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Production and Immunogenicity of Hypoallergenic Codon-Optimized DNA Vaccine Encoding Mature Der p 1 Allergen

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Abstract

Background: Genetic vaccination with plasmid DNA encoding allergens is a promising potential approach for the treatment or prevention of allergy. Nonetheless, because the allergens expressed can display immunoglobulin (Ig) E reactivity, methods to deliver hypoallergenic variants can minimize the risk of type 2 helper (T_H2) cell priming after DNA immunization.
Methods: A humanized synthetic gene encoding mature *Dermatophagoides pteronyssinus* group 1 (Der p 1) allergen was cloned into the pHIS expression vector carrying unmethylated CpG 2006 (CpG 2006) motif but devoid of signal sequence. The immunogenicity of this DNA construct was compared in naïve mice with that of recombinant ProDer p 1 protein adjuvanted with alum.
Results: Codon optimization of the cDNA encoding mature Der p 1 markedly improved allergen expression. Mature Der p 1, expressed intracellularly in Human Embryonic Kidney 293 cells (HEK 293 cells) transfected with codon-optimized Der p 1 cDNA (pHIS-mHuDer p 1), was shown to be hypoallergenic as it displayed no IgE reactivity. Intradermal vaccinations of naïve Balb/C mice with pHIS-mHuDer p 1 elicited an allergen-specific T_H1 response characterized by the production of specific IgG2a, a very low amount of specific IgG1, and no specific IgE. Lipoplex formulation with cationic liposome composed of lecithin, N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP) and cholesterol not only accelerated the induction of T_H1 response but also increased its intensity.
Conclusion: A codon-optimized DNA vaccine encoding mature Der p 1 in a lipoplex formulation could represent a promising hypoallergenic vaccine candidate for safer immunotherapy against house dust mite allergy.

Key words: Hypoallergen. Der p 1. Codon-optimized DNA vaccine. Mature Der p 1.

Resumen

Antecedentes: La vacunación genética con el ADN plasmídico que codifica los alérgenos constituye una posible y prometedora estrategia de abordaje para el tratamiento o la prevención de la alergia. Sin embargo, dado que los alérgenos expresados pueden mostrar reactividad de la inmunoglobulina (Ig) E, los métodos capaces de producir variantes hipoalergénicas pueden minimizar el riesgo de sensibilización de los linfocitos T cooperadores de tipo 2 (T_H2) tras la vacunación con ADN.
Métodos: Se clonó un gen sintético humanizado que codifica el alérgeno Dermatophagoides pteronyssinus de grupo 1 (Der p 1) maduro en el vector de expresión pHIS portador del motivo CpG 2006 (CpG 2006) no metilado pero desprovisto de secuencia señal. Se comparó la inmunogenicidad de esta construcción de ADN en ratones no sometidos previamente a experimentación con la de la proteína ProDer p 1 recombinante potenciada con alumbre.
Resultados: La optimización del codón del ADNc que codifica el alérgeno Der p 1 maduro mejoró notablemente la expresión de este último. El alérgeno Der p 1 maduro, expresado intracelularmente en células de riñón embrionario humano 293 (células HEK 293) transfectadas con ADNc de Der p 1 (pHIS-mHuDer p 1) con codón optimizado, demostró ser hipoalergénico dado que no mostró reactividad IgE. La vacunación intradérmica con pHIS-mHuDer p 1 de ratones Balb/C no sometidos previamente a experimentación desencadenó una respuesta T_H1 específica del alérgeno caracterizada por la producción de IgG2a específica y una cantidad muy pequeña de IgG1 específica, así como por la ausencia de IgE específica. La formulación lipoplex con liposomas catiónicos compuestos por lecitina, N-[1-(2,3-dioleoiloxi)propil]-N,N,N-trimetilamonio metilsulfato (DOTAP) y colesterol no solo aceleró la inducción de la respuesta T_H1 sino que también aumentó su intensidad.
Conclusión: Una vacuna de ADN con codón optimizado que codifica el alérgeno Der p 1 maduro en una formulación lipoplex podría representar una prometedora vacuna hipoalergénica para una inmunoterapia más segura frente a la alergia a los ácaros del polvo doméstico.

Palabras clave: Hipoalérgeno. Der p 1. Vacuna de ADN con codón optimizado. Der p 1 maduro.

Introduction

Allergic reactions are symptomatic responses to a normally innocuous environmental antigen such as pollen, animal dander, and house dust mites (HDMs). This exacerbated immune-mediated disorder is characterized by the production of allergen-specific immunoglobulin (Ig) E, chronic airway inflammation through neutrophil and eosinophil recruitment, mucus production, and variable airflow obstruction with airway hyperresponsiveness (AHR) [1]. The prevalence of allergic asthma, the most common allergic disease, has increased dramatically in the last 50 years, with rates ranging from 5% to 30% worldwide [2].

HDMs are a predominant source of inhalant allergens worldwide, notably in tropical countries. In industrialized countries, mite sensitization affects between 15% and over 20% of the population [2]. The most common species, *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, produce more than 20 different allergen groups, which are classified according to sequence homology and biological function [3].

Experimental evidence suggests that allergen-specific type 2 helper (T_H2) cells play a central role in each of the 3 steps of the allergic response (sensitization and immediate and late phases) through the secretion of interleukin 4 (IL-4), IL-5, IL-9, and IL-13, probably in addition to other recently identified cytokines such as IL-25, IL-31, and IL-33 [4,5]. Consequently, new immunotherapeutics capable of inducing T_H1 or regulatory T cells would provide a valuable means of preventing allergy or shifting the T_H2-biased immune response to a nonpathogenic reaction.

