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Functional Deimmunization of Interferon Beta-1b by Identifying and Silencing Human T Cells Epitopes

Amin Moradi Hasan-Abad[1], Elham Adabi[1], Esmaeil Sadroddiny[1], Mohammad Reza Khorramizadeh[1], Mohammad Ali Mazlomi[1], Saeed Mehravar[2], and Gholam Ali Kardar[1,3]

[1] Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran
[2] Department of Epidemiology and Statistics, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
[3] Immunology, Asthma and Allergy Research Institute, Tehran University of Medical Sciences, Tehran, Iran

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ABSTRACT

Interferonbeta-1b (IFNβ-1b) developed as therapeutic protein for the treatment of multiple sclerosis (MS). Studies have been shown that Long-term usage of this protein can lead to the development of anti-drug antibodies (ADAs) and this phenomenon cause total loss or reduced efficacy of IFNβ-1b. The aim of this study was to predict and silence IFNβ-1b T-cells epitopes by in silico methods and genetic engineering.

Based on bioinformatics studies we identified optimal sets of conservative point mutations for eliminating T-cells epitopes in IFNβ-1b protein. Four synthetic genes with desirable mutation constructed and PET26b+ was used as an expression vector in E. coli. The expression of this proteins confirmed by SDS-PAGE and Western blotting, consequently, IFNβ-1b proteins was purified by His-tag chromatography. To determined activity of mutants' variants anti-proliferative and anti-viral activity compared to wild form was evaluated using MTT assay in A549 and Vero cells lines respectively. Also the immunogenicity of mutant proteins compared with Betaseron measured in BALB/c mice.

The in vitro bioactivity analysis demonstrated that functional activities of all mutant proteins were maintained and is the same as biological activity of Betaseron. Pharmacokinetic studies suggest that, in engineered proteins that contain substitution of Histidine to Glutamic Acid at position 131 (mut 2 and mut 1+2) antibodies response reduced by about 50%, as compared to that for Betaseron.

Computational analysis expedites identification and prediction of epitopes in therapeutic protein, therefore, we used immunoinformatic tools for modification of dominant T-cell epitope in IFNβ-1b protein, and this strategy has capacity to create proteins which have naturally reduced immunogenicity.

Keywords: Anti-drug antibodies; Computational protein design; Deimmunization; Interferon-beta-1b; T-cell epitope; Therapeutic protein

Corresponding Author: Gholam Ali Kardar, PhD; Immunology, Asthma and Allergy Research Institute, Tehran University of Medical Sciences, Tehran, Iran. Tel: (+98 21) 61472147, Fax: (+98 21) 6642 8995, E-mail: gakardar@tums.ac.ir

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NTRODUCTION

Human interferon beta (hIFNβ) is a cytokine that produced in response to viral infections and possess a wide range of anti-inflammatory properties. Recombinant human IFNβ (rhIFNβ) was the first effective compound in Relapsing-Remitting MS (RRMS). The mechanism of rhIFNβ action in MS patients is very complex and multifactorial and up to now remained unknown. It was suggested that rhIFNβ might down regulate the expression of pro-inflammatory cytokines and simultaneously increase expression of anti-inflammatory agents. Moreover, rhIFNβ treatment may limit the trafficking of inflammatory cells across the blood-brain barrier.[1] Overall rhIFNβ products are effective in MS treatment and decrease the number and severity of relapses, slow down the progression of MS and improves patients' Condition.[2] Since 1990s, three types of rhIFNβ have been approved and there are in the market: Avonex (Biogen Indec, USA) and Rebif (Merck Serono, USA), Betaferon/Betaseron (Schering AG, Germany and Berlex, USA). rhIFNβ-1a, the effective substance of Avonex and Rebif, are produced in ovarian cells of the Chinese hamster. Its amino acid sequences is quite identical to the hIFNβ protein and is glycosylated. rhIFNβ-1b, the effective substance in Betaferonis produced in E. coli, this form is non-glycosylated and has a Met-1 deletion and a Cys-17 to Ser substitution when compared to the hIFNβ protein. Due to a stabilizing effect of the glycosylation on protein structure, rhIFNβ-1b is less stable in solution and is very prone to aggregation than rhIFNβ-1a. Also, its biological activities is ten times less than rhIFNβ-1a.[3] Therapeutic proteins have properties that make them attractive as new drugs, but their applications can be limited by the appearance of Anti-Drug Antibodies (ADAs), these ADAs may cause a spectrum of clinical adverse effects including neutralizing protein biological activity and loss of efficacy, accelerating drug clearance that lead to shorten their half-life in the blood circulation, and cause life-threatening immune responses from anaphylactic reactions in rare cases to more common delayed infusion-like reactions.[4-7]All three forms of commercially available rhIFNβ leading to induction of the ADAs response in MS patients. Betaferon as the most immunogenic form of rhIFNβ induced ADAs in up to 90% of Patients. Other immunogenic product forms is Rebifwith 20-40% and Avonex with less than 20% of ADAs positive patients.[8] Several factors are involved in the high immunogenicity of Betaferon, these factors include the primary sequence of the protein, lack of Carbohydrate chain that shields the epitopes, amino acid modifications and method of administration including the highest dose and frequency of injections compared to the other drugs forms.[9] Protein engineering is a rapidly growing field of biotechnology science that gives the ability of changing proteins structure to achieve desired performance. The strongest and most extensive protein engineering methods is site directed-mutagenesis (SDM) that leading to exact changes in specific amino acids in a protein at DNA levels. The specific amino acids would be identified based on the three-dimensional structure of proteins that obtain through X-ray diffraction and NMR techniques.[10] Previous studies had shown many performed efforts to improve the pharmacological properties of hIFN-β. Amartya et al improved the physical and functional properties of IFNβ-1b compounds by PEGylation strategies, employment of -poly(ethylene glycol) polymers that bioconjugate to IFNβ-1b, leading to better solubility, less aggregation, prolonged blood circulation and a decreased immunogenicity response versus modified protein.[11] In 2003 a new variant of hIFN-β with improved solubility were produced that enhanced hydrophilic property of the protein surface though exchange of hydrophobic amino acids (Leu5, Phe8, Phe15, Leu47, Phe50, Leu106, Phe111, Leu116, Leu120, Phe156) with one hydrophilic amino acid that contains hydroxyl groups such as Ser, Thr and Tyr.[12] N-terminal replacement in amino acids 1 to 28 of hIFN-β with the same amino acid position of interferon - alpha, enhanced antiviral, antiproliferative and immunomodulatory property of this protein.[13] In another study has been mentioned changing asparagine 25 to aspartate, numbered in accordance with native hIFN-β, through a point mutation increased IFNβ-1b activity and this protein analog was suitable for treatment of MS.[14] Also the unnatural amino acid insertion in hIFN-β structure was carried out to improve physio-chemical properties of this protein.[15] Glycoengineering of hIFN-β and addition one sequences as a glycosylation site, increase the solubility of its and improved biologic activity of the protein.[16,17]The aim of this study was to predict and Silence IFN-β-1b T-cells epitopes by in silico

methods and genetic engineering. We identified two major T-cell epitopes by bioinformatics tools and it seemed possible that we could eliminate them by mutagenesis. Our approach to silence epitopes was to exchange surface-exposed residues that are commonly involved in antibody binding.

