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Clinical validation of the "*in silico*" prediction of immunogenicity of a human recombinant therapeutic protein

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Abstract Antibodies elicited by protein therapeutics can cause serious side effects in humans. We studied immunogenicity of a recombinant fusion protein (FPX) consisting of two identical, biologically active, peptides attached to human Fc fragment. EpiMatrix, an *in silico* epitope-mapping tool, predicted promiscuous T-cell epitope(s) within the 14-amino-acid carboxy-terminal region of the peptide portion of FPX. On administration of FPX in 76 healthy human subjects, 37% developed antibodies after a single injection. A memory T-cell response against the above carboxy-terminus of the peptide was observed in antibody-positive but not in antibody-negative subjects. Promiscuity of the predicted T-cell epitope(s) was confirmed by representation of all common HLA alleles in antibody-positive subjects. As predicted by EpiMatrix, HLA haplotype DRB1*0701/1501 was associated with the highest T-cell and antibody response. In conclusion, *in silico* prediction can be successfully used to identify Class II restricted T-cell epitopes within therapeutic proteins and predict immunogenicity thereof in humans.

Introduction

Virtually all therapeutic proteins induce some level of antibody response. Protein-induced immune responses can vary from low-level, low-affinity and transient IgM antibodies to high-level, high-affinity IgG antibodies. Antibody-related clinical sequelae also vary from none to severe, and can occasionally be associated with life threatening side effects [1]. Antibody analyses from a number of clinical studies strongly suggest that serious side effects are associated with high levels of IgG antibodies [2–6]. Such an antibody response is T-lymphocyte driven and includes isotype switching and affinity maturation [7]. T-helper cells, a subset of T-lymphocytes that specifically recognize epitopes presented by antigen presenting cells (APCs) in the context of MHC (major histocompatibility complex) Class II molecules, are the major drivers of the mature antibody response. Protein therapeutics that express MHC Class II

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restricted T-helper epitopes are likely to elicit more frequent and mature antibody responses with IgG as predominant isotypes. These T-helper epitopes can be represented as linear sequences comprising 8 to 12 contiguous amino acids that fit into the MHC Class II binding groove. Over the last 10 years, a number of computer algorithms have been developed and used for detecting Class II epitopes within protein molecules of various origins (De Groot and Berzofsky [8] and the accompanying issue of *Methods* [9]). Such "*in silico*" predictions of T-helper epitopes have already been successfully applied in attempts to increase immunogenicity and efficacy of vaccines [10–12].

The relationship between T-cell epitopes and immune response has also been the subject of a number of investigations in the field of protein therapeutics. In some cases, therapeutic proteins have also been screened for T-helper epitopes in an attempt to evaluate their potential immunogenicity [13–16]. Obviously, reliable *in silico* prediction of helper epitopes would be of significant value in development of protein therapeutics. Such predictions would make it possible to meaningfully rank candidates at the pre-clinical stage of drug development or to reengineer proteins to make them less immunogenic. Furthermore, individuals at higher risk of developing T-cell-driven antibody responses to the protein therapeutic could be identified prospectively using HLA (human leukocyte antigen) typing, if certain HLA can be associated with T-cell response and higher neutralizing antibody titers, as recently described by Barbosa et al. [14]. The use of these methods in the context of clinical trials of protein therapeutics is rather recent and deserves further exploration.

In this study, we describe the *in silico* evaluation of a protein therapeutic in terms of its T-helper epitope content. At the time of the epitope analysis, this protein had also been administered to human subjects in a clinical trial. We were therefore, able to test subsequent immune responses *in vivo* and compare them with the EpiMatrix predictions of immunogenicity.

Materials and methods

Human fusion protein

A recombinant human fusion protein (FPX) consisting of a human Fc fragment fused with two identical 24-amino-acid peptides was generated as described previously [17]. Briefly, phage display peptide libraries (Dyax Corp.) were employed and panned against the targets. After several rounds of selection, the resulting target specific-binding clones were recovered and converted into peptide-Fc fusion proteins (by expressing the active peptides in *Escherichia coli* as fusions to the Fc portion of human IgG1). One of the peptide-Fc fusion proteins (termed FPX) with two identical 24-amino-acid peptides attached to the amino-terminal end of the human germ line Fc-γ fragment was chosen for the study.

Generation of FPX peptide fragments

Solid-phase peptide synthesis

In addition to the whole fusion protein, three peptides spanning amino acids 1–10 (aa 1–10), 11–24 (aa 11–24) and 1–24 (aa 1–24) of the FPX peptide portion were synthesized. These peptide fragments were prepared at 0.2-mmol scale on a Symphony peptide synthesizer (Protein Technologies Inc., Tucson, AZ) or Odessey peptide synthesizer (CEM, Matthews, NC) employing Fmoc/OtBu protection strategy. All Fmoc amino acids were purchased from Midwest Biotech (Fishers, IN). Preloaded Fmoc-Glu (OtBu), Fmoc-Trp (Boc), Fmoc-Pro, Fmoc-Leu HMP resins were purchased from Midwest Biotech (Fishers, IN).

Purification

Crude peptide and HPLC pooled fractions were analyzed by analytical RP-HPLC with a Vydac (Hesperia, CA) 214TP™ C18 column using linear gradients of 0–60% ACN in 0.1% aqueous TFA over 30 min, and a flow rate of 0.6 ml/min. The large-scale RP-HPLC was carried out with a Vydac 218TP C18 column (Vydac) by using a linear gradient of 5 to 40% ACN in 0.1% aqueous TFA over 50 min with a flow rate of 20 ml/min.

Mass spectral analysis

HPLC-MS was performed with an API 150 (PerSeptive Biosystems) in conjunction with Waters analytical HPLC system (Waters Corporation, Milford, MA) by using YMC ODS-AQ C18 column, with a linear gradient of 0% to 60% ACN in 0.1% aqueous TFA over 12 min with a flow rate of 0.6 ml/min.

Human subjects and FPX dosing

The primary objective of this study was to assess the safety and tolerability of FPX. The study included standard safety monitoring (e.g., collection of adverse experience reports; repeated clinical chemistry, hematology, urinalysis, electrocardiograms and other assessments typical for this stage of development) as well as assessment of the pharmacokinetic properties of FPX. Healthy human female and male subjects were dosed with FPX in a Phase 1, single dose, and placebo-controlled, randomized, blinded, sequential dose escalation study. All subjects provided informed consent for participation in the study including HLA typing, anti-FPX antibody assays and T-cell assays. The study enrolled 76 subjects aged 18 to 55 years. In total, 36 subjects received a single dose of FPX intravenously and 40 subjects received FPX subcutaneously. Blood samples for analysis of anti-FPX antibodies were collected from all subjects on day 1 prior to dosing and on day 42 after dosing. In addition, eleven antibody-positive and 4 antibody-negative subjects agreed to provide additional blood samples for the *in vitro* PBMC studies with the FPX peptide fragments and HLA typing at the 6 months follow-up visit.

Prediction and characterization of T-helper epitopes

The peptide portion of the FPX fusion protein (FPX peptide) was screened for potential immunogenicity using previously published EpiMatrix System [18,19]. Briefly, the 24-amino-acid sequence was parsed into overlapping 9-mer frames where each frame overlaps the last by eight amino acids. Each frame was then scored for predicted binding to each of eight common Class II HLA alleles (DRB1*0101, DRB1*0301, DRB1*0401, DRB1*0701, DRB1*0801, DRB1*1101, DRB1*1301, and DRB1*1501). Due to their prevalence and their difference