DNA vaccination has been shown to induce antiallergic immune responses through the recruitment of allergen-specific T_H1 cells, $CD8^+$ cells and the establishment of a T_H1 cytokine milieu, notably by interferon γ (IFN-γ) production [6]. These allergen-specific immune responses have been found to be efficient in preventing and/or reverting the T_H2-biased allergic response in prophylactic and/or therapeutic use, respectively [7-9]. DNA vaccine formulations can thus be optimized by improving their immunogenicity and therefore their efficacy, notably through the adaptation of codon usage to increase allergen expression [10,11], the incorporation of immunostimulatory sequences (ISSs) in the plasmid backbone [12,13], or the use of signal sequences to enhance the stability of messenger RNA to improve translational efficiency [14]. However, cloning complementary DNA (cDNA) encoding native allergen downstream of the strong signal peptide could induce the priming of T_H2 response through specific IgE production, leading to undesirable T_H2 sensitization [15]. The creation of hypoallergenic DNA vaccines with very weak IgE reactivity and based on deliberate allergen gene fragmentation or mutation is a promising approach to circumventing this potential problem.

Within the group of HDM antigens, group 1 mite allergens are among the most important allergenic molecules, firstly because the vast majority of HDM-allergic patients develop specific IgE to these allergens and secondly because 50% to 100% of IgE reactivity to HDM extract is directed toward these proteins [16,17]. The group 1 allergen Der p 1, from *D pteronyssinus*, is a cysteine proteinase synthesized as a PreProDer p 1 precursor, comprising a signal peptide, a prosequence, and a mature protein of 18, 80, and 222 amino acid residues, respectively [18]. The prosequence has been shown to play a critical role in the appropriate adoption of correct Der p 1 folding as this propeptide acts as a chaperonin [19]. The IgE reactivity of this allergen has been shown to be strictly dependent on allergen conformation [11,20].

The present study reports the production and immunogenicity of a hypoallergenic DNA vaccine encoding Der p 1. In this DNA construct, cDNA encoding Der p 1 lacking a leader peptide and prosequence was synthesized based on human codon usage and cloned into a CpG motif–containing expression vector.

Material and Methods

Preparation of Humanized Der p 1 cDNA

To generate human codon-optimized cDNA encoding full-length mature Der p 1, 8 primers with oligonucleotides with a 20 base-pair (bp) overlap were designed and synthesized for polymerase chain reaction (PCR) amplification. The humanized Der p 1 sequence was designed according to the human codon usages from the GenBank database (http://www.kazusa.or.jp/codon). In this synthetic DNA construct, only the codon with the highest frequency was selected. The different oligonucleotides had the following sequences: HuD1 (5'-ACCAACGCCTGCAGCATCAACGGCAACGCCCCCGCCGAGATCGACCTGCGGCAGATGCGGACCGTGACCCCCATCCGGATGCAAGGCGGCTGCGGCAGCT-3') (Pst I restriction site is underlined), HuD2 (5'-CTGCTCGGCCAGGTCCAGGCTCTGGTTCCGGTAGGCCAGGTAGGCGCTCTCGGTGGCGGCCACGCCGCTGAAGGCCCAGCAGCTGCCGCAGCCGCCTTGC-3'), HuD3 (5'-GCCTGGACCTGGCCGAGCAGGAGCTGGTGGACTGCGCCAGCCAGCACGGCTGCCACGGCGACACCATCCCCAGAGGCATCGAGTACATCCAGCACAACGG-3'), HuD4 (5'-ATGCCGAACCGCTGGGCGTTGGGCCGCCGGCAGCTCTGCTCCCGGGCCACGTACCGGTAGTAGCTCTCCTGCACCACGCCGTTGTGCTGGATGTACTC-3'), HuD5 (5'-CCCAGCGGTTCGGCATCAGCAACTACTGCCAGATCTACCCCCCCAACGCCAACAAGATCCGGGAGGCCCTGGCCCAGACCCACAGCGCCATCGCCGTG-3'), HuD6 (5'-GCTGATATCCGTTGTCGCGCTGGATGATGGTCCGGCCGTCGTATGCCGGAAGGCGTCCAGGTCCTTGATGCCGATGATCACGGCGATGGCGCTGTGGG-3'), HuD7 (5'-CGCGACAACGGATATCAGCCCAACTACCACGCCGTGAACATCGTGGGCTACAGCAACGCCCAGGGCGTGGACTACTGGATCGTGCGGAACTCCTGGGACA-3') (Eco RV restriction site is underlined), HuD8 (5'-CAGGATCACCACGTAGGGGTACTCCTCGATCATCATCAGGTCGATGTTGGCGGCGAAGTAGCCGTAGCCGTTGTCGCCCCAGTTGGTGTCCCAGGAGTTCCGCACG-3').

Pairs of oligonucleotides with an overlapping sequence were used in a first PCR reaction to amplify the fragments HuD1-HuD2, HuD3-HuD4, HuD5-HuD6, and HuD7-HuD8 in order to generate HuD12, HuD34, HuD56, and HuD78 of 180 bp, 180 bp, 180 bp, and 186 bp, respectively. In a second round of PCR, HuD12-HuD34 and HuD56-HuD78 were used to obtain HuD1234 (340 bp) and HuD5678 (346 bp) fragments. Finally, in the third round of PCR, HuD1234 and HuD5678 were used to obtain full-length humanized mature Der p 1 (666 bp).

