

Original article

Optimization of codon usage is required for effective genetic immunization against Art v 1, the major allergen of mugwort pollen

Background: As the major allergen of mugwort pollen, Art v 1 is an important target for specific immunotherapy. However, both recombinant protein as well as a gene vaccine for Art v 1 failed to be immunogenic in mice. In order to improve immunogenicity we focused on genetic immunization because interspecific differences of codon usage have been shown as an obstacle for effective induction of immune responses with gene vaccines encoding infectious pathogens.

Objective: In order to find out, whether codon usage might also be used to improve genetic immunization with allergen genes, the response against a gene vaccine expressing the wild-type gene of Art v 1 (pCMV-wtArt) was compared with a synthetic codon-optimized vector with human codon usage (pCMV-humArt).

Methods: Balb/c mice were injected intradermally with pCMV-wtArt or pCMV-humArt. *In vitro* expression levels of both constructs were compared in transfection experiments. Total immunoglobulin G (IgG), IgG1, IgG2a and IgE antibodies were analyzed by enzyme-linked immunosorbent assay and the anaphylactic activity of the sera was determined by allergen-specific degranulation of rat basophil leukemia-2H3 cells.

Results: No immune response was detectable with the gene vaccine expressing the wildtype Art v 1, but immunization with pCMV-humArt revealed a strong and allergen-specific induction of antibody responses. The antibodies recognized both the recombinant as well as the purified natural (glycosylated) Art v 1 molecule. The response type was Th1-biased, as indicated by high levels of IgG2a antibodies. Expression analysis with B16 mouse melanoma cells transfected with pCMV-humArt or pCMV-wtArt revealed an impaired expression of the wild-type vector but normal translation after recoding.

Conclusion: The results demonstrate that optimization of codon usage offers a simple way to improve immunogenicity and therefore should be routinely considered in the development of gene vaccines for the treatment of allergy.

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Pollen from mugwort (*Artemisia vulgaris*) is one of the main allergen sources in central Europe and parts of Asia during late summer. Among patients suffering from pollinosis, the incidence of allergic disease caused by mugwort pollen is between 10 and 14% (1, 2). As the major allergen of mugwort pollen, Art v 1 (3) represents an important target for the conventional specific immunotherapy (SIT) as well as for novel therapy concepts such as genetic desensitization.

The latter has emerged as an important new vaccination strategy and a powerful alternative to protein-based vaccination against pathogens, tumors and even allergic diseases (4, 5). Intramuscular or intradermal injection of

plasmid DNA encoding clinically relevant allergens can induce immune responses with a Th1 bias and promote the formation of IFN- γ producing CD4⁺ T cells (6) thus stimulating B cells to synthesize immunoglobulin G (IgG)2a antibodies and inhibit IgE production (7–11).

Furthermore, genetic immunization offers a palette of modulating possibilities to enhance the immunogenicity of plasmid constructs (12).

One of these modulating approaches is based on the fact that most amino acids are encoded by more than one codon, and codon usage varies from organism to organism. Therefore, differences in the codon usage concerning a heterologous gene and the transfected cell can have a

drastic effect on protein expression with a significant influence on the immunogenicity of a gene vaccine (13–17).

Whereas genetic vaccines encoding the native sequences of various allergens induced reliable immunogenicity and seroconversion (6–8, 10, 18), no immune response was detectable after genetic immunization with the wildtype sequence of the Art v 1 gene.

In the present work we demonstrate that a negative codon usage-effect impaired the immunogenicity of a gene vaccine encoding the allergen Art v 1. Recoding of the vaccine with a synthetic gene sequence based on the human codon usage increased the expression level of the antigen and thus enhanced the immunogenicity of the construct.

Materials and methods

Recoding of Art v 1 DNA

The humanized gene was synthesized in a template-free polymerase chain reaction (PCR) using three overlapping oligonucleotides (125-mer 1–115, 128-mer 100–228 and 147-mer 195–330) spanning the entire Art v 1 sequence (Fig. 1). The 125-mer contained a 5 prime attachment 5' CACCGAATTC3' incorporating a *EcoRI* restriction site; the 147-mer contained a 5 prime attachment 5' CGACTCTCTAGA3' incorporating a *XbaI* restriction site for directed subcloning.

Codons were adapted according to the codon usage tabulated from GenBank (<http://www.kazusa.or.jp/codon>) for the optimal human codon preference (Table 1) (19). No deviations from strict adherence to optimized *Homo sapiens* codon usage were accepted. The alignment of the novel codon-modified Art v 1 sequence (GeneBank accession number AF502559) with the wildtype-expressing sequence (Gene Bank accession number AF493943) is demonstrated in Fig. 1.

Plasmid constructions

Two plasmid vectors, utilizing either the wildtype nucleotide sequence of Art v 1 (pCMV-wtArt) or a codon-optimized Art v 1 sequence (pCMV-humArt), were constructed.

For the wildtype construct pCMV-wtArt, PCR amplification of Art v 1 cDNA was conducted with primers incorporating a 5' *EcoRI* and 3' *XbaI* restriction sites. The amplified PCR product was cut with *EcoRI* and *XbaI* and ligated to the pCMV-Tpa mammalian expression vector. Among other features, this vector contains a CMV immediate early promoter-enhancer region and a tissue plasminogen activator leader sequence for efficient secretion of the mature protein (20).