MATERIALS AND METHODS

Bioinformatics Methods

T-cell Epitopes Prediction by the Immune Epitope Database and Analysis Resource (IEDB)

Human T-cell epitope prediction carried out by IEDB server (http://www.iedb.org/). After presentation of hIFNβ-1b protein sequence to IEDB tools several regions recognized as a human T-cell epitopes, residues that are important for HLA binding in these regions identified and exchanged to a proper amino acid by point mutation.

B-cell epitopes prediction

New epitope creation in Mutant variants of IFNβ-1b analyzed by B-cell epitope prediction tools. The linear or continuous B-cell epitope prediction was carried out using the online BepiPred-1.0 (http://www.cbs.dtu.dk/services/BepiPred).
Discontinuous B-cell epitope Prediction of IFNβ-1b and mutants based on three dimensional structures of these proteins, performed by DiscoTope Server 2.0(www.cbs.dtu.dk/services/DiscoTope), this software utilizes calculation of surface accessibility of residues and estimated epitope propensity score for each amino acid.

The study was approved by the local ethics committee [N. IR.TUMS.IAARI.REC.1394.28186].

Secondary and Tertiary Structure Prediction

Effect of mutations on protein structure analyzed by structure homology-modelling .Secondary structure prediction PSI-PRED server (http://bioinf.cs.ucl.ac.uk/psipred/) was used to predict and analyze the secondary structure of variants proteins. The 3D structure of the mutants proteins were generated by using the SWISS-MODEL server (https://swissmodel.expasy.org/) and visualized by Rasmol software.[18]

Microorganism strains and Expression Vectors

DH5α and BL21 (DE3) strains of E. coli were used as hosts for cloning and protein expression, respectively. pET26b+ (Novagen, USA) was used as expression plasmids. Four hIFNβ-1b gene variants was designed regarding to codon optimized gene in the previously reported patent.[19] The synthetic hIFNβ-1b (wild) genes sequence modified with substitution of Tyrosine to Serine at position 155 (mut 1), Histidine to Glutamic Acid at position 131 (mut 2) and (mut 1+2) that contain both previously modification. These gens synthesized and inserted into the XhoI and MscI cloning sites of pET-26b+ vector by GenScript Company (USA). E. coli BL21 (DE3) were transformed using CaCl2 method and transformed clones were selected on Luria-Bertani (LB) medium with kanamycin. Plasmid verification was carried out by colony PCR: the PCR reactions were performed with Taq DNA polymerase and forward primer: 5' TAATACGACTCACTATAGGG 3' (T7 promoter), reverse primer: 5' GCTAGTTATTGCTCAGCGG 3' (T7 terminator) as follows: 95°C for 3 min, 95°C for 30 s, 55°C for 30 s, 72°C for 1 min, repeat for 30 cycles; 72°C for 10min, 4°C hold. Sequence of each constructs was analyzed by automated DNA sequencing in GenScript Company (USA).

Protein Induction and Preparation of Periplasmic Protein

Transformed BL21 bacteria was selected and grown in LB medium with 50 µg kanamycin at 37ºC, after optical density between 0.9 -1 cells were induced with final concentration of 0.2 mM isopropyl-b-D-thiogalacto-pyranoside (IPTG) (Fermentas, USA) and protein expression was carried out at 25 ºC for 16-18 hours. In continues cells were harvested by centrifugation at 7000 g for 30 min at 4°C and Periplasmic proteins was extracted through osmotic shock as previously described.[20] Periplasmic proteins dialyzed against D-PBS overnight at 4°C. Final samples that contain hIFNβ-1b and variants analyzed by SDS-PAGE.

Protein Purification

The hIFNβ-1b and other variants proteins in this study contain a C-terminal hexahistidine tag. The amino acid sequence of the tag that appended to the C-terminus of these proteins is LEHHHHHH. The periplasmic solution that contains soluble hIFNβ-1b and variants was clarified over a 0.45 µm filter and

purified by Ni^{+2} affinity chromatography accordingly on Ni-IDA Resin column protocol (Pars Tous, IRAN). Final fractions of chromatography purification were pooled and dialyzed against D-PBS overnight at 4°C. All Proteins sample concentrated by molecular weight cut-off ultrafiltration devices 10 kDa (Sartorius, USA). Protein density were determined by the Bradford's method using bovine serum albumin as standard[21] and hIFNβ-1b and variants confirmed by Western blotting.

UV Spectroscopy

In room temperature or 25°C, UV spectra (λ=250–360 nm) of 200 µg/mL of hIFNβ-1b and variant solutions were recorded at 8.5 mm path length quartz cuvettes using a spectrophotometer (Eppendorf Biophotometer). Corresponding PBS buffer (pH 7.2) was used as a blank. Optical density (OD) of all samples measured in presence or absence of 0.01% (w/v) SDS.[22]

SDS-PAGE and Western blot

The extracted protein solutions of all hIFNβ-1b samples analyzed by SDS-PAGE according to the methods of Laemmli.[23] The purified hIFNβ-1b and variants were combined with a reducing 5x sample-loading buffer (Tris-Glycine 5×SDS sample buffer plus 10% β mercaptoethanol), heated at 95°C for 5 min, and applied to 15% polyacrylamide gels. Gels were stained and analyzed for protein using Coomassie brilliant blue R-250 dye (Merck, Germany). For Western blotting, proteins were electrophoretically transferred to polyvinylidene difluoride membranes (PVDF, Roche, Germany). PVDF membrane which was loaded with the transferred proteins, was blocked with 5% skimmed milk (Sigma-Aldrich, USA) dissolved in PBS containing 0.05% Tween-20. As the primary antibody a 1:2000 dilution of Mouse anti Human IFN beta antibody (Bio-Rad, UA) was used. Goat Anti-Mouse IgG HRP (Abcam, UK) was diluted 1:2000 served as the secondary antibody. The complex was developed by the addition of the horseradish peroxidase substrate diaminobenzidine (DAB, Roche, Germany)