Plasmid Construction and Preparation

Two DNA vaccine constructs were generated to produce mature Der p 1 based on its wild-type (mWt) and humanized (mHu) cDNA sequences. To produce the vector encoding mWt Der p 1 (pHIS-mWtDer p 1) (reference control), the authentic DNA Der p 1 sequence was amplified from a plasmid pPICZα-Der p1w1 using specific primers containing 5' Xho I (forward: 5'-GATCTCGAGATGACTAACGCCTGCAGT ATCAATGG-3'; restriction site is underlined) and 3' Kpn I (reverse: 5'-GGTTGGTACCCTACAGGATCACCACGTA GGGGTACTC-3'; restriction site is underlined). The mature codon-optimized synthetic Der p 1 cDNA was amplified with the following primers containing Xho I (forward) and Kpn I (reverse) restriction sites: 5'-GATCTCGAGATGACCAACG CCTGCAGCATCAACGG-3' and 5'-GGTTGGTACCCTA CAGGATCACCACGTAGGGGTACTC-3' (restriction sites are underlined). Both amplified fragments were restricted with Xho I and Kpn I and cloned into a pHIS plasmid vector (CSL Ltd., Melbourne, Australia) digested with the same enzymes to get pHIS-mHuDer p 1.

To generate expression vectors encoding fusion Der p 1-green fusion protein (GFP), the mWtDer p 1 and mHuDer p 1 PCR products were amplified with specific primers (wt forward: 5'-GATCTCGAGATGA CTAACGCCTGCAGTATCAATGG-3' and reverse 5'-TTGGTACCAAGAGAATGACAACTATGATATTC; humanized forward: 5'-GATCTCGAGATGACCAACGCCT GCAGCATCAACGG-3' and reverse: 5'-TTGGTACCAACA GGATCACCACGTAGGGGTACTC-3', (Xho I (GGTACC) and Kpn I (GGTACC) restriction sites are underlined). The amplified DNA pieces were digested with Xho I and Kpn I and cloned into Xho I-Kpn I restricted pEGFP-N1 vector, upstream of the GFP reporter gene. All the final DNA constructs were produced and purified under lipopolysaccharide-free conditions using GigaPrep Kits (QIAGEN, Hilden, Germany).

In Vitro Transfection

HEK 293 cells were cultured onto 6-well plates in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, California, USA) supplied with 10% fetal bovine serum (FBS) (Invitrogen) at a density of 5×10^5 cells/well. On the following day, the cells reached 90% confluence for the transfection assays. The different plasmid DNA samples were diluted in 250 µL of serum-free DMEM medium, gently mixed, and incubated with 250 µL of a diluted solution of Lipofectamine 2000 (Invitrogen), 2.5 µl of Lipofactamine 2000 diluted in 247.5 µL of Opti-MEM I (Invitrogen). The lipoplexes (1 µg of each DNA) were added to each well containing cell and medium and the plates were gently mixed and incubated at 37°C in a CO_2 incubator for 48 hours.

Detection of Protein Expression

Transfected cells were lysed with lysis buffer (0.3 M NaCl, 50 mM Tris, pH 7.5, 1 mM phenylmethanesulphonylfluoride (PMSF), 0.5% v/v Triton×100, 0.1% v/v leupeptin, 0.1% v/v aprotinin) for 30 minutes on ice and the cell lysate was clarified by centrifugation. The different supernatants were loaded onto 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels. The various protein bands were then transferred onto a nitrocellulose membrane using a semi-dry Trans-Blot transfer apparatus (Bio-Rad, Hercules, California, USA). The membrane was saturated with 5% skimmed milk and incubated with a mouse-anti Der p 1 polyclonal serum at a dilution of 1 to 2000 for 1 hour. The membrane was washed with phosphate buffer saline tween (PBST) (PBS, 0.5%, Tween 20) and incubated for 1 hour with an anti-mouse IgG horseradish peroxidase (HRP) conjugated secondary antibody (dilution 1:5000) (KPL, Silver Spring, Maryland, USA). The immunoreactive bands were detected by chemiluminescent HRP substrate (Millipore, Billerica, Massachusetts, USA) as substrate and exposed to X-ray film. The expression of Der p 1-GFP fusion proteins was detected by fluorescence light microscopy (Biozero KEYENCE, Osaka, Japan), flow cytometry analysis with a FACSCalibur flow cytometer (BD Biosciences, Bedford, Massachusetts, USA) and western blotting using anti-GFP monoclonal antibody (Rockland, Gilbertsville, Pennsylvania, USA) at a dilution of 1 to 2000.

IgE Reactivity of Recombinant Der p 1

The plates were coated with anti-human IgE antibody (KPL) (500 ng/well), at 4°C overnight. The next day, the plates were washed and incubated with blocking buffer for 1 hour at 37°C. Sera from HDM-allergic patients with IgE to natural Der p 1 were diluted with blocking buffer (1:5) and incubated for 1 hour at 37°C. Nonallergic human serum was used as a control. Lysate from HEK 293 cells expressing mature Der p 1 was then added to blocking buffer and incubated for 1 hour at 37°C. Immune complexes were detected with biotinylated anti-Der p 1 monoclonal antibody 4C1 (Indoor Biotechnologies, Cardiff, UK) and incubated at 37°C for 1 hour and followed with streptavidin-horseradish peroxidase conjugate (SAv-HRP) (BD Biosciences) for 30 minutes. Antigen-antibody complexes were detected after the addition of tetramethylbenzidine (TMB) substrate (BD Biosciences). The plate was measured in an enzyme-linked immunosorbent assay (ELISA) reader at an optical density (OD) of 450 nm.