Analogously to the wildtype gene, the 350 bp fragment generated in the recoding process of Art v 1 was ligated into an *EcoRI-XbaI* digested pCMV-Tpa expression vector. The resulting plasmid was designated pCMV-humArt and encodes Art v 1 by using the most frequent codons commonly found in highly expressed human genes (Table 1).

Cloning was controlled by sequencing all constructs in an Abi Prism™ Genetic Analyzer (Perkin Elmer, Norwalk, CT).

										Section 1																					
	(1)	1				10				20					30				40					57							
wtArt	(1)	ATGGCTGGTTCAAAGTTGTGTG	GAAAAGACAAGCAAGACG										TATTCGGGTAAAGTGCGAC																		
humArt	(1)	ATGGCCGGCAGCAAGCTGTGCG	GAGAAGACCAGCAAGACCTACAGCGGCAAGTGCGAC																												
Art v 1	(1)	Met	Ala	Gly	Ser	Lys	Leu	Cys	Glu	Lys	Thr	Ser	Lys	Thr	Tyr	Ser	Gly	Lys	Cys	Asp											
										Section 2																					
	(58)	58								70					80				90					100				114			
wtArt	(58)	AACAAGAAATGTGACAAAAAGTGTATAGAGTGGGAGAAAGCGCAACATGGTGTGCTTGT																													
humArt	(58)	AACAAGAAGTGC	GACAAAGAAGTGCA										TCGAGTGGGAGAAAGGCCAGCACGGCGCCTGC																		
Art v 1	(58)	Asn	Lys	Lys	Cys	Asp	Lys	Lys	Cys	Ile	Glu	Trp	Glu	Lys	Ala	Gln	His	Gly	Ala	Cys											
										Section 3																					
	(115)	115								120					130				140					150				160			171
wtArt	(115)	CACAAGAGAGAAAGCCGGCAAAGAAAGTTGCTTTTGCTACTTTGACTGTTCCAAATCG																													
humArt	(115)	CACAAGCGGGAGGCCGGCAAGGAGAGCTGCTTCTGCTACTTCGACTGCAGCAAGAGC																													
Art v 1	(115)	His	Lys	Arg	Glu	Ala	Gly	Lys	Glu	Ser	Cys	Phe	Cys	Tyr	Phe	Asp	Cys	Ser	Lys	Ser											
										Section 4																					
	(172)	172								180					190				200					210				228			
wtArt	(172)	CCTCCTGGAGCAACACCAGCGCCTCCTGGTGCAGCTCCTCCCCAGCTGCTGGCGGC																													
humArt	(172)	CCTCCAGGCGCCACCCAGCCCACTGGCGCGCGCCCTCCACCCGCCCGCGCGGC																													
Art v 1	(172)	Pro	Pro	Gly	Ala	Thr	Pro	Ala	Pro	Pro	Gly	Ala	Ala	Pro	Pro	Pro	Pro	Ala	Ala	Gly	Gly										
										Section 5																					
	(229)	229								240					250				260					270				285			
wtArt	(229)	TCTCCGTACCTCCCGCTGATGGTGGCTCACCACCTCCTCCAGCTGATGGTGGATCT																													
humArt	(229)	AGCCCCAGCCCACTGCCGACGGCGGCAGCCACCTCCACCTGCCGACGGCGGCAGC																													
Art v 1	(229)	Ser	Pro	Ser	Pro	Pro	Ala	Asp	Gly	Gly	Ser	Pro	Pro	Pro	Pro	Pro	Ala	Asp	Gly	Gly	Ser										
										Section 6																					
	(286)	286								300					310				320					330							
wtArt	(286)	CCTCCTGTAGATGGTGGCTCTCCACCTCCTCCGTCCACTCACTAA																													
humArt	(286)	CCACCCGTGGACGGCGGCAGCCCCCCCCCCCCAGCACCACTAA																													
Art v 1	(286)	Pro	Pro	Val	Asp	Gly	Gly	Ser	Pro	Pro	Pro	Pro	Ser	Thr	His	***															

Figure 1. Alignment of the wtArt and humArt sequence with the protein consensus sequence. Differences in the nucleic acid sequences are marked with shadows.

