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Recombinant Production of Carrier Proteins

Well-Characterized CRM_{197} Essential for the Development of Many Conjugate Vaccines

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Within the rapidly growing vaccine space, conjugate vaccines—polysaccharide antigens covalently linked to carrier proteins—have been shown to be effective against several bacterial pathogens.

Conjugate vaccines use carrier proteins to increase the immunogenicity of antigens (e.g., peptides, oligosaccharides, polysaccharides) and other haptens. These antigens are poor immunogens unless conjugated to proteins.

The role of the carrier protein is to enhance immunogenicity by providing T-cell epitopes via MHC Class II presentation to T-helper cells. Carrier proteins both increase the magnitude of the immune response as well as engender B-cell "memory."

The number of carrier proteins used in licensed vaccines is relatively limited and includes tetanus toxoid, diphtheria toxoid, CRM_{197} (a nontoxic mutant of diphtheria toxin), *Haemophilus influenzae* protein D, and *Neisseria* outer membrane protein. Access to clinically-proven, safe, and efficacious carrier proteins is critical for research in the field of conjugate vaccines.

CRM_{197}, which has a single amino acid substitution of glutamic acid for glycine, is a well-characterized carrier protein and is utilized in a number of approved conjugate vaccines for diseases such as meningitis and pneumococcal bacterial infection. It is best known for its use in Pfizer's conjugate vaccine for pneumococcal infection, Prevnar®, as well as HibTiter® (*Haemophilus influenzae* b) and Novartis' meningococcal vaccine Menveo®.

Improved Recombinant Expression

CRM_{197}, which has been traditionally produced by fermentation of *Corynebacterium diphtheriae* C7 (β_{197}) tox (-) strain, is secreted into the extracellular medium, recovered after separation of biomass, and purified using filtration and chromatographic steps. Typical expression levels tend to be low (~200 mg/L).

The use of a native pathogenic strain presents a further complication for production on a larger scale since the biosafety level 2 (BSL-2) nature of this organism requires a production facility with the appropriate level of containment.

The crystal structure of diphtheria toxin (DT) reveals that the molecule consists of three domains: a catalytic domain (fragment A), a transmembrane domain, and a receptor-binding domain (both in fragment B). Mild trypsinization and reduction of the native molecule in vitro results in two fragments, A and B. Fragment A is a NAD+ binding enzyme that inhibits protein synthesis.

A similar proteolytic cleavage, or nicking, occurs in vivo. After nicking, the chains are still held together by a disulfide bond linking the two fragments. CRM_{197} is similarly highly susceptible to proteolytic cleavage. Consequently, high level production of high-quality CRM_{197} is extremely challenging.

Pseudomonas fluorescens is a useful host for high-level production of recombinant proteins. Utilizing this expression platform, Pfenex has developed a process to produce high levels of CRM_{197} using a BSL-1 *P. fluorescens* production strain and also filed a Biologics Master File at the U.S. FDA.

Pfenex scientists employ a high-throughput, combinatorial biology- driven approach to generate and rapidly screen up to 1,000 expression strain constructs in approximately four to five weeks.

The nature of the *P. fluorescens* organism, molecular biology toolbox, and work flow resulted in a production strain achieving expression titers of CRM_{197} in excess of 10 times that achieved with the native organism.

In addition, by exploiting a range of secretion leaders and periplasmic expression, inclusion body formation was avoided, leading to expression of highly pure soluble CRM_{197}, which is identical to the native form, making it a preferred solution for conjugate vaccine development.

Comparability and Bioequivalence Studies

A series of comparability studies were performed, comparing both the structure and function of *P. fluorescens* recombinant CRM_{197} to the protein produced in the native organism, *C. diphtheriae*. The characteristics of CRM_{197} produced in *P. fluorescens* appear identical to that made by the native organism. Conjugate Epitope density, measured by acid hydrolysis and RP-HPLC, showed that the conjugation achieved with recombinant CRM_{197} is indeed comparable to that obtained from the native organism. Peptide-mass fingerprinting after digestion with LysC (*Figure 1*) shows a highly similar pattern of masses for the resulting peptides.

Additional bioanalytical characterization studies demonstrate comparable secondary structure of the recombinant CRM_{197} to that of the native form. Circular dichroism spectra (*Figure 2*) are comparable to the reported spectra of native CRM_{197}. K2D analysis of the spectra indicates that the secondary structure contains 31% alpha helix, which is consistent with x-ray crystallographic analysis of diphtheria toxin.

In vivo Comparability Analysis

In addition to comparative physicochemical analysis of the recombinant CRM_{197} and the native form, in vivo studies were also performed. Conjugates made by chemically linking recombinant CRM_{197} produced in *P. fluorescens* to pneumococcal capsular polysaccharides induced antibody levels similar to that of Prevnar, further supporting suitability of the *P. fluorescens* product as a clinically useful vaccine carrier protein.

The design for this in vivo study contained two arms. Arm 1 was injected with Prevnar, while Arm 2 was injected with a 10-valent pneumococcal polysaccharide-CRM_{197} conjugate vaccine currently under development by an undisclosed vaccine company. The results (*Figure 3*) demonstrated comparable immune responses, as indicated by IgG and opsonophagocytic activity (OPA) assays.

This tutorial has demonstrated that *P. fluorescens* CRM_{197} is similar in both structure and function to the protein produced in *C. diphtheriae*. The availability and use of recombinant CRM_{197} is currently allowing researchers to actively explore vaccines that previously would not have been possible or economically feasible due to high internal development costs and long timelines required to produce this complex protein.

The use of the latest advances in recombinant protein expression technology for the production of well-established carrier proteins will enable the rapid translation of novel conjugate vaccine-based product concepts into the clinic. The result will strengthen the overall vaccine research industry by allowing ideas to be validated in a shorter time span.

Currently, Pfenex CRM_{197} is being used by several companies including the Serum Institute of India in their conjugate vaccine programs. Multiple *P. fluorescens* CRM_{197}-based conjugate vaccines are currently in various stages of clinical trials in the U.S., Europe, and Asia.

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