Lipoplex Preparation for DNA Vaccination

To prepare the liposomes, 500 µg lipoid S75 (Lipoid GmBH, Ludwigshafen, Germany), 10 µg DOTAP (Sigma-Aldrich, St. Louis, MO, USA) and 6 µg cholesterol (Sigma-Aldrich) were mixed and dissolved in chloroform. The solvent was evaporated in a rotary evaporator at 60°C until the lipid film was dry. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (10 mM, pH 7.0) was added to dissolve the lipid film. The liposomes were then extruded using a LIPEX Extruder (Northern Lipids Inc, Burnaby, Canada) through a 600-nm polycarbonate filter (6 times) and then through a 200-nm polycarbonate filter (9 times). The size of the liposomes, measured with a Nanoparticle Sizer (Malvern, Herrenberg, Germany), was estimated at 200 nm and stored at 4°C.

Mice

BALB/c female mice aged 6 to 8 weeks old were purchased from the National Laboratory Animal Centre at Mahidol University, Thailand. Annual care and experimental procedures were carried out in accordance with Chulalongkorn University guidelines.

Immunization of Mice

Groups of 5 mice were immunized with 100 μg naked pHIS-mHuDer p 1 or 50 μg of lipoplex DNA. Control mice were vaccinated with a pHIS-empty vector (negative control), recombinant ProDer p 1 formulated with alum (10 μg of protein, protein/alum (Sigma-Aldrich) (ratio, 1:20), or liposome alone. The mice were anesthetized with inhaled anesthetic AERRANE (isoflurane) (Baxter, Deerfield, Illinois, USA) and vaccinated intradermally on days 0, 7, 14, 21, and 28. The control mice immunized with recombinant ProDer p 1 formulated with alum were intraperitoneally injected following the same schedule. Sera were collected on days 0, 7, 14, 21, and 28 to measure allergen-specific antibody responses.

Determination of Mouse-Specific IgG2a, IgG1 and IgE Antibodies

Der p 1-specific antibody response in mice models was determined by ELISA. The plates (NUNC Maxisorp, Wiesbaden, Germany) were coated overnight with 500 ng of recombinant ProDer p 1 in 0.1 M sodium carbonate buffer, pH 9.5. They were then saturated with blocking buffer (3% skimmed milk in PBS) for 1 hour at 37°C and subsequently incubated with individual mice sera diluted at 1:40, 1:40, and 1:20 for IgG2a-, IgG1-, and IgE-specific antibody detection, respectively. The plates were washed with washing buffer (PBS containing 0.05% Tween 20) and incubated with biotin-conjugated anti-mouse IgG2a, IgG1, or IgE (BD Biosciences) at a dilution of 1:250 in blocking buffer for 1 hour at room temperature, followed by 30-minute incubation with SAv-HRP diluted at 1:250 at room temperature. Antigen-antibody complexes were detected after addition of TMB substrate. Plate absorbance was read at OD_{450} nm using an ELISA plate reader (Multiscan EX plate reader; Pittsburgh, Pennsylvania, USA). All the serum samples were tested in duplicate.

Statistical Analysis

The Mann Whitney test was used to analyze the results for the IgE reactivity assay and Der p 1-specific IgG2a values between experimental groups. Statistical significance was set at $P<.05$.

Results

Expression of the Humanized Mature Der p 1 DNA Construct

As it has been demonstrated that codon usage optimization drastically improves the expression of recombinant ProDer p 1 in mammalian cells [21] we designed synthetic mature Der p 1 cDNA based on humanized codon usage for DNA vaccination.

The 666 bp full-length humanized Der p 1 cDNA was generated by a series of 3 PCR reactions and 8 overlapping oligonucleotides that covered the mature Der p 1 sequence. Sequence alignment between the authentic (GenBank P08176) and the synthetic mature Der p 1 cDNA showed that more than 70% of nucleotides were modified when compared with mWtDer p 1 cDNA, resulting in the modification of approximately 75% (167/222) of the codons (Figure 1).

In order to evaluate whether this DNA vaccine construct can produce mature Der p 1, HEK 293 cells were transfected with pHIS-mHuDer p 1 and mature Der p 1 expression was compared by western blotting with that obtained after transfection with the authentic mature Der p 1 cDNA (pHIS-mWtDer p 1). As a positive control, HEK 293 cells were transfected with DNA plasmid coding for humanized ProDer p 1 (pNIV4868) As shown in Figure 2, pHIS-mHuDer p 1 induced a higher production of mature Der p 1 than the plasmid containing the authentic mature Der p 1 cDNA. As expected, the mature Der p 1 migrated as a 30 kD molecule whereas a 35 kD band corresponding to ProDer p 1 was detected after transfection (Figure 2). Mature Der p 1 was strictly produced intracellularly whereas ProDer p 1 was also detected in the supernatant as the ProDer p 1 cDNA was cloned downstream of a strong signal sequence [11].

