts than *D. siboney*. No clear relationship was found between microbial levels and concomitant mite culture development. The source of these contaminant microorganisms was tracked to mite inoculums.

Conclusion: These data could be useful for the establishment of permissible microbial limits for the mite culture process.



P4.10 - Process Development of Therapeutic Vaccines based on Recombinant Glycoproteins produced with Animal Cell Technology

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During development of novel biotechnological products, critical quality attributes and process parameters should be established. In this way the use recombinant DNA technology to express these molecules, allows not only the improvement of process yields and economy, but also regulatory acceptance of such products. Moreover expression of recombinant glycoproteins in mammalian cells is being increasingly used for complex biomolecules, when expression in bacteria and yeast do not provide functional products. These expression systems could be also advantageously applied to the development of certain allergen vaccines.

The process development approach should address in the first place the up-stream stage, stressing the cell line selection, screening and optimization of tailored protein-free media and supplements for specific cell lines, selection of the fermentation mode, improving protein expression and monitoring folding problems. Proteomic tools can be helpful in these studies. Also, issues of down-stream process development, as selection and optimization of the isolation step (ex. affinity purification) and other chromatographic polishing steps should be addressed, taking into account regulatory requirements. Finally, the structural and functional characterization of final and intermediate products is also an essential goal, relevant to define the required quality parameters as purity, identity and biological activity. In this work we illustrate this development approach applied to therapeutic vaccines for cancer treatment based on two different glycoproteins: HER-1 (extracellular domain of EGF receptor) and 1E10 (murine anti-idiotypic antibody that mimics N-glycolyl-GM3 gangliosides) expressed in human (HEK-293) and murine hybridoma cell lines, respectively. Both products are now being used in different clinical protocols in Cuba and in other countries.



P4.11 - Development and validation of a Western Blot assay for identifying antigens in the Outer Membrane Protein Complex of *Neisseria meningitidis*

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Introduction: The Outer Membrane Protein Complex (OMPC) from *Neisseria meningitidis* B has been used as a pro Th1 adjuvant in experimental anticancer and allergen vaccines. Identity tests are usually required for bio-pharmaceuticals to be carried out mainly in final products. Particularly, for vaccines, identity tests may be based on the antigen-antibody interaction. The aim of this work was to develop and validate a Western Blot assay, based on monoclonal antibodies, as an identity test for OMPC antigens, both in intermediate products and in alum-adsorbed vaccines.

Methods: It was evaluated the feasibility of implementing this technique using monoclonal antibodies for the identification of antigenic proteins P1.15 and P1.4, in OMPC and finished adsorbed product, respectively. Anti mouse IgG conjugated to Peroxidase was used as the secondary antibody.

Results: The validation parameters: Specificity, Detection Limit, Repeatability, Intermediate Precision, Reproducibility and Robustness fulfilled the defined acceptance criteria.

Conclusions: Western blot can be reliable used as an identity test for OMPC antigens in alum-adsorbed vaccine formulations.



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P4.12 - *Development of a lyophilization cycle and a procedure for drying rubber stoppers to increase the shelf life of hygroscopic allergen formulations*

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Introduction: The stability of a lyophilized product during storage can be compromised by a high level of residual moisture. Thus, the control of residual moisture requires special attention, particularly on the stage of secondary drying of the lyophilization process to ensure keeping residual humidity of the lyophilized product at the desired level. Rubber bromobutyl stoppers are commonly used for lyophilized products, which help to keep a low humidity during storage, acting as a barrier to the water vapor transference. However, rubber stoppers itself have a certain content of water that can be transferred to the lyophilized product, then, a low product humidity value at the end of lyophilization process does not ensure maintaining an acceptable level during storage. In this work it was developed a lyophilization cycle for hygroscopic allergenic formulations of House Dust mites *Dermatophagoides pteronyssinus*, *Dermatophagoides siboney* and *Blomia tropicalis* (VALERGEN, BIOCEN, Cuba).

Methods: Lyophilization process was performed in an industrial-scale lyophilizer (USIFROID SMH-100) under GMP aseptic conditions. Residual moisture was measured by the Karl Fisher method according to US Pharmacopeia.

Results: An increase of the temperature of the secondary drying stage at the lyophilization process, to 35°C was able to decrease the humidity content of the final product to a desired level (below 5 % according to European Pharmacopeia monograph) without increasing unnecessarily the secondary drying time, and not affecting the product's biological activity. A thermal treatment procedure of rubber stoppers in oven at a temperature of 105°C after the sterilization cycle in autoclave and before the lyophilization process, was implemented, demonstrating that 2 hours of drying at these conditions were sufficient to remove the amount of water incorporated during the sterilization cycle, and even to decrease the humidity to a value below the initial one, prior to autoclaving. In this way we avoid the transference of water from de stopper to the product and ensure longer shelf-life stability, which have being demonstrated by on-going stability studies.



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P5.1 - Challenges in design of an appropriate genotoxicity package for allergen products

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The registration process for new chemical entities intended for pharmaceutical and diagnostic use includes the requirement for an assessment of genotoxic potential, specifically ICH S2A and S2B (1995, 1997). For new biological entities, genotoxicity testing is often not required. This may be because the entities are identical to normal human peptides and proteins, or because the molecules are so large they will not be expected to penetrate the cells. However, many allergen drug products are converted to allergoids, to enhance the safety profile. As allergoid formation involves chemical modification of a biological entity, the product could be considered a new chemical entity and the required safety testing is considered comparable to that for conventional pharmaceuticals. Allergy Therapeutics have evaluated such a product and identified an optimal approach for assessment of genotoxicity using a combination of in vitro and in vivo studies to best evaluate all genotoxic agents.



P5.2 - A murine model of allergic respiratory inflammation provoked by Dermatophagoides siboney allergens as a tool for preclinical evaluation of therapeutic antiallergic vaccines.

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Introduction: Allergic respiratory diseases are a major health issue worldwide. Animal models, specially based on mice, have been developed in the last years allowing a better understanding of the physiological and immunological mechanisms underlying allergy, and as a proof-of-concept test for evaluating possible therapeutic or prophylactic agents. The aim of this work was to develop a murine model of allergic respiratory inflammation, using a clinically relevant allergen, from the endemic Cuban House Dust Mite species: *Dermatophagoides siboney*.

Methods: Animals were injected intraperitoneally with two doses of the allergen and further, exposed every day to different concentrations of aerosolized allergenic extract (100-500 ug/ml Der s 1), during a maximum of two weeks. Two different mice strains were tested: C57BL6 and Balb/C, which have been reported before as prone to develop Th2 response.

Results: Mice exposed to allergens exhibited high levels of serum specific IgE and IgG1 antibodies, featuring a typical Th2 response. Serum eosinophil levels were also incremented as compared to the control group. The histopathological examination of the lungs showed clear symptoms of acute inflammatory response, mucus production and remodeling of the airways, similar to features observed in human asthmatic patients. The response was stronger in C57BL6 strain than in Balb/C and was dependent of the exposure time.

Conclusions: This sensitization model provokes an allergic response in mice, resembling the human allergic respiratory disease. Thus, it can be a useful tool for preclinical evaluation of novel therapeutic agents, and particularly as a challenge test model for therapeutic vaccines.



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P5.3 - Influence of a proteoliposome adjuvanted allergen vaccine on to an earlier response against *Neisseria meningitidis*

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Introduction: A novel anti-allergic therapeutic vaccine candidate is based on purified allergens of *Dermatophagoides siboney* House Dust Mite and proteoliposome (PL) of *Neisseria meningitidis* as immuno-stimulatory adjuvant. A major potential benefit provided by this vaccine would be enhancement of efficacy of allergen vaccination, reducing the number of injections required for that treatment. The PL is a component of the anti-meningococcal vaccine (VAMENGOC-BC, Finlay Institute, Havana), therefore, this study aimed at assessment of the influence of the antiallergic vaccine on to an earlier response against *N. Meningitidis* induced by prophylactic vaccination.