Table 1. Recoding of the Art v 1 gene

Amino acid	Art v 1 codons	<i>n</i> for Art v 1	Human codon frequency/1000	Optimized codon	Human codon frequency/1000 of the optimized codon
Ala	GCT	7	18.6	GCC	28.6
	GCA	2	16.0		
	GCC	1	28.6		
	GCG	2	7.6		
Arg	AGA	1	11.5	CGG	11.9
Asn	AAC	1	19.5	AAC	19.5
Asp	GAT	3	22.3	GAC	26.0
	GAC	3	26.0		
Cys	TGT	5	9.9	TGC	12.2
	TGC	3	12.2		
Gln	CAA	1	11.8	CAG	34.6
Glu	GAA	3	29.1	GAG	40.8
	GAG	2	40.8		
Gly	GGT	7	10.8	GGC	22.9
	GGA	2	16.3		
	GGC	5	22.9		
His	CAT	1	10.4	CAC	14.9
	CAC	2	14.9		
Ile	ATA	1	7.1	ATC	21.5
Leu	TTG	1	12.5	CTG	40.2
Lys	AAA	5	24.0	AAG	32.9
	AAG	7	32.9		
	ATG	1	22.3		
Met	ATG	1	22.3	ATG	22.3
Phe	TTT	2	16.9	TTC	20.4
Pro	CCT	12	17.3	CCC	20.0
	CCG	2	7.0		
	CCA	5	16.7		
	CCC	2	20.0		
Ser	TCA	3	11.7	AGC	19.3
	TCG	2	4.5		
	TCC	2	17.4		
	TCT	3	14.6		
	AGT	1	11.9		
Thr	ACA	2	14.8	ACC	19.2
	ACT	1	12.8		
	ACG	1	6.2		
Trp	TGG	1	12.8	TGG	12.8
Tyr	TAT	1	12.0	TAC	15.6
	TAC	1	15.6		
Val	GTA	1	7.0	GTG	28.9

The codons and their frequency of occurrence of the wild type Art v 1 gene (*n* for Art v 1 = the number of the respective codons as used in the wild-type Art v 1) are compared with the codon frequencies of highly expressed human genes (human codon frequency per thousand). The codon usage data for human proteins are derived from published results (19). The codon-optimized Art v 1 gene always represents the optimal codon concerning the human codon usage (expressed for all optimized codons in the rightmost column).

DNA preparation

Large scale purification of the expression vectors were carried out with Endo Free Plasmid Giga kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The Endotoxin level in the plasmid DNA was < 3 EU/ml as measured by the Pyroquant (*Limulus* amoebocyte lysate) assay (Pyroquant Diagnostik, Moerfelden, Germany). Plasmid DNA was analyzed by agarose gel electrophoresis and quantified by spectrophotometry (OD260/

OD280 ratio > 1.8). The plasmid DNA was stored in endotoxin-free H₂O at -20°C.

Expression of Art v 1 *in vitro*

5 × 10⁵ B16 mouse melanoma cells were transfected with 1.8 µg of pCMV, pCMV-wtArt, pCMV-humArt or pCMV-β (beta-galactosidase, gb:U02451) using Superfect (Qiagen) as described by the manufacturer. Cells and supernatants were harvested 16 h after transfection. The production of Art v 1 was measured in supernatants of cells and normalized to the activity of the beta-galactosidase gene, which was measured via Galacto-Star reporter gene assay according to the protocol of the manufacturer (Applied Biosystems, Foster City, CA).

Expression levels were analyzed in an enzyme-linked immunosorbent assay (ELISA) against serum-free supernatants of transfected cells. In brief, supernatant containing 100 ng protein, either from cells transfected with pCMV-wtArt or pCMV-humArt, was coated overnight at 4°C onto ELISA-plates. After blocking with 0.5% bovine serum albumin (BSA)-phosphate-buffered saline tween (PBST) (PBS + 0.5% BSA + 0.2% Tween-20), plates were incubated with appropriate dilutions of Art v 1-specific serum for 1 h at room temperature. The Art v 1-specific serum was obtained by immunization with purified natural Art v 1 protein (see 'animals and immunization' below). After washing with PBS, the plates were then incubated with an anti-mouse IgG HRP conjugated antibody (Bio-Rad, Hercules, CA) diluted 1 : 1000 in blocking buffer for 1 h at room temperature. The assay was developed with Luminol (BM chemiluminescence substrate; Boehringer Mannheim, Mannheim, Germany) diluted 1 : 1 in H₂O. Chemiluminescence (photon counts/s) was quantified by using a Lucy I ELISA-plate luminometer (Anthos-Labtec, Salzburg, Austria).

Animals and immunization

Female, 6–10 weeks old BALB/c mice were obtained from the animal breeding facilities in Himberg, Austria, and maintained at the central animal care facility at the University of Salzburg according to the local guidelines for animal care.

Groups of mice (*n* = 6) were immunized i.d. into the shaved back with a total of 100 µg of plasmid DNA in a volume of 200 µl sterile PBS, once a week for three consecutive weeks.

For protein immunization, mice (*n* = 6) were injected with 5 µg of purified natural Art v 1 together with 100 µl Al(OH)₃ (Serva, Heidelberg, Germany, 2 mg/ml) in a total volume of 150 µl sterile PBS three times in weekly intervals.

Analysis of serum antibody titers

Black 96-well high-bind immunoplates (Greiner, Kremsmünster, Austria) were coated by overnight incubation at 4°C with 100 ng/well recombinant Art v 1 or purified natural Art v 1 (Art v 1, purified from mugwort pollen). Plates were blocked with blocking buffer (PBS + 0.5% BSA + 0.2% Tween-20) for 1 h at room temperature.

Sera were serially diluted in blocking buffer, transferred to the coated microtiter plates, incubated for 1 h at room temperature and then washed.