To confirm intracellular expression of mature Der p 1, authentic as well as codon-optimized mature Der p 1 cDNA was fused to the GFP reporter gene. Expression of Der p 1-GFP fusion proteins in transfected HEK 293 cells was detected by fluorescence microscopy, flow cytometry, and western blotting. Unlike unfused GFP expression, which displays a homogeneous intracellular GFP distribution, mHuDer p 1-GFP expression was clustered within the cell (spot phenotype) (Figure 3A). These experiments also confirmed that mature Der p 1 cDNA humanization is beneficial for allergen expression as an approximately 5-fold increase in expression (compared with the production of authentic Der p 1-GFP) was observed by flow cytometry (Figure 3B); furthermore, a 52 kD band corresponding to synthetic Der p 1-GFP was clearly detected by anti-GFP antibodies (this did not occur with natural Der p 1-GFP, Figure 3C). These results support the idea that codon optimization is critical for mature Der p 1 expression.

Hypoallergenicity of Mature Der p 1 Produced Intracellularly

Indirect ELISA was performed to determine the IgE binding activity of mature Der p 1 following transfection with the corresponding DNA vaccines. Four sera from HDM-sensitized patients (skin prick test wheal size of greater than 5 mm with pseudopod formation) and 3 sera from healthy individuals (negative skin prick test) were used to evaluate the IgE reactivity of mature Der p 1 from lysate of pHis-mHuDer p 1–transfected HEK cells. In contrast to the ProDer p 1, which has IgE-binding activity, the mature Der p 1 did not show any IgE binding reactivity ($P=.0286$, Figure 4) suggesting that the intracellular expression of mature Der p 1 leads to the production of a hypoallergenic variant of Der p 1.

Hypoallergenic DNA Vaccine Encoding Der p 1 Induces a Specific T_H1 Response

To determine whether this mHuDer p 1 DNA with CpG motif vaccine elicits anti-T_H2 allergic responses, naïve mice were intradermally immunized with this DNA construct formulated or not with Lipoid S75/DOTAP/cholesterol liposome. The humoral response was compared with that induced after intraperitoneal administration of recombinant ProDer p 1 formulated with alum (Figure 5). ProDer p 1/alum immunization triggered a typical allergen-specific T_H2 response

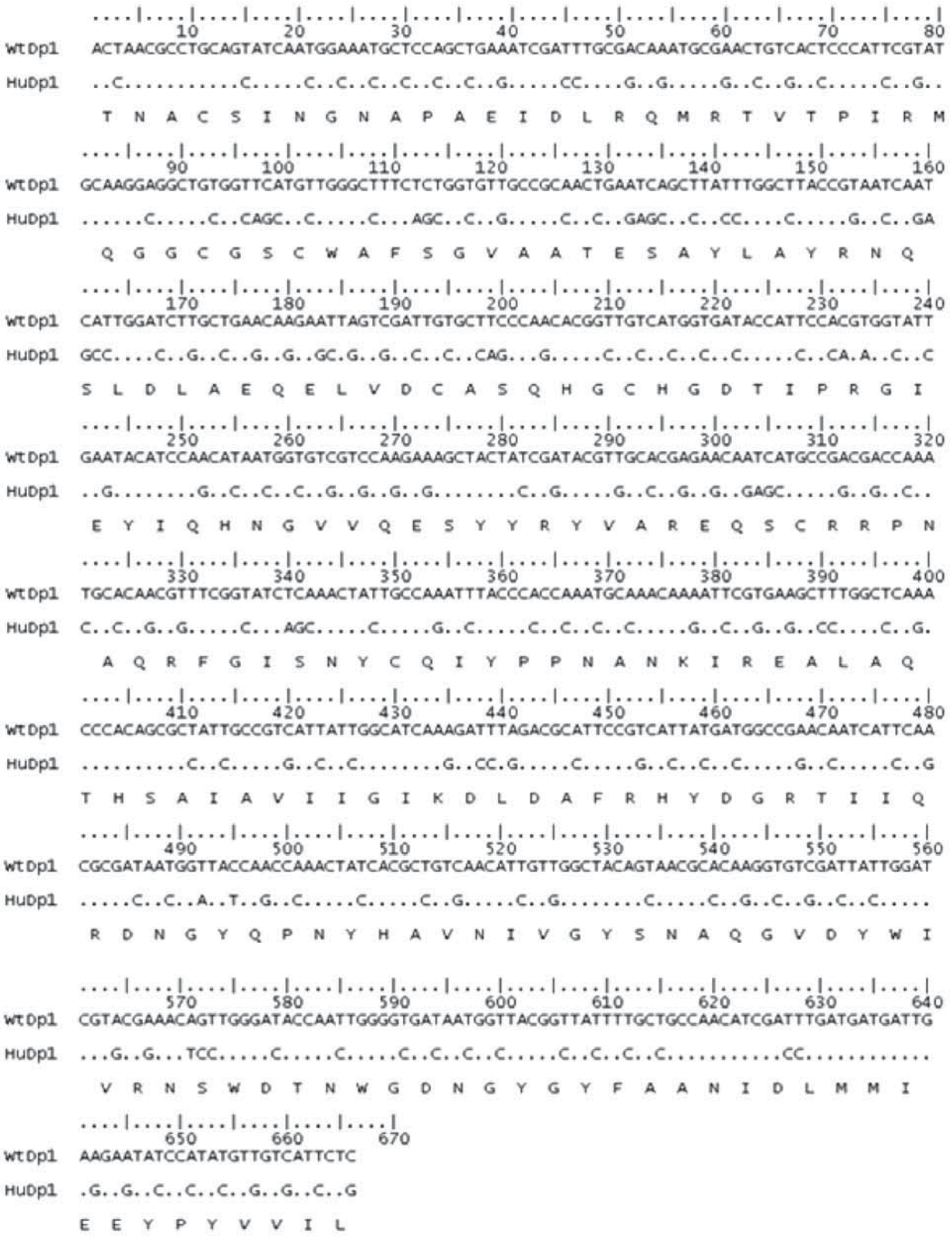

Figure 1. Sequence alignments of wild-type (wt) Der p 1 (GenBank P08176) and human codon-optimized (mHu) Der p 1 complementary DNA. Similarities in nucleic acid sequences are shown as dots. Der p 1 indicates major *Dermatophagoides pteronyssinus* group 1 allergen.