Methods: It was measured the PL-specific IgG antibody response, including IgG1 and IgG2a subclasses, before and after the administration of three doses of the allergen vaccine (2 µg Der s 1, each) in Balb/C mice vaccinated previously with two doses of VAMENGOC-BC. The allergen specific IgG and subclass antibody response was also evaluated.

Results: The administration of the PL-containing allergen vaccine in these mice showed only a slight dose-dependent increase on PL-specific IgG, IgG1 and IgG2a antibodies. Unexpectedly, previous immunization with VAMENGOC was associated to a significant increase of the allergen-specific IgG, IgG1 and IgG2a antibody response induced by the later administration of the allergen vaccine (ANOVA, $p < 0.05$). Current results confirmed that the highest IgG2a and IgG1 response to the allergen vaccine was obtained after the third dose.

Conclusion: These results sustain the safety of this novel anti-allergic vaccine with regard to its lack of negative influence to anti-meningococcal response.



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P5.4 - Preclinical evaluation in mice of the immunogenicity of a novel anti-allergic vaccine, based on *Neisseria meningitidis* proteoliposome, as adjuvant.

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Introduction: Proteoliposomes (PL) from *Neisseria meningitidis* serogroup B have been reported as a potent vaccine adjuvant, inducing a Th1-skewed response. The aim of this work was the preclinical evaluation of immunogenicity of a novel anti-allergic vaccine candidate based on purified allergens from *Dermatophagoides siboney* mite and PL as adjuvant, both components adsorbed onto Aluminum hydroxide.

Methods: Balb/c mice were administered with 3 doses containing 2 µg of allergen each at one week intervals by subcutaneous route. The allergen-specific antibody response was assessed determining serum levels of IgE, IgG1, and IgG2a by ELISA. Additionally, IL-4, IL-5, IFN γ and IL-10 cytokine levels were measured in broncho-alveolar lavage (BAL) by ELISA, in mice subjected previously to aerosolized allergen challenge.

Results: The vaccine induced a mixed IgG1 and IgG2a antibody response in naïve mice. The induction of IgG2a was PL dependent. The administration of the vaccine prevented the development of allergic response in mice subjected to allergen exposure by inhalant route. In this regard, vaccinated mice showed lower levels of serum IgE, IL-4, and IL-5 (Th2 cytokines) in BAL and lower eosinophil counting in blood as compared to controls. Histological examination of lungs showed also a diminished allergic inflammatory response in vaccinated mice in contrast to mice which were administered with the conventional formulation of Alum-adsorbed allergen.

Conclusions: These results indicate the potential superiority of the PL-adjuvanted vaccine candidate regarding the induction of a protective anti-allergic immune response.



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P5.5 - Shelf-life stability study of a novel adjuvanted and adsorbed House Dust Mite allergen vaccine

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Introduction: Evaluation of shelf-life stability of pharmaceutical products is required during the pharmaceutical development phase, prior to advancing to clinical trials. The objective of this work was to test the stability of a new experimental allergen vaccine of *Dermatophagoides siboney* adjuvanted with Outer Membrane Vesicles (OMV) of *Neisseria meningitidis* and adsorbed into Alum hydroxide.

Methods: The ICH methodology established for stability studies for biological products was followed. Samples of three pilot scale GMP batches were stored at 4 °C and assayed at 0,3,6,9,12,18 and 24 months. Possible desorption from alum gel was monitored, testing the supernatant for allergenic activity (IgE-inhibition ELISA), Der s1 content (MAb-ELISA) and total protein content. Preservation of antigen's integrity was checked by SDS-PAGE and Western-Blotting after forced desorption. Other tests were applied for measuring preservative content, pH stability, and sterility. Acceptance limits matched those used for product release. Since, a potency test is not yet established for this new vaccine, allergen-specific immunogenicity in Balb/C mice was determined at the beginning and end of the study.

Results: After 24 months no deviations of quality specifications were detected in any parameter. Although a slight tendency toward increasing the allergen activity and Der s 1 content in the supernatant was noted, it was not statistically significant (regression analysis, $p < 0.05$). The immunogenicity test showed the expected outcome regarding induction of allergen-specific IgG, IgG1 and IgG2a antibodies (the later is dependent of the OMV adjuvant effect), similarly to initial results.

Conclusion: This study proved the vaccine stability during 24 months as a basis for approval of a reliable expiration period.



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P6.1 - The Paul-Ehrlich-Institut: An Example of Quality Management in a European Regulatory Agency

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The Paul-Ehrlich-Institut (PEI) is the German authority responsible for registration, marketing authorization and batch testing of biological medicinal products for human and veterinary use. These procedures are supplemented by scientific advices, authorization of clinical trials, pharmacovigilance and regulatory inspections and require a process-based Quality Management System (QMS). Such a system has been implemented and accredited by the German accreditation body Chemistry (DACH). The QMS of the PEI is based on International Organization for Standardization (ISO) norms, especially the ISO 17025 (General requirements for the competence of testing and calibration laboratories) with relevant elements of ISO 9001, and on national and European regulatory guidelines. The Quality policy of the PEI is established on working with qualified personal, and includes the application of actual quality assurance guidelines, use of validated methods, documentation of operation procedures, systematic audits by internal and external auditors, and continuous improvement by annual reviews. For applicants these tools ensure high quality, efficacy and reliability for these administrative procedures. Confidentiality towards marketing authorisation holders and transparency towards other authorities of the European and international network are self-evident attributes for this mission. Twenty-five of thirty sections (laboratories and regulatory areas), five administrative units, three units of drug safety and one unit of legal affairs are covered by PEI-QM-System. Annual reviews, the establishment of performance indicators and mutual interest in practical improvement are main tools for the detection of weaknesses and the refinement of the system. The department of Allergology is embedded in this QM-System and contributes significantly to the availability of high quality allergen products for in vivo diagnosis and immunotherapy on the German and European market.



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P6.2 - Methodology for obtaining the Sanitary License of Pharmaceutical Operations for Allergen Vaccines in BIOCEN.

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Introduction: Manufacturing of medicine products, in addition to Product Registration, requires a Sanitary License of Pharmaceutical Operations (SLPO) by the National Regulatory Agency. The objective of this work was to describe the technical documentation, regulatory requirements and methodology followed for obtaining the SLPO for allergen products in Cuba, and particularly, our experience with allergen vaccines from House Dust Mites *Dermatophagoides pteronyssinus* (VALERGEN-DP), *D. Siboney* (VALERGEN-DS) and *Blomia tropicalis* (VALERGEN-BT), in a context of an industrial complex with different biopharmaceutical products and facilities and a unified Quality Management System (QMS).

Methods: There were separate licensing process for the Active Substance (API) and finished product (lyophilized allergen extracts), the last one in a multi-product facility. A multidisciplinary specialist's team was created to prepare Master Files, containing general organization data, personnel training program, biosafety hygiene and occupational health requirements, facility and equipment characteristics, production flow, description of QMS, definition of critical control points, validation program, corrective and preventive action and Change Control systems, process documentation (SOPs, batch records, etc) according to SLPO regulations and GMP certification system.

Results: As a result of this process, and following the Pharmaceutical Inspection by the National Regulatory Agency (CECMED), both SLPOs (for API and finished product) were granted. Thus, BIOCEN became the first institution that is officially licensed in Cuba for manufacturing Allergen Products for in-vivo tests and injection immunotherapy.