Horseradish peroxidase-conjugated detection antibodies goat anti-mouse IgG1 (Serotec, Oxford, UK), goat anti-mouse IgG2a (Serotec) or rat anti-mouse IgE (Clone LO-ME-3; Serotec) were diluted 1 : 1000 in blocking buffer and reacted for 1 h at room temperature. The assay was developed with Luminol

(BM chemiluminescence substrate; Boehringer Mannheim) diluted 1 : 1 in H₂O. Chemiluminescence (photon counts/s) was quantified by using a Lucy I ELISA-plate Luminometer (Anthos-Labtec).

For end-point titer determinations any well with a luminescence > 3 SD above background (calculated using > 20 wells containing no primary antibody) was scored as positive. The end-point titer of total serum antibody bound to the plates was determined by using a standard curve generated with known dilutions of a high titered antiserum (won by immunization with the purified natural Art v 1 protein as described above).

Rat basophil leukemia cell mediator release

Rat basophil leukemia (RBL)-2H3 cells were plated in 96 well tissue culture plates (4×10^4 /well) and incubated for 24 h at 37°C using 7% CO₂. Passive sensitization was performed by incubation with the different Art v 1-specific sera at a final dilution of 1 : 30 for 2 h. To remove unbound antibodies, the cell layer was washed three times in Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 0.5 mM MgCl₂, 1.8 mM CaCl₂, 0.4 mM NaH₂PO₄, 5.6 mM D-glucose, 12 mM NaHCO₃, 10 mM HEPES and 0.1% BSA, pH 7.2). Cross-linking of the Fc_ε R-bound IgE and subsequent degranulation of RBL cells was induced by adding 100 µl purified natural Art v 1 (1.5 µg/ml) in Tyrode's buffer for 30 min in a humidified atmosphere at 37°C. Supernatants were analyzed for β-hexosaminidase activity by incubation with 80 µM 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (Sigma, Taufkirchen, Germany) in citrate buffer (0.1 M, pH 4.5) for 1 h at 37°C. The reaction was stopped by addition of 100 µl glycine buffer (0.2 M glycine, 0.2 M NaCl, pH 10.7) and fluorescence was measured at $\lambda_{\text{ex}} : 360/\lambda_{\text{em}} : 465$ nm using a fluorescence microplate reader (Spectrafluor, Tecan, Austria). Results are reported as percentage of total β-hexosaminidase released after addition of 1% Triton X-100.

Results

Construction of a codon-optimized Art v 1 gene

Most of the hitherto published gene vaccines encoding allergens displayed normal immunogenicity after intradermal or intramuscular injection (6, 8–10, 21). In contrast, constructs encoding Art v 1 revealed no or only marginal immune responses. Immunogenicity of the Art v 1 gene vaccine could not be enhanced by proven modulations such as the addition of an eukaryotic leader sequence (22), coinjection of the cytokine-plasmid adjuvant GM-CSF (23) or cationic liposome mediated intradermal gene delivery (24) (data not shown). We therefore investigated whether the codon usage of this plant sequence was responsible for the observed effect.

The choice of synonymous codons in both prokaryotic and eukaryotic genes is known to be strongly biased with clear differences in codon usage between taxonomically distant organisms, even among genes encoding related proteins (25, 26).

Analysis of the wild-type sequence of Art v 1 shows, that the codon usage frequencies of this plant gene are quite different from those prevalent in the human genome. For example two Ala, one Gln, one Ile, two Pro, two Ser and one Thr (Table 1) of Art v 1 are encoded

by triplets which are almost never used in human genes (19). The remaining amino acids also display a codon bias different from those in human genes, though not as dramatic.

For recoding the Art v 1 gene (3), the majority of the codons were modified (72.4%). No deviations from strict usage of optimized codons were allowed (Table 1). The human optimized Art v 1 (Fig. 1) shows a high GC content (71%) and a distinct codon bias for guanosine and cytosine at the third codon position (86.9% G or C at the third codon position) compared with the wildtype Art v 1 gene with 53% GC and only 35.5% G or C at the third codon position.

In vitro expression of wild-type Art v 1 is suboptimal and can be increased by codon optimization

To analyze the *in vitro* expression from both Art v 1 genes, the vector construct with the synthetic gene and the plasmid encoding wild-type Art v 1 were transfected into the B 16 mouse melanoma cell line. The co-transfected expression plasmid pCMV-β, coding for the reporter gene beta-galactosidase served as an internal standard for transfection. Supernatants were prepared from cell suspensions and then analyzed by ELISA and Western blotting.

For quantitative analysis of the expression level, 100 ng protein extract, either from cells transfected with pCMV-wtArt or pCMV-humArt, were coated onto ELISA-plates and detected with anti-Art v 1 specific antibody. Compared with the wild-type gene, the expression level of the codon-optimized Art gene was about 180-fold higher (Fig. 2). Only trace amounts of Art v 1 could be detected after transfection with the wild-type construct.

Western blotting with the same protein extracts and antibodies revealed a polypeptide corresponding to the recombinant Art v 1 only in the protein extracts from B 16 cells transfected with pCMV-humArt, but not in those transfected with pCMV-wtArt (data not shown).