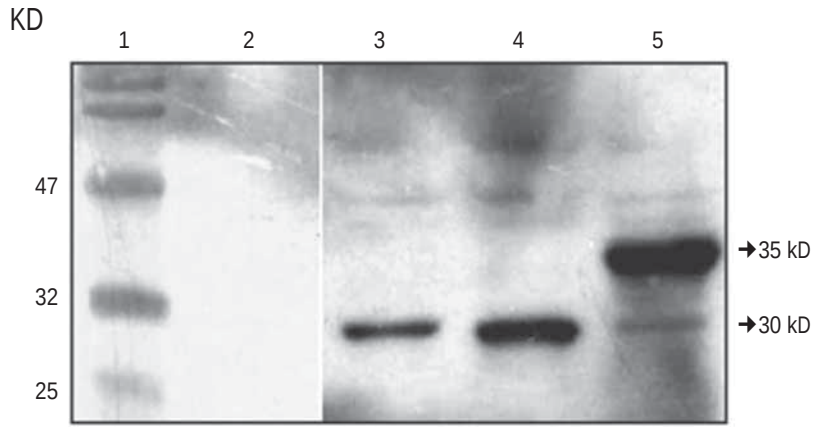

Figure 2. Recombinant Der p 1 expression after HEK 293 cell transfection. Lane 1, molecular weight marker. Lane 2, nontransfected cell (negative control). Lane 3, cell lysate from pHIS-mWtDer p 1–transfected cells. Lane 4, cell lysate from pHIS-mHuDer p 1–transfected cells. Lane 5, cell lysate from pNIV4868-transfected cells (plasmid producing ProDer p 1, positive control [11]). One representative experiment out of 3 is shown. Der p 1 indicates major *Dermatophagoides pteronyssinus* group 1 allergen; HEK 293 cells, Human Embryonic Kidney 293 cells.