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P6.3 - The new regulation for therapy allergens as approach to extend the marketing authorisation to Named Patient Products (NPP) in Germany

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According to the European and German regulations allergen products for in vivo diagnosis and immunotherapy are medicinal products. They are subjected to a marketing authorization procedure if manufactured by a method involving an industrial process. However, a significant proportion of the allergen products for immunotherapy marketed in Germany are named patient products (NPP). These products are manufactured individually on the basis of a prescription. According to the German Medicinal Products Act these individually manufactured products do not require a marketing authorisation. Therefore no independent evaluation of the quality, safety and efficacy is performed by the competent authority. Thus, the possibility remains that patients are treated with products not being efficacious resulting in a progression and worsening of the allergic disease. To address this problem Germany implemented a new regulation extending the demand for a marketing authorisation on the majority NPPs by November 2008. The approval of these products will be performed in three different steps: (i) by May 2009 the individual NPPs have been notified to the authority. The notification includes information on the manufacturing and testing of the products as well as the SPC. (ii) by October 2009 the bulks at the latest step prior to mixing or filling of the NPPs are subjected to the official batch release. (iii) by December 2010 marketing authorisation approvals have to be submitted. Depending on a justification to be provided a transition period of up to seven years may be granted on a case by case basis to allow generation of additional clinical data.



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P6.4 - Harmonization with ICH Q10 guidelines in the development, production, and commercialization of allergen products

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Introduction: Allergen vaccines face the challenge of full ICH compatibility as other biopharmaceutical products. ICH Q10 Guidelines, which describes the requirements for the implementation of the Pharmaceutical Quality System (PQS), including development, technology transfer, commercial production and product discontinuation, has been recommended for adoption to the regulatory bodies of European Union, Japan and USA. The objective of this work was to identify the opportunities of improvement within the Quality Management System (QMS) implemented in the National Center of Bioproducts (BIOCEN), regarding the ICH Q10 guidelines, and particularly, concerning its application to allergen products.

Results: This work identified the weaknesses and strengths of the QMS of BIOCEN in the whole life cycle of the allergen products. The critical points and the design space definition were analyzed for this specific type of products according to Quality Risk Management. The system for change management was analyzed too, and the methodology and program for harmonization with the ICH Q10 was prepared. The Risk Analysis of the allergen production was prepared to identify the general improvement opportunities.

Conclusion: This study will guarantee the ICH Q10 harmonization in BIOCEN QMS in the development, technology transfer, commercial manufacturing and product discontinuation of allergen products.



P7.1 - Evaluation of grass pollen specific immunotherapy: Long-time evaluation of sublingual or supralingual routes

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Introduction: The aim of the study was to confirm or refute a difference between sublingual or supralingual longterm specific immunotherapy with standardized grass pollens allergen extract. Patients, who been the previous one-year double-blind, placebo-controlled, randomized study were enrolled in the open randomized study which succeeded the next 3 years.

Methods: The study treatment was performed either by sublingual or supralingual specific immunotherapy. The maintenance phase of immunotherapy with a maximum tolerated therapeutic dose was continued all the time of the second study in the patients with active treatment in the previous study.

Results: Both routes of administration are effective according to subjective clinical parameters and drug consumption. No statistically significant difference between sublingually and supralingually treated patients was observed at the end of the first study. After long-term SIT, the clinical improvement was accompanied by the significant reduction of skin prick test wheal diameters. The cumulative allergen dose or SIT duration influenced the rise of specific IgG levels whereas specific IgE levels remained unchanged. Adverse effects were limited to a small number of generally mild local and/or systemic reactions.

Conclusion: The administration of allergens via the oral mucosa is clinically effective, favoring the sublingual rather than supralingual route. The significant therapeutic effect of sublingual and supralingual SIT was achieved for a period of 3 years or longer time.



P7.2 - Long-term Study of Hyposensitisation Therapy by means of Depot Allergen Drugs (Spring Trees and Grasses) till the Stage of Complete Remission.

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Introduction: The objective of this study was the 5 years follows up of 300 allergic patients subjected to allergen immunotherapy (AIT). AIT was indicated in following allergic cases: rhino-conjunctivitis and bronchial asthma (67% vs. 33%). The objectives of AIT were reduction of disease severity, improvement of patient's life quality, reduction of pharmacotherapy (i.e. decrease of risk, side effects and higher costs).

Methods: AIT is contraindicated: In immunopathologic diseases (especially collagenoses, autoimmunity of severe immunodeficiencies), malignity, by lack of cooperation of patient, treatment by beta-blocks (i.e. risk of low efficiency of administered adrenalin), severe bronchial asthma poorly controlled (FEV-1 below 70% of appropriate value especially treated by systemic corticosteroids), in more situations when adrenalin administration is contraindicated (e.g. ICHS) and generally in children below 7 years old. Allergens of spring trees were administered to 80 patients (48% M vs. 52% F). Allergens of grasses were administered to 220 pts (55% M vs. 45% F). Initiation treatment was started in both cases during the autumn period.

Results: Efficiency of AIT can be reliably evaluated only according to clinical status improvement documented by score of symptoms (i.e. nose, eye and bronchial), frequency of rescue medication use, reduction of pharmacotherapy and lung function examination in case of bronchial asthma. Help criteria in evaluation of AIT efficiency can be using by skin tests and laboratory assessment of specific antibodies (IgE & IgG). Efficiency of AIT was evaluated and documented at least once a year, post termination of pollen season. Recommended duration of AIT is 3-5 years. Long-term improvement was observed already after 2 years administration by 12%, after 3 years in 63% and after 5 years in 92 % of patients.

Conclusion: Allergen immunotherapy is the only possibility of causal influence of allergic disease. This treatment consists of administration of gradually increasing doses of allergen to maintenance dose, which is administered repeatedly in a certain time interval. This way sufficient cumulative dose of allergen is provided, which results in extinction of complaints by repeated exposure to such allergen.



P7.3 - IgG4 allergen-specific antibodies are inversely correlated to IgE mediated allergic response in asthmatic patients allergic to domestic mites

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Introduction: Immediate allergic reactions are mediated by IgE antibodies specific to environment allergens. Induction of IgG4 allergen-specific antibodies can play a blocking role of the allergic response in patients subjected to allergen immunotherapy (therapeutic vaccination). These antibodies could be induced also by natural exposure to high allergen levels. The aim of this work was to determine allergen-specific IgE and IgG4 antibodies in a population of Cuban allergic patients.

Methods: The study was carried out in 120 adult patients, with positive Skin-Prick-Test (SPT) responses to at least one of the 3 most relevant species of domestic mites in our country: Dermatophagoides pteronyssinus (Dp), D. siboney and Blomia tropicalis. SPT was performed with VALERGEN allergenic extracts (BIOCEN, Cuba) at a concentration of 20 000 BU/ml. Allergen-specific IgE and IgG4, as well as, total IgE levels were measured by ELISA.

Results: The IgE response was similar in intensity between mite species, whereas greater IgG4 response was observed to Dp. A significant correlation ($p < 0.05$) was detected between the skin reaction size and total or allergen-specific IgE, as expected. On the other hand, the IgG4 response was inversely correlated to specific IgE, and, in the case of patients with high titers, IgG4 was even negatively correlated to the skin reaction size, suggesting to play an anti-allergic protecting role in these patients.

Conclusion: Allergen-specific IgG4 antibodies can be relevant for a better diagnosis of the patient's clinical allergic status and an attractive target for monitoring the success of immunotherapy.



P7.4 - Efficacy and safety of subcutaneous immunotherapy using standardized House Dust Mite vaccines in the treatment of allergic asthma in a Cuban population. Results from Double-Blind Placebo Controlled clinical trials.

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Introduction: Specific Immunotherapy is an effective treatment for rhinitis and mild to moderate asthma. Allergic sensitization to House Dust Mites *Dermatophagoides pteronyssinus*, *D. siboney* and *Blomia tropicalis* has been described before in Cuba, as strongly linked to asthma. The aim of this work was to evaluate the efficacy and safety of standardized allergen vaccines of these 3 mite species (VALERGEN, BIOGEN, Cuba), in asthmatic patients.

