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Enhanced sublingual immunotherapy by TAT-fused recombinant allergen in a murine rhinitis model

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ABSTRACT

Allergen-specific sublingual immunotherapy (SLIT) is well known as an effective and non-invasive route to induce allergy desensitization. The goal of this study was to investigate whether a TAT-fused recombinant allergen could enhance SLIT efficacy. BALB/c mice sensitized to the main allergen (Che a 3) of *Chenopodium album* pollen were treated sublingually either with rChe a 3 (100 μg/dose) or rTAT-Che a 3 (100 μg/dose), two times per week for eight weeks. SLIT with rTAT-Che a 3 led to significantly greater allergen-specific IgG2a than rChe a 3; however, neither rTAT-Che a 3 nor rChe a 3 affected allergen-specific IgE or IgG1 antibody levels. In addition, interleukin 4 (IL-4) levels in re-stimulated splenocytes from the rTAT-Che a 3 mice were significantly lower than in those from the rChe a 3 mice, while interferon-γ (IFN-γ) was significantly greater in the rChe a 3 mice than in the rTAT-Che a 3 mice. Furthermore, sublingual administration of rTAT-Che a 3 induced significantly greater TGF-β secretion in re-stimulated splenocytes than administration of rChe a 3. Accordingly, SLIT with rTAT-Che a 3 led to significantly greater expression of TGF-β- and Foxp3-specific mRNAs in the splenocytes than in those from the rChe a 3 mice. Our findings demonstrate that TAT-fused rChe a 3 suppressed the allergic response through preferential enhancement of systemic regulatory T-cell (Treg)-mediated immunity responses, likely by facilitating allergen capture and presentation by sublingual Langerhans-like dendritic cells.

1. Introduction

The increasing prevalence of allergic diseases has become a public health problem in many developed and developing countries [1]. Allergen-specific sublingual immunotherapy (SLIT) is well known as an effective and non-invasive method to induce allergy desensitization [2–6]; however, current vaccines for SLIT require further enhancement of allergen presentation to sublingual immune cells. Second-generation vaccines using well-defined recombinant allergens, rather than allergen extracts from natural sources, are under development [7,8]. During SLIT, the allergens are captured within the sublingual mucosa by local Langerhans-like dendritic cells (DCs) following phagocytosis, macropinocytosis, or receptor-mediated endocytosis [9,10]. Currently, new vaccine formulations, which take into account the appropriate delivery systems to improve allergen targeting to the sublingual immune cells, are being investigated. In particular, muco-adhesive vaccines have been shown to increase SLIT efficacy by enhancing the contact duration of the allergen with the sublingual mucosa [11,12].

The TAT protein transduction domain (TAT-PTD), an 11-amino acid arginine- and lysine-rich peptide derived from the human immunodeficiency virus type 1 (HIV-1), has been demonstrated to cross cellular membranes, both alone and linked to therapeutic biomolecules [13,14]. Several groups have shown the effectiveness of the TAT protein transduction domain (PTD) as a delivery vehicle for a wide variety of

Abbreviation: SLIT, sublingual immunotherapy; Che a 3, main allergen of *Chenopodium album* pollen; rChe a 3, recombinant Che a 3; TAT, HIV viruse-TAT protein transduction domain; rTAT-Che a 3, recombinant TAT-fused Che a 3; IPTG, isopropyl-β-D-thiogalactopyranoside; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; BSA, bovine serum albumin; HRP, horseradish peroxidase; Far- and near-UV CD, circular dichroism (CD) spectroscopy

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biomolecules including peptides, proteins, anti-cancer therapeutic cargos, and antisense oligonucleotides, both *in vivo* and *in vitro* [15–17]. The TAT-PTD, with six arginine and two lysine residues, is a highly cationic peptide. Mechanistic studies have shown that the positive charge of TAT plays a key role in its transduction. Various studies have suggested that an initial strong electrostatic interaction between the positively-charged TAT-PTD and the negatively-charged plasma membrane of eukaryotic cells induces endocytic pathways leading to the cellular internalization of TAT [18,19].

Herein, we hypothesized that a TAT-fused recombinant allergen might enhance SLIT *via* allergen crossing of sublingual endothelium or prolongation of the contact between the positively-charged allergen and the negatively-charged sublingual mucosa. To investigate, we used a mouse model sensitized to the main allergen (Che a 3) of *Chenopodium album* (*C. album*) pollen to compare recombinant TAT-Che a 3 (rTAT-Che a 3) to free recombinant Che a 3 (rChe a 3) in SLIT.

2. Materials and methods

2.1. Animals

Female, 6–8 weeks old BALB/c mice were purchased from the Razi Vaccine and Serum Research Institute) Mashhad, Iran(. All the animal experiments and handling procedures were approved by the Institutional Animal Ethics Committee (No. 87542) of the Mashhad University of Medical Sciences.

2.2. Antigens

Recombinant Che a 3 was expressed in *E. coli* BL21 Codon Plus (DE3) RIL cells (Stratagene, USA) and purified by Ni-NTA chromatography as described previously [20]. The recombinant TAT-Che a 3 fusion protein (rTAT-Che a 3) was produced as describe below.