Antiviral Assays of hIFNβ-1b Variants

Vero cells are sensitive to infection with poliovirus.[24] Specific antiviral activity of hIFNβ-1b variants were assessed by using the Vero /Polio virus cytopathic effect assay.[25] Vero cells, African green monkey kidney, (NCBICode: C101, purchased from the Pasteur Institute of Iran) were seed on 96-well microtiter plates (20000 cells/well) in 0.1 mL of complete DMEM medium supplemented with 10% FBS. Vero cells that were treated with Betaseron used as a positive control and non-treated Vero cells used as a negative control, Purified human hIFNβ-1b (wild) and variants added to the Vero cells in serial dilutions, initiating at 25 Units/mL and incubated at 37°C, 5% CO2 for 24 h. polio virus (received from Department of Virology, TUMS, Tehran, Iran) was added to each well (200 pfu/20 µL) at 25°C for 1 h. After virus adsorption, the cell monolayers were washed with PBS to removed remaining virus, and then wells filled with 0.1 mL/well fresh medium. Microplate then incubated at 37 °C for 24 h after addition of 20 µL of 10 mg/mL methylthiazolyldiphenyl-tetrazolium bromide (MTT) stock solution (Sigma, USA) to each well, incubation was continued for 4 h then 100 µL of DMSO (Sigma, USA) was added to each well, and the plate was read at 570 nm in a 96- well plate reader to determine mean OD for each group.

Anti-Proliferative Activity with MTT Assay

MTT assay was used to evaluate the antiproliferative effect of recombinant hIFNβ-1b variants, as previously described.[26] A549, Lung Carcinoma Cell Line, (NCBICode: C137, purchased from the Pasteur Institute of Iran) were plated in 96-well plates at 4000 cells/well, in 100 µL DMEM medium supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and 25 mM of Hepes buffer solution. Commercially available Betaseron used as a positive control and non-treated A549 cells used as a negative control. Purified human hIFNβ-1b variants added to the A549 cells in serial dilutions, initiating at 62.5 ng/mL, which is equivalent to 2000 Units/mL of Betaseron activity, then cells were allowed to grow for 4 days and cell proliferation measured by MTT assay.

Anti-Drug Antibodies Induction in Animal

Female BALB/c mice were purchased from Pharmacology Animal Lab (TUMS, Tehran, Iran). All animal experiments were approved by the animal care committee of the Tehran University of Medical Sciences [IR.TUMS.IAARI.REC.1394.28186]. The female 6-7 week-old BALB/c mice weighing (about

14-16 g) were divided equally into five groups (6 mice per group). Mice were injected with Betaseron, rhIFNβ-1b (wild) or hIFNβ-1b variants at three dose of 1 µg/mL per week for two months. Serum samples were collected via the retro-orbital route, on different days during the 2 treatment months (days 0, 21, 35, 49, 63). To prevent interference of ADA-drug complexes in antibody determination, Serum samples were obtained 72 h after the third weekly administration before the next injection of hIFNβ-1b on Days 21, 35, 49 and 63. After last day (day 63) all mice were sacrificed. Bloods samples were clotted and serum was separated through centrifugation, this serum was stored at −20°C until analysis.

Anti-drug Antibodies Assay

ADAs measured by direct ELISA, enzyme immunoassay plates were coated overnight at 4°C with 2 µg/mL hIFNβ-1b (wild) protein in phosphate-buffered saline (PBS). Plates were blocked using skim milk (Sigma-Aldrich, USA) for 2 h at room temperature on a shaker. After plate washing using PBS containing 0.05% Tween 20 (Sigma-Aldrich, USA) pH 7.4, Serum samples were diluted in 1/100 and add to plates wells, then incubate for 2 h at room temperature on a shaker. Plates were subsequently washed three times then incubated with Goat anti-mouse IgG (Abcam, UK) at a 1/2000 dilution for 1 h. Plates were again washed three times, followed by 1 h incubation with HRP-cogitated rabbit anti- Goat IgG (Abcam, UK) at a 1/2000 dilution. Plates were developed for 15 min using TMB substrate (Thermo Fisher) and were read at a wavelength of 450 nm and a reference wave length of 630 nm by ELISA reader.

Statistical Analysis

All statistical analyses were performed using SPSS 24 software. Pharmacokinetic factors were calculated using Excel. The mean values ± standard deviations (SD) were used to express the results. (Each dilution of IFN was tested in triplicate).

RESULTS

Prediction of T-Cell Epitopes

Since protein drugs are developed, identification and removing of T-cell epitope for decreasing immunogenicity of these new drugs becomes important application of IEDB. T cells epitopes prediction by IEDB tools were shown that there are conserved T cell epitopes mainly in the 121-132 and 151-162 regions of the protein, which had potential for the development of ADAs (Figure 1). Because Peptide sequence in these regions displays a high degree of sequence homology with other animal species such as monkey, mouse and pig (Figure 1) probably this region are very important for IFNβ activity. For elimination of T-cell epitopes we selected amino acids that existed in other animal IFNβ protein that when replaced with residue in hIFNβ-1b, this region of protein would not be recognized as T-cell epitopes by IEDB Tools (Figure 2).

Prediction of B-Cell Epitopes

The B-cell epitope prediction tools were used to analyze effect of mutation on hIFNβ-1b immunogenicity. The linear epitope prediction analysis by BepiPred, predicted these mutation regions recognized by low affinity compare to reference structures (Table 1). Also discontinuous B-cell epitopes analysis using the DiscoTope server show that this region were not predicted as an epitope in comparison with reference protein (Table 2).

Secondary and Tertiary Structure Prediction

The secondary structure of full length of hIFNβ-1b and variants is predicted by PSI-PRED, and the results showed in (Figure 3). The 3D structure of the hIFNβ-1b and variants proteins was successfully modeled using the SWISS-MODEL server and visualized by Rasmol software. The best tertiary models predicted for all proteins, which have five separated helix, are shown in (Figure 4).

Gene Synthesis and Expression Vectors Construction

Four synthetic hIFNβ-1b genes constructed and existent of mutations and identity (absence of unwanted mutations) and integrity of the genes were confirmed by automated DNA sequencing of the recombinant plasmid (data not shown). Finally four expression vector that encodes an N-terminal pelB sequence in frame with the hIFNβ-1b sequence followed by a poly-histidine tag successfully constructed.