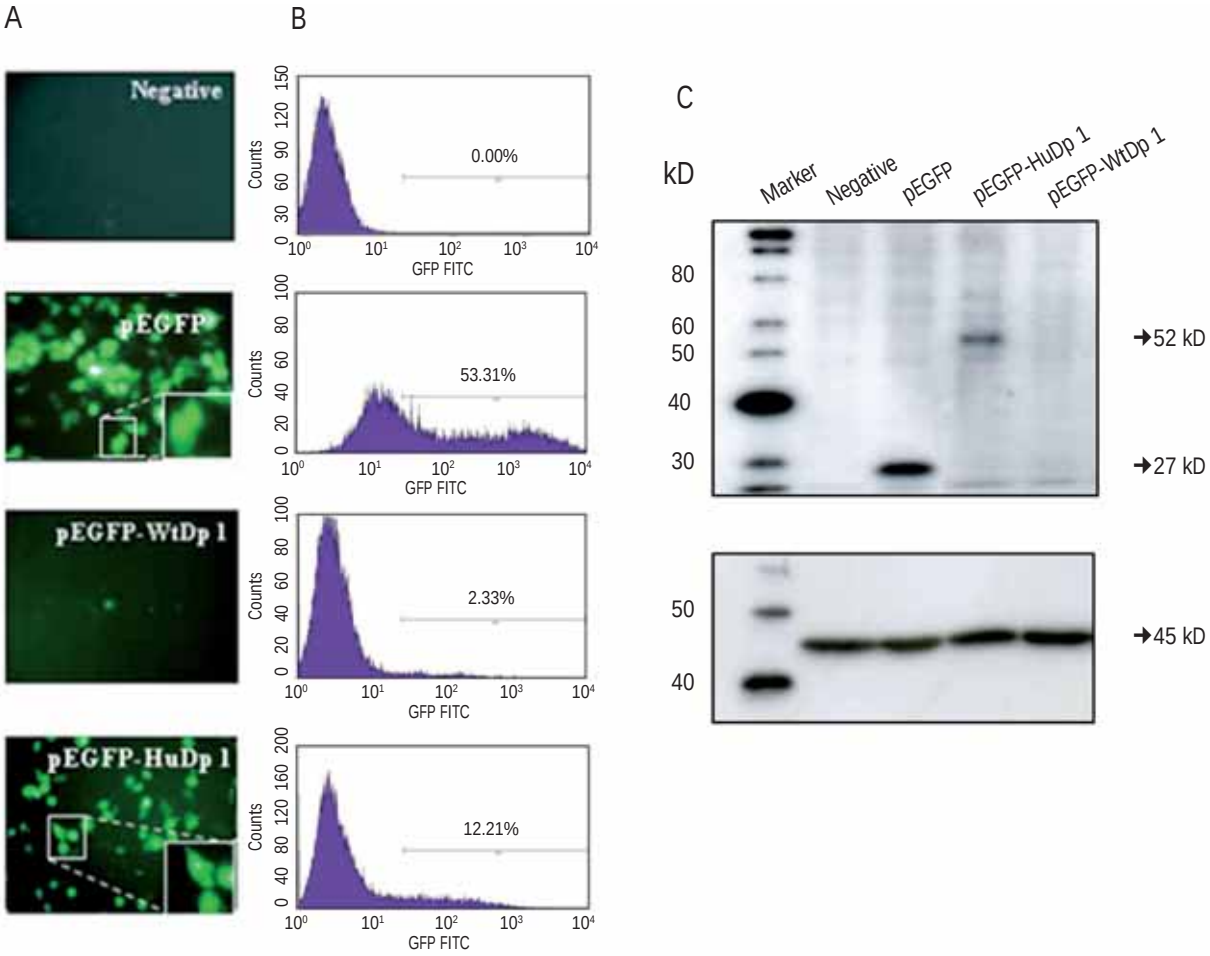

Figure 3. Der p 1-green fluorescent protein (GFP) expression in HEK 293 cells. Detection by fluorescence microscopy (panel A), flow cytometry analysis (panel B), and western blot (panel C) of GFP or Der p 1-GFP after transfection of HEK 293 cells with a pEGFP-N1–empty vector, pEGFP-mHuDer p 1, or pEGFP-mWtDer p 1. The percentages of GFP-positive cells, calculated in 100 000 cells, are shown in panel B. The detection of ß-actin protein expression (45 kD) used to control protein loading is shown in the lower part of panel C. One representative experiment out of 3 is shown. Der p 1 indicates major *Dermatophagoides pteronyssinus* group 1 allergen; HEK 293 cells, Human Embryonic Kidney 293 cells.

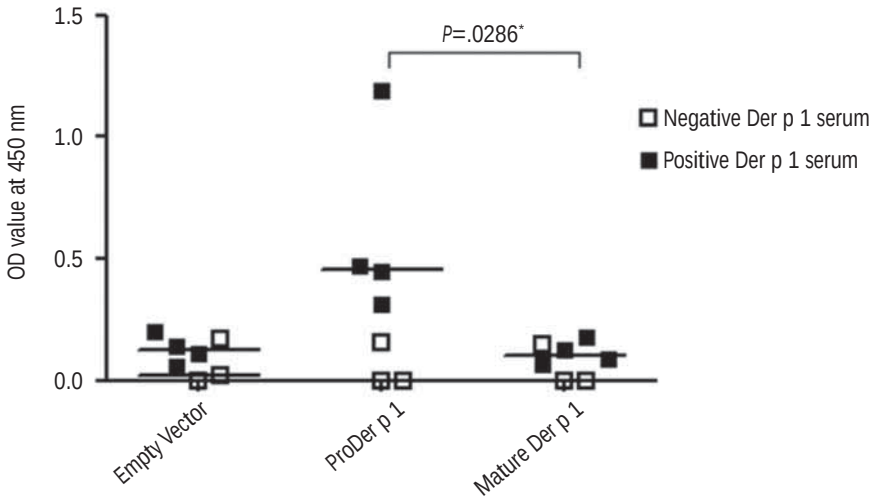

Figure 4. Immunoglobulin (Ig) E binding activity of mature Der p 1. The IgE reactivity of ProDer p 1 and mature Der p 1 was determined using enzyme-linked immunosorbent assay with sera from house dust mite–sensitized patients (n=4) and negative sera (n=3). To select sera patients (patients with positive skin prick tests) with positive or negative IgE to Der p 1, a direct Der p 1 IgE binding assay was performed using anti-human IgE as a coating antibody. The optical density (OD) at 450 nm corresponded to the level of specific Der p 1 Abs. Der p 1 indicates major *Dermatophagoides pteronyssinus* group 1 allergen.
*P=.0286 compared with IgE binding of ProDer p 1 and mature Der p 1.

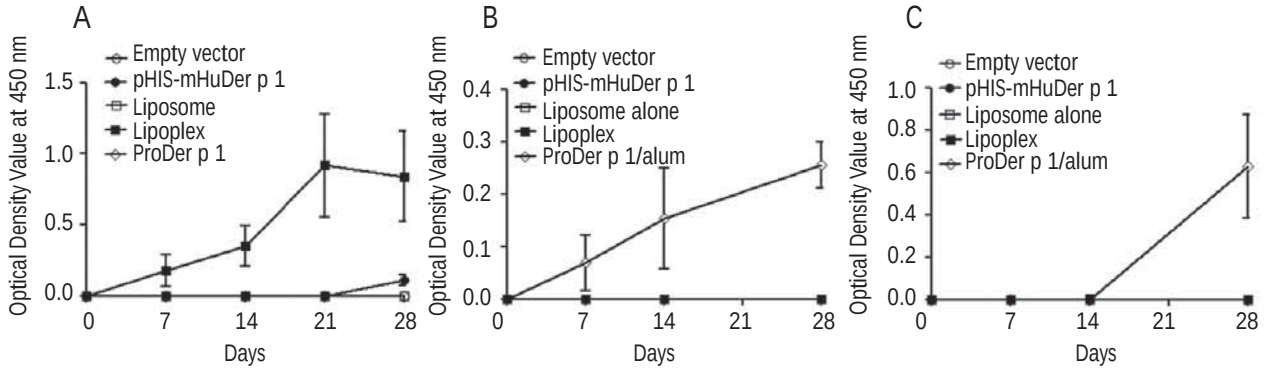

Figure 5. Specific antibody response after immunization of mice with ProDer p 1/alum, naked pHIS-mHuDer p 1, lipoplex (liposome-pHIS-mHuDer p 1), liposome, or empty vector alone. Der p 1–specific immunoglobulin (Ig) G2A (Figure 5A), IgE (Figure 5B), and IgG1 (Figure 5C) were detected in the serum of all the mice by enzyme-linked immunosorbent assay. The data were plotted as means (SD) (n=5 in each group). The difference between the lipoplex-vaccinated group and the ProDer p 1 group, the naked DNA group, the liposome only group, and the empty vector group at each week was significant at P<.001. Der p 1 indicates major *Dermatophagoides pteronyssinus* group 1 allergen.