Methods: Three separate DBPC clinical trials were performed in 40 asthmatic patients each, with variable polysensitization, but positive predominant Skin Prick Test (SPT) to the vaccine allergen, respectively. Half of patients received the active treatment consisting of subcutaneous injections with increasing doses. The total one year cumulative dose was 63035 BU, in an average of 20.5 injections.

Results: The treatment was effective in the reduction of clinical symptoms (up to 32%, CI_{95%}: 28-36%; $p=0.0006$) and medication intake (23%, CI: 18-28%), as compared to control treatment. The skin sensitivity to the allergens decreased significantly ($p=0.0001$), with regard to the beginning of the treatment. Immunotherapy with Dp induced also desensitization to Ds, and vice versa, while no cross-effect was observed to Bt. The reduction of skin sensitivity was correlated ($p<0.05$) to clinical outcome. Improvement of the lung function was observed, as a modest PEF increase and reduction of PEF daily variability ($p<0.05$). SIT was effective in 71% of patients. The frequency of local adverse reactions was 2.4 % of injections.

Conclusions: The overall results indicate that immunotherapy using VALERGEN standardized vaccines is effective for the control and amelioration of the allergic asthma in our population.



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P7.5 - Associated factors to adverse reactions to immunotherapy with mite allergen extracts in allergic diseases

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Introduction: The purpose of this study was to evaluate the factors associated with the appearance of adverse reactions to a conventional schedule of subcutaneous immunotherapy with standardized aqueous allergen extracts of house dust mites.

Methods: A descriptive, transversal study was designed, in 206 adults, age ranged 18 to 65 years, with a clinical diagnosis of allergic asthma, rhinitis or conjunctivitis, who were treated with a conventional schedule of subcutaneous immunotherapy with mite allergen extracts (*Dermatophagoides pteronyssinus*, *Dermatophagoides siboney* and *Blomia tropicalis*), standardized in Biological Units (BIOCEN, Cuba). Associations between prognostic factors (age, sex, allergic disease, severity, skin prick test, allergenic composition, exact time in the schedule that the reactions took place) and adverse reactions were estimated.

Results: There were 28 reactions (20 local and 8 systemic) in 16 patients. 87.5% were female and the predominant age group was between 18 and 27. The most frequent allergic disease diagnosed was asthma with perennial symptoms (62.5%). The greater frequency of reactions was among patients with moderate asthma (70 %). Most reactions took place at the escalating phase (81.2%, $p=0, 0004$). A mean wheal diameter of 6-8 mm was found by skin prick test, in patients who reacted. The greatest frequency of reactions was found with mixed vaccines containing contained two mite species ($p=0, 02$).

Conclusions: An individualized evaluation of the aforementioned factors allows could be helpful in prevention of adverse reactions to allergen immunotherapy and a better acceptance and compliance of the treatment by the patients.



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P7.6 - The practice of allergen specific immunotherapy by Cuban allergologists

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Introduction: Immunotherapy with allergens in allergies is used in Cuba since the '50s of the last century. Its practice has presented changes in relation to extract obtaining, dosing and treatment duration.

Method: A national descriptive study with the objective of investigating the daily practice of immunotherapy by Cuban allergologists, from January 2008 to June 2009.

Results: 120 allergologists, with a mean age of 44.2 and more than 10 years of experience in the field, were surveyed in all the provinces in the country. For the immunotherapy formulation, extracts in Biological Units (BU), Protein Nitrogen Units (PNU) and Weight - Volume Unit (WVU) were used. BU extracts employed by 100% of the allergologists were from the Centro Nacional de Biopreparados, Cuba (BIOCEN) with a good clinical result and low adverse reaction index. 76.6% of the allergologists used the schedule specified by the industry, 71.6% indicated a mixture in BU with extracts of local production (PNU and WVU). 70.8% used a mixture with more than 5 antigens and slow schedules. 81.6% followed schedule and dosage, according to personal experience. The highest number of favourable clinical results was reported for BIOCEN extract and schedule. 100% of allergologists stated higher safety to the use of industrial extracts (BIOCEN).

Conclusions: There is a variety of methods used by Cuban allergologists in the preparation and administration of immunotherapy with allergens. The most accepted extract is the one from BIOCEN.



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P7.7 - Clinical Trial of Sublingual Immunotherapy in asthmatic Cuban patients using a standardized allergen vaccine of *Dermatophagoides siboney*

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Introduction: Specific immunotherapy using mite allergen vaccines is considered an effective treatment for allergic asthma. Sublingual route has the potential for decreasing the risk of systemic reactions and improving compliance. The objective was to determine the therapeutic effect and safety of sublingual immunotherapy using a standardized vaccine of *Dermatophagoides siboney* (VALERGEN-DS, BIOCEN, Cuba) in Cuban asthmatic patients.

Methods: A Double-Blind Placebo-Controlled study included 40 adult patients, with mild to moderate asthma and allergic sensitization to Ds. Half of patients were randomized to placebo. Treatment consisted of sublingual drops with escalating dosage up to 2000 BU; 107 applications were administered in each patient, 21 of them corresponding to the increment phase with a daily frequency. In the maintenance phase the vaccine was administered twice per week during 12 months.

Results: The treatment was very effective in decreasing clinical symptoms (up to 37%) and medication (29%), compared to conventional medication in the control group. Allergen-specific skin reactivity, as measured by the wheal diameter, and PEF variability decreased significantly ($p < 0.05$). Clinical outcome was correlated with reduction of skin reactivity. Overall, the treatment was considered effective in 70% of patients. Efficacy of the sublingual treatment was similar to a previous trial with subcutaneous route. However, safety was clearly superior: no product-related systemic reactions were reported. Local reactions were reported in only 0.36% of administered doses.

Conclusion: This study indicates that sublingual immunotherapy with VALERGEN-DS is an effective and safe treatment for allergic asthma in Cuba and support its introduction in Allergy Services in our Healthcare System.



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P7.8 - Cost-Effectiveness of Subcutaneous Immunotherapy with Mite Vaccines containing Depigmented and Polimerized Allergen Extracts plus Beclomethasone Dipropionate versus Beclomethasone Dipropionate in children with asthma in Bogota-Colombia

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Objective: To estimate the cost and clinical implications of using subcutaneous immunotherapy (SIT) with mites vaccines containing depigmented and polymerized allergen extracts plus beclomethasone dipropionate (BDP) for the treatment of children with asthma.

Methods: The Course of asthma moderate and severe children between 13 and 14 years old, treated with SIT plus BDP or with BDP alone was modeled using discrete event simulation, used in health economic evaluations, allowing modeling at the level of the individual patient; 1000 pairs of identical patients were simulated 100 times. Population data was obtained from children who participate in prevalence study of asthma in Bogotá (ISAAC III) . Rates of asthma attacks were based on patient's Peak Expiratory Flow which varies according to patient's treatment. Physician visits, hospitalization and emergency room visits rates were based on published data. Only direct medical costs were considered updating the costs from 1998 to 2007 Colombian Pesos (COP) using the Consumer Price Index (CPI).

Results: SIT plus BDP decreased the number of asthma attacks during 3 years from 1709 to 515 at a net cost of COP 3,315,959 to COP 590,215 per patient, and reduced the number of physician visits, hospitalizations and emergency room visits at a higher cost than BDP during 3 years of analysis

Conclusion: The health authorities should analyze the results of SIT plus BDP as a treatment that will generate savings in the long term compare to BDP and that will bring improvement of health children population and in health government system.



P7.9 - Monitoring of antibody-responses during grass pollen extract immunotherapy and after five years of discontinuation with recombinant allergens

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Introduction: Allergen-specific immunotherapy is considered as the only allergen-specific and disease-modifying treatment of allergy. Our aim was to study in detail allergen-specific antibody responses in patients receiving grass pollen-specific injection immunotherapy.