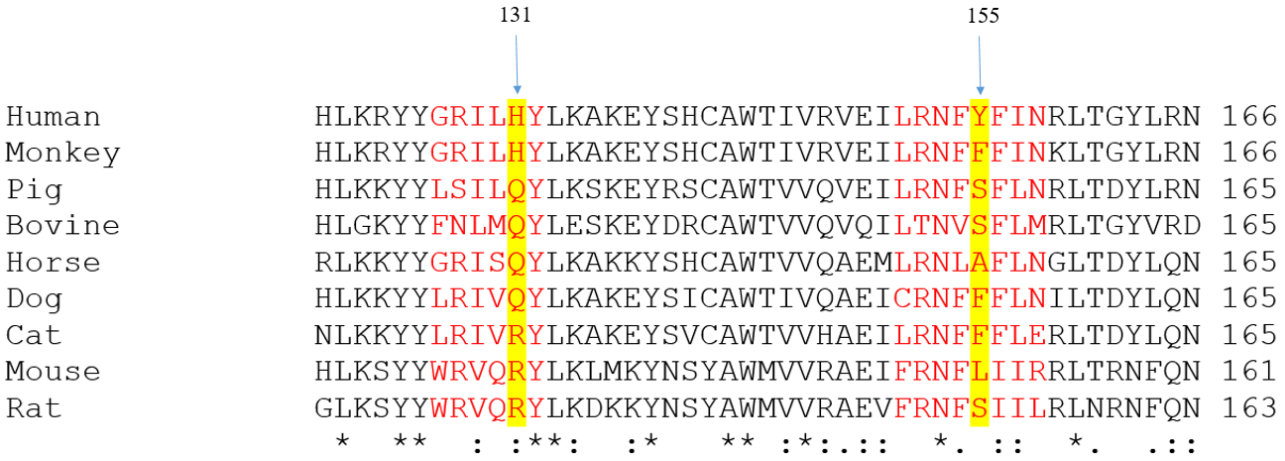

Figure 1. A sequence alignment of C-terminal amino acid residues in mammalian IFNβ proteins in a study on functional deimmunization of interferon beta-1b by identifying and silencing human T cells epitopes. Homology of the sequence of ^{121}HLKRYYGRILHY^{132} and ^{151}LRNFYFINRLTG^{162} residues to that of all known mammalian IFNβ species are shown. Residues that are T cell epitopes are demonstrated in red and residues highlighted in yellow were selected for mutation. The key below denotes conserved sequence (*), semi-conservative mutations (:).

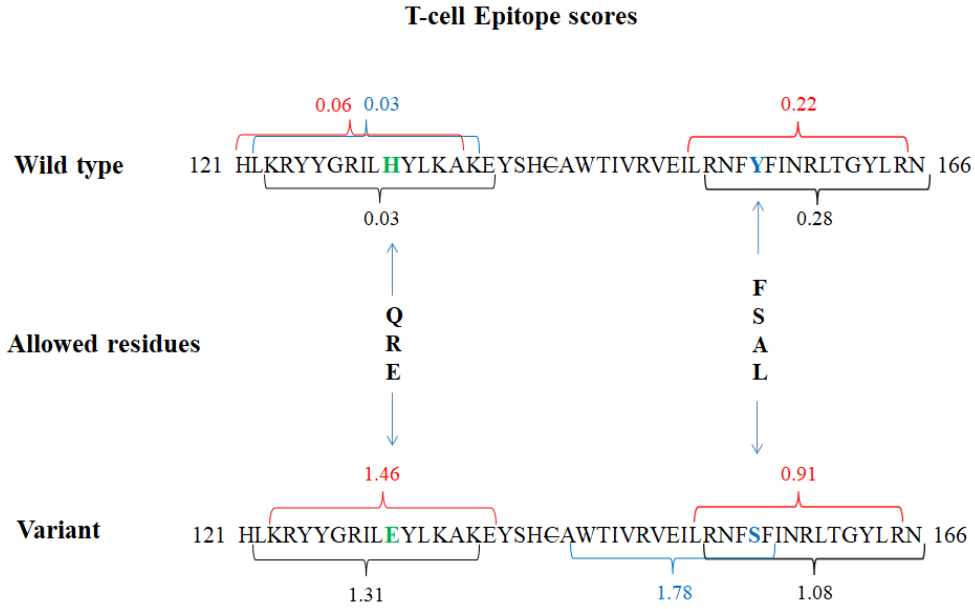

Figure 2. Summarized IEDB-MHC class II binding prediction results for hIFNβ-1b T cell epitopes in a study on functional deimmunization of interferon beta-1b by identifying and silencing human T cells epitopes. We employed IEDB tools to score each 15-mer peptide for potential immunogenicity, in Interferon-β-1b residues 121-166. IEDB server recognized rather than five peptides as immunogenic by several of the 8 most representative MHC II alleles that are related to anti-drug-antibodies (ADAs) response to hIFNβ-1b. We substituted a single amino acid in each position (based on sequence homology) and analyzed sequence and structure of these variants to identify which residues are acceptable for minimizing epitope score.
IEDB: The Immune Epitope Database and Analysis Resource.
***Low percentail scores represent high affinity binding to MHC class II molecules.Y: Tyrosine; H: Histidine; S: Serine; E: Glutamic acid**

Table 1. Prediction of continuous B-cell linear epitopes performed by BepiPred server before and after amino acid substitutions in position 131 and 155 in a study on functional deimmunization of interferon beta-1b by identifying and silencing human T cells epitopes

Amino acid*	position	Method used	Score
HIS	131	bepipred-1.0	-1.269
GLU	131	bepipred-1.0	-0.946
TYR	155	bepipred-1.0	-1.620
SER	155	bepipred-1.0	-1.260

*Tyr: Tyrosine; HIS: Histidine; SER: Serine; GLU: Glutamic acid

Table 2. Prediction of discontinuous B-cell epitopes by DiscoTope server before and after amino acid substitutions in position 131 and 155 in a study on functional deimmunization of interferon beta-1b by identifying and silencing human T cells epitopes

Amino acid*	position	Method used	Propensity score
HIS	131	DiscoTope-2.0 Server	-7.007
GLU	131	DiscoTope-2.0 Server	-7.556
TYR	155	DiscoTope-2.0 Server	-11.432
SER	155	DiscoTope-2.0 Server	-11.127