Recoding makes the Art v 1 gene vaccine immunogenic

The efficacy of recoding with respect to immunogenicity was tested with an Art v 1 gene vaccine encoding a secreted form of the allergen. This type of antigen presentation is superior to intracellular translation of the gene product and proved to enhance humoral immunogenicity (12, 20, 22). For that purpose the human tissue plasminogen activator leader sequence was used to target translation into the endoplasmic reticulum (ER) thus ensuring the subsequent secretion of the antigen.

Figure 3 demonstrates that genetic immunization with the wild-type gene for Art v 1 induced neither antibodies against the purified natural Art v 1 nor against the recombinant molecule. However, recoding of Art v 1 led

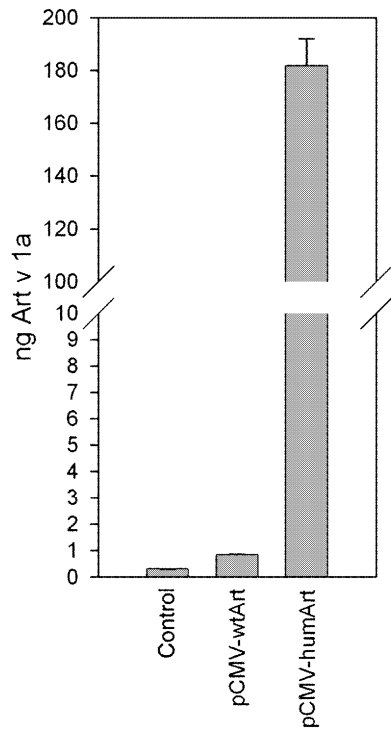


Figure 2. Expression of Art v 1 in the B16 melanoma cell line. Compared to transfection with a control vector lacking any insert, pCMV-wtArt induced only marginal expression of Art v 1. Transfection with the recoded gene vaccine pCMV-humArt elicited a strong expression of the gene product in the supernatant.

to a strong immune response after two booster injections with the typical Th1-biased antibody profile of intradermal genetic immunization (high titers of IgG2a but no IgE antibodies). Furthermore, genetic immunization induced antibodies, which obviously recognized both the purified natural as well as the recombinant Art v 1 to the same extent.

The protein-based immune response induced by the purified natural Art v 1 was mainly restricted to IgG1 and IgE antibodies indicating the Th2 bias of the immune reaction against the natural allergen (data not shown).

The recoded Art v 1 gene vaccine is 'non-allergenic'

The lack of IgE antibody induction after genetic immunization against both forms of the antigen points to the 'non-allergenic' property of the Art v 1 gene vaccine. We tested this assumption with an effector cell test measuring the allergen-specific degranulation of rat basophil leukemia cell line (RBL-2H3). For this purpose RBL-2H3 cells were passively sensitized with murine sera, degranulation was stimulated by cross-linking of receptor-bound antibodies with purified natural Art v 1 and detected via the release of β -hexosaminidase (Fig. 4). Sera from mice immunized with purified natural Art v 1 displayed high lysis values indicating the strong anaphylactic properties of the natural Art v 1 protein and the Th2 character of the protein immunization. In contrast, genetic immunization with either pCMV-wtArt or pCMV-humArt did not stimulate basophil cell release.

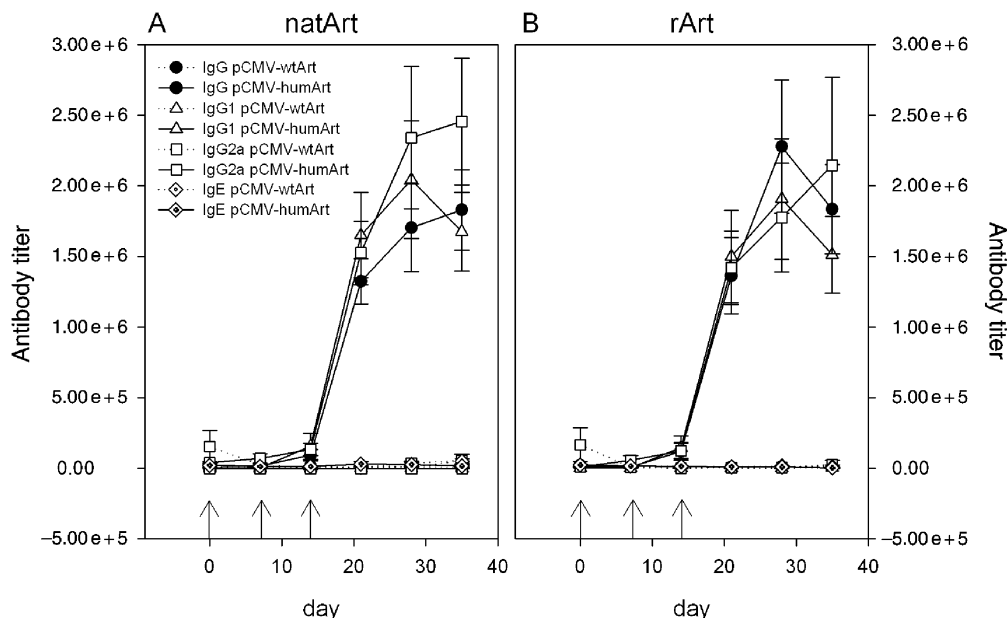


Figure 3. Art v 1-specific antibody responses. Balb/c mice ($n = 6$) were immunized i.d. with pCMV-wtArt or pCMV-humArt, three times in a 1 week interval. Total immunoglobulin G (IgG), IgG1 and IgG2a titers against natural purified Art v 1 (natArt, panel A) or recombinant Art v 1 (rArt, panel B) were measured on day 0, 7, 14, 21, 28, 35 and 42. Immunizations are indicated by arrows, data are shown as mean $\pm$ SEM of end-point titers of each group.