characterized by the production of specific IgE and IgG1 and by the absence of specific IgG2a. By contrast, mice injected with 100 μg naked mHuDer p 1 DNA developed a specific T_H1 response as shown by both a statistically significant production of specific IgG2a (P<.001) detected in the sera and the absence of an allergen-specific IgE and IgG1 antibody response. More interestingly, the combination of 50 μg pHIS-mHuDer p 1 DNA with liposome drastically accelerated the development of the specific IgG2a response and increased the intensity of the anti-Der p 1 antibody response.

Discussion

Several DNA-based methods of immunization including unmethylated CpG oligonucleotides, CpG DNA conjugated to a protein allergen and plasmid DNA encoding allergen were shown to be effective in animal models to downregulate the allergen-specific T_H2 response by, mainly, the induction of T_H1 responses characterized by the production of IgG2a antibodies and T_H1 cytokines such as IFN-γ production [22].

DNA vaccines for the treatment or prevention of HDM allergy need to be based at least on group 1 allergens due to

the very high prevalence of IgE reactivity to these allergens in HDM-allergic patients [14,15].

Several concurring preclinical studies on Der p 1-based DNA vaccine candidates have highlighted the prophylactic and/or therapeutic potential of these vaccines against HDM allergy. Indeed, vaccinations with plasmids encoding mature Der p 1 [23-26], ProDer p 1 [10], and Der p 1 T-cell epitopes [27,28] have notably been seen to decrease total and Der p 1-specific IgE, upregulate specific IgG2a, activate IFN-γ production, reduce IL-4, IL-5, and IL-13 secretion, and impair eosinophilia in the lungs as well as airway hyperresponsiveness and mucus production.

It is noteworthy that mature Der p 1 or ProDer p 1 cDNA is usually cloned downstream of strong leader peptides such as the mite allergen Der p 5 [25,26], murine Ig kappa light chain [24], or the varicella zoster virus glycoprotein E [10]. The addition of a leader sequence in the DNA constructs drives protein secretion, leading to allergen presentation through major histocompatibility complex class I and II molecules to activate both $CD4^+T_H$ cells and $CD8^+$ cytotoxic T cells. However, the use of a strong signal peptide could lead to an enhanced level of circulating allergen that primes T_H2-skewed responses, as has previously been demonstrated for the design of a Der p 2–based DNA vaccine [13].

Consequently, hypoallergenic DNA vaccines displaying very low IgE reactivity but retaining T-cell reactivity are highly desirable to prevent priming and potential anaphylactic side effects. The disruption of conformational IgE-binding epitopes by point mutations or truncation, forced ubiquitination or the targeting of allergens to lysosomal/endosomal compartments could prevent the risk of allergen sensitization [29,30]

To successfully produce a correctly folded recombinant Der p 1 with IgE reactivity and proteolytic activity similar to those of the natural allergen in animal cells, cDNA encoding ProDer p 1, carrying authentic or heterologous signal peptide, must be used. Indeed, the absence of Pre-Pro sequences in Der p 1-based DNA constructs has been found to induce not only the intracellular sequestration of Der p 1 but also, strikingly, to prevent the allergen from adopting a correct fold and consequently displaying enzymatic activity [9,31,32] as the prosequence acts as an intramolecular chaperonin which is critical for appropriate Der p 1 folding [19]. Based on these findings, we decided to clone codon-optimized cDNA that encoded only mature Der p 1 (222 amino acids) in order to design a safer Der p 1-based DNA vaccine with hypoallergenic properties. Our results show, as expected, that codon optimization drastically influenced the production of mature Der p 1 and that the intracellular expression of this allergen drastically reduced its IgE reactivity. Accordingly, the inappropriate folding of Der p 1 could extensively prevent any putative T_H2 priming following DNA vaccination.

This new Der p 1 DNA vaccine candidate maintained its ability to induce allergen-specific T_H1 response as pHIS-mHuDer p 1 immunizations prevented the production of anti-Der p 1 IgE and IgG1 and induced a specific IgG2a response. As expected, the administration of pHis-mHuDer p 1 as a lipoplex formulation induced a faster and stronger pro-T_H1 antibody response. Moreover, thanks to the lipoplex approach, the amount of DNA can be reduced at least 2-fold compared with naked DNA immunizations. It is now well recognized that the combination of cationic liposomes with DNA significantly increases the potency of activation of innate immunity [33,34]. In future experiments, the prophylactic and therapeutic potential of pHis-mHuDer p 1-based lipoplexes will be evaluated in a Der p 1 sensitization murine model. Alternatively, such lipoplexes will be also combined with recombinant ProDer p 1 as co-immunizations of DNA and protein vaccines have been shown to suppress allergic responses via the induction of specific T regulatory cells or the cross-priming of $CD8^+$ T cell responses [35,36].

Moreover, as electroporation has been seen to drastically improve the immunogenicity of DNA-based vaccines [37,38], the mode of delivery will also be evaluated to vaccinate mice with pHis-mHuDer p 1 in further studies.

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