*Tyr: Tyrosine; HIS: Histidine; SER: Serine; GLU: Glutamic acid

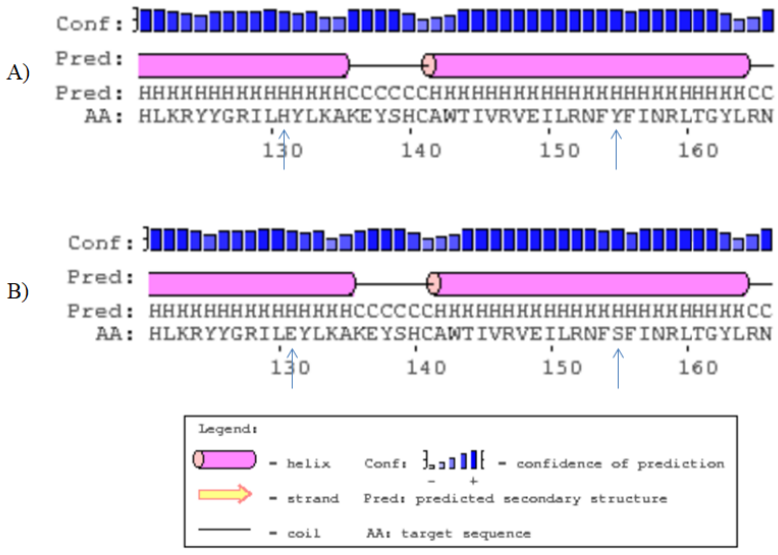

Figure3. PSI-PRED secondary structure prediction for the hIFNβ-1b and variants sequence in a study on functional deimmunization of interferon beta-1b by identifying and silencing human T cells epitopes. A) Secondary structure of the hIFNβ-1b B) Secondary structure of mut1+2 that contain both mutations.. It shows that mutant's forms had same secondary structure with hIFNβ-1b (wild).

PSIPRED: PSI-blast based secondary structure prediction; Y: Tyrosine; H: Histidine; S: Serine; E: Glutamic acid

Table 3. Physicochemical Characteristics of hIFNβ-1b and other variants by UV spectroscopy method in a study on functional deimmunization of interferon beta-1b by identifying and silencing human T cells epitopes

Parameter	wild	mut 1	mut2	mut 1+2
OD350	0.0061	0.0081	0.0058	0.0073
OD280/OD260	2.14	2.31	2.40	2.26
OD280/OD260 + 0.01% SDS*	2.65	2.71	2.82	2.78

* 0.01% (w/v) SDS was added to the rhIFNβ-1a preparations before analysis

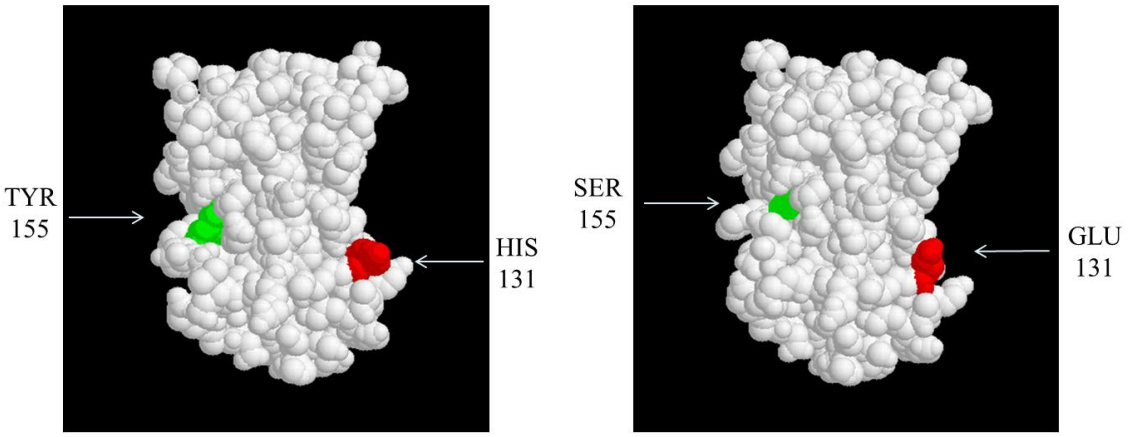

Figure 4. Two Rasmol pictures of hIFNβ-1b. 3D structure of hIFNβ-1b (wild) and mut1+2 predicted using SWISS-MODEL server and PDB File visualized by Rasmol software in a study on functional deimmunization of interferon beta-1b by identifying and silencing human T cells epitopes, The protein sequence of hIFNβ was considered for homology modeling using SWISSMODEL server to model the wild type. Similar step was followed for modeling mut1+2 where in Tyr at 155th position was substituted by Ser and also His at 131th position was substituted by Glu. (The space-filling model, the protein is shown in white and mutation site be chromatic).
Tyr: Tyrosine; HIS: Histidine; SER: Serine; GLU: Glutamic acid

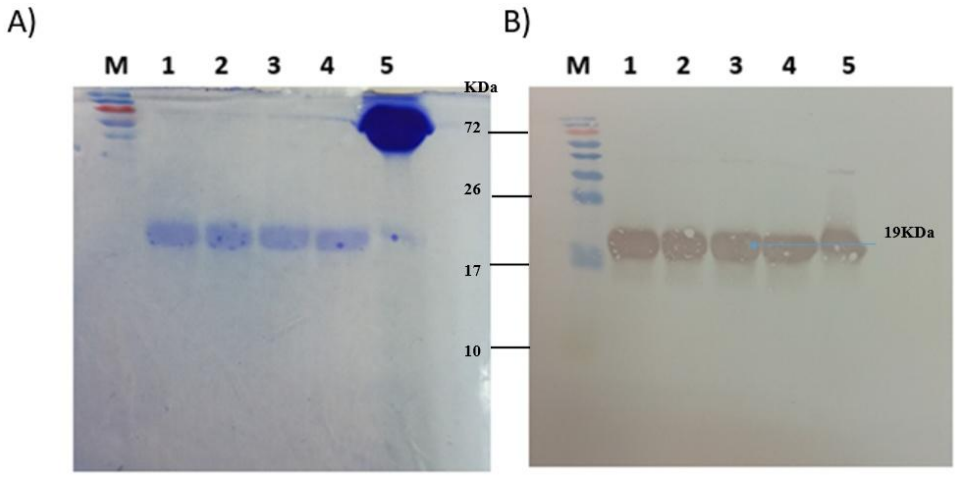

Figure 5. SDS-PAGE gels under reducing conditions and B) the corresponding Western blots of the gels under reducing conditions of the three structural variants of hIFNβ-1b in a study on functional deimmunization of interferon beta-1b by identifying and silencing human T cells epitopes. Numbers on the midline represent band positions (in kDa) of the molecular weight markers (Lane M). Lane 1- 4 are wild, mut1, mut2 and mut1+2 respectively, lane 5 is standard hIFNβ-1b (Betaseron).