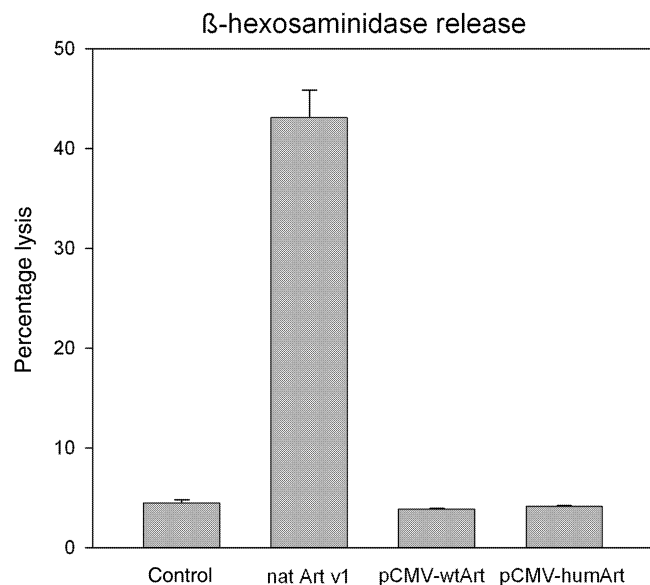


Figure 4. Rat basophil leukemia (RBL) cell release assay with Art v 1-specific antibodies. Allergen-specific degranulation of RBL cells was monitored by β -hexosaminidase release assay. The RBL-2H3 cells were passively sensitized with sera obtained on day 42 (28 days after the last DNA injection, 35 days after immunization with natural purified Art v 1). Cross-linking of receptor-bound antibodies was performed with 1.5 μ g/ml natural purified Art v 1. Control ($n = 6$), sera of untreated animals; natArt ($n = 6$), sera of mice immunized s.c. twice with 1-week interval (5 μ g natural purified Art v 1 adsorbed to $\text{Al}(\text{OH})_3$ in a total volume of 150 μ l sterile phosphate-buffered saline); pCMV-wtArt and pCMV-humArt ($n = 6$) are sera from animals immunized with these constructs. Results are expressed as mean $\pm$ SEM.

Discussion

In this study, we have shown that recoding a gene vaccine encoding the major mugwort allergen Art v 1 (3) towards mammalian codon usage results in improved immunogenicity. Furthermore the study clearly demonstrates that the specific codon usage of plant allergen genes can lead to an inhibited translation of these proteins in mammalian cells.

With respect to the correlation between increased antigen expression and increased antibody responses after genetic immunization (27) increased expression of plant protein *in vivo* is the likely explanation for the increased immunogenicity of the codon-optimized Art v 1 gene vaccine as demonstrated in the current study.

Mammalian codon usage pattern is different from those of a variety of microorganisms and yeasts (28) and also differs remarkable from the codon usage of *A. vulgaris* (Table 1). The differences in codon choice have been attributed to differences in the populations of isoacceptor tRNAs and to differences in modified nucleotides at the anticodon wobble position (26, 29). The

codon usage pattern is considered to be related with the translational efficiency of the gene but do not affect the nature of the protein synthesized (26, 29, 30).

In wild-type Art v 1 several amino acids are encoded by triplets which are almost never used in human genes and also the remaining amino acids display a non-human codon bias with low GC-content at the third codon position (wtArt 35.5%, human consensus 60.8%). Presumably one or more of these wild-type codons are poorly translated in the cell types in which Art v 1 expression is required for induction of antibody *in vivo*.

In the synthetic, humanized Art v 1 sequence all inferior triplets were changed to the optimal human codon pattern but resulted in the same amino acid sequence as in the wild-type protein. The optimized gene shows a GC content (71% GC, 86.9% G or C at the third codon position) which is indicative of efficient translation in mammalian cells (31).

Transfection followed by an efficient translation of the gene of interest is one of the major prerequisites of successful genetic immunization (4, 32). In the case of inefficient translation immunomodulatory stimuli (we used the addition of an eukaryotic leader sequence, coinjection of the cytokine-plasmid adjuvants GM-CSF or cationic liposome mediated intradermal gene delivery) have no effect on the immunogenicity of the constructs.

However, changing the codon usage of a gene vaccine towards mammalian codon usage, thus improving protein expression as shown by *in vitro* transfection, obviously is sufficient to overcome nonimmunogenicity of a wild-type gene vaccine.

Our data demonstrating the positive effect for recoding the allergen gene of Art v 1 are in agreement with recent publications reporting enhanced immunogenicity and protection against infectious pathogens after changing the codon usage of the gene vaccines (13, 14, 17). They prove this approach being highly relevant for the concept of desensitization methods based on genetic immunization (6–8, 10, 18). It can be assumed, that most of the hitherto used gene vaccines from various allergen sources are suboptimal with respect to their codon usage and its effect on the immunogenicity of the constructs.