Methods: Sera were obtained from grass pollen allergic patients having received one course of injection immunotherapy (SCIT) with grass pollen extract (SCIT group, n = 12) or only anti-inflammatory treatment (non-SCIT group, n=7) before treatment, after five months of treatment and after five years. Specific IgE, IgG1-IgG4, IgM, IgA and light chain responses were monitored using purified major and minor recombinant timothy grass and birch pollen allergens.

Results: After one course of SCIT but not after anti-inflammatory treatment we found increases of IgG1>IgG4>IgG2 antibody responses in particular against the major grass pollen allergens but no relevant increases against the other tested allergens. No relevant induction of allergen-specific IgA, IgM or IgG3 responses were found. Increases of allergen-specific IgG responses were accompanied by allergen-specific kappa and lambda light chain binding in the SCIT group. Inhibition of allergen-dependent basophil degranulation was only obtained with SCIT group sera containing therapy-induced allergen-specific IgG antibodies. A decrease of specific IgE levels against the major timothy grass pollen allergens Phl p 1 and 5 occurred in the SCIT group after the grass pollen season but not in the non-SCIT group. After five years allergen-specific (Phl p 1, Phl p 5) IgE and IgG antibody levels had returned to baseline levels in the SCIT group.

Conclusion: Our results demonstrate that only SCIT but not anti-inflammatory treatment prevents boosts of allergen-specific IgE production in allergic patients.

Supported by grant 813003 of the Austrian Research Promotion Agency and by a research grant from BIOMAY, Vienna, Austria and the Christian Doppler Research Association, Austria.



P7.10 - Therapeutic effect in asthmatic adults treated with sublingual immunotherapy with a standardized allergen vaccine of *Dermatophagoides pteronyssinus*

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Introduction: Sublingual immunotherapy (SLIT) has been regarded as a practical alternative to subcutaneous immunotherapy (SCIT). During the last 20 years, increasing evidence on the clinical efficacy and safety of SLIT in rhinitis and asthma has been provided. Objective: To evaluate the therapeutic effect and safety of a *Dermatophagoides pteronyssinus* (Dp) standardized allergen vaccine (VALERGEN-DP, BIOGEN) for SLIT in asthmatic patients.

Methods: A DBPC clinical trial was performed in 40 adults with mild to moderate asthma. After a 4-week baseline phase, patients were randomized to placebo or active treatment, consisting on sublingual drops with increasing daily doses for 3 weeks and maintenance doses twice a week for 12 months. Maximum dose was 2000BU. The effect was evaluated with symptom/medication diary cards, peak expiratory flow (PEF) measures and skin reactivity. Adverse reactions were classified according to WAO.

Results: After 12 months, SLIT significantly reduced clinical symptoms (up to 35%) and medication (30%), with respect to the placebo group. Allergen-specific skin sensitivity was also reduced significantly ($p < 0.05$). Similarly to SCIT, SLIT with Dp was able to induce desensitization towards *D. siboney*. A slight improvement of the respiratory function with reduction ($p < 0.005$) of PEF variability was noted. Overall, 75% of patients reported clinical improvement. These figures are similar to those reported by SCIT with the same vaccine. Moreover, safety was clearly superior. No specific systemic reactions were reported. Local reactions were described only in 0.58% of administrations.

Conclusion: These results support the efficacy and safety of SLIT with VALERGEN-DP for asthma treatment in our population and endorse its large-scale introduction in our healthcare system.



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P7.11 - Clinical Trial of sublingual immunotherapy with a standardized allergen vaccine of *Blomia tropicalis* in asthmatics patients

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Introduction: Sensitization to *Blomia tropicalis* (Bt) is very frequent in tropical countries and has been associated with allergic asthma. Nevertheless, clinical studies of immunotherapy with *Blomia* allergen vaccines are very scarce. The efficacy of immunotherapy by sublingual route (SLIT) in asthma has been reviewed in a Cochrane meta-analysis. The aim of this work was to assess the therapeutic effect and safety of SLIT using a standardized vaccine of Bt (VALERGEN-BT, BioCen) in Cuban asthmatic patients with a predominant sensitization to Bt.

Methods: A phase II DBPC trial was performed in 40 adults with mild to moderate asthma. Half of patients were randomized to placebo. Treatment consisted of sublingual drops with escalating dosage up to 2000 BU. A total of 110 applications were administered in each patient, 21 of them corresponding to the increment phase. In the maintenance phase the vaccine was administered twice per week during 12 months.

Results: The treatment was very effective in decreasing clinical symptoms (up to 42%) and medication intake (29%), compared to placebo. Allergen-specific skin reactivity, as measured by the wheal diameter, and PEF variability decreased significantly ($p < 0.05$). In general, the treatment was clinically effective in 85% of patients. No product-related systemic reactions were reported and local reactions appeared in only 0.36% of administered doses. These results were similar to what has been described in analogous studies using allergens of *D. siboney* or *D. pteronyssinus* and the same dosage schedule.

Conclusion: This study indicates that SLIT with VALERGEN-BT is an effective and safe treatment for allergic asthma and supports its introduction in our Healthcare System.



P7.12 - IgE/IgG4 ratio as a possible surrogate marker of clinical efficacy during allergen immunotherapy

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Introduction: Allergen immunotherapy induces IgG4 antibodies with blocking effect, and in the long-terms reduces or prevents the seasonal raising of IgE antibodies. The problem of finding immunological suitable surrogate markers of clinical efficacy during IT is currently very pertinent. The aim of this work was to evaluate the allergen-specific IgE/IgG4 ratio as a paraclinical efficacy marker.

Methods: IgE and IgG4 antibodies to *Dermatophagoides pteronyssinus*, *D. siboney* and *Blomia tropicalis* were measured by in-house ELISA in 120 asthmatic patients subjected to SCIT with standardized allergen vaccines (VALERGEN, BIOGEN) of each mite in three separate DBPC clinical trials (40 patients in each trial, half receiving placebo). Antibody titres were expressed in relative units, normalized and averaged between the three trials. Size effect was calculated as the Standard Mean Difference (SMD) between the active and placebo groups, and averaged using meta-analysis tools.

Results: After 6 months there was a significant increase of IgG4 antibodies ($p < 0.05$), whereas no significant change was noted for IgE. At 12 months the IgG4 increase was even greater and the reduction of IgE achieved significance. The IgE/IgG4 ratio was the immunological variable with the greatest size effect value (SMD=0.81 CI₉₅%:0.71-0.91) since it combined the IgG4 increase and IgE decrease, showing a figure close to the clinical effect (symptom-medication SMD = 1.2 CI₉₅%:0.7-1.7). IgE/IgG4 ratio was significantly correlated to the clinical variable ($r=0.23$, $p=0.04$) and to the reduction of skin reactivity on a per patient basis.

Conclusion: These results support the use of this serological marker for evidencing the immunological changes during IT and possibly, for predicting patient's clinical improvement.



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P7.13 - Double-Blind Placebo-Controlled Clinical Trials of Subcutaneous Immunotherapy for evaluating the efficacy and safety of standardized House Dust Mites allergen vaccines in Cuban asthmatics

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Introduction: Subcutaneous allergen immunotherapy (SCIT) has been used for almost a century for management of allergic disorders, including perennial allergic rhinitis and asthma, using allergen extracts of diverse quality and, frequently, ill-defined composition. Only in the last two decades the use of standardized allergens is increasingly prevailing. Objective: To assess the efficacy and safety of SCIT with standardized allergen vaccines of common in Cuba mite species *Dermatophagoides pteronyssinus* (Dp), *D. siboney* (Ds), and *Blomia tropicalis* (Bt), in asthmatic patients.

Methods: Three DBPC trials were carried out, with a total of 120 patients (40 in each trial, respectively). Half patients were randomized to receive active placebo. All individuals were diagnosed with mild/moderate asthma caused by sensitization to Dp, Ds or Bt. SCIT consisted on subcutaneous injections up to 6000 UB (12ug Der p1) per dose, using allergen products VALERGEN (BIOCEN, Cuba), with an average of 18.8 injections per patient.