Protein Expression, Purification and Western Blotting

Plasmids were transformed into BL21 (DE3) cells for protein production. This E. coli strain contain a chromosomal copy of the T7 RNA polymerase gene under control of the lacUV5 promoter. IPTG addition leading to transcription of T7 RNA polymerase and expression of the hIFNβ-1b gene. The N-terminal pelB via the Sec-dependent transport pathway target the translated polypeptide in its unfolded state to the E. coli periplasm.[27] The N-terminal pelB signal peptide was cleaved, also protein folding and disulfide bond formation carried out by chaperones and disulfide bond isomerases respectively in the periplasm. After cleavage of the pelB secretion signal an amino-terminal serine was generated, resulting in the mature hIFNβ-1b protein sequence. Purification was carried out by Ni^{2+} affinity chromatography. Because expression of hIFNβ-1b and variants was very low in the periplasmic fraction, therefore we report yields as the amount of rhIFNβ-1b recovered after the chromatography step. Yields after purification and several concentrations was range from 201.5 µg/mLto 254µg/mLfor all proteins, protein density were determined by the Bradford's method. The expressed hIFNβ-1b protein was composed of 165 residues with a calculated mass of ~18 kDa and Polyhistidine-tag was ~1 kDa. SDS-PAGE analysis showed a single protein band for hIFNβ-1b and variants at approximately 19 kDa (Figure 5). Under reducing SDS-PAGE, the observed molecular weights for proteins bands were in agreement with the expected molecular weights, indicating a proper cleavage of the pelB signal peptides. Furthermore, Western blot analysis confirmed that all protein bands were specifically recognized by the antibody against IFN-β (Figure 5). Also there are no detectable high molecular weight aggregates by SDS-PAGE.

UV Spectroscopy

Presents of disulfide bonds and three aromatic residues, tryptophan, tyrosine or phenylalanine within the sequence of hIFNβ-1b caused an absorbance peak at 280 nm in all UV spectra of samples. Protein aggregation leading to increase in light scattering of samples in UV spectroscopy. Light scattering intensity determined by Shape, size and number of aggregate particles in samples. For aggregation analysis of all sampels, the optical density (OD) at 350 nm and the ratio OD 280 nm to OD 260 nm were determined (Table 3). A higher OD350 and a lower OD280:OD260 is indicative of aggregation.[28] Addition of 0.01% SDS to all samples leading to reduce the OD350 and increased of OD280:OD260 that indicated non-covalent protein bonds of hIFNβ-1b Aggregations (Table 4).This data show that all three hIFNβ-1b variants have equal Physicochemical Characteristic with wild hIFNβ-1b protein.

Comparison of hIFNβ-1b and Variants mediated Antiviral and Antiproliferative Activity

Two important biologic property of hIFNβ-1b are antiviral and antiproliferative activity. The antiproliferative activity of hIFNβ-1b and three variants was assessed by using A549 cells, which are sensitive to IFN-β as other type I IFNs. A549 cell proliferation measured by the MTT assay, all three variants and wild proteins displayed activity in approximately equivalent to that of the commercially obtained Betaseron (Figure 6). Antiviral Activity of all variants was tested by cytopathic effect (CPE) inhibition method on Vero cells exposed to polio virus (Figure 7). Vero cells were treated with hIFNβ-1b and three variants and infected with polio virus in a dose-dependent manner. As shown in (Figure 8), hIFNβ-1b and three variants inhibited CPE formation of polio virus on Vero cells in compare with standard Betaseron (positive control). Result show that antiviral activity was equivalent to that of the commercially obtained Betaseron.

Anti-drug Antibodies Assay

Antibody responses against hIFNβ-1b (wild), three variants and Betaseron after 2 month of treatment (3 times per week) in Female BALB/c mice were tested as described. All test proteins induced ADAs in mice as the human protein is foreign to the murine immune system in this setting. All mice that received Betaseronand hIFNβ-1b (wild) formed significantly very high immunogenicity compared to animals administered with the three variants. The immunogenicity in BALB/c mice at different days measured by Direct ELISA and results shown in (Table 4).

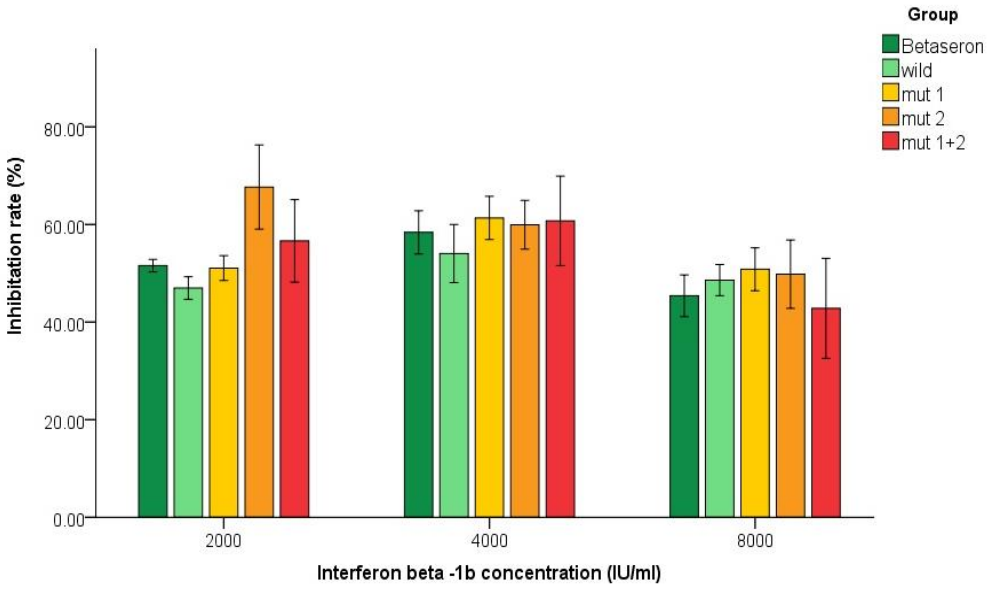

Figure6. Antiproliferative activity of hIFNβ-1b (wild), Betaseron (standard) and variants in a study on functional deimmunization of interferon beta-1b by identifying and silencing human T cells epitopes. MTT assay was used to evaluate the antiproliferative effect of recombinant hIFNβ-1b variants. The results were reported as the mean OD for each group in MTT assay. Error bars represent standard deviations from triplicate measurements.