2.2.1. Recombinant TAT-Che a 3 plasmid construction

To produce rTAT-rChe a 3, DNA was amplified by PCR from the *pET21b*$^{+}$*-rChe a 3* vector described previously [20], using the following primers: 5′-GGAAAGCTT*GGCAGAAGAAGCGGAGACAGCGACGAAGAG-CT*ATGGCTGCTGAGGATACACCTC-3′ and 5′-ATTCTCGAGATGGCTGC-TGAGGATACACCTC-3′; the underlined sequences are *HindIII* and *XhoI* restriction sites respectively, and italicized letters correspond to the TAT sequence. The PCR product was cloned into the *HindIII* and *XhoI* cloning sites of the *pET21b* + expression vector (Novagen, USA) and expressed in *E. coli* BL21 (DE3) cells. A clone containing the desired construct was verified by sequence analysis.

2.2.2. Expression and purification of rTAT-Che a 3

100 μL aliquots of overnight cultures of BL21 cells carrying the plasmids were aliquoted into 100 mL of LB medium containing 50 μg/mL of ampicillin. The cultures were grown at 18 °C and induced at OD_{600} = 0.5 with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 12 h. To purify the recombinant fusion protein, the cells were harvested by centrifugation at 9000 × *g* at 4 °C for 10 min and then resuspended in 10 mL of lysis buffer containing 50 mM Tris–HCl, pH 7.8, 5 mM imidazole, and 500 mM NaCl. The bacterial cell walls were disrupted by three freeze-thaw cycles in liquid nitrogen and 37 °C. The supernatants and insoluble fractions were separated by centrifugation. Ni-NTA sepharose columns (Amersham, UK) were used to purify the recombinant proteins according to the manufacturer's instructions. The elution fractions were electrophoresed by SDS–PAGE to identify the fraction(s) containing the fusion protein. The fractions containing rTAT-rChe a 3 were dialyzed overnight against phosphate buffer (pH 7.4), using 12 kDa molecular weight cut-off dialysis membranes (Sigma, Germany).

2.3. SDS-PAGE and western-blotting

Allergen purity was analyzed by 15% SDS-PAGE under non-reducing conditions. The acrylamide gels were stained with Coomassie Brilliant Blue G-250 (Merck, Germany). For western blotting, following SDS-PAGE, purified proteins were transferred onto PVDF membranes (Immobilon P, Millipore Corp., Bedford, USA) at 0.3 A, 100 V (using Bio-Rad System, USA), for 15 min at 4 °C. After blocking in 2% BSA, pH 7.4, overnight at 4 °C, the membranes were incubated overnight at 4 °C with pooled sera from five patients allergic to *C. album* pollen, diluted 1:3 in blocking buffer. Human-specific IgE was detected by a biotinylated goat anti-human-IgE antibody (KPL, USA) diluted 1:1000 in blocking buffer for 3 h at room temperature, and then with HRP-conjugated streptavidin (Bio-Rad, USA) diluted 1:20,000 for 1 h at room temperature, followed by development with a Chemiluminescence Western Blotting kit (ParsTous, Iran). A Syngene (UK) apparatus was used for image acquisition. A pooled sera from non-allergic individuals was applied as a negative control.

2.4. Circular dichroism

Far- and near-UV CD analyses were performed on a Jasco-815 spectropolarimeter (Jasco, Tokyo, Japan) as previously described [21]. Briefly, spectra were determined from samples with concentrations of 0.01% in 20 mM phosphate buffer, pH 7.4, using a 1 mm path length cuvette. Far- and near-UV CD spectra were obtained over wavelength ranges of 190–240 and 250–350 nm. The secondary structures of the proteins were analyzed by SELCON3, CONTIN, and CDSSTR to estimate the relative proportions of α-helices, β-sheets, turns, and random coils [22,23].

2.5. Fluorescence spectroscopy

The fluorescent spectra were determined using an F-2500 spectrofluorimeter (Hitachi, Japan) as previously described [21]. Briefly, slit widths were set at 5 nm, and sample emission spectra were determined using a 1.0-cm quartz cuvette. The excitation wavelengths were 280 and 295 nm at 5 nm steps and emission scans were performed at wavelengths of 220 to 500 nm.

2.6. Experimental procedure

2.6.1. Sensitization phase

The effect of the TAT-fused recombinant allergen on SLIT efficacy was tested in a therapeutic *in vivo* model of rChe a 3-sensitized mice, according to a previously-described protocol [11] (Fig. 1). Briefly, BALB/c mice were sensitized to rChe a 3 by means of two intraperitoneal injections with 10 μg of rChe a 3 adsorbed onto 2 mg of aluminum hydroxide (Sigma, Saint Quentin Fallavier, France) in 150 μL of PBS on days 0 and 14. Starting on day 21, the mice were challenged for 20 min with 1% (*w/v*) rChe a 3 on four consecutive days using an aerosol delivery system (Omron CX3 nebulizer, Netherlands). Control mice were sensitized with PBS and 2 mg of aluminum hydroxide. On day 25, to evaluate sensitization, blood samples were collected through the retro-orbital sinus, and based on rChe a 3-specific IgE and IgG1 levels, the mice were stratified into treatment groups (n = 5).

2.6.2. Sublingual immunotherapy

SLIT was started on day 28, twice a week for eight weeks by applying 100 μg/dose of rChe a 3 or rTAT-Che a 3, in a total volume of 25 μl, under the tongue. The sham-treated group received PBS. Furthermore, a not-sensitized, not-treated group was included as the control group. On days 90 and 91 all groups were challenged for 20 min with aerosolized rChe a 3 (1%) as described previously. The blood samples from the retro-orbital plexus were collected weekly during the sensitization and immunotherapy stages. Mice were euthanized at the