Furthermore, the recoded Art v 1 gene vaccine displayed the following features, which should be required for any gene vaccine intended for allergy protection or treatment:

(i) The immune response type is Th1-biased as demonstrated by the distribution of IgG1 and IgG2a antibody subclass responses. In addition, supernatants of spleen cells stimulated with Art v 1 revealed increased levels of IFN- γ in animals immunized with the gene vaccine (data not shown). This indicates the recruitment of Th1 cells which are assumed to prevent from an allergic reaction and/or can balance an established Th2 type response (6–8, 11, 18, 33).

(ii) In contrast to protein immunization, the gene vaccine stimulated no induction of IgE antibodies, thus

the risk of anaphylactic side effects upon immunization is minimized. This was confirmed by the assay measuring degranulation of rat basophil cells, which proved the codon-optimized Art v 1 gene vaccine to be functionally nonallergenic.

(iii) Genetic immunization with the recoded Art v 1 gene induced antibodies which recognized both, the glycosylated wildtype molecule as well as the bacterial recombinant counterpart in a similar manner thus indicating that protein epitopes play an important role in the immune response against Art v 1. With respect to genetic desensitization it may be crucial that B cells, which have been activated by genetic immunization in a Th1-biased context, can recognize the naturally occurring allergen for further presentation and activation processes.

(iv) The recoded (humanized) Art v 1 gene vaccine was immunogenic not only in the mouse system but also in the rabbit animal model (data not shown). Together with the

fact, that there is no difference in the most preferred codons for amino acids between *Homo sapiens* and *Mus musculus* (19), this indicates a general applicability of this approach in mammals.

In conclusion, the present work demonstrates that codon-optimization should be routinely considered as a simple method to overcome the hurdles of nonimmunogenicity for the development of safe and efficient gene vaccines for allergy immunotherapy.

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References

- CORNILLON J, BERNARD JP, GUEHO E, TOURAINE R. Estivo-autumnal pollinosis. *Rev Fr Allergol* 1972;**12**:313–322.
- SPIEKSMAN FT, CHARPIN H, NOLARD N, STIX E. City spore concentrations in the European Economic Community (EEC). IV. Summer weed pollen (*Rumex*, *Plantago*, *Chenopodiaceae*, *Artemisia*), 1976 and 1977. *Clin Allergy* 1980;**10**: 319–329.
- HIMLY M, JAHN-SCHMID B, DEDIC A, KELEMEN P, WOPFNER N, ALTMANN F et al. Art v 1, the major allergen of mugwort pollen, is a modular glycoprotein with a defensin-like and a hydroxyproline-rich domain. *Faseb J* 2003;**17**: 106–108.
- GURUNATHAN S, KLINMAN DM, SEDER RA. DNA vaccines: immunology, application, and optimization. *Annu Rev Immunol* 2000;**18**:927–974.
- SEDER RA, GURUNATHAN S. DNA vaccines – designer vaccines for the 21st century. *N Engl J Med* 1999;**341**: 277–278.
- RAZ E, TIGHE H, SATO Y, CORR M, DUDLER JA, ROMAN M et al. Preferential induction of a Th1 immune response and inhibition of specific IgE antibody formation by plasmid DNA immunization. *Proc Natl Acad Sci USA* 1996;**93**: 5141–5145.
- SLATER JE, PAUPORE E, ZHANG YT, COLBERG-POLEY AM. The latex allergen Hev b 5 transcript is widely distributed after subcutaneous injection in BALB/c mice of its DNA vaccine. *J Allergy Clin Immunol* 1998;**102**:469–475.
- HSU CH, CHUA KY, TAO MH, LAI YL, WU HD, HUANG SK et al. Immunoprophylaxis of allergen-induced immunoglobulin E synthesis and airway hyperresponsiveness in vivo by genetic immunization. *Nat Med* 1996;**2**:540–544.
- HOCHREITER R, HARTL A, FREUND J, VALENTA R, FERREIRA F, THALHAMER J. The influence of CpG motifs on a protein or DNA-based Th2-type immune response against major pollen allergens Bet v 1a, Phl p 2 and *Escherichia coli*-derived beta-galactosidase. *Int Arch Allergy Immunol* 2001;**124**:406–410.
- HARTL A, KIESSLICH J, WEISS R, BERNHAUPT A, MOSTBOCK S, SCHEIBLHOFFER S et al. Immune responses after immunization with plasmid DNA encoding Bet v 1, the major allergen of birch pollen. *J Allergy Clin Immunol* 1999;**103**: 107–113.
- HARTL A, KIESSLICH J, WEISS R, BERNHAUPT A, MOSTBOCK S, SCHEIBLHOFFER S et al. Isoforms of the major allergen of birch pollen induce different immune responses after genetic immunization. *Int Arch Allergy Immunol* 1999;**120**:17–29.
- LEITNER WW, HAMMERL P, THALHAMER J. Nucleic acid for the treatment of cancer: genetic vaccines and DNA adjuvants. *Curr Pharm Des* 2001;**7**: 1641–1667.
- LIU WJ, ZHAO KN, GAO FG, LEGGATT GR, FERNANDO GJ, FRAZER IH. Polynucleotide viral vaccines: codon optimization and ubiquitin conjugation enhances prophylactic and therapeutic efficacy. *Vaccine* 2001;**20**:862–869.
- STRATFORD R, DOUCE G, ZHANG-BARBER L, FAIRWEATHER N, ESKOLA J, DOUGAN G. Influence of codon usage on the immunogenicity of a DNA vaccine against tetanus. *Vaccine* 2000;**19**: 810–815.
- NAGATA T, UCHIJIMA M, YOSHIDA A, KAWASHIMA M, KOIDE Y. Codon optimization effect on translational efficiency of DNA vaccine in mammalian cells: analysis of plasmid DNA encoding a CTL epitope derived from microorganisms. *Biochem Biophys Res Commun* 1999;**261**:445–451.
- ANDRE S, SEED B, EBERLE J, SCHRAUT W, BULTMANN A, HAAS J. Increased immune response elicited by DNA vaccination with a synthetic gp120 sequence with optimized codon usage. *J Virol* 1998;**72**:1497–1503.
- LEDER C, KLEINSCHMIDT JA, WIETHE C, MULLER M. Enhancement of capsid gene expression: preparing the human papillomavirus type 16 major structural gene L1 for DNA vaccination purposes. *J Virol* 2001;**75**:9201–9209.
- PENG HJ, SU SN, CHANG ZN, CHAO PL, KUO SW, TSAI LC. Induction of specific Th1 responses and suppression of IgE antibody formation by vaccination with plasmid DNA encoding Der f 11. *Vaccine* 2002;**20**:1761–1768.
- NAKAMURA Y, GOJOBORI T, IKEMURA T. Codon usage tabulated from international DNA sequence databases: status for the year 2000. *Nucleic Acids Res* 2000;**28**:292.