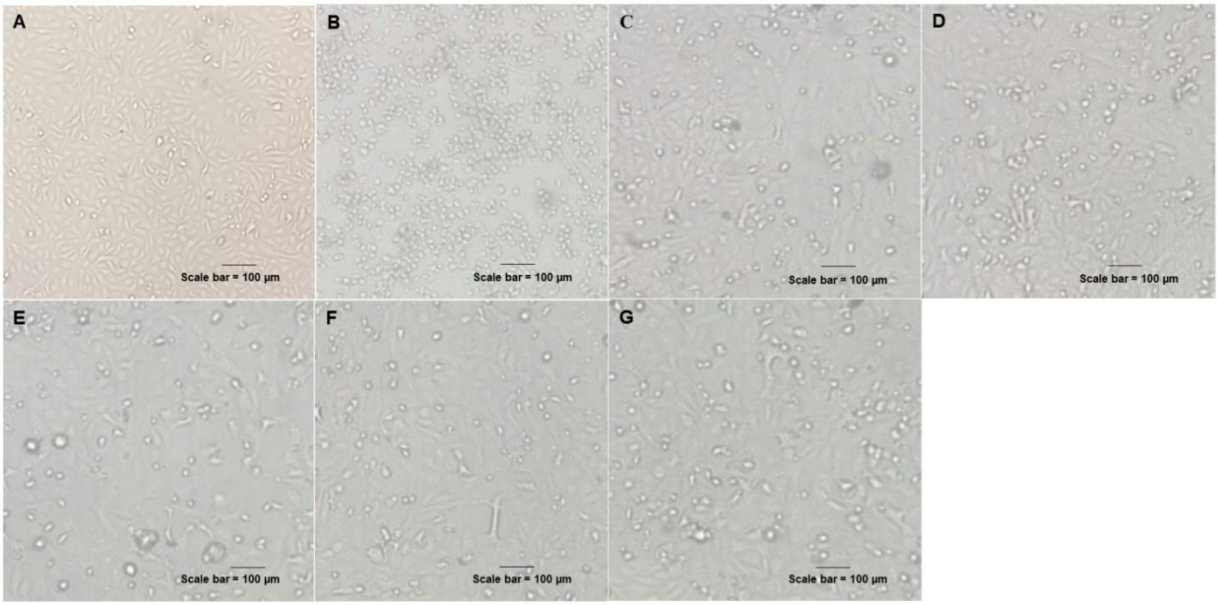

Figure 7. Morphological changes of Vero cells following infection with polio virus in a study on functional deimmunization of interferon beta-1b by identifying and silencing human T cells epitopes. (A) Cell control, Vero cells without polio virus and IFNβ. (B) Virus control,Vero cells exposed to polio virus shows CPE. (C) Vero cells exposed to polio virus with Betaseron. (D) Vero cells exposed to polio virus with hIFNβ-1b,wild. (E) Vero cells exposed to polio virus with mut1. (F) Vero cells exposed to polio virus with mut2. (G) Vero cells exposed to polio virus with mut1+2. Lower percent of CPE is seen in Vero cells in presence of hIFNβ-1b.

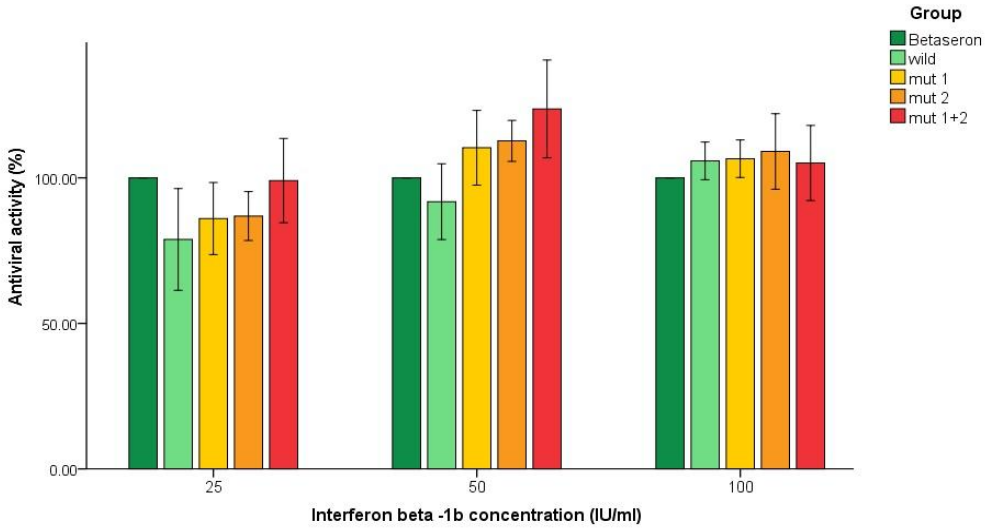

Figure 8. Antiviral activity of hIFNβ-1b (wild), Betaseron (standard) and variants in a study on functional deimmunization of interferon beta-1b by identifying and silencing human T cells epitopes. Antiviral activity of Betaseron was considered as 100%, each bar represents the mean ± SD of triplicate samples of three independent experiments.

Table 4. Immunogenicity of hIFNβ-1b and other variants groups in BALB/c mice in a study on functional deimmunization of interferon beta-1b by identifying and silencing human T cells epitopes.

Group	Day 0	Day 21	Day 35	Day 49	Day 63
Betaseron	0.018+0.008	0.126+0.027	0.383+0.082	0.498+0.111	0.664+0.124
wild	0.022+0.006	0.114+0.053	0.179+0.074	0.503+0.081	0.619+0.085
mut 1	0.038+0.003	0.131+0.072	0.207+0.115	0.431+0.085	0.521+0.151
mut 2	0.029+0.005	0.048+0.017	0.115+0.056	0.090+0.017	0.141+0.063
mut 1+2	0.017+0.004	0.083+0.036	0.089+0.040	0.148+0.092	0.193+0.086
***p* values comparison in Day 63**		mut 1	*mut 2	*mut 1+2	Betaseron
wild		p=0.838	p<0.001	p<0.001	p=0.996

Data is presented as an average of triplicate measurements; error bars represent standard deviations. *Significantly different ($p < 0.001$) from wild group. Data Analysis were done using repeated measure one-way Analysis Variance (ANOVA) on day 63.

