

# IDENTIFICATION OF A NOVEL *Mycobacterium tuberculosis* 24 KDA SECRETED LIPOPROTEIN RELEVANT TO CELLULAR IMMUNITY AND VACCINE DESIGN

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## Introduction

Tuberculosis is a major health problem, and no effective vaccine for endemic regions is available (1). The objective of the study was to identify and characterize novel mycobacterial protein antigens with relevance to cellular immune responses and subunit vaccine design.

## Materials and Methods

Identification of a novel mycobacterial protein antigen was performed by screening of a genomic *Mycobacterium leprae*  $\lambda$ gt11 DNA library by *M. leprae* reactive human CD4+ T cell clones as probes. T cell clones were established from healthy donors immunized with killed *M. leprae* by the limiting dilution technique. DNA sequencing: dideoxy chain termination method. T cell epitope mapping was based on screening antigen reactive T cell clones for proliferative response against synthetic peptides. MHC restriction analysis was performed by combining results from HLA typing, blocking with anti HLA antibodies and panel studies.

## Results

We present the complete amino acid sequence of a novel *M. tuberculosis* protein antigen common to *M. leprae* and the vaccine strain *M. bovis* BCG with a

deduced molecular weight of 24.1 kD. The 233 amino acid long reading frame contains a signal peptide sequence for secretion and a consensus motif for lipid conjugation. The complete gene was identified within the Sanger Centre data base (UK) by searching with a 123 bp C terminal DNA sequence isolated from a genomic *M. leprae*  $\lambda$ gt11 library by expression of an epitope recognized by human T cell clones. The same T cell clones were used to confirm the deduced molecular mass in a T cell Western analysis as well as define the peptide epitope recognized (LVRASIDL) and its MHC restriction (HLA-DRw53). In addition, we have shown that such T cell specificities are present in the peripheral memory T cell repertoire against mycobacteria.

## Discussion

Several lines of evidence favour secreted protein antigen from *M. tuberculosis* as subunit vaccine candidates (2). The consensus sequences identified suggest that the mature protein is a secreted antigen probably located to the mycobacterial cell wall as a lipoprotein. In conclusion, we have identified and described the primary structure of a novel secreted *M. tuberculosis* lipoprotein antigen with relevance to human cellular immunity and subunit vaccine design.

Trabajos seleccionados del Congreso Biotecnología Habana '97, diciembre 1-6 de 1997

Selected papers from Congress Biotecnología Habana '97, December 1-6, 1997.

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## USE OF THE P64K PROTEIN AS A CARRIER FOR VACCINE FORMULATIONS

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## Introduction

Several proteins have been used as carriers to activate thymus-dependent immunity against poor immunogenic peptide and polysaccharide (PS) antigens, but only group B meningococcal outer membrane proteins (OMP), tetanus toxoid (TT), diphtheria toxoid (DT)

and its mutant CRM 197 have reached significant application on vaccine development.

Toxoids carry lot-to-lot variations in physical and chemical properties as a consequence of detoxification process, introducing variability into the manu-

facturing process. The OMP is an expensive and complex mixture of antigens of undefined composition, which constitutes an additional difficulty for consistency of the conjugation process.

The humans are world-wide immunised with DPT vaccine early in the infancy, and receive several booster doses with TT and DT in their life. Therefore, they are primed with these antigens. In Cuba and in several Latin-American countries, the children are also immunised with the antimeningococcal vaccine from 2-3 months of age.

Regarding the immunisation status of the vaccine recipients, the use of the same proteins as carriers for multiple PS and peptide conjugates may let to excessive antitoxin production and epitope suppression of anti-PS or anti-peptide responses (1-3).

According to this, it is desirable the evaluation of new antigens to be used as carrier proteins for conjugate vaccines under development.

In our laboratory, we have obtained the recombinant P64k protein expressed at high level in *Escherichia coli*. The P64k (594 amino acid residues) is a physical-chemical well-characterised membrane protein of *Neisseria meningitidis*, with known three-dimensional structure and epitope mapping. The recombinant protein has been recognised, on ELISA and western-blot, by sera of convalescent Cuban, Brazilian and Norwegian patients, and along with the immunogenicity of the natural protein in humans, the recombinant P64k also generates high antibody titers in mice, rabbits and monkeys (4).

Here it is shown the progress towards the application of the P64k as a vaccine carrier in different vaccine formulation.

## Materials and Methods

### Antigen conjugation

Peptides conjugates were made by the glutaraldehyde conjugation method (5) and PS were conjugated by the carbodiimide method (6) using the adipic acid dihydrazide (ADH) as the spacer arm.

## Results and Discussion

### Peptide conjugates

Peptide D20-3 (22 amino acid residues) from the membrane protein of Dengue virus, conjugated to different carrier proteins was used to immunise Balb/c mice. After the second dose, the IgG level against dengue was similar using TT and P64k as carriers and significantly higher than the titer obtained with BSA-P64k conjugate or the peptide alone.

Decapeptide GnRH (human chorionic gonadotropin release hormone) conjugated to BSA, P64k, CSP and peptides carrying T helper epitopes from

Conjugate	Epidim weight / Animal weight (g)	Testicle weight / Animal weight (g)	Prostate weight / Animal weight (g)
poly GnRH-P64k	2.85 ± 0.56	2.93 ± 0.34	0.59 ± 0.17
poly GnRH-BSA	4.74 ± 2.34	8.78 ± 8.69	2.11 ± 1.94
GnRH-DT	6.73 ± 2.42	13.30 ± 7.20	4.47 ± 2.90
GnRH-TT	4.64 ± 1.36	7.34 ± 3.79	2.32 ± 1.49
GnRH-CSP	3.52 ± 1.60	6.50 ± 6.03	1.29 ± 0.96
CSP-GnRH	4.59 ± 1.23	8.29 ± 2.23	4.20 ± 3.47
GnRH-BSA	6.56 ± 1.88	17.00 ± 11.00	6.32 ± 6.47
Placebo	7.62 ± 2.09	20.80 ± 2.50	11.60 ± 6.90

tetanus and diphtheria toxins showed significantly higher inhibition of growth in the pigs epidim, testicle and prostate, when P64k was used as a carrier. See Table.

Epidermal growth factor (EGF) conjugated either to TT or P64k elicited 90 % seroconversion in NMRI mice, while with EGF alone only 20 % of animals seroconverted. The TT-P64k conjugate also showed an increase in survival times in mice transplanted with EAT cells compared with the control mice treated only with Freund's adjuvant (7).

African green monkeys immunised subcutaneously with two doses of EGF-P64k conjugate, adjuvated, with aluminium hydroxide, developed 2-10 fold higher antibody titers than EGF conjugated to monoclonal antibodies T3 and B7, used as carrier proteins.

### Polysaccharide conjugates

Conjugates of the P64k linked to the capsular PS of *N. meningitidis* group C (PS-C), obtained both by protein or PS-C activation with carbodiimide, were compared immunising Balb/c mice. The PS-C alone and the Cuban antimeningococcal vaccine VA-MENGOC-BC (containing PS-C and OMP complex of *N. meningitidis* group B) were used as controls. All the preparations gave significant but different titers against group C strain and showed bactericidal activity.

### Clinical trial

A pilot clinical trial was conducted including two groups of 5 patients with cancer immunised with two doses of EGF-P64k and EGF-TT conjugates. All patients seroconverted and the antibody titers were similar in both group of patients.

In conclusion, the P64k is at least as good carrier as the antigens currently used in vaccine formulations, lacking its inhibitory effect on the generation of immunological response and manufacturing inconveniences.

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