Results: After 12 months, the active group experienced a significant reduction of clinical symptoms (40%, CI_{95%}:36-44) and medication intake (29%, CI:25-34), with regard to placebo group. Skin reactivity decreased significantly in 3.2 log (CI:3.0-3.4) of allergen concentration. SCIT with Ds induced desensitization towards Dp and vice versa, but no for Bt. The treatment increased specific IgG4 antibodies with regard to the pre-treatment values ($p < 0.05$). Per-patient clinical efficacy was 83%. Severe systemic reactions were not reported, the frequency of adverse events related to the product was 2.6% of injections.

Conclusion: The general outcome of these trials added evidence supporting the efficacy and safety of industrially manufactured standardized allergen vaccines.



P7.14 - Cross-protection to Tyrophagus putrescentiae Allergy by Local Nasal Immunotherapy using Strips of Dermatophagoides pteronyssinus

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Introduction: House dust mite *Dermatophagoides pteronyssinus* (Dp) is a major source of aeroallergens for patients with allergic asthma. The mite Group 2 major allergens are highly allergenic and show the highest rates of serum specific IgE positivity in atopic patients. We have demonstrated that local nasal immunotherapy (LNIT) with Dp-coated strips could reduce the serum levels of Dp-specific IgG1 and IgE and up regulate IgG4 within 4 months. Whether, the Tyr p2 specific Igs were modulated by LNIT with Dp, was determined in this study.

Results: The results showed that there was significant reduction of Der p2 specific IgE (0.54 ± 0.23 vs. 0.25 ± 0.09 IU/ml, $p < 0.01$), IgG1 (0.98 ± 0.15 vs. 0.73 ± 0.12 IU/ml, $p < 0.01$) and upregulation of IgG4 (1.56 ± 0.25 vs. 2.13 ± 0.27 IU/ml, $p < 0.01$) after LNIT with Dp allergen strips. There was also significant reduction of Tyr p2 specific IgE (0.44 ± 0.19 vs. 0.32 ± 0.09 IU/ml, $p < 0.05$), IgG1 (0.76 ± 0.21 vs. 0.45 ± 0.18 IU/ml, $p < 0.01$) and upregulation of IgG4 (0.78 ± 0.31 vs. 1.32 ± 0.21 IU/ml, $p < 0.01$) after LNIT with Dp allergen strips. In comparison with the group of buffer control, the changes of serum concentration of Igs were significantly reduced after LNIT with Der p ($p < 0.001$) for specific IgE to Der p2 and Tyr p2 ($p = 0.001$), respectively.

Conclusion: Since both IgE and IgG to Tyr p2 and Der p2 were significantly modulated by Dp allergen strips. It is conceivable that there might be cross-protective antibodies production to group 2 mite allergens after LNIT with Dp allergen strips.



P8.1 - Recombinant Der p 10 as a diagnostic tool to identify patients with genuine shrimp sensitization

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Introduction: Tropomyosins represent important cross-reactive allergens in mites, invertebrates and in particular in crustacean, which are consumed as sea food. Aim of this study was to express biologically active recombinant house dust mite tropomyosin, Der p 10 and to study its usefulness for diagnosis of allergies to invertebrates.

Methods: A cDNA encoding Der p 10 was obtained from *D. pteronyssinus* RNA by reverse transcription and PCR. Recombinant Der p 10 was expressed in *E. coli*, purified to homogeneity and characterized by mass spectroscopy and circular dichroism. rDer p 10 was tested for IgE reactivity with sera from 1322 *D. pteronyssinus* allergic patients. Cross-reactivity of Der p 10 with other invertebrate tropomyosins was studied using anti-rDer p 10 rabbit antibodies and by IgE immunoblot inhibition experiments. The allergenic activity of Der p 10 was evaluated with RBL cells, which had been transfected with human Fc ϵ RI.

Results: rDer p 10 was expressed and purified as a folded protein. It reacted with IgE from 15.2% of mite allergic patients and showed broad cross-reactivity with invertebrate tropomyosins. Testing of Mediterranean patients with rDer p 10 and major house dust mite allergens (Der p 1, 2, 5, 7, 21, 23) identified a subgroup of patients. These patients showed IgE reactivity to Der p 10 but not to the tested major mite allergens and therefore seemed to have a genuine sensitization to crustacean. Sera from these patients induced degranulation of RBL cells upon stimulation with Der p 10.

Conclusions: Diagnostic testing with rDer p 10 and major house dust mite allergens allows identification of a subgroup of patients with genuine crustacean sensitization which does not seem to be suitable for immunotherapy with house dust mite allergens.

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P8.2 - Sensitivity and Specificity of Skin Prick Test with standardized allergen extract of *Dermatophagoides pteronyssinus* in adults

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Introduction: Skin Prick Test (SPT) using standardized mite allergen extracts can be very sensitive and reliable diagnostic tool. IgE sensitization to domestic mites has been identified worldwide as a major cause of asthma and respiratory allergic diseases. In Cuba, the most relevant species are: *Dermatophagoides pteronyssinus* (Dp), *D. siboney* and *Blomia tropicalis*. Objective: To determine the diagnostic efficacy of SPT, using an standardized allergen extract of Dp, of national production (VALERGEN-DP, BIOCEN) and two other well known foreign commercial products (A and B).

Methods: An open clinical trial was carried out, on 50 allergic symptomatic patients and 50 non-allergic individuals. Age range: 16-50 years. The allergic symptoms were defined using a questionnaire, being related to allergy to indoor/house dust (asthma, rhinitis and/or conjunctivitis). Replicate punctures were performed in both arms of the patients using a concentration of 20 000 BU/mL (40|jg/mL Der p 1).

Results: The wheal area produced by VALERGEN-DP was 43.5 mm², a value slightly greater than with products A and B. Nevertheless, the difference was significant only for product A ($p = 0.002$). Despite this difference, the diagnostic coincidence among the three products was 98-99 %. Sensitivity and Specificity were 82% and 98%, respectively, and no significant differences ($p = 0.05$) were detected between products for these parameters. ROC analysis confirmed that the cut-off diameter of 3 mm rendered the optimal results.

Conclusion: Therefore, VALERGEN-DP is similar to other commercial allergen extracts, regarding diagnostic efficacy in our adult population.



P8.3 - IgE response to sweet orange (*Citrus sinensis*) fruit in Cuban allergic patients

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Introduction: Allergy to sweet orange (*Citrus sinensis*) has been described as a relatively frequent food allergy in Cuba. Nevertheless, there have been no in-vivo or in-vitro studies characterizing IgE response to the fruit allergens. The aim of this work was to estimate the diagnostic sensitivity of Skin-Prick (SPT) and Prick-Prick (PPT) Tests regarding Oral Provocation Test (OPT), and to characterize the IgE allergen binding profile.

Method: 24 adult patients with symptoms of allergic reactions provoked by eating or handling the fruit, were selected. SPT, PPT and OPT were performed on all patients. For SPT, two separate extracts of peel and pulp, were used, at 1mg/mL protein concentration. Serum IgE Specificity of 16 positive patients was studied by Immunoblotting.

Results: 22 (92%) patients resulted positive to OPT. Only 16 (67%) were positive to PPT using pulp and 14 (58%) using peel. Positive results were even less for SPT: 11 (46%) and 10 (42%), respectively for pulp and peel extracts. A significant concordance value ($\kappa=0.3$, $p=0.03$) was found only between OPT and PPT to pulp. IgE binding patterns to pulp and peel were similar. Major bands were detected at 26kDa (75% of patients reacted) and 17kDa (54%), corresponding presumably to Cit s1 and Cit s2 allergens. Another major band, previously unreported, was observed at 30-31KD showing reactivity to 71% of patient's sera.

Conclusions: Both skin tests are inadequately sensitive, suggesting the use of OPT as the diagnostic tool of choice. A new 30KD major allergen candidate has been found.



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P8.4 - Skin Prick Tests Results In Children from Middle Black Sea Region, Turkey

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Objective: The aim of this study was to determine the spectrum and frequency of positive reactions to common allergens in allergic children by skin prick tests in Middle Black Sea Region of Turkey.

Material and Methods: In the study, we retrospectively evaluated skin prick test results of 739 children (aged 2-17) with allergic diseases history (asthma, allergic rhinitis, allergic conjunctivitis, atopic dermatitis) who showed positive reaction to at least one allergen in the skin prick tests performed between May 2003 to April 2005 in the Middle Black Sea Region, Turkey.

Results: In 339 of 739 patients we obtained positivity to at least one of the allergens tested. The highest positive sensitivity of allergens in the skin prick tests were house dust mite [*D. farinae*, *D. pteronyssinus* %97 (n= 719)], secondly plant pollens (grass, weed, tree) sensitivity was positive in 226 (30.6%) patients. Thirdly and fourthly fungus (*Alternaria alternata*, *Aspergillus fumigatus*, *Cladosporium herbarum*, *Penicillium notatum*) and cockroach sensitivity have been found %12 (n = 89) and %12.3 (n= 91) respectively.

Conclusion: We suggest these results will give some help to diagnose and management of allergic diseases in children of this region.



P8.5 - Evaluation of a soy extract (Glycine max) for skin prick test in a Cuban population

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Introduction: The increase of soy consumption favours allergic sensitization to this food allergen source. Skin Prick Test (SPT) can be a valuable tool for the diagnosis of food allergy, particularly when standardized extracts are used. We aimed to evaluate the diagnostic accuracy and clinical safety of soy allergenic extract for SPT, in a Cuban population, calculating the specificity, sensitivity and effectiveness related to clinical manifestations of the disease, as well as, to identify the appropriate protein concentration for the best diagnosis of food allergy and the possible adverse reactions.

Methods: Thirty patients were included with the clinical diagnosis of soy food allergy, and 30 supposedly non-allergic. All individuals were subjected to SPT with dialyzed soy extract prepared either from defatted and grounded soy beans or from soy flour.

Results: Of the total number of patients clinically allergic to soy, 60% were positive to the soy bean extract and only 43% to the soy flour extract. In the control group all the patients were negative for both extracts ($p < 0.05$). The specificity for both extracts and dilutions (0.5 mg/ml and 1 mg/ml total protein) was 100%. The soy bean extract at 1 mg/ml showed the highest effectiveness value: 80%. The analyzed sera of positive patients recognized predominantly a band of approximately 20 kDa, by IgE Western Blotting, compatible with the molecular weight of the Soy Tripsin Inhibitor a known major allergen of this plant.

Conclusions: the soy extract prepared from beans showed the highest sensitivity and the better protein concentration for SPT was 1 mg/ml. SPT was specific and safe. Nevertheless a large proportion of patients showed false-negative results indicating that SPT can not replace completely the clinical assessment.



P8.6 - A possible relationship between the atopic status of children with asthma and allergic rhinitis and HSV1 infection

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Introduction: This study investigated the association between HSV1 infection and atopy by comparing seropositivity to HSV1 in atopic children with asthma and allergic rhinitis and in non-atopic children.

Methods: 249 children randomly selected from the university outpatient pediatric clinics were prospectively enrolled in the study between September 1 and November 30, 2007. Serum samples were examined using the virus neutralization test (VNT) for HSV1 Immunoglobulin G (IgG) seropositivity. Skin prick tests (SPTs) were performed to determine atopic status.

Results: HSV1 IgG seropositivity was significantly higher in atopic children (56.8%) with asthma and allergic rhinitis than in the age-matched non-atopic children group (30.4%) ($p < 0.001$). Although the occurrence of atopy was higher in seropositive girls (57%) than in seropositive boys (47%), the difference was not significant ($p = 0.329$).

Conclusions: These results support a possible relationship between the atopic status of children with asthma and allergic rhinitis and HSV1 infection.



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P8.7 - Carpet Beetle (*Anthrenus verbasci*, Linnaeus 1767): A New Seasonal Indoor Allergen

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Introduction: Sensitization of children to indoor allergens has been demonstrated to be one of the major risk factors for development of allergic diseases, especially for asthma and allergic rhinitis.

Case Report: A 5-year-old boy presented with 4 year history of recurrent rhinitis during winter seasons. Although the serum total IgE level was high, no atopy was detected to common regional allergens by skin prick tests. Patient's house was infested with larva and adult forms of a carpet beetle. The insect was identified as *Anthrenus verbasci*. Skin test with *A.verbasci* was planned but intradermal injection was not accepted by the parents. Eradication of the beetle from the patient's house was followed by a complete clinical improvement. Intradermal injection of the extract was performed in patients who admitted to the pediatric allergy department and we obtained one positive result out of 19 patients which probably supports our opinion of defining *A. Verbasci* as a new allergen.

Conclusion: Seasonal infestation of houses with *A.verbasci* might have given rise to a new source of indoor allergens in the Black Sea Region of Turkey and in similar geographical areas, as well.



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P8.8 - Diagnostic efficacy of Nasal Provocation Test with a standardized allergen extract of *Blomia tropicalis*

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Introduction: Sensitivity and Specificity values have been used as indicators of efficacy or accuracy of diagnostic tests. Nasal Provocation test (NPT) is the "gold standard" for diagnosis of mite-associated perennial allergic rhinitis. *Blomia tropicalis* (Bt) is a mite species, known as an important sensitizing agent in tropical countries. The objective of this work was to determine the diagnostic accuracy of the NPT using a standardized allergen extract of Bt (VALERGEN-BT, BIOGEN, Cuba) in Bt-sensitized patients with perennial allergic rhinitis.

Methods: This open controlled clinical trial was carried out on 50 patients with clinical history of perennial allergic rhinitis related with exposure to house dust and positive Skin Prick Test (SPT) to Bt; and 50 healthy non-allergic volunteers with negative SPT to this mite. NPT was performed in all investigated subjects using five increasing concentrations of the allergen extract (2 BU/mL, 20 BU/mL, 200 BU/mL, 2 000 BU/mL and 20 000 BU/mL). Dose was increased only if the previous one rendered a negative response.

Results: A high significant correlation was shown ($r = 0.94$, Spearman) between NPT inverse positive concentration and SPT reaction size in rhinitic patients. Concerning the diagnostic accuracy of the NPT, the following values were calculated: 70%, 100% and 85%, for sensitivity, specificity and efficiency, respectively, as referred to SPT. There were registered 20 adverse reactions, all located in the respiratory tract, corresponding to allergic rhinitis (60%) and asthma (40%).

Conclusions: NPT with a standardized allergen extract of *Blomia tropicalis* can be used as an accurate test for the diagnosis of allergic rhinitis provoked by sensitization to this mite.