20. SCHEIBLHOFFER S, CHEN D, WEISS R, KHAN F, MOSTBOCK S, FEGEDING K et al. Removal of the circumsporozoite protein (CSP) glycosylphosphatidylinositol signal sequence from a CSP DNA vaccine enhances induction of CSP-specific Th2 type immune responses and improves protection against malaria infection. *Eur J Immunol* 2001;**31**: 692–698.
21. ROY K, MAO HQ, HUANG SK, LEONG KW. Oral gene delivery with chitosan – DNA nanoparticles generates immunologic protection in a murine model of peanut allergy. *Nat Med* 1999;**5**: 387–391.
22. WEISS R, DURNBERGER J, MOSTBOCK S, SCHEIBLHOFFER S, HARTL A, BREITENBACH M et al. Improvement of the immune response against plasmid DNA encoding OspC of *Borrelia* by an ER-targeting leader sequence. *Vaccine* 1999;**18**:815–824.
23. XIANG Z, ERTL HC. Manipulation of the immune response to a plasmid-encoded viral antigen by coinoculation with plasmids expressing cytokines. *Immunity* 1995;**2**:129–135.
24. GREGORIADIS G, SAFFIE R, SOUZA JB. Liposome-mediated DNA vaccination. *Febs Lett* 1997;**402**:107–110.
25. GRANTHAM R, GAUTIER C, GOUY M, JACOBZONE M, MERCIER R. Codon catalog usage is a genome strategy modulated for gene expressivity. *Nucleic Acids Res* 1981;**9**:r43–74.
26. IKEMURA T. Correlation between the abundance of *Escherichia coli* transfer RNAs and the occurrence of the respective codons in its protein genes. *J Mol Biol* 1981;**146**:1–21.
27. BARRY MA, JOHNSTON SA. Biological features of genetic immunization. *Vaccine* 1997;**15**:788–791.
28. IKEMURA T. Codon usage and tRNA content in unicellular and multicellular organisms. *Mol Biol Evol* 1985;**2**:13–34.
29. IKEMURA T. Correlation between the abundance of yeast transfer RNAs and the occurrence of the respective codons in protein genes. Differences in synonymous codon choice patterns of yeast and *Escherichia coli* with reference to the abundance of isoaccepting transfer RNAs. *J Mol Biol* 1982;**158**:573–597.
30. ZOLOTUKHIN S, POTTER M, HAUSWIRTH WW, GUY J, MUZYCZKA N. A “humanized” green fluorescent protein cDNA adapted for high-level expression in mammalian cells. *J Virol* 1996;**70**: 4646–4654.
31. HAAS J, PARK EC, SEED B. Codon usage limitation in the expression of HIV-1 envelope glycoprotein. *Curr Biol* 1996;**6**:315–324.
32. DONNELLY JJ, ULMER JB, SHIVER JW, LIU MA. DNA vaccines. *Annu Rev Immunol* 1997;**15**:617–648.
33. ADEL-PATIENT K, CREMINON C, BOQUET D, WAL JM, CHATEL JM. Genetic immunisation with bovine beta-lactoglobulin cDNA induces a preventive and persistent inhibition of specific anti-BLG IgE response in mice. *Int Arch Allergy Immunol* 2001;**126**:59–67.