DISCUSSION

Immunogenicity is an important issue in the development of therapeutic protein. In M.S patients who are using Betaseron, ADAs is a common phenomenon that reported in an increasing number of studies.[8] Today these antibodies molecularly characterized very well, and the mechanism that leading to Abs production relatively understood. Previous studies show that ADAs recognized several epitope at the characterized sequence of hIFNβ-1b. These antibodies bind to the epitopes ^{1}MSYNLLGFLQRS^{12}, ^{121}HLKRYYGRILHY^{132}, ^{151}LRNFYFINRLTG^{162} and in this regions interfered with hIFNβ function. This epitopes region display a high degree of sequence homology in other animal species, for instance Peptide sequence ^{121}HLKRYYGRILHY^{132} and ^{151}LRNFYFINRLTG^{162} are much conserved across mammals such as monkey, mouse and pig (Figure1) replacement or deletion of this regions severely impairs hIFNβ activity.[29,30] This regions contain residues that has important role in

protein, for example deletion or replacement of K and Y in HLKRYYGRILHY sequence (D Helix of hIFNβ) decreases antiviral activity and protein inactivation respectively.[31,32] Based on the crystal structure of the hIFNβ - IFNAR 1and 2 complex, this regions of hIFNβ interact directly with hIFNβ Receptor or contribute to the formation of hIFNβ dimmers.[32] Gneiss *et al* indicated that Binding Antibody (BAbs) and Neutralizing antibody (NAbs) bind to hIFNβ-1b at residues 1-12, 121-132 and 151-162, BAbs and NAbs recognized residues 121-132 with equal frequency but NAbs significantly bind to residues 1-12 and 151-162 and interfered with the bioactivity of hIFNβ.[30] Consequently epitopes identification provided a rational molecular basis to figure out the adverse effects of ADAs in MS patients. A previous study has been shown that a single substitution in a T-cell epitopes can reduce immunogenicity of these peptides.

Today computer technology can use for identification of immunogenic T-cell epitopes. In our study IEDB tools were used to identify and silence of T-cell epitopes in hIFNβ-1b sequence. IEDB tools by several MHC II alleles recognized two important regions as most immunogenic epitopes in this protein. Each position in this epitopes structurally analyzes by Swiss-Model server and visualized by Rasmol software to identify which residues are exposed in protein surfaced. Based on sequence homology analysis we selected tow specific mutations for minimizing epitope score in these regions. Two residues in this sequence selected and replaced with other amino acids; Tyrosine in position 155 that replaced with Serine: Y155S (mut1) and Histidine in position 131 that substituted with Glutamic Acid: H131E (mut2). In these two positions residues are exposed in protein surface and bioinformatics tools show that after mutations, these regions probably do not identified as epitope by T Cells. Further analysis indicated that these amino acid substitution did not lead to creating new epitopes in this protein (data not shown). Measurement of the antibody response versus a human protein in animals is very difficult, because immunogenicity in humans is not sufficiently predictive compare to immunogenicity in animals. Nonetheless we examined the immune response to hIFNβ-1b and three variants in BALB/c mice, our results were shown that the synthesis of hypoimmunogenic hIFNβ-1b variants that have a single amino acid modification in a T cell epitope can effectively reduce immune reactivity to the hIFNβ-1b protein in mice. Results showed that H131E mutation (mut2 and mut1+2) lead to decreasing of Ab response to hIFNβ-1b and production of Ag-specific IgG were severely reduced and the difference between these groups are significant (Table 4)

Our result is compatible to the previous study which has been shown the single amino acid change in dominants epitopes lead to immunogenicity reduction and cannot effects on subdominant epitope for processing and presentation.[33] Alteration in Ag presentation has been associated with conformation changes, including protein unfolding and the loss of stabilizing disulfides.[34,35]The protein conformation of the all variants proteins was presumed to be correct, as the hIFNβ-1b variants had identical antiproliferation and antiviral activity in cell lines (Figures 8 and 10). Our result is in contrast to published reports that indicate Ab responses to H131A mutation in dominant T cell epitopes are not affected after modification of this epitopes in hIFNβ-1b.[29] In that study Histidine was modified to Alanine at position 131 inhIFNβ-1b and BALB/c mice were immunized with this variant (i.p. in alum on days 1, 3 and 10). Subsequently, lymph node cell proliferation as a marker of epitope responsiveness was tested and T cell proliferation were detected. Alanine is a nonpolar and aliphatic amino acid whereas Glutamic Acid (in our variants) is polar and have negative charge, difference between this tow amino acid and also route of immunization may have led to these discrepant results.[36] In this report we created a new recombinant protein of hIFNβ-1b: one variant not only has hIFNβ-1b biologic function but also is hypoimmunogenic protein. So far, the mechanisms underlying this effect is not completely described but previous studies indicated Immunogenicity is the result of a series of processes and interactions between peptide and MHC class II molecules which have been shown peptide/MHC complexes with high-affinity tend to be immunogenic.[37,38] Antigen presentation pathway started by antigen take up into endosomal compartments of APC that are specialized cells. Antigens after proteolysis bind to empty MHC class II cleft, HLA-DM that is an enzyme present in endosomes, release CLIP peptide from MHC class II and stabilizes peptide/MHC complexes for cell surface presentation, where this Antigenic peptides examined by specific TCR.[39] Hall et al hypothesis said that kinetic stability of peptide/MHC complexes and sufficient remaining on the cell surface is a key

determinant of immunogenicity of one peptide, finally outcome is recognition by a specific TCR and consequently T cell activation. Other studies also have been shown that kinetic stability of the peptide/MHC complex and avidity of the TCR for the peptide/MHC ligand are very important for T cell activation.[38] As we have shown in this study in Immunodominant epitopes we can decreased peptide/MHC complexes affinity with substitution of one amino acid by the other that has different biochemical property, this may be leading to differential Ag processing, HLA-DM editing and inefficient presentation by dendritic cells. This results show that by exact identification and modification of immunodominant T cell epitopes, new variants of interested protein with vastly modified immune response characteristics can be produced. One limitation in our study was polymorphism in MHC II proteins, As T-cell epitopes are presented through the highly polymorphic MHC II proteins, it seems difficult to identify and remove all T-cell epitopes in therapeutic protein.

In summary the purpose of this study was to identify and remove human T-cell epitopes in hIFNβ-1b. We identified two major epitopes and their possible elimination by mutagenesis. Three novel hIFNβ-1b proteins which consist the single amino acid modification and one contain two amino acid exchanges in the immunodominant epitopes were constructed, expressed and purified. The variant displayed 100% of control antiproliferation and antiviral activity in cell lines. When Mice immunized with hIFNβ-1b variant molecule that containing H131E substitution, had significantly reduced Ab response. This demonstrates that a single amino acid exchange within an immunodominant epitope can eliminate an immune response to a whole protein.

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