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P8.9 - Diagnostic efficacy of the Skin-Prick-Test, using allergen extracts of House Dust Mites in Cuban allergic children

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The Skin Prick Test (SPT) efficacy using VALERGEN allergenic extracts from the most common House Dust Mites in Cuba, was previously demonstrated in Cuban adult population. However, it is regarded that the relevance of the specific diagnostics, for the etiological management of allergic diseases, is greater during the first years of life. This work aimed to determine the sensitivity and specificity of SPT, using standardized allergenic extracts (VALERGEN, BIOCEN, Cuba) of *Dermatophagoides pteronyssinus* (Dp), *D. siboney* (Ds) and *Blomia tropicalis* (Bt) in Cuban children. A controlled trial was carried out on 50 allergic symptomatic children and 50 non-allergic volunteers, ages 2-15 years. SPT was performed according to Nordic Guidelines. Two different doses were used (20 000 and 2000 UB/ml) in order to find the optimal one. The highest sensitivity values were obtained for the highest dose: 92%, 86% and 52% for Dp, Ds and Bt, respectively. The specificity values ranged from 90 to 94%. The mean wheal diameter was similar between the 3 allergens (4.2-5.2mm). The relatively low sensitivity values found for Bt indicate that the allergic symptoms in children should be provoked mostly by the other two mites. Nevertheless, the pooled diagnostic result of the three products, revealed a higher sensitivity value (96%) than for each product individually, keeping the same specificity. No side reactions were observed. It is concluded that SPT using VALERGEN extracts, is effective and safe as a diagnostic tool in Cuban children, and it is recommended the use of a whole panel of mite extracts, simultaneously.



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P8.10 - Diagnostic accuracy of the Skin Prick Test with standardized allergen extracts of domestic mites in children of a Cuban inner province.

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Introduction: The allergen-specific diagnosis can be accomplished readily by Skin Prick Test (SPT). The diagnostic accuracy of SPT in children using standardized allergen extracts of the most locally relevant domestic mites: *Dermatophagoides pteronyssinus* (Dp), *D. siboney* (Ds) and *Blomia tropicalis* (Bt) has been assessed before in Cuba only in the capital city, located in a coastal area. The objective of this work was to evaluate the diagnostic accuracy of these products in a paediatric population of the biggest Cuban inner city: Camaguey, 600km far away from Havana.

Materials and Methods: An open controlled clinical trial was carried out. Both, the clinically allergic and control groups included 70 children from 6 to 14 years, who were patients at the "Eduardo Agramonte Piña" Pediatric Hospital. Allergic patients were selected according to clinical symptoms of rhinitis or asthma associated to House Dust. SPT was performed in both groups with standardized allergen extracts VALERGEN-DP, DS and BT, respectively (BIOCEN, Cuba) at a concentration of 20 000 BU/mL. Wheal diameter (d) was measured. Tests were considered positive for $d > 3\text{cm}$.

Results: The diagnostic Sensitivity and Specificity values for Dp were: 99% and 97%, respectively. For Ds the sensitivity was 94 % and specificity 97%. Bt was positive in 89% of allergic patients and in 4% of the non-allergic group for a Sensitivity value of 84% and a Specificity of 94%. The positive predictive value for Bt was 92 %, and the negative one was 86 %.

Conclusions: The SPT using allergen extracts of Dp, Ds and Bt showed to be sensitive and specific in children of the Cuban inner city, with a similar diagnostic accuracy as reported for coastal areas. Sensitization to Bt appears to be slightly less associated to clinical respiratory allergy to House Dust.



P8.11 - Plasmid vectors for the expression of complete allergen-specific human IgE, IgG1 and IgG4 antibodies

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Introduction: Allergen-specific immunotherapy induces IgG antibodies which can block the IgE recognition of allergens, the central event in allergic inflammation. Our aim was to construct plasmid vectors suitable for the expression of human allergen-specific monoclonal IgE, IgG1 and IgG4 antibodies to investigate their relevance in defined models of allergic inflammation.

Methods: The plasmid vector pLNOH2 was engineered to express human Phl p 5-specific IgE, IgG1 and IgG4 antibodies by grafting of genomic DNAs encoding constant regions of IgE, IgG1 and IgG4. These constructs together with a plasmid expressing the light chain of the Phl p 5-specific IgE Fab were co-expressed in COS-7 cells. The Phl p 5-specific monoclonal antibodies (rhuMabEP5, rhuMabG1P5, rhuMabG4P5) were analyzed regarding allergen and isotype-specificity by ELISA. The allergenic activity of Phl p 5 was studied by loading RBL-2H3 cells expressing human-Fc ϵ RI with rhuMabEP5 and Phl p 5.

Results: We report the construction of vectors for the expression of human Phl p 5-specific IgE, IgG1 and IgG4 with identical paratopes. rhuMabEP5 cross-reacted with group five allergens in natural grass pollen extracts and was used in effector cells assays showing that the allergenic activity of Phl p 5 is independent of allergen-oligomerization.

Conclusion: The described expression vectors should allow the production of functional human monoclonal IgE, IgG1 and IgG4 antibodies of any desired specificity and will represent useful tools to investigate mechanisms underlying IgE-mediated allergies and to develop diagnostic and therapeutic strategies for allergic diseases.

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P5.6 - Comparison between *Blomia tropicalis* and *Dermatophagoides pteronyssinus*-induced murine models of allergic asthma

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Introduction: The levels of allergic asthma have increased significantly in recent decades (Allergy.64:1083-1092, 2009). The mites *Dermatophagoides pteronyssinus* (Dp) and *Blomia tropicalis* (Bt) are the most important sensitizing agents related to asthma causation (Allergy Asthma Proc. 30: 166-70 2009). Studies have been shown different patterns of immune response to these mites in humans. In addition, few studies have been done in experimental models of asthma comparing these two allergens (J Clin Immunol. 24:533-41, 2004.).

Objective: To evaluate the murine models of experimental allergic asthma to the mites Bt and Dp.

Methods: AJ mice were sensitized with 10µg of Bt or Dp adsorbed on aluminum hydroxide on days 0 and 7. On days 8,10,12,14 they were challenged intranasally with 10µg of Bt or Dp diluted in saline. A group of animals received saline as negative control. On day 15 the animals were sacrificed and BAL was collected for determination of cellularity, cytokines and eosinophilic peroxidase (EPO). Lung tissue was collected for histopathology.

Results and Conclusions: Eosinophils were more observed in animals sensitized with Bt and neutrophils was more observed in animals sensitized with Dp. Quantification of EPO in BAL and lung was observed in both groups, being higher in animals sensitized with Bt. The Bt animals produced more IL-5 in BAL. Hystopathology evaluation of the lung showed a higher density of cells in animals sensitized with Bt. These results show that sensitization by these mites, modulates differently the mice immune system. We also showed higher eosinophil numbers in Bt-sensitized animals and neutrophils in Dp-sensitized animals, contrasting with Sato e cols. (2004), work which found more eosinophils in Dp and neutrophils in Bt.



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P8.12 Evaluation of peanut allergenic extracts for skin prick test in a Cuban population

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Introduction: In adulthood the most common food allergen is peanut. Skin Prick Test (SPT) can be a valuable tool for the diagnosis of food allergy, particularly when standardized extracts are used. We aimed to evaluate the diagnostic accuracy and clinical safety of peanut allergenic extracts for SPT, in a Cuban population, calculating the specificity, sensitivity and effectiveness related to clinical manifestations of the disease, as well as, to identify the appropriate protein concentration for the best diagnosis of food allergy and the possible adverse reactions.

Methods: Thirty patients were included with the clinical diagnosis of peanut food allergy, and 30 supposedly non-allergic. All individuals were subjected to SPT with dialyzed peanut extracts prepared either from raw or roasted peanut, respectively.

Results: Of the total number of patients clinically allergic to peanut, 53.3% were positive to the roasted peanut extract and only 43.3% to the raw peanut extract. In the control group all the patients were negative for both extracts ($p < 0.05$). The specificity for both extracts and dilutions (0.5 mg/ml and 1 mg/ml total protein) was 100%. The roasted peanut extract at 1 mg/ml showed the highest effectiveness value: 72.7%. The analyzed sera of positive patients recognized predominantly a band of approximately 17 kDa, by IgE Western Blotting, compatible with the molecular weight of the Ara h 2 and Ara h 6 major allergens of this plant.

Conclusion: It was concluded that the toasted peanut extract showed the highest sensitivity and the better protein concentration for SPT was 1 mg/ml. SPT was specific and safe. Nevertheless a large proportion of patients showed false-negative results indicating that SPT can not replace completely the